Identifying new deubiquitinases involved in the circadian clock

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ABSTRACT

Circadian rhythms reflect daily variations in our physiological state such as sleep, body temperature and hormonal secretion. At the cellular level, they are driven by a molecular clock formed by clock proteins cycling in transcriptional/translational feedback loops. Deubiquitinases can regulate this mechanism by modulating the function, localization and stability of clock proteins. In fact, our lab and others have found that Ubiquitin Specific Peptidase 2 (USP2) interacts with and deubiquitinates multiple clock proteins. Surprisingly, *Usp2* KO mice have only mild alterations to their locomotor activity rhythms, which prompted us to identify other clock-regulating deubiquitinases that might have a redundant function with USP2. We investigated this using two complementary approaches: 1) a targeted approach, where specific candidate deubiquitinases were knocked down individually in a primary mouse cell line expressing a bioluminescent circadian reporter; 2) a global approach, where all known deubiquitinases were screened for their role in the clock in either a wildtype or a *Usp2* knockdown background. Interestingly, we found that the loss of one of our four candidate deubiquitinases, USP8, dampened the bioluminescence rhythms of mammalian cells. Furthermore, our global approach identified 40 new deubiquitinases possibly involved in cellular circadian rhythms. We observed that reducing their levels considerably altered the period or the amplitude of cellular rhythms. Most importantly, the knockdown of seven of these deubiquitinases in combination with *Usp2* deficiency further altered the period or the amplitude of the rhythms, suggesting a possible overlapping role of these deubiquitinases with USP2. Overall, our work provides multiple new avenues to pursue regarding the roles of deubiquitinases in the molecular clock and improves our global understanding of circadian rhythms regulation.

RÉSUMÉ

Les rythmes circadiens consistent en des variations quotidiennes observées dans une multitude de processus physiologiques, incluant le sommeil, la température corporelle et les sécrétions hormonales. Ces rythmes sont générés par une horloge cellulaire formée par des protéines interagissant entrent elles dans des cycles négatifs de rétroaction transcriptionnelles/traductionnelles. Ces protéines de l'horloge sont précisément régulées, notamment par des modifications post-traductionnelles comme l'ubiquitination. Les déubiquitinases peuvent modifier ou retirer ces signaux d'ubiquitination afin de moduler la fonction, la localisation et la stabilité de leurs substrats. L'une d'entre elle, la peptidase spécifique à l'ubiquitine 2 (USP2) interagit et déubiquitine plusieurs protéines de l'horloge. Cependant, les souris *Usp2* KO ne démontrent que de légères altérations de leurs rythmes d'activité locomotrice. Ce phénotype nous a incité à identifier de nouvelles déubiquitinases ayant un rôle partiellement redondant avec USP2. Pour ce faire, nous avons utilisé deux approches complémentaires : 1) une approche ciblée, où l'expression de déubiquitinases candidates a été diminuée individuellement dans des cellules primaires de souris exprimant un rapporteur circadien bioluminescent ; 2) une approche globale, où toutes les déubiquitinases humaines connues ont été criblées pour leur rôle dans l'horloge dans des cellules contrôles ou réduites en *Usp2*. Nous avons constaté que la réduction de l'expression de l'une des quatre déubiquitinases candidates, *Usp8*, atténuait l'amplitude des rythmes de bioluminescence de cellules de mammifères. De plus, notre approche globale a identifié que la réduction de l'expression de 40 déubiquitinases altérait considérablement la période ou l'amplitude des rythmes de bioluminescence. Plus précisément, la combinaison de la réduction d'*Usp2* avec celle de sept de ces déubiquitinases a amplifié ces altérations de la période ou de l'amplitude des rythmes, ce qui

suggère que ces déubiquitinases ont un rôle dans l'horloge possiblement redondant avec USP2. Globalement, ces travaux offrent de nouvelles pistes à explorer concernant les rôles de la déubiquitination dans l'horloge moléculaire et améliorent notre compréhension globale de la régulation des rythmes circadiens.

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CONTRIBUTION OF AUTHORS

Namasthée Harris-Gauthier generated all reporter cell lines used throughout this project, which include U2OS *Per2*-Luc and *Bmal1*-Luc cells as well as the generation of PER2::Luc mouse embryonic fibroblasts. *Per2*-Luc and *Bmal1*-Luc vectors were already cloned by Christine Kirady. Namasthée Harris-Gauthier also developed and performed all cellular transfections of siRNAs and bioluminescence recordings. She also performed all the analyses described in this thesis.

For the first approach of the project, Namasthée Harris-Gauthier performed all bioinformatic research on DUBs with some help from Marie-Ève Cloutier. She also participated in the design of the quantitative qPCR, luciferase degradation assay and cell viability assay and performed all these experiments.

For the second approach, Namasthée Harris-Gauthier developed the siRNA screening protocol and performed all experiments. The siRNA library against human DUBs was created and provided by Dr. El Bachir Affar.

Nicolas Cermakian supported and helped the design of all experiments. He also provided his expertise in molecular biology as well as in circadian rhythms throughout the project. He also advised on techniques and analyses. Finally, he provided laboratory space and necessary equipment for all experiments described in this thesis, as well as all reagents.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of Variance

ARF-BP1: ARF-Binding Protein 1 (also called HECT, UBA and WWE Domain-Containing Protein 1 or HUWE1)

BLASTP: Basic Local Alignment Search Tool – Protein

BMAL1: Brain and Muscle ARNT-Like 1

Bmal1-Luc: Luciferase gene under the control of *Bmal1* promoter

CEZANNE: Cellular Zinc-finger Anti-NF-κB

CHX: Cycloheximide

CKI: Casein Kinase I

CLOCK: Circadian Locomotor Output Cycles Kaput

CRY: Cryptochrome

DMEM: Dulbecco's Modified Eagle's Medium

dsiRNA: Duplexed Small Interfering RNA

DUB: Deubiquitinase

FBXL: F-box and Leucine Rich Repeat Proteins

GEO: Gene Expression Omnibus

JAMM: JAB1/MPN/MOV34 Metalloproteases

KO: Knockout

LSD1: Lysine-Specific Demethylase 1A

MAGEL2: Melanoma-Associated Antigen Gene (MAGE) Protein Family Member L2

MEF: Mouse Embryonic Fibroblast

MINDY: Motif Interacting with Ub-containing Novel DUB Family

MJD: Machado-Joseph Domain-Containing Proteases

NOT: Non-Stop

NPAS2: Neuronal PAS Domain Protein 2

OTU: Ovarian Tumor Proteases

PAM: Protein Associated with Myc (also called Myc-Binding Protein 2 or MYCBP2)

PCR: Polymerase Chain Reaction

PDP1: PAR Domain Protein 1

PER: Period

PER2::Luc: PER2 Luciferase fusion protein

Per2-Luc: Luciferase gene under the control of *Per2* promoter

PI: Propidium Iodide

PKC: Protein Kinase C

PTM: Post-Translational Modification

REV-ERB: Reverse Erythroblastosis Virus Protein

RLU: Relative Luminescence Unit

ROR: Retinoic Acid Receptor-Related Orphan Receptor

SAGA: Spt-Ada-Gcn5 Acetyltransferase

SCN: Suprachiasmatic Nucleus

siRNA: Small Interfering RNA

SPSB: Sp1A/Ryanodine Receptor Domain and SOCS Box-Containing

STUB1: STIP1 Homology and U-Box-Containing Protein 1

TNF: Tumor Necrosis Factor

TIM: Timeless

TRAF: TNF Receptor-Associated Factor

TRAF: TNF Receptor-Associated Factor 2

U2OS: Human U-2 Osteosarcoma Epithelial Cells

UBE2O: Ubiquitin-Conjugating Enzyme E2 O

UBE3A: Ubiquitin Protein Ligase E3A

UCH: Ubiquitin C-terminal Hydrolase

USP: Ubiquitin Specific Peptidase

VRI: Vrille

WT: Wild-Type

ZUP1: Zinc Finger-Containing Ubiquitin Peptidase 1

β-TRCP: Beta-Transducin Repeat-Containing Protein

INTRODUCTION

Circadian rhythms reflect physiological changes that occur over the course of a day, enabling a majority of organisms to anticipate and adapt to daily environmental changes such as the day-night cycle (Vitaterna et al., 2001). Their disruption, as occurs in shift-workers, have been shown to be involved in the development of multiple disorders, including cancer, mental illness and sleep disorders, making it necessary to improve our knowledge of their mechanisms (Dibner et al., 2010).

These daily physiological rhythms can also be observed at a cellular level. In fact, they are driven by a molecular clock composed of clock genes and proteins forming a negative transcriptional-translational feedback loop that generates 24-hour rhythmic gene expression in most tissues of the body (Dibner et al., 2010; Srikanta and Cermakian, 2021). The great robustness of this mechanism relies on the fine tuning and high regulation of clock proteins. Post-translational modifications (PTMs) such as phosphorylation and ubiquitination can impact the stability, the cellular localization and the function of targeted proteins (Crosby and Partch, 2020; Hirano et al., 2016a). Although far less studied, deubiquitination was also shown to play an important role in clock modulation (Srikanta and Cermakian, 2021).

Ubiquitin-Specific Peptidase 2 (USP2) is the most studied deubiquitinase (DUB) in the field of circadian rhythms. In mice, it interacts with and regulates many clock proteins (Scoma et al., 2011; Tong et al., 2012; Yang et al., 2012; Yang et al., 2014). However, *Usp2* KO mice only exhibit slight period changes of locomotor activity rhythms and some minor defects in light response and clock genes expression (Scoma et al., 2011; Yang et al., 2012). Although significant, these effects were not as strong as predicted. This suggest that another deubiquitinase with overlapping function might rescue *Usp2* deficiency. Indeed, similar redundancies between

proteins in the clock were already shown to exist, notably amongst clock proteins such as CRYs and PERs, but also between clock regulators such as kinases and ubiquitin ligases (D'Alessandro et al., 2017; DeBruyne et al., 2007; Lee et al., 2009; Shi et al., 2010; van der Horst et al., 1999; Zheng et al., 2001).

Thus, we hypothesized that one or more DUBs are involved in the clock mechanism through a role partly redundant with USP2. Using circadian bioluminescent reporters to track changes in cellular rhythms, our goal was to identify and characterize new DUBs whose function in the clock may be overlapping with USP2. We aimed to address this through two complementary approaches:

Targeted approach: To identify candidate DUBs through bioinformatic research on the suprachiasmatic nucleus (SCN) expression patterns, rhythmicity and USP2 similarities of all DUBs. To subsequently assess the role of identified candidate DUBs in the molecular clock by knocking down these DUBs in both WT and *Usp2* KO cell lines expressing a circadian bioluminescent reporter and to investigate their involvement in the clock.

Global approach: To identify DUBs involved in the cellular circadian clock by knocking down all known DUBs in *Usp2* knocked down cell lines expressing a circadian bioluminescent reporter.

BACKGROUND

Circadian rhythms and the molecular clock

Circadian rhythms can be observed at behavioral and physiological levels, where certain activities and biological processes vary over the course of a day. These rhythms are essential for most species to synchronize their physiology to environmental changes such as the daily light-dark cycle (Vitaterna et al., 2001). In fact, circadian rhythms are both endogenous and entrainable by external cues like food intake and light (Dibner et al., 2010). The SCN, a small hypothalamic structure at the base of the brain, is thought to be the master clock of the body. It can notably integrate light inputs with its endogenous clock to transmit circadian information to other peripheral clocks dispersed throughout the body (Partch et al., 2014).

Circadian rhythms can also be observed at a cellular level. In fact, around 55% of all mouse protein-coding genes are thought to be expressed in a circadian manner in at least one tissue in the body (Zhang et al., 2014). This rhythmicity is mainly due to a self-sustained cellular clock formed by a set of clock genes and proteins cycling every 24 hours (**Figure 1**) (Dibner et al., 2010). The cycle begins with Brain and Muscle ARNT-Like 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK), two transcription factors that heterodimerize to promote the transcription of *Period* (*Per1/2/3*) and *Cryptochrome* (*Cry1/2*) genes (Srikanta and Cermakian, 2021). Once translated into proteins, PERs and CRYs heterodimerize and translocate into the cell nucleus where they repress their own expression through the inhibition of BMAL1/CLOCK-mediated transcription (Srikanta and Cermakian, 2021). The BMAL1/CLOCK complex also regulates the transcription of orphan nuclear receptors Reverse Erythroblastosis Virus Proteins α and β (REV-ERBα/β) and Retinoic Acid Receptor-Related Orphan Receptors α, β and γ (RORα/β/γ), which are respectively repressors and activators of *Bmal1* transcription and

form additional regulatory loops (Guillaumond et al., 2005; Preitner et al., 2002; Sato et al., 2004). BMAL1/CLOCK and REV-ERBα also control the cyclic expression of many clock-controlled genes, thereby having an important influence on many cellular pathways such as the cell cycle and the immune response (Dibner et al., 2010; Ikeda et al., 2019; Labrecque and Cermakian, 2015). A tight and robust regulation of the molecular clock is thus essential to the proper function of most tissues.

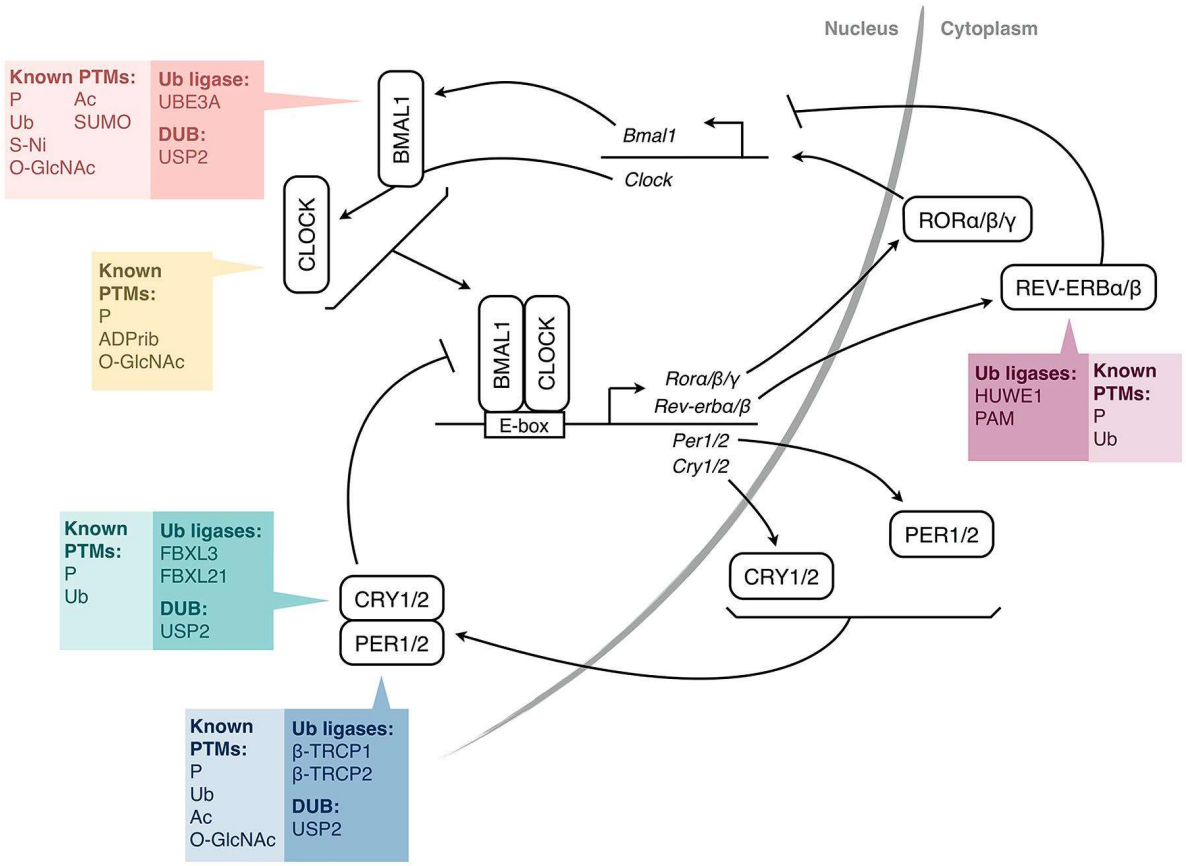


Figure 1. Simplified molecular mechanism of the mammalian circadian clock. Most clock proteins are known to be modified at a post-translational level mainly through phosphorylation (P), ubiquitination (Ub), acetylation (Ac), O-GlcNAcylation (O-GlcNAc) and SUMOylation (SUMO). **(Figure adapted from Stojkovic et al. (2014) under the Creative Commons Attribution Licence (CC BY))**

Redundancy in the clock

Redundancy between genes occurs in many pathways in biology and most importantly in the circadian clock mechanism. For instance, major loss of locomotor activity rhythms in constant darkness was only observed in Neuronal PAS Domain Protein 2 (*Npas2*) and *Clock* double KO mice, but not when they were knocked out individually (DeBruyne et al., 2007). A similar effect was observed for other pairs of clock genes, such as *Cry1*/*Cry2* and *Per1*/*Per2* (van der Horst et al., 1999; Zheng et al., 2001). BMAL1 also have a redundant partner, called BMAL2. However, since *Bmal2* expression is under the control of BMAL1, knocking out *Bmal1* alone in mice leads to the downregulation of both genes and accordingly, these mice are arrhythmic (Shi et al., 2010).

Redundancy was also observed in other clock-related proteins. For instance, the individual loss of ubiquitin ligases Beta-Transducin Repeat-Containing Protein 1 or 2 (β-TrCP1 or β-TrCP2) in mice only led to mild changes in their circadian behavior, but a double knockout completely disrupted their locomotor activity rhythms (D'Alessandro et al., 2017). Similarly, the inactivation or mutation of both Casein Kinases Iδ and Iε (CKIδ and CKIε) resulted in a complete loss of bioluminescence rhythms in primary mouse fibroblasts, while their individual inactivation only led to slight period changes (Lee et al., 2009).

Post-translational modifications

Most clock proteins are known to be modified at a post-translational level (Crosby and Partch, 2020; Hirano et al., 2016a). Indeed, PTMs have been largely studied in the circadian clock due to their extensive control of the interaction, activity, cellular localization and degradation rate of target proteins. Accordingly, PTMs such as phosphorylation, acetylation,

ubiquitination and SUMOylation are important modulators of the clock pace and robustness (Cardone et al., 2005; Duguay and Cermakian, 2009; Gallego and Virshup, 2007; Srikanta and Cermakian, 2021; Stojkovic et al., 2014). Accordingly, many kinases and ubiquitin ligases have already been identified as key regulators of the clock (Busino et al., 2007; Dardente et al., 2008; Eide et al., 2005; Maier et al., 2009; Mekbib et al., 2019; Ohsaki et al., 2008).

Ubiquitination

Ubiquitination relies on the covalent attachment of a small 8.5 kDa ubiquitin protein which usually takes place at lysine residues (Komander and Rape, 2012). Although a single ubiquitin signal can be attached to targets, multiple ubiquitin moieties can be connected together in various architectures (Komander and Rape, 2012). Three processes achieved by three different types of enzymes lead to ubiquitination. First, ubiquitin is activated thought ATP conjugation by E1 ubiquitin-activating enzymes (Schulman and Harper, 2009). Then, E2 ubiquitin-conjugating enzymes can process ubiquitin before it is transferred to the targeted protein (Ye and Rape, 2009). Finally, E3 ubiquitin ligases confer specificity by transferring the ubiquitin signal to precise substrate proteins (Deshaies and Joazeiro, 2009).

The ubiquitination of clock proteins can lead to their rapid degradation. In fact, PER proteins have very short half-lives and their polyubiquitination is thought to lead to their rapid degradation (D'Alessandro et al., 2015; D'Alessandro et al., 2017). Ubiquitin ligases are thus important regulators of the clock. Accordingly, multiple E2 and E3 enzymes were shown to regulate the levels of clock proteins and to maintain the pace and robustness of circadian rhythms. Ubiquitin ligases β-TrCP1 and β-TrCP2 both ubiquitinate PER proteins and destabilize them (D'Alessandro et al., 2017). Indeed, β-TrCP1/2 double mutant mice exhibit an increased period of locomotor activity rhythms (D'Alessandro et al., 2017). CRY proteins oscillations are

precisely regulated by F-Box and Leucine-Rich Repeat Protein 3 and 21 (FBXL3 and FBXL21), which were shown to function through counterbalancing processes (Busino et al., 2007; Godinho et al., 2007; Hirano et al., 2013; Siepka et al., 2007; Yoo et al., 2013). *Fbxl3* mutant mice were consistently showed to have a longer period of locomotor activity rhythms (Godinho et al., 2007; Siepka et al., 2007), while *Fbxl21* KO or mutant mice have a free-running period similar to or slightly shorter than WT (Hirano et al., 2013; Yoo et al., 2013).

BMAL1 is also regulated by multiple ubiquitin ligases. Ubiquitin Protein Ligase E3A (UBE3A), Ubiquitin-Conjugating Enzyme E2 O (UBE2O), TNF Receptor-Associated Factor 2 (TRAF2) and STIP1 Homology and U-Box-Containing Protein 1 (STUB1) all destabilize BMAL1 *in vitro* by promoting its ubiquitin-dependent degradation (Chen et al., 2018a; Chen et al., 2018b; Gossan et al., 2014; Ullah et al., 2020). Similarly, SIAH2, Sp1A/Ryanodine Receptor Domain and SOCS Box-Containing 1 and 4 (SPSB1 and SPSB4), ARF-Binding Protein 1 (AFR-BP1) and Protein Associated with Myc (PAM) are multiple E3 ubiquitin ligases shown to ubiquitinate and destabilize REV-ERBα (DeBruyne et al., 2015; Mekbib et al., 2019; Yin et al., 2010).

Deubiquitinases

It is now clear that ubiquitin ligases are important modulators of clock proteins by precisely timing their degradation. However, ubiquitination is a reversible process that can be counterbalanced by antagonist enzymes. Deubiquitinases or DUBs are therefore also of great interest since they are essential in balancing ubiquitination and controlling protein homeostasis (**Figure 2**) (Clague et al., 2019).

DUBs are involved in all ubiquitin regulatory processes, ranging from the modification or removal of ubiquitin signals to the recycling and processing of ubiquitin (**Figure 2**) (Clague et al., 2019). Over a hundred DUBs have been identified in mammals and are further divided in seven families based on their structure. Ubiquitin-Specific Proteases (USPs) form the largest family with more than 50 members. (Clague et al., 2019). Other smaller families include

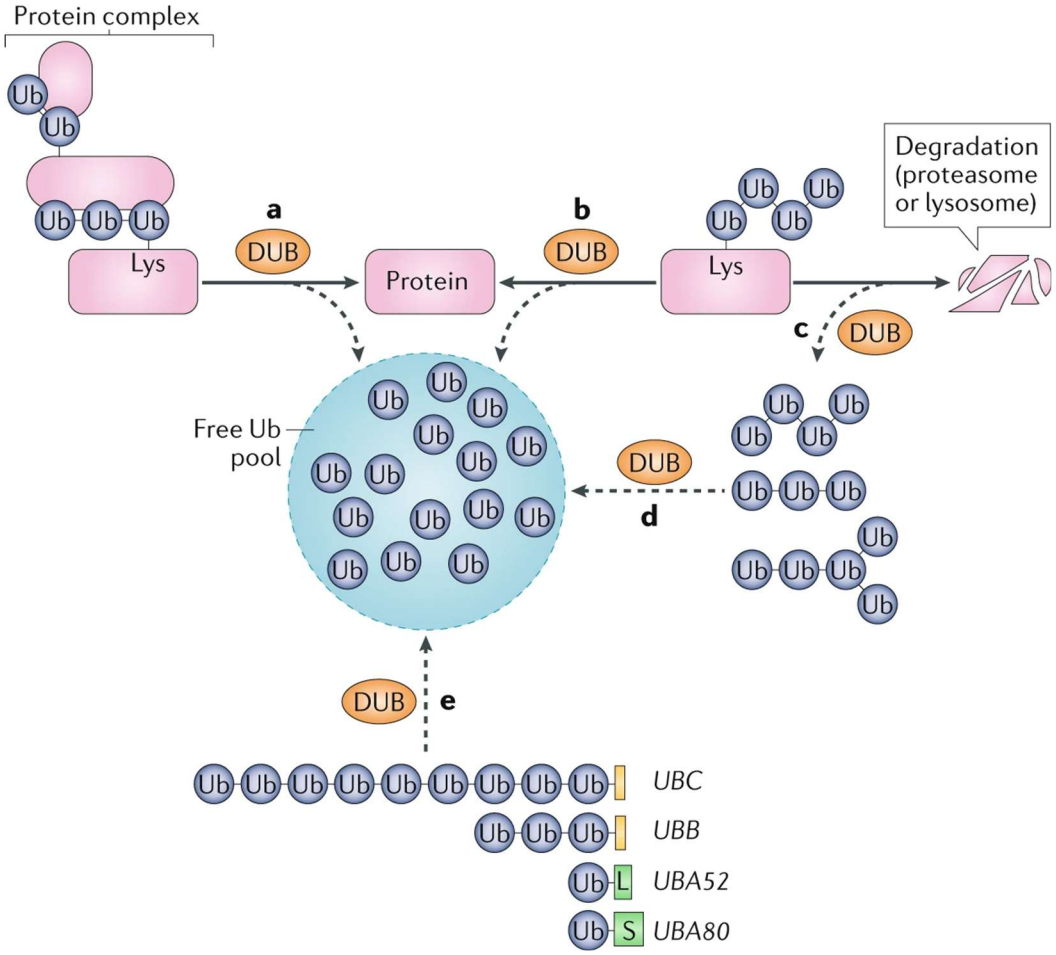


Figure 2. Major roles of DUBs. DUBs are essential for ubiquitin (Ub) homeostasis in the cell. Their main roles involve **a.** the modulation of the function and interaction of targeted proteins, **b.** the stabilization of targeted proteins, **c. d.** the recycling of ubiquitin and **e.** the processing and synthesis of new ubiquitin. Both UBC and UBB encode multiple copies of ubiquitin, while UBA52 and UBA80 encode ubiquitin fused with ribosomal subunits. Dashed arrows show entry into ubiquitin pools resulting from DUB cleavage; solid arrows indicate the substrate protein fate.

JAB1/MPN/MOV34 Metalloproteases (JAMMs), Ovarian Tumor Proteases (OTUs), Machado-Joseph Domain-Containing Proteases (MJDs), Ubiquitin C-terminal Hydrolases (UCHs), Motif Interacting with Ub-Containing Novel DUB Family (MINDY) and Zinc Finger-Containing Ubiquitin Peptidase 1 (ZUP1) (Clague et al., 2019). DUBs play critical roles in most cellular processes and their disruption or loss are linked to several diseases such as cancer (Bonacci and Emanuele, 2020). However, only a few studies so far have looked at their role in the circadian clock.

USP2

USP2 is the most studied DUB involved in circadian rhythms. Its transcript is expressed rhythmically in most mammalian tissues which include the SCN (Kita et al., 2002; Oishi et al., 2005; Scoma et al., 2011; Storch et al., 2002; Yan et al., 2008; Zhang et al., 2014). It was later shown to interact with multiple clock proteins including PER1, CRY1 and BMAL1 (Scoma et al., 2011; Tong et al., 2012; Yang et al., 2012), although it only interacts directly with PER1 (Yang et al., 2012). More specifically, USP2 deubiquitinates BMAL1 and CRY1 to promote their stability (Scoma et al., 2011; Tong et al., 2012). It also controls PER1 nuclear localization through its deubiquitination (Yang et al., 2014).

Based on these findings, it was expected that *Usp2* KO mice would exhibit clear circadian dysfunctions. Indeed, such disruptions were previously observed in mice lacking ubiquitin ligases such as β-TrCP1/2, FBXL3 and FBXL21 (Godinho et al., 2007; Hirano et al., 2013; Siepka et al., 2007; Yoo et al., 2013). However, *Usp2* KO mice have an overall functional clock, as they only exhibit a slightly longer period of locomotor activity rhythms in constant darkness and some minor alterations to the expression of their clock genes in the SCN (Scoma et al., 2011; Yang et al., 2012). Interestingly, *Usp2* KO mice show slower entrainment of the clock

to phase advances of the light-dark cycle, but were better than WT in entraining to phase delays (Yang et al., 2012), suggesting that USP2 might be involved in the light response pathway.

Other deubiquitinases involved in the mammalian clock

USP7

USP7 (also called Herpes Virus-Associated Ubiquitin-Specific Protease or HAUSP) was shown to interact with CRY proteins, although it was only found to promote the stability of CRY1 (Hirano et al., 2016b; Papp et al., 2015). However, the circadian phenotypes upon the knockdown or the overexpression of *Usp7* in mammalian cells is inconsistent across studies. The knockdown of *Usp7* was first shown to lengthen the period of bioluminescence rhythms in immortalized mouse embryonic fibroblast (MEFs) and in a human osteosarcoma cell line (U2OS) (Papp et al., 2015). In contrast, one year later, *Usp7* knockdown in MEFs and NIH3T3 mouse fibroblasts resulted in a significant reduction in the period of bioluminescence rhythms (Hirano et al., 2016b). Accordingly, *Usp7* overexpression led to the opposite effect (Hirano et al., 2016b). These discrepancies might arise from a differential interaction of USP7 with CRY1 and CRY2, as the deficiency of either clock protein leads to opposite cellular phenotypes (Baggs et al., 2009; Maier et al., 2009). Interestingly, USP7 was shown to be itself negatively regulated by an E3 ubiquitin ligase called Melanoma-Associated Antigen Gene (MAGE) Protein Family Member L2 (MAGEL2), which was previously shown to repress CLOCK/BMAL1 activity (Carias et al., 2020; Devos et al., 2011).

USP9X

USP9X was found to deubiquitinate and stabilize BMAL1 (Zhang et al., 2018). Accordingly, BMAL1 target genes *Per2* and *Cry1* were downregulated upon *Usp9x* knockdown

in a mouse neuroblastoma cell line (Zhang et al., 2018) Its knockdown in reporter U2OS cells did not result in a significant period change, but rather in a reduction of the amplitude of bioluminescence rhythms (Zhang et al., 2018).

USP14

PER proteins are heavily polyubiquitinated and the balance of this ubiquitination is critical to maintain the precise 24-hours pace of the circadian clock (D'Alessandro et al., 2015; D'Alessandro et al., 2017). In fact, USP14 was found to downregulate the polyubiquitination levels of PER1 and PER2 (D'Alessandro et al., 2017). A dominant-negative form of *Usp14* expressed in a primary mouse cell line decreased the period of bioluminescence rhythms in a dose-dependent manner (D'Alessandro et al., 2017).

UCHL1

The Ubiquitin C-terminal Hydrolase L1 (UCHL1) is one of the most abundant protein in the brain (Day and Thompson, 2010). In fact, it is mostly expressed in neuronal cells (Day and Thompson, 2010) and its transcript is highly expressed in the SCN (Dong et al., 2005). A *Uchl1* spontaneous mutation in mice revealed disruptions in locomotor activity rhythms in both a light-dark cycle and in constant darkness (Pfeffer et al., 2012). These mice also have trouble adjusting their clock to phase delays of the light-dark cycle (Pfeffer et al., 2012). Given these phenotypes and its high level of expression in neurons, UCHL1 might be involved in the clock regulation or in clock outputs in the brain, although further investigation is needed to confirm its role precisely in the molecular clock.

Deubiquitinases involved in the Drosophila clock

The core clock mechanism is highly conserved between animal phyla, notably between the mammalian and the *Drosophila* system. In the *Drosophila* clock, a complex composed of two transcription factors named CLOCK and CYCLE (homologous to mammalian CLOCK and BMAL1) activate the transcription of clock genes including *timeless (tim), period (per), PAR domain protein 1ε (pdp1ε) and vrille (vri)*. PER and TIM proteins then inhibit their own expression by repressing CLOCK/CYCLE activity (Williams and Sehgal, 2001). Additional regulatory loops involve the respective activation and repression of *clock* expression by PDP1ε and VRI (Ozkaya and Rosato, 2012).

PER, TIM and CLOCK were all shown to be ubiquitinated and regulated by various ubiquitin ligases (Srikanta and Cermakian, 2021). Some DUBs were also identified as important modulators of the *Drosophila* clock (Srikanta and Cermakian, 2021). Indeed, USP8, a DUB closely related to USP2 (Clague et al., 2013), deubiquitinates CLOCK and represses its transcriptional activity (Luo et al., 2012). Interestingly, *Usp8* knockdown in clock neurons lengthened the period of locomotor activity rhythms (Luo et al., 2012).

More recently, NON-STOP (NOT) was also found to regulate the *Drosophila* clock. It is part of the deubiquitinating module of the Co-Activator Spt-Ada-Gcn5 Acetyltransferase (SAGA) complex which regulates histone modifications (Helmlinger and Tora, 2017). The NOT-mediated deubiquitination of histone 2B at both *tim* and *pdp1ε* loci promotes their transcription (Bu et al., 2020). Indeed, flies with a clock neurons-specific knockdown of *not* were shown to have a lengthen period of activity rhythms and reduced expression of clock genes (Bu et al., 2020; Mahesh et al., 2020).

No studies to date have looked at the role of *Usp8* or *Usp22*, the mammalian homolog of *not*, in the mammalian clock. Nevertheless, histone 2B mono-ubiquitination was found to regulate the transcription of clock genes in mouse livers (Tamayo et al., 2015). As for *Usp8*, it was shown to be embryonically lethal in mice (Niendorf et al., 2007), suggesting the need for *in vitro* studies or for a more targeted *in vivo* approach to study its role in the mammalian clock.

As little is still known about the roles of DUBs in the clock mechanism, especially when compared to their counteracting enzymes, ubiquitin ligases, it is highly relevant to identify other DUBs involved in circadian rhythms. A cellular approach, as the one we are taking in our research, is indeed an efficient way to rapidly identify such clock components and could lead to future *in vivo* studies of DUBs.

METHODOLOGY

Rhythmic expression of DUBs in the SCN

We used the online database research tool CircaDB (Pizarro et al., 2013a) to identify DUBs that are rhythmically expressed in the SCN. The JTK_cycle analysis (Hughes et al., 2010) was performed to find significant rhythmic genes using a cut-off q value of 0.05. The analysis was performed on two SCN microarray datasets (Panda et al., 2002; Zhang et al., 2014).

Expression of DUBs in the SCN

To analyze the expression of DUBs in the SCN, we used three different mouse SCN RNA sequencing datasets and one mouse SCN microarray dataset (data accessible at NCBI GEO database (Edgar et al., 2002), accessions GSE72095, GSE70391, GSE70384 and at ArrayExpress repository (Athar et al., 2019), accession MTAB7496). Since the sampling was achieved at different times of day, we averaged the levels of expression of each gene across all time points. In case different genotypes were used in the study, we only analyzed the results for the WT mice. For each dataset, we ranked DUBs based on their expression levels. We then averaged the ranks of each DUB, resulting in a global ranking of the expression of DUBs in the SCN. We used rankings instead of the absolute levels of gene expression due to the variability between each dataset. We also only analyzed DUBs that were identified in all three datasets.

Protein alignments of DUBs with USP2

To assess the similarities of DUBs with USP2, we aligned the amino acid sequences of all DUBs with USP2 using the NCBI BLASTP tool (Boratyn et al., 2013). We used USP2-69 isoform as a comparison, but similar results were also obtained with the USP2-45 isoform. We ranked DUBs based on their expectation value (E value), which represents a more significant

alignment when closer to 0. This value takes into account the length of the sequences and represents the expectation of finding an alignment by random chance. The percentage of identities of amino acids with USP2 was also analyzed.

Generation of mouse embryonic fibroblasts (MEFs)

USP2+/-; PER2::Luc males with USP2-/-;PER2::Luc females were bred to generate *Usp2* KO embryos and USP2+/+;PER2::Luc males and females were bred to generate WT embryos. *Usp2* heterozygous males were used since *Usp2* KO males are subfertile (Bedard et al., 2011). Following each breeding night, females were monitored for the presence of a vaginal plug, indicating embryonic day 0.5. At embryonic day 13.5, pregnant dams were euthanized, and embryos were harvested. MEFs were generated as described (Tan and Lei, 2019). Briefly, embryos were individually dissected, and the head, heart and liver tissues were removed and kept on ice for later genotyping. The remaining tissue was cut in small pieces using a clean razor blade. Trypin-EDTA solution (0.25%; Wisent) was added to each dish and incubated 10 minutes at 37°C. The digested tissue was then filtered using a 40 µm cell strainer and the resulting solution was pelleted by centrifugation. Fresh Dulbecco's Modified Eagle Medium (high glucose DMEM, Gibco) supplemented with 10% NuSerum IV (Corning) was used to resuspend the cells. The suspension was pelleted again by centrifugation. The cells were resuspended in supplemented DMEM, transferred in a 75 cm^2 flask and incubated at 37°C and 5% CO_2 until confluent. These cells were considered at passage 0.

The leftover tissues were used to genotype each cell line. The DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen). Polymerase chain reaction (PCR) was used to identify cell lines that were either WT or *Usp2* KO and heterozygous for PER2::Luc.

Generation of U2OS reporter cell lines

Human U-2 osteosarcoma epithelial cells (U2OS) were a kind gift of Dr. El Bachir Affar. They were chosen due to their suitability for transfection, but also as they were shown in many studies to exhibit robust and sustained circadian rhythms and were frequently used in screens for clock modulators (Maier et al., 2009; Tamai et al., 2018; Zhang et al., 2009).

U2OS cells were reverse transfected in 6-well plates with a PGL-4.16 vector (Promega, Madison, WI, USA) containing the luciferase gene (*luc2*) under the control of either the *Per2* promoter (*Per2*-Luc) or the *Bmal1* promoter (*Bmal1*-Luc) using the Lipofectamine 2000 transfection reagent (Invitrogen). One day later, cells were passaged and split in new 6-well plates. Since the vector also expresses an *E. coli* hygromycin resistance gene (*hyg*), successfully transfected cells were selected by a treatment with Hygromycin B (1 mg/ml; Gibco) until the death of all control cells. Surviving colonies were passaged and single clones were isolated by serial dilutions, expanded and frozen for future use. A lower concentration of Hygromycin B (0.3 mg/ml) was used for the subsequent culture of these cells. Bioluminescence recordings of either *Per2*-Luc or *Bmal1*-Luc rhythms were analyzed to choose the best rhythmic clones for future assays.

Cell culture

MEFs and U2OS cells were cultured in high glucose DMEM (Gibco) supplemented with 10% NuSerum (IV or I; Corning), 2 mM L-glutamine (Wisent) and 1 mM sodium pyruvate (Wisent) and incubated at 37°C and 5% CO_2. Cells were passaged before they reached confluence. MEFs were not cultured beyond the tenth passage.

MEFs and U2OS dsiRNAs transfection

Duplexed small interfering RNA (dsiRNA) transient knockdowns were achieved using three different dsiRNAs against each candidate DUB (IDT) in order to remove possible confounding effects due to non-specific interference of dsiRNAs. Furthermore, knocked down samples were compared to cells treated with a non-specific dsiRNA (**Table 1**) used at the same concentration (IDT) in order to remove any confounding effects of RNA interference activation.

Table 1. List of dsiRNAs used in the knockdowns of candidate DUBs. Name of all dsiRNAs used for individual knockdowns of DUBs (IDT). A single nontargeting dsiRNA was used as a negative control. The siRNA #3 against human *Usp2* was also used to knockdown *Usp2* in the DUB screen.

Target	siRNA #1	siRNA #2	siRNA #3
Mouse *Usp8*	mm.Ri.Usp8.13.1	mm.Ri.Usp8.13.2	mm.Ri.Usp8.13.3
Mouse *Usp21*	mm.Ri.Usp21.13.1	mm.Ri.Usp21.13.2	mm.Ri.Usp21.13.3
Mouse *Usp22*	mm.Ri.Usp22.13.1	mm.Ri.Usp22.13.2	mm.Ri.Usp22.13.3
Mouse *Uchl1*	mm.Ri.Uchl1.13.1	mm.Ri.Uchl1.13.2	mm.Ri.Uchl1.13.3
Human *Usp8*	hs.Ri.USP8.13.1	hs.Ri.USP8.13.2	hs.Ri.USP8.13.3
Human *Usp2*	hs.Ri.USP2.13.1	hs.Ri.USP2.13.2	hs.Ri.USP2.13.3
Non-specific control	Universal Negative Control (DS NC1)		

PER2::Luc MEFs (both WT and *Usp2* KO cell lines) or U2OS were passaged and resuspended at either $0.1x10^6$ cells per milliliter or $0.3x10^6$ cells per milliliter respectively before being reverse transfected in 24-well plates using the Lipofectamine 2000 transfection reagent (Invitrogen). Individual dsiRNAs (**Table 1**) were used at a final concentration of 10 nM (or 20 nM for *Usp22*) and diluted in opti-MEM media (Gibco) prior to the transfection. In the case of U2OS knockdown experiments, a pool of three *Usp2* dsiRNAs (**Table 1**) was used at a total concentration of 10 nM and compared to cells treated with 10 nM of the non-specific control.

Medium was changed to supplemented DMEM 24 hours post-transfection. Cells were assayed for bioluminescence or mRNA knockdown 48 hours post-transfection, and for cell viability or degradation assays 72 hours post-transfection.

Quantitative Real-Time PCR

RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer instructions. cDNA was generated from RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was analyzed via quantitative real-time PCR (qRT-PCR) using a SYBR Green qPCR Master Mix (Biorad). Following the manufacturer protocol, the reactions were first incubated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min. Refer to **Table 2** for gene specific primer sequences. Results were analyzed with the

Table 2. qPCR primers used for knockdown efficacy and for clock genes expression. All qPCR primers were validated for efficiency using standard curves and for specificity using a dissociation curve analysis.

mRNA Target	Primer sequences (5'→3')
Mouse *β-Actin*	GAACCCTAAGGCCAACCGTG
	GGTACGACCAGAGGCATACAGG
Mouse *Usp8*	CAGCACCAAGAATACCAAGTTTTG
	AGATCTTCTGCGCGCTGTG
Mouse *Usp21*	CTACAATGACTCCCGCGTTTCC
	TTGGTAGAACAGCACGTAGCCC
Mouse *Usp22*	GGCTTGGAAGATGCAAGGTGTTG
	AGCAGTTCCAGCTCCCGTTTAG
Mouse *Uchl1*	GCTCCTCGGGTTTGTGTCTGC
	CAACACTTTGTTCAGCATCTCGG
Human *β-Actin*	GAGAATTCATGATATCGCCGCGCTCGTC
	TGCTGGAAGTTGGATGTGGCT
Human *Usp8*	TCCTACGATGGCAGGTGGAA
	CAGCCCACCGTAGTGATTTGAA
Human *Usp2*	AAGCCAACATGCTGTCGCTG
	AACCGCTTCAGATGGAGCAC
Human *Cry1*	TGCTGGAAGTTGGATGTGGCT
	TAGGACAGGCAAATAACGCCTGA
Human *Cry2*	AACTGAACTCCCGCCTGTTT
	GGGTCACTCCCCATTCCTTG
Human *Per2*	CAGGAGAAGCTGAAGCTGC
	GGACTGTCTTCCTCATATGG
Human *Bmal1*	TGGAAGAAGTTGACTGCCTGGAAGG
	GGCCCAAATTCCCACATCTGAAGTTAC
Human *Rev-erbα*	TGCCATGTTTGACTTCAGCG
	GTTCTTCAGCACCAGAGCC

$2^{-\Delta\Delta Ct}$ method using *β-actin* as the endogenous control and the non-specific dsiRNA treatment as the calibrator.

Bioluminescence recording and analysis

Forty-eight hours post-transfection, cells were synchronized by the addition of dexamethasone (Sigma) at a final concentration of 100 nM. After two hours, media was changed to DMEM without phenol red supplemented with 10% NuSerum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES (Wisent), and 0.1 mM D-Luciferin (Cedarlane). Plates were sealed and incubated in a LumiCycle (Actimetrics) at 37°C where bioluminescence was recorded for 4 or 5 days.

Bioluminescence traces were analyzed using the LumiCycle Analysis software (Actimetrics, version 3). The first peak was excluded from the analysis. Raw rhythms were detrended using a 24-hour running average. The parameters of the oscillations were estimated by fitting a sine wave function to the detrended rhythms using the equation:

$$L = A(\sin(2\pi f + \varphi)(\exp(-t/d))$$

Where L is the luminescence, A is the amplitude, f is the frequency of the sine wave, φ is the phase, t is the time and d is the damping rate.

For MEFs recordings, five distinct WT and *Usp2* KO cell lines were used as biological replicates. For U2OS recordings, a total of nine replicates were achieved through three independent experiments.

Cell viability assay

Both WT and *Usp2* KO MEFs were transfected with dsiRNAs targeting *Usp8* following the same protocol as above. Seventy-two hours post-transfection, the cells and their respective

supernatant were collected and pelleted down by centrifugation. The pellets were washed once before being resuspended in cell sorting buffer. Before reading each sample, propidium iodide (PI) solution (Biolegend) was added at a final concentration of 10 µg/ml. Samples were read on a C6 flow cytometer (Accuri) and analyzed using FlowJo (version 10). The following gating strategy (**Figure 3a-f**) was used to identify PI positive cells, which correspond to dead cells.

1. FSC-A/SSC-A

   Identification of cells based on size and granularity respectively (**Figure 3a,d**)

2. FSC-A/FSC-H

   Identification of single cells based on area and height of the size parameter (**Figure 3b,e**)

3. PI/SSC-A

   Identification of PI positive cells (**Figure 3c,f**)

The percentage of PI positive cells was drawn from the number of single cell (**Figure 3b,e**). Three distinct WT and *Usp2* KO cell lines were used as biological replicates and three technical replicates were averaged for each biological replicate.

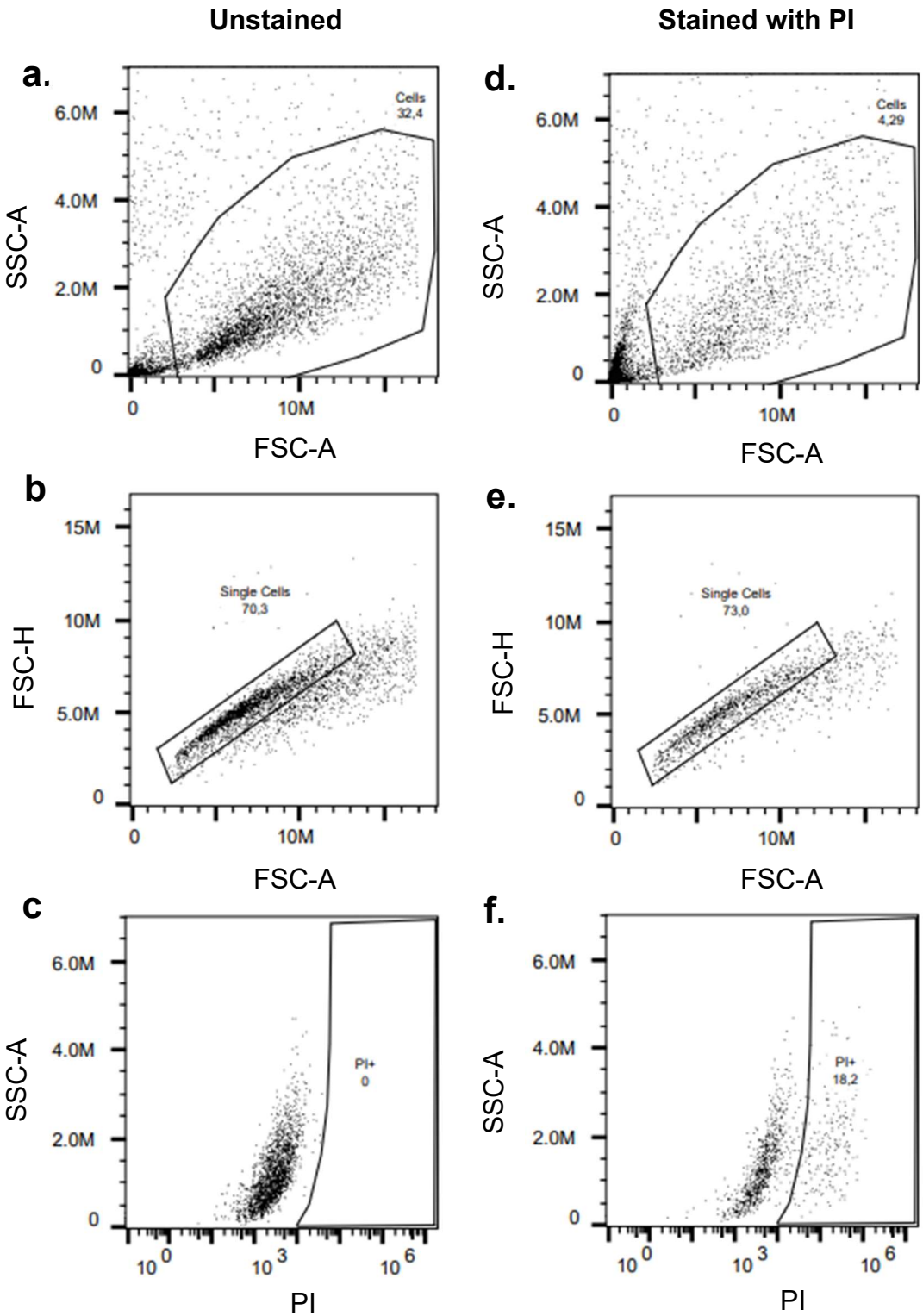


Figure 3. Flow cytometry gating strategy. Three gates used to identify PI positive cells, which represent dead cells. **a. b. c.** Unstained control sample. **d. e. f.** PI positive control where a proportion of cells have been damaged. **a. b.** Exclusion of cellular debris based on size and granularity. **b. e.** Selection of single cells bases on size ratios. **e. f.** Isolation of PI positive cells based on fluorescence intensity.

PER2::Luc degradation assay

Both WT and *Usp2* KO MEFs were transfected with dsiRNAs targeting *Usp8* following the same protocol as above. Seventy-two hours post-transfection, cells were treated with cycloheximide (CHX; Millipore Sigma) at a final concentration of 50 μg/ml. Using the Firefly Luciferase Assay Kit 2.0 (Biotum), cells were lysed 0, 2, 4 and 6 hours after CHX treatment following the manufacturer protocol. PER2::Luc bioluminescence for each sample was measured using the Orion II (Berthold) plate reader. Three distinct WT and *Usp2* KO cell lines were used as biological replicates and were averaged over two technical replicates each. Results were normalized with a DMSO sample for all conditions. Every sample was further normalized against their corresponding luminescence at time 0. The half-life for each sample was measured by a one phase decay analysis performed with Graph-Pad PRISM (version 9.1, GraphPad Software, Inc.).

Clock genes expression following Usp8 knockdown

Both WT and *Usp2* KO MEFs were transfected with dsiRNAs targeting *Usp8* following the same protocol as above. Forty-Eight hours post-transfection, cells were synchronized for 2 hours with 100 nM dexamethasone. Zero, 18, 24, 30 and 36 hours after the synchronization, cells were lysed in Trizol reagent and RNA was isolated according to the manufacturer instructions. cDNA was generated from RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression of *Per2*, *Bmal1* and *Rev-erbα* was analyzed by qRT-PCR using a SYBR Green qPCR Master Mix (Biorad). Following the manufacturer protocol, reactions were first incubated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1min. Refer to **Table 2** for gene specific primer sequences.

Results were analyzed with the $2^{-\Delta\Delta Ct}$ method using *β-actin* as the endogenous control and a time 0 non-specific dsiRNA treatment as the calibrator. Three distinct WT and three distinct *Usp2* KO cell lines were used as biological replicates. For the RT-qPCR, three technical replicates were averaged for each sample.

For the single time point analysis of *Per2* expression, we normalized the samples to the treatment with the non-specific dsiRNA. Four distinct WT and four distinct *Usp2* KO cell lines were used as biological replicates. Three technical replicates were averaged for each sample in the RT-qPCR assay.

siRNA library transfection

A small interfering RNAs (siRNAs) library against 111 DUBs (Sigma) was a kind gift from Dr. El Bachir Affar. This library is composed of two pooled siRNAs against each of the 111 human DUB, as well as two distinct pools of non-specific siRNAs used as negative controls (**Table 3**). *Usp2* knockdown was achieved by using a single dsiRNA (IDT) at a final concentration of 10 nM and was compared to cells treated with the non-specific dsiRNA (IDT) at the same final concentration (**Table 1**). *Cry1* knockdown using a pool of two siRNAs (Sigma) was used as a positive control because it was shown to produce consistent phenotypes in the bioluminescence rhythms of U2OS cells (Maier et al., 2009; Zhang et al., 2009).

DUB siRNAs were transiently transfected in both *Usp2* knocked down and control *Bmal1*-Luc or *Per2*-Luc U2OS cells following a similar protocol as previously described on page 31. Briefly, each siRNA pool (Sigma) against DUBs were used at a final concentration of 50 nM. Cells were passaged and resuspended at $0.5x10^6$ cells per milliliter before being reverse transfected in white 96-well plates using the Lipofectamine 2000 transfection reagent

(Invitrogen). Medium was changed to supplemented DMEM the next day and bioluminescence was be recorded 48 hours post-transfection.

Table 3. List of siRNAs used in the DUB screen. Names and sequences of both siRNAs used against each human DUB (Sigma). Two pools of two non-specific siRNAs were used as negative controls. A pool of two siRNAs against *Cry1* was used as a positive control.

Target	Name	siRNA #1	Name	siRNA #2
Control #1	Human Non-Target 1	UGGUUUACAUGUCGACUAA	Human Non-Target 2	UGGUUUACAUGUUGUGUGA
Control #2	Human Non-Target 3	UGGUUUACAUGUUUUCUGA	Human Non-Target 4	UGGUUUACAUGUUUUCCUA
Cry1	SASI_Hs01_00192668	CCUUUAUGGGCAACUGUUA	SASI_Hs01_00192669	CCGAUUUGGUUGUUUGUCA
Fam63A	SASI_Hs01_00094199	CAGACUUGGUAAUGUCCUU	SASI_Hs01_00094200	GGGUGAACUUAGCGUCUUU
Fam63B	SASI_Hs02_00308987	CCAUCAUCACCCAGAAUGA	SASI_Hs01_00060574	CUCAGAAUUUCAUCUUCGA
Fam188A	SASI_Hs01_00110290	CACAGAUCGCUCUCCUUCA	SASI_Hs01_00110291	CCUUGAUAGAUCCUGUAUA
Fam188B	SASI_Hs01_00139144	CUCAUCACCUCAUCACCGA	SASI_Hs01_00139145	CUCUACUUGCCUGGUGGUA
Bap1	SASI_Hs01_00105395	CGUGAUUGAUGAUGAUAUU	SASI_Hs01_00105396	CCAUCAACGUCUUGGCUGA
Uchl1	SASI_Hs01_00178415	GGACAAGAAGUUAGUCCUA	SASI_Hs01_00178416	GGCCAAUAAUCAAGACAAA
Uchl3	SASI_Hs01_00200423	CUGAUUCAUGCUAUUGCAA	SASI_Hs01_00200424	GCAUCUCUAUGAAUUAGAU
Uchl5	SASI_Hs01_00142742	CAGUUAUGUUCCUGUUAAU	SASI_Hs01_00142743	GAAGCAUAAUUAUCUGCCU
Usp1	SASI_Hs01_00204271	GUAUACUUCAGGUAUUAUA	SASI_Hs01_00204272	CCAUACAAACAUUGGUAAA
Usp2	SASI_Hs01_00149958	GACCUAAGUCCAACCCUGA	SASI_Hs02_00337104	CUAAGAGACCUGGACUUAA
Usp3	SASI_Hs01_00023593	CGGAUAAACUUUAAUACCU	SASI_Hs01_00023595	CAUUACACAGCAUACGCAA
Usp4	SASI_Hs01_00113975	CACUACACUGCAUAUGCGA	SASI_Hs01_00113976	GAGAAUCACAGGUUGAGGA
Usp5	SASI_Hs02_00319396	CUGUCAAGCUGGGCACCAU	SASI_Hs02_00319397	CUAAUGAAGUGUUCCGCUU
Usp6	SASI_Hs01_00167628	GUGUUGAUGCCAAUAACCA	SASI_Hs01_00167629	GAGAAUGGGAGACAUAUAA
Usp7	SASI_Hs01_00079539	GACGUUUCGAAUAGAGGAA	SASI_Hs01_00079540	GACUUUGAGAACAGGCGAA
Usp8	SASI_Hs02_00339089	CCUUUGACAAGAGCACGAA	SASI_Hs01_00136039	GAGAAUGGGACCACUGAAU
Usp9X	SASI_Hs01_00026227	GUCGUUACAGCUAGUAUUU	SASI_Hs02_00308595	CUGUGAUUCAGCAACUCUA
Usp9Y	SASI_Hs01_00081909	GUAGUGAUUUACACGAUGA	SASI_Hs02_00338066	CUUACUAAGAGCCACACUA
Usp10	SASI_Hs01_00213007	GUCAUUGAACCCAGUGACA	SASI_Hs01_00213008	GUUCUAAUGUGGAGGCGGA
Usp11	SASI_Hs01_00148685	CAGAGAUGAAGAAGCGUUA	SASI_Hs01_00148686	GAUUCUAUUGGCCUAGUAU
Usp12	SASI_Hs01_00167303	GAAACUCUGUGCAGUGAAU	SASI_Hs01_00167305	CAUCAGAUAUCUCAAAGAA
Usp13	SASI_Hs01_00108438	CUGAAUACUUGGUAGUGCA	SASI_Hs01_00108439	GAGCUAUUUGCAUUCAUCA
Usp14	SASI_Hs01_00089059	CAAUAAUUGUGGAUACUAU	SASI_Hs01_00089060	GAUAUUGGCUCCAAUAAUU
Usp15	SASI_Hs01_00059894	CUCUUGAGAAUGUGCCGAU	SASI_Hs01_00059895	CACAAUAGAUACAAUUGAA
Usp16	SASI_Hs01_00210886	CAUCUUUGGUGGUGAACUA	SASI_Hs02_00304059	GAGAAACUUCGAGAUGCGA
Usp17L9P	SASI_Hs02_00324631	GAAAUUCCUUCAAGAGCAA	SASI_Hs02_00324632	GGAAAUUCCUUCAAGAGCA
Usp17L2	SASI_Hs02_00372940	CUAUCAUUGCGGUCUUUGU	SASI_Hs02_00372941	CAACAAACUUGCCAAGAAU
Usp17L6P	SASI_Hs02_00494866	GCCUAUCAUUGUGGUGUUU	SASI_Hs02_00494867	CAGGCAACAAGAUUGCCAA
Usp17L1P	SASI_Hs02_00517496	CUAUCAUUGCGGUCUUUGU	SASI_Hs02_00517497	GCAACAAACUUGCCAAGAA

Usp18	SASI_Hs01_00221412	GCUUCAAUGACUCCAAUAU	SASI_Hs01_00221413	GUCAUUACUGUGUCUACAU
Usp19	SASI_Hs01_00130241	CACAAGAUGAGGAAUGACU	SASI_Hs01_00130242	GCAAGUUCUGCAUUGGUCA
Usp20	SASI_Hs01_00033438	GGACUUAUCACUGCCCAUU	SASI_Hs01_00033439	GGUUCUACGUGUCCCGCGA
Usp21(Iso-1)	SASI_Hs01_00193567	CCAACUUAGCCCGUUCCAA	SASI_Hs01_00193568	GACAAGAUGGCUCAUCACA
Usp21(Iso-2)	SASI_Hs01_00177787	CCAACUUAGCCCGUUCCAA	SASI_Hs01_00177788	GACAAGAUGGCUCAUCACA
Usp22	SASI_Hs02_00347438	CAAAGCAGCUCACUAUGAA	SASI_Hs02_00347439	CUGAUCAACCUUGGGAACA
Usp24	SASI_Hs02_00347524	GCACAAUACUGUGACCGUA	SASI_Hs02_00347525	GAAACUCAGGGUUGAUACU
Usp25	SASI_Hs02_00344762	CUAUGGUUCCGGUCCCAAA	SASI_Hs01_00191397	GAAAGAUUACCUCACGGUA
Usp26	SASI_Hs01_00043149	CUACAGAAGUCUAACAGGA	SASI_Hs01_00043150	CCAUCUUGGGAAGACUCUA
Usp27X	SASI_Hs02_00394607	GAUACUGAGAGAUUUCUUU	SASI_Hs02_00394608	CCUGUAUUACGGAGGUAUA
Usp28	SASI_Hs01_00077918	GACCUUACUCAUGAUAACA	SASI_Hs01_00077919	GACACUAUUGGGCCUAUAU
Usp29	SASI_Hs01_00032719	GAAAGAAGCUCUCAUUGAA	SASI_Hs01_00032720	GAAUAACGAGCAAGUUUAU
Usp30	SASI_Hs01_00155679	CACGAAUUAUUCCAUGUCA	SASI_Hs01_00155680	CUAGUCAACACAACCCUAA
Usp31	SASI_Hs01_00020641	GCAUUCAGGUGUGUCCAUU	SASI_Hs01_00020642	GAGUCAUCCCUUUCAAGUA
Usp32	SASI_Hs01_00086230	GAUAAUCAGCCAUUAGUAA	SASI_Hs01_00086231	GGAACUAUGUUAUACGGGA
Usp33	SASI_Hs01_00180288	CACAGAUCCUUCCAUCAAA	SASI_Hs01_00180289	GAAGUGUUAUUUCAGACAU
Usp34	SASI_Hs01_00089169	GGAUCUAGCAAUGAGGUUA	SASI_Hs02_00346129	GAUCUUAGGGCUGAAGUAA
Usp35	SASI_Hs02_00353962	GCAAGAUUGGUCUCAUCAA	SASI_Hs02_00353963	CUGUUAAGAAGUUCAGCAU
Usp36	SASI_Hs02_00357787	CUAAGACGGUGAAGCUGAA	SASI_Hs01_00194136	CGUAUAUGUCCCAGAAUAA
Usp37	SASI_Hs01_00018875	CAGCUAAGUCAUAACAUUA	SASI_Hs02_00354323	CUUGUCUAUUGACAAAGUA
Usp38	SASI_Hs01_00189218	GAGAGAUAGUCCCAGUGCA	SASI_Hs02_00360065	GUGAAACUUCUUUACAGGA
Usp39	SASI_Hs01_00034674	CAUAUGAUGGUACCACUUA	SASI_Hs01_00034675	CAAUGAUUAUGCCAACGCU
Usp40	SASI_Hs01_00145783	CACUGAAAGAACUUCUGAU	SASI_Hs02_00351527	CACAUGUCUUUCCAGCUAA
Usp41	USP41-36	GUACGUGCAUCCUUGUGUA	USP41-13	UUGUUCAGGGCUCAUCAGU
Usp42	SASI_Hs01_00078970	CAGUCUACCUCGAACGCAU	SASI_Hs01_00078971	GUUAAUAGGUCCUCAGUGA
Usp43	SASI_Hs02_00367061	GUGAUCUUGGUUGAACUGU	SASI_Hs02_00367062	GUGAAAGGCAGAAGCAUUA
Usp44	SASI_Hs02_00309548	GAAGGAUACUAAUGGGUAA	SASI_Hs02_00309549	CAACAAAUCAAAUACCAUA
Usp45	SASI_Hs02_00315256	CCAGUUUACAUCUAUGGAA	SASI_Hs02_00315257	CACUACACUGCUUAUGUGA
Usp46	SASI_Hs01_00080807	GUCUCAAUGGUCUGGCUGU	SASI_Hs01_00080808	GGUAUCACUCCGAGUCUCA
Usp47	SASI_Hs01_00112225	GCAUAUAUGCUGAUCUAUA	SASI_Hs01_00112227	CAUGCAAGUUUCUGCUAGA
Usp48	SASI_Hs01_00185218	CUUUAUGUCUCUAUUGGAA	SASI_Hs01_00185219	CUACUUAUGUCCAAGCACU
Usp49	SASI_Hs01_00055585	CUGAAACACUUUGAGGAGA	SASI_Hs02_00352112	CUCAGAAGUGGUGCUGCUU
Usp50	SASI_Hs02_00373416	CACUACACUGCUUUCUGCA	SASI_Hs02_00373417	GGAAAUAUCCUAAAUACAA
Usp51	SASI_Hs02_00372924	CAUAGUGUUUCUACCACCA	SASI_Hs02_00372925	CAAAGCUACCAGGAGUCUA
Usp52	SASI_Hs02_00329594	CUGUCUACCUGUUCCAUAU	SASI_Hs02_00329595	CAGUUUGACAUGAAUUGGA
Usp53	SASI_Hs01_00234222	GAACCAAGUUUAGAAGUGA	SASI_Hs01_00234224	CAGCUAAGUUAAGUCACAU
Usp54	SASI_Hs02_00366408	GACUUAGCAGAAGAUGUUA	SASI_Hs02_00366409	CCAAGUAUUGUUAAGCCAA
Usp55	SASI_Hs02_00340306	CCAUAUUCAUGUUGCACUU	SASI_Hs01_00128479	GUAUCGAGCAAAUAAUCAU
Cyld	SASI_Hs02_00309208	GAACGAUGUAGAAUAUUAU	SASI_Hs02_00309209	GAACAGAUUCCACUCUUUA
Dub3	SASI_Hs02_00372940	CUAUCAUUGCGGUCUUUGU	SASI_Hs02_00372941	CAACAAACUUGCCAAGAAU
Atxn3	SASI_Hs01_00043300	GAAGAAUUAGCACAACUAA	SASI_Hs01_00043301	CAAAUGAUGGCUCAGGAAU

Atxn3L	SASI_Hs02_00379672	CAUUAUCAUCUAUGGGAUA	SASI_Hs02_00379673	CAAUUAACAUGGAUUUCAU
Josd1	SASI_Hs02_00346483	CGGGAUACGCUGCAAGAGA	SASI_Hs01_00072064	CACUUCAGACCAAAGGCUA
Josd2	SASI_Hs01_00128488	GGUGGACGGUGUCUACUAC	SASI_Hs01_00128489	GCAACUAUGAUGUCAAUGU
Josd3	SASI_Hs01_00119698	GACAUCUGAUGCUGUGGAA	SASI_Hs01_00119700	GAUAAAUCAGGAAUAGAUU
Tnfaip2	SASI_Hs01_00033556	GGAAGAAAUACACAUAUUU	SASI_Hs01_00033557	GGAUGUUACCAGGACAUUU
Otud7B	SASI_Hs01_00089029	CAACUAAUUGGCUCAUCAA	SASI_Hs01_00089030	CUGAUCAAGCUUGCCUCAA
Otud7A	SASI_Hs01_00055782	GUCCUAGCCCAUAUAUUAA	SASI_Hs01_00055783	GACUUGAUCGAGCAGGCAA
Otud4	SASI_Hs02_00323440	UAAUUUAUCGGGAACCAAA	SASI_Hs02_00323441	GUUUCUCCUUCACAAGUAA
Parp11	SASI_Hs01_00173656	CACAAUCAAACACAUGAAU	SASI_Hs02_00353319	GUGUUCAGUUAGCAGUGAA
Otud6A	SASI_Hs01_00018704	CAUUGAAUCUGUCGUCGAA	SASI_Hs01_00018705	GAGUUCCUGCCCUUCUUCA
Yod1	SASI_Hs01_00052848	GAAAUAUGUGUAGUGGAUA	SASI_Hs01_00052849	GUAACUUCCCUGAUCCAGA
Otud6B	SASI_Hs01_00222466	GAAGUCAGACCGCUGAGUA	SASI_Hs02_00348712	CUACUAAGGAGAAUAAGAU
Otud5	SASI_Hs01_00052828	CACUAUAAUUCAGUGGUGA	SASI_Hs01_00052829	GGACUUUACCACCUACAUU
Otub1	SASI_Hs01_00080525	GCAAGUUCUUCGAGCACUU	SASI_Hs02_00350395	CCUGGAAGUUCCUUAGGGA
Otub2	SASI_Hs01_00010701	CAGAGUGCCUCGGACCACA	SASI_Hs01_00010702	GACAUCAAAGACUUCUGCA
Otud1	SASI_Hs01_00270248	CUGAUUUCUCACAGUGUAA	SASI_Hs01_00270249	GAUGUUUCAUGAUAGCUUU
Otud3	SASI_Hs02_00347304	GAAAUCAGGGCUUAAAUGA	SASI_Hs02_00347305	GUAGUGAUUCAUCAACUUA
Zranb1	SASI_Hs01_00155779	GAACUUGAAGUAGACUUUA	SASI_Hs02_00350242	GAAUCGUCCUUCUGCCUUU
Vcpip1	SASI_Hs02_00357704	GUAACUGCCUUUCAGGGAA	SASI_Hs01_00165636	GAUGUAUGGUCUUAUGCAA
Stambp	SASI_Hs01_00182263	CCAAAGAAUAUACAGAAUA	SASI_Hs01_00182264	CAAAGUGCUGGGUCUGAUU
Stambpl1	SASI_Hs01_00241436	GAGUUAGCCCGAGGUCAAA	SASI_Hs01_00241437	CAAUUCCUUGCUGAAUGUA
Brcc36	SASI_Hs01_00177040	GUCAGAAUUGUUCACAUUC	SASI_Hs01_00177042	CAUCUUACGACGUUCUGAU
Cops5	SASI_Hs02_00342404	CCAUUUGUAGCAGUGGUGA	SASI_Hs01_00209042	GUGAAUCUUGGCGCCUUUA
Cops6	SASI_Hs02_00342398	GAUCUUCCUGUCAGCGUUU	SASI_Hs01_00117938	CACACUGGUUGGUCAACUU
Eif3H	SASI_Hs01_00072290	CAAGGAUCUCUCUCACUAA	SASI_Hs01_00072291	CAAAUAUCACCUUUGAGUA
Eif3F	SASI_Hs01_00246686	GAGGAUGUACUGUCUGGAA	SASI_Hs02_00336223	CCGAUGACUUUGAGACCAU
Ifp38	SASI_Hs01_00017774	CAUAGUGUUGCAAUAUGCA	SASI_Hs01_00017775	CAUAUGAGCAUCAAAGCCU
Psmd14	SASI_Hs02_00340316	GGUCUUAGGACAUGAACCA	SASI_Hs01_00024446	GUGAUUGAUGUGUUUGCUA
Prpf8	SASI_Hs01_00015918	CACGUAUCAAGAUUGGACU	SASI_Hs01_00015919	GGAUUAUGAUGCGCCGAGA
Psmd7	SASI_Hs02_00334491	CGUGUUGUUGGUGUGCUUU	SASI_Hs02_00334492	CACUUGUUACGAGAUAUCA
Cezanne	SASI_Hs01_00089029	CAACUAAUUGGCUCAUCAA	SASI_Hs01_00089030	CUGAUCAAGCUUGCCUCAA
Cezanne2	SASI_Hs01_00055782	GUCCUAGCCCAUAUAUUAA	SASI_Hs01_00055783	GACUUGAUCGAGCAGGCAA
Mpnd	SASI_Hs01_00162447	CCGAGAUGCUGCUGGUGGA	SASI_Hs01_00162448	GGAGUGAGGUCGUGGGUUA
Mysm1	SASI_Hs02_00317976	CUUACGAGAUAUUGACACA	SASI_Hs02_00317977	GACAAGAUGGAUAAUAGAA
Senp1	SASI_Hs01_00206194	GAAACAGCCGAAGUCUUUA	SASI_Hs01_00206195	GACAUUUGGACCGAUCUUU
Senp2	SASI_Hs01_00048880	GGAAUAAGUGACUAUCCAA	SASI_Hs02_00354976	GCUGUAACAGAGAUGAUUU
Senp3	SASI_Hs01_00092168	GUGCAUUGGUCCCUCAUCU	SASI_Hs01_00092171	CUGAUGCCAGCAUCCUCAU
Senp5	SASI_Hs02_00366591	CUGGUAACCAAAGGAUAUA	SASI_Hs01_00030279	GUGCUAAGACCAAGUUCAA
Senp6	SASI_Hs01_00034500	CAUACAGAUGGCUUAAGCA	SASI_Hs01_00034502	CCAUUAUUAAGAACGUCAA
Senp7	SASI_Hs02_00312204	CAAUAGCAGUGAUUGUGGA	SASI_Hs02_00312205	CAGUAUUAUGCCUAGUAAU
Senp8	SASI_Hs01_00195113	CAGUUCAUCAAGUGCACUA	SASI_Hs01_00195114	CCAACUUAUUUGAACAUUU

Bioluminescence recordings and analysis

Forty-eight hours post-transfection, cells were synchronized by the addition of dexamethasone at a final concentration of 100 nM. After two hours, media was changed to DMEM without phenol red supplemented with 10% NuSerum I, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 0.1 mM D-Luciferin. Plates were sealed and incubated in a Synergy 4 (Biotek) plate reader maintained at 37°C where bioluminescence was recorded every 20 minutes for 5 days.

Bioluminescence traces were analyzed using the Chronostar analysis software (Maier et al., 2021). The first day was excluded from the analysis. Raw rhythms were detrended using a 24-hour running average (**Figure 4a**). The period and amplitude of the rhythms were estimated by fitting a sine wave function to the raw data (**Figure 4b**), as described above.

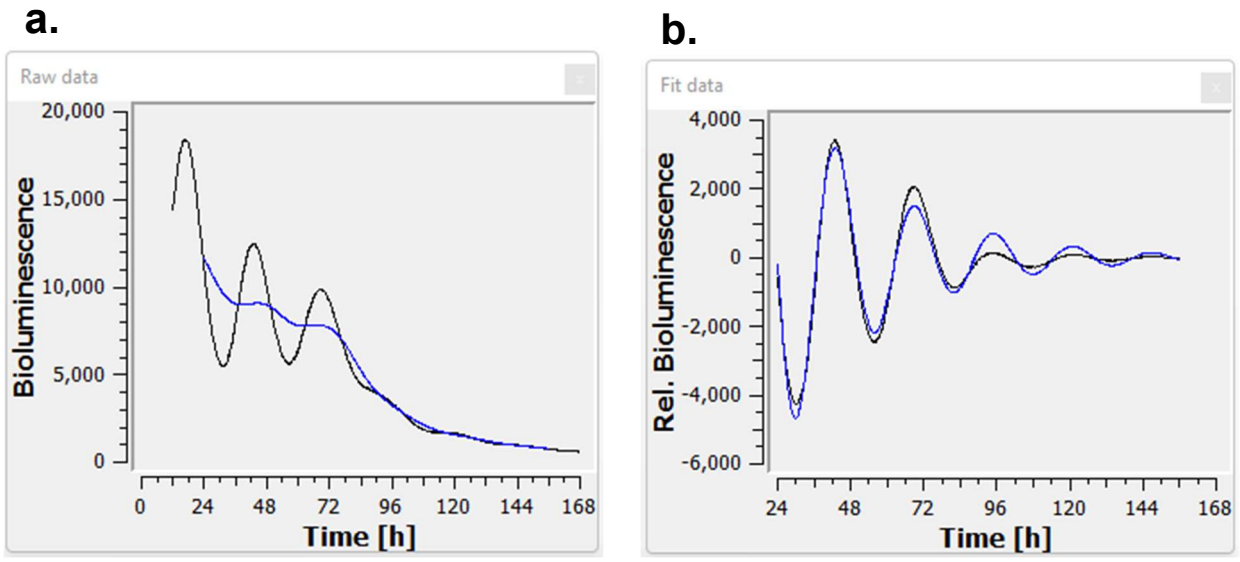


Figure 4. Analysis of bioluminescence rhythms using the Chronostar software (Maier et al., 2021). a. The first day of recording was excluded from the analysis. To detrend the raw rhythms (black line), a 24-hours running average was applied (blue line). **b.** The period and amplitude of the rhythms were estimated using a sin wave function fitted to the data (black line: detrended rhythm, blue line: sin wave fit).

Since only 96 samples could be run at the same time, the DUB siRNA screen was achieved in five different experiments, half of which used *Bmal1*-Luc U2OS cells, and the other half, *Per2*-Luc cells. Therefore, both positive and negative controls were included in each of the experiment and used for the analysis. For each plate, we normalized the rhythm parameters of each DUB knockdown using the mean period and amplitude of the two non-specific siRNA controls. A cut-off of ±1 hour was used to identify period hits. A cut-off value of ± 75% relative luminescence unit (RLU) was used to identify amplitude hits. For a DUB to be identified, these cut-off values needed to be met for both *Per2*-Luc and *Bmal1*-Luc rhythms. Furthermore, we used the same period and amplitude cut-off values to identify possible USP2-redundant DUBs amongst previously identified hits. We used ± 1 hour and ± 75% relative luminescence unit (RLU) between DUB knockdown in the control background versus in the *Usp2* knockdown background to identify these DUBs. Again, for a DUB to be identified, this cut-off value needed to be met for both *Per2*-Luc and *Bmal1*-Luc rhythms.

Statistical analysis

For each experiment, error bars represent the SEM. A two-way analysis of variance (ANOVA) was performed on most data using Graph-Pad PRISM (version 9.1, GraphPad Software, Inc.). JASP (version 0.14.1) was used to perform three-way ANOVAs for the clock genes expression following *Usp8* knockdown. If a significant interaction between factors was found significant, simple effects were analyzed for significance. Otherwise, main effects of both factors were analyzed. In both cases, a Dunnett's tests for multiple comparisons were conducted using the non-specific dsiRNA treatment as the control, with the exception of the analysis of the screen controls, where a Tukey's test was conducted to compare all treatments together. A p value < 0.05 was considered significant.

RESULTS

Database analyses to identify candidate DUBs

Using a targeted approach, we wanted to identify candidate DUBs to test their role in the clock. We searched in published and available RNA sequencing and microarray datasets for rhythmic expression of DUBs in the central pacemaker in the brain, the SCN, as well as their levels of expression in this brain region. We also compared the protein sequences of DUBs with that of USP2 to identify the DUBs most similar to USP2. Taken together, these analyses allowed

Table 4. DUBs found to be expressed rhythmically in the SCN. Two SCN microarray datasets (Panda et al., 2002; Zhang et al., 2014) were analyzed with CircaDB (http://circadb.hogeneschlab.org/). Using the JTK_cycle rhythmic analysis (Hughes et al., 2010), only genes with a q value less than 0.05 were selected as being expressed rhythmically. DUBs were ordered based on this q value. In both datasets, sampling was achieved when mice were kept in constant darkness.

Dataset	Affymetrix Mouse SCN 1.0ST	Affymetrics Mouse SCN (MAS4 preprocessing)
SCN sampling interval	Every 2 hours over 48 hours	Every 4 hours over 48 hours
Total mice per time point	3	10
Gene Symbol	q values	
Usp38	1.000	0.001
Otub1	0.263	0.002
Otud5	0.597	0.002
Usp36	0.003	N/A
Usp9x	0.381	0.011
Usp22	0.016	1.000
Otud4	1.000	0.019
Usp2	1.000	0.023
Usp29	0.028	1.000
Uchl1	0.097	0.039
Uchl5	1.000	0.039
Otud6b	1.000	0.046

us to identify potential clock-related DUBs as well as DUBs structurally close to USP2.

1. Rhythmic expression of DUBs in the SCN

Following a CircaDB analysis, we found a total of 12 DUBs that were expressed with a significantly rhythmic pattern in the SCN (**Table 4**). As expected, *Usp2* was among these genes (**Table 4**). It is also to note that both datasets did not provide similar results and identified different DUBs as being rhythmic. This discrepancy could be due to different sampling intervals as well to different numbers of biological replicates in the two experiments.

2. Expression of DUBs in the SCN

Following our analysis of three mouse SCN RNA sequencing datasets and one mouse SCN microarray dataset, we compiled the 20 most expressed DUBs in the SCN (**Table 5**). Interestingly, many of the DUBs most studied with regards to circadian rhythms are found in this table (*Uchl1*, *Usp9x*, *Usp7* and *Usp8*). We also observed that some of these DUBs were also found to be rhythmically expressed in the SCN, notably *Otub1*, *Otud5*, *Usp9x*, *Usp22*, *Uchl1* and *Otud6b* (**Table 4 and 5**).

Table 5. Most expressed DUBs in the SCN. Three SCN RNA sequencing datasets and one SCN microarray dataset were analyzed for the levels of expression of mouse DUBs in the SCN. DUBs were ranked in each datasets from the most expressed to the least. The four ranks of each DUBs were averaged to give the final mean rank. A small mean rank is associated with a greater SCN expression.

Gene Symbol	Mean rank
Uchl1	6.8
Usp11	7.5
Cyld	13.3
Usp47	13.5
Usp34	16.0
Cltc	18.3
Usp7	20.5
Usp32	20.8
Usp24	21.8
Usp20	22.3
Usp22	22.5
Cops5	23.5
Usp14	23.5
Usp9x	23.5
Usp31	26.3
Otub1	27.0
Otud5	27.0
Usp10	28.0
Usp8	28.3
Otud6b	28.8

3. Similarities of DUB amino acid sequences with USP2

As expected, all DUBs identified as similar to USP2 were USPs, since they all share a common structure (**Table 6**) (Clague et al., 2019). USP21 was the most closely related to USP2 (**Table 6**), which is concordant with previous phylogenetic results (Clague et al., 2013). USP8 was also found to be highly similar to USP2 (**Table 6**).

Table 6. DUBs most similar to USP2. All DUB protein sequences were aligned to USP2-69 using the BLASTTP tool from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). DUBs were ranked based on the expectation value (E value) representing the significance of the match. Percentage identities between sequences are also shown.

Gene Symbol	Expectation Value (E value)	% Identities
Usp21	1.99×10^{-111}	49.4%
Usp8	1.16×10^{-74}	42.1%
Usp50	7.88×10^{-64}	37.4%
Usp3	2.61×10^{-51}	36.8%
Usp27	7.21×10^{-48}	30.3%
Usp22	2.26×10^{-46}	30.3%
Usp15	1.46×10^{-43}	44.2%
Usp36	9.24×10^{-43}	45.4%
Usp32	1.91×10^{-41}	31.3%
Usp19	3.19×10^{-41}	29.5%
Usp11	6.7×10^{-40}	39.9%
Usp42	1.1×10^{-39}	42.5%
Usp17lC	1.19×10^{-38}	42.6%
Usp33	9.46×10^{-38}	32.1%
Usp17lA	1.81×10^{-31}	40.0%
Usp51	4.7×10^{-31}	42.8%
Usp4	1.09×10^{-29}	43.9%
Usp17lD	2.83×10^{-25}	39.9%
Usp17lE	1.05×10^{-10}	43.8%
Usp17lB	1.86×10^{-7}	35.4%

Knockdown of candidate DUBs in PER2::Luc MEFs

Based on its similarity with USP2 (**Table 4**), its high level of expression in the SCN (**Table 5**) and its role in the *Drosophila* clock (Luo et al., 2012), USP8 was our first choice of candidate for our specific approach. Since USP21 was the DUB most similar to USP2 (**Table 6**) (Clague et al., 2013), we chose it as our second candidate. USP22 was also chosen since it was found to be part of the top DUBs in all three of our analyses (**Tables 4,5 and 6**) and due to the role of its *Drosophila* homolog, NOT, in clock regulation (Bu et al., 2020; Mahesh et al., 2020). Finally, since *Uchl1* is highly expressed in the SCN (**Table 5**), have a rhythmic expression in this tissue (**Table 4**) and seems necessary for circadian behaviors in mice (Pfeffer et al., 2012), we included it as our last DUB candidate.

To test these four candidates, we decided to use *Usp2* KO mice that were already available to us in our lab in order to generate MEFs expressing a PER2::Luc fusion protein as a circadian bioluminescent reporter.

1. Usp8

In the case of *Usp8* knockdown, where all three dsiRNAs led to a strong decrease in *Usp8* mRNA levels (**Figure 5a**), we did not detect any significant differences in PER2::Luc period following a two-way ANOVA (Interaction: $F(3,32)=0.1899$; $p=0.9025$, *Usp2* KO: $F(1,32)=0.1616$; $p=0.6903$, si*Usp8*: $F(3,32)=0.4385$; $p=0.7270$) (**Figure 5b,c,d**). No significant effects were observed for the damping rate of the rhythms (Interaction: $F(3,32)=1.080$, $p=0.3716$, *Usp2* KO: $F(1,32)=0.1593$, $p=0.2160$, si*Usp8*: $F(3,32)=0.2251$, $p=0.1014$) (**Figure 5f**). The most interesting result came from the amplitude analysis. A two-way ANOVA showed no interaction of *Usp8* knockdown and *Usp2* KO ($F(3,32)=0.9808$, $p=0.4140$) on the amplitude of PER2::Luc oscillations. However, we found a significant increase of amplitude in *Usp2* KO cells compared

to WT (F(1,32)=19.54, p=0.0001). Most importantly, we found an amplitude main effect of *Usp8* dsiRNA treatments (F(3, 32)=4.208, p=0.0129), from which a Dunnett's test for multiple

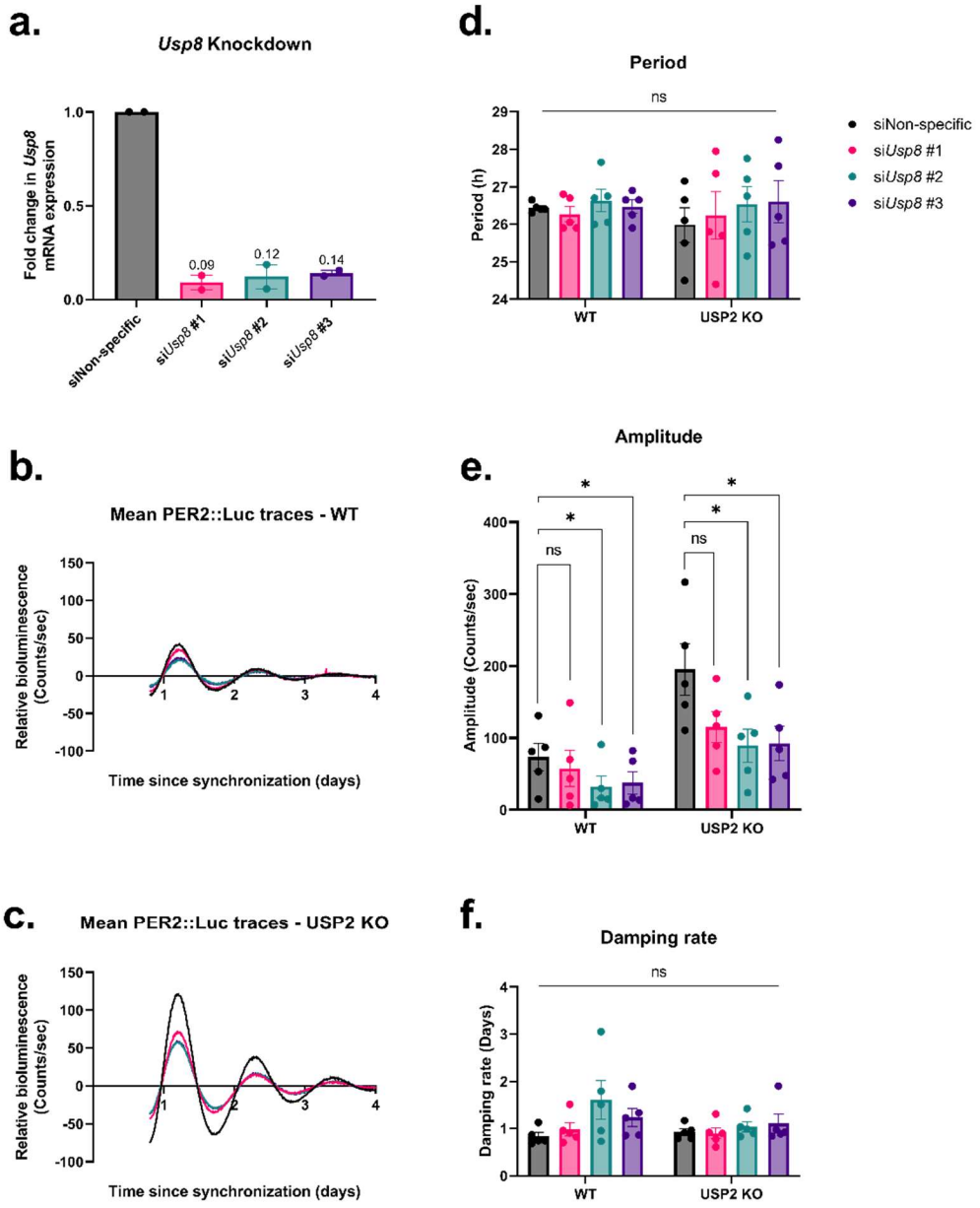


Figure 5. *Usp8* knockdown in WT and *Usp2* KO PER2::Luc MEFs. a. Three dsiRNAs targeting *Usp8* were transfected and led to a major reduction in *Usp8* mRNA level. **b.c.** PER2::Luc mean bioluminescence traces following *Usp8* knockdown in both WT and *Usp2* KO MEFs respectively. **d.** Period of the PER::Luc bioluminescence rhythms. A two-way ANOVA found no significant effect of *Usp8* knockdown, nor of *Usp2* KO. **e.** Amplitude of the PER2::Luc bioluminescence rhythms. A two-way ANOVA found main effects of both *Usp8* knockdown and *Usp2* KO. A Dunnett's test for multiple comparisons found a significant decrease in amplitude following treatment with the second and third dsiRNAs against *Usp8*. **f.** Damping rate of the PER2::Luc bioluminescence rhythms. A two-way ANOVA found no significant effect of *Usp8* knockdown, nor of *Usp2* KO. (n = 5, * $p<0.05$, ns: >0.05).

comparisons confirmed a significant decrease in amplitude following the treatment with the second and the third dsiRNAs against *Usp8* (p=0.0100 and p=0.0165) (**Figure 5e**).

2. Usp21

Following *Usp21* knockdown, we only observed an efficient reduction in mRNA levels with two of the three dsiRNAs (**Figure 6a**). However, since we had a ~80% reduction with two distinct dsiRNAs, we decided to move forward to record cellular rhythms. We did not detect any significant changes in period when we conducted a two-way ANOVA (Interaction: F(3,32)=0.02719, p=0.9938; *Usp2* KO: F(1,32)=0.01770, p=0.8950; si*Usp8*: F(3,32)=2.848, p=0.0530) (**Figure 6b,c,d**). Similarly, no significant effects were observed for the damping rate of the rhythms (Interaction: F(3,32)=0.6313, p=0.6002, *Usp2* KO: F(1,32)=0.08063, p=0.7783, si*Usp8*: F(3,32)=0.4040, p=0.7511) (**Figure 6f**). Finally, a two-way ANOVA showed no interaction of *Usp21* knockdown and *Usp2* KO (F(3,32)=0.3031, p=0.8229) on the amplitude of PER2::Luc oscillations (**Figure 6e**). Main effects analysis revealed a significant increase of amplitude in *Usp2* KO cells compared with WT (F(1,32)=8.069, p=0.0078) (**Figure 6e**), which is consistent with what we observed upon the knockdown of *Usp8* (**Figure 5**). However, we found no significant main effect of *Usp21* knockdown (F(3,32)=0.1591, p=0.9230) (**Figure 6e**).

3. Usp22

Usp22 knockdown with three distinct dsiRNAs led to a sufficient reduction in mRNA expression when used at a higher concentration of 20 nM, compared to 10 nM for the other DUBs (**Figure 7a**). Again, we did not detect any significant changes in period when we conducted a two-way ANOVA (Interaction: F(3,32)=0.04760, p=0.9860; *Usp2* KO: F(1,32)=0.1.121, p=0.2979; si*Usp8*: F(3,32)=1.566, p=0.2171) (**Figure 7b,c,d**). No significant effects were observed for the damping rate of the rhythms in the case of the interaction as well as

for *Usp22* dsiRNA treatments (Interaction: F(3,32)=0.3595; p=0.7826; si*Usp8*: F(3,32)=0.4040, p=0.7511) (**Figure 7f**). However, we did find a significant main effect of *Usp2* KO on the

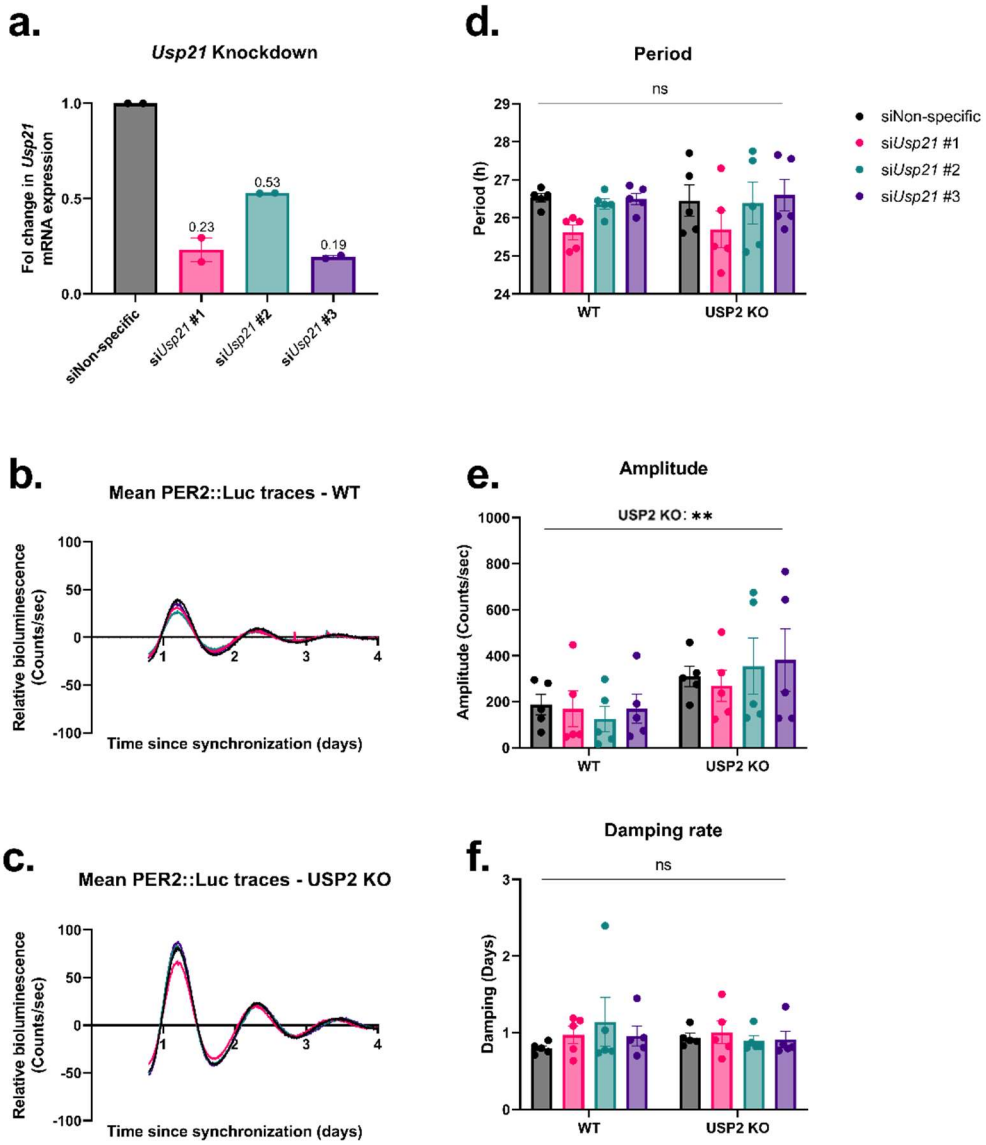


Figure 6. ***Usp21*** **knockdown in WT and** ***Usp2*** **KO PER2::Luc MEFs. a.** Three dsiRNAs *targeting Usp21* were transfected. Only two of the three treatments led to a good reduction in *Usp21* mRNA level. The third dsiRNA only achieved 47% knockdown of gene expression. **b.c.** PER2::Luc mean bioluminescence traces following *Usp21* knockdown in both WT and *Usp2* KO MEFs respectively. **d.** Period of the PER::Luc bioluminescence rhythms. A two-way ANOVA found no significant effect of *Usp21* knockdown, nor of *Usp2* KO. **e.** Amplitude of the PER2::Luc bioluminescence rhythms. A two-way ANOVA only found a significant main effect of *Usp2* KO. **f.** Damping rate of the PER2::Luc bioluminescence rhythms. A two-way ANOVA found no significant effect of *Usp21* knockdown, nor of *Usp2* KO. (n = 5, ** p<0.01, ns: >0.05).

damping rate, as *Usp2* KO cells were more robust throughout time ($F(1,32)=9.175$, $p=0.0049$) (**Figure 7f**). Finally, a similar analysis showed no interaction of *Usp22* knockdown and *Usp2* KO on the amplitude of PER2::Luc oscillations ($F(3,32)=1.600$, $p=0.2093$) (**Figure 7e**). Main effects analysis revealed a significant increase of amplitude in *Usp2* KO cells compared to WT ($F(1,32)=28.62$, $p<0.0001$) (**Figure 7e**), which is again consistent with what we saw in both *Usp8* and *Usp21* knockdowns (**Figures 5 and 6**). However, we found no significant main effect of *Usp22* knockdown on the amplitude of the rhythms ($F(3,32)=2.058$, $p=0.1262$) (**Figure 7e**).

4. Uchl1

Uchl1 knockdown was well achieved with all three dsiRNAs (**Figure 8a**). However, we did not find any significant changes in period when we conducted a two-way ANOVA (Interaction: $F(3,31)=0.6126$, $p=0.6120$; *Usp2* KO: $F(1,31)=2.186$, $p=0.1494$; si*Usp8*: $F(3,31)=1.826$, $p=0.1629$) (**Figure 8b,c,d**). Similarly, no significant effect was observed for the damping rate of the rhythms (Interaction: $F(3,31)=0.1863$; $p=0.9049$, *Usp2* KO: $F(1,31)=0.5455$, $p=0.4657$, si*Usp8*: $F(3,31)=0.8104$, $p=0.4978$) (**Figure 8f**). Finally, no significant interaction of *Uchl1* knockdown and *Usp2* KO was found regarding the amplitude of the oscillations ($F(3,31)=0.09189$, $p=0.9640$) (**Figure 8e**). Main effects analysis revealed a significant increase of amplitude in *Usp2* KO cells compared with WT cells ($F(1,31)=6.371$, $p=0.0169$) (**Figure 8e**), a result we obtained in all analyses (**Figures 5-7**). However, no significant changes in the amplitude of the rhythms upon *Uchl1* knockdown were found ($F(3,31)=0.5705$, $p=0.06386$) (**Figure 8e**).

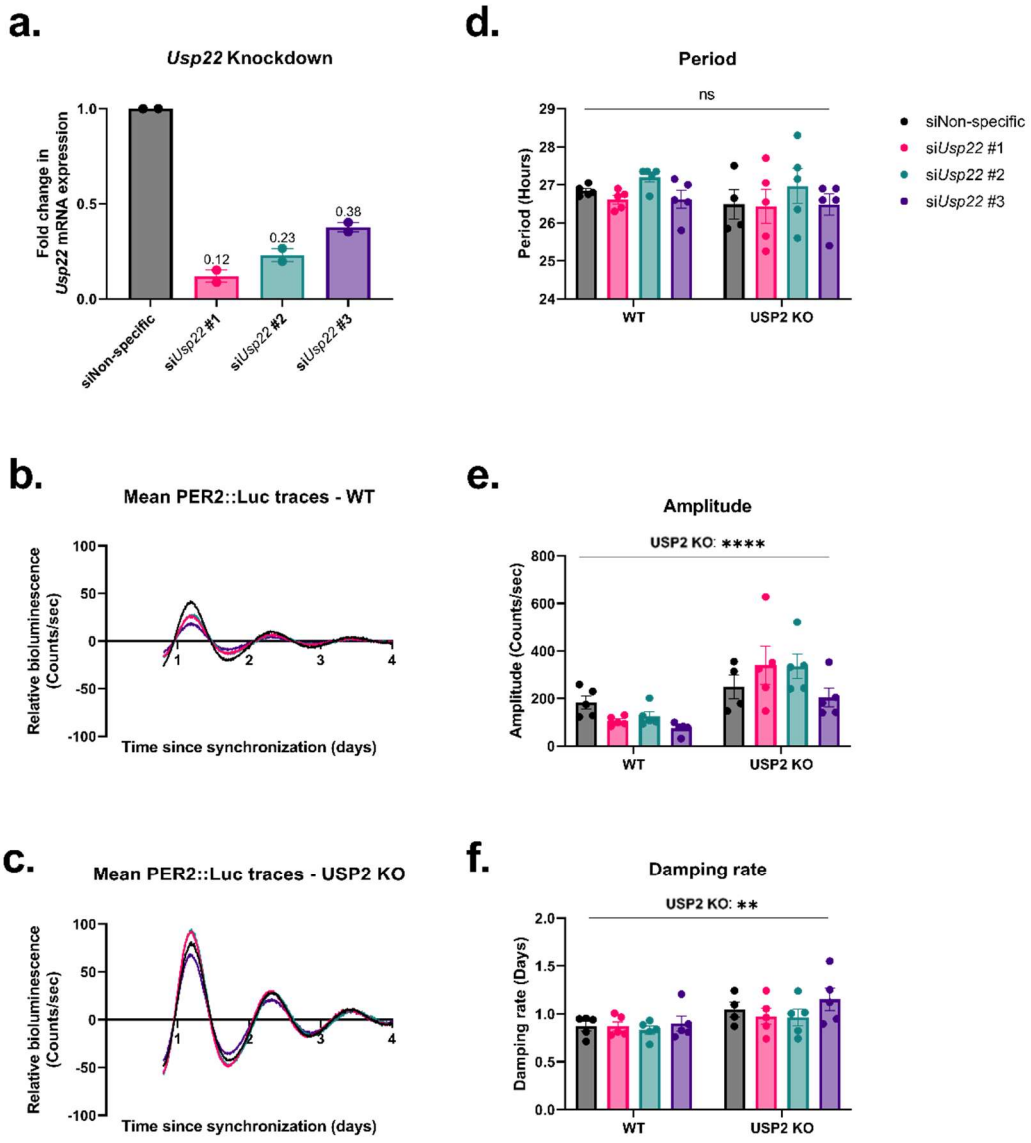


Figure 7. *Usp22* knockdown in WT and *Usp2* KO PER2::Luc MEFs. a. Three dsiRNAs targeting *Usp22* were transfected and led to a good reduction in *Usp22* mRNA level when used at 20 nM. **b.c.** PER2::Luc mean bioluminescence traces following *Usp22* knockdown in both WT and *Usp2* KO MEFs respectively. **d.** Period of the PER::Luc bioluminescence rhythms. A two-way ANOVA found no significant effect of *Usp22* knockdown, nor of *Usp2* KO. **e.** Amplitude of the PER2::Luc bioluminescence rhythms. A two-way ANOVA only found a significant main effect of *Usp2* KO. **f.** Damping rate of the PER2::Luc bioluminescence rhythms. A two-way ANOVA found a significant main effect of *Usp2* KO. (n = 5, **** $p<0.0001$, ** $p<0.01$, ns: >0.05).

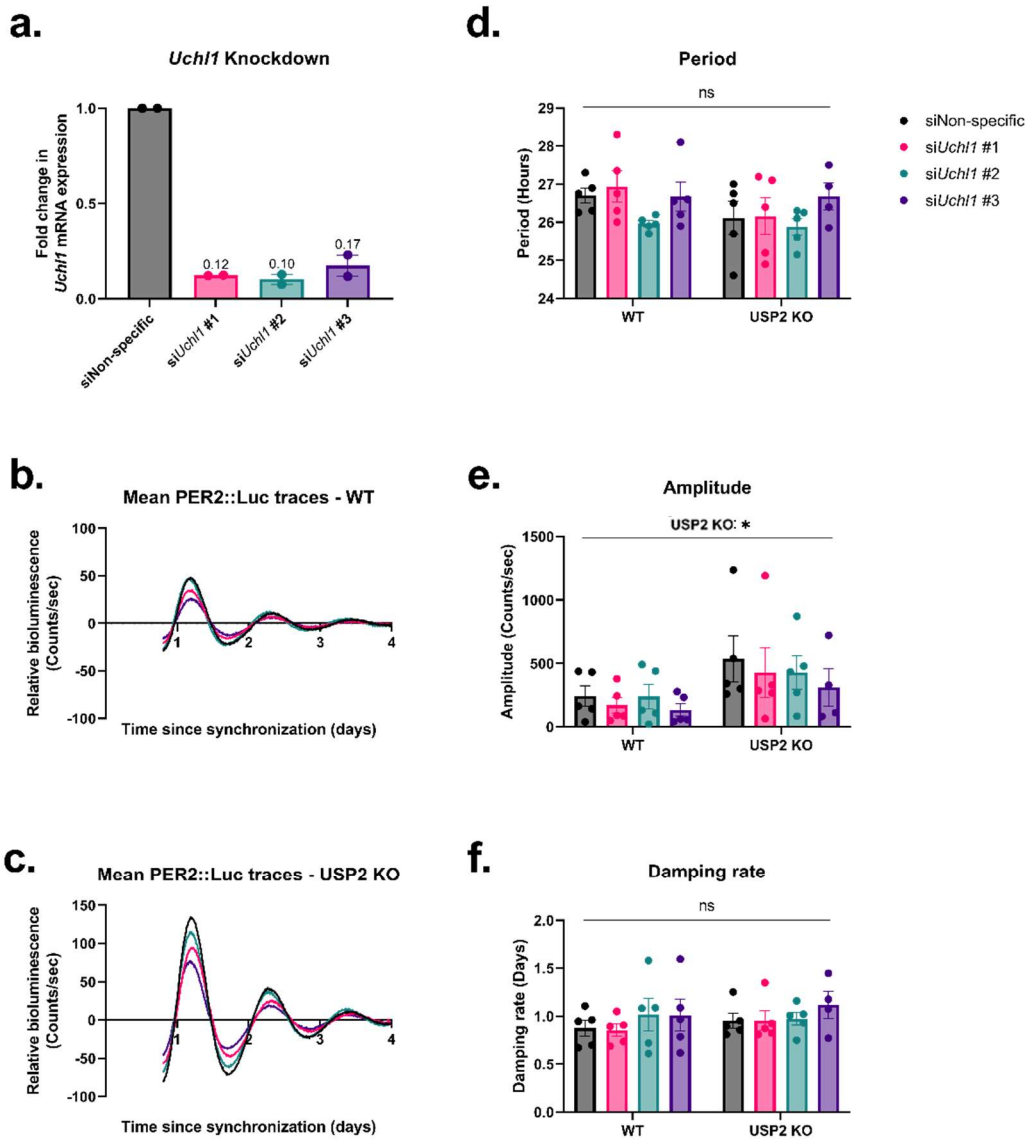


Figure 8. *Uchl1* knockdown in WT and *Usp2* KO PER2::Luc MEFs. a. Three dsiRNAs targeting *Uchl1* were transfected and led to a good reduction in *Uchl1* mRNA level. **b.c.** PER2::Luc mean bioluminescence traces following *Uchl1* knockdown in both WT and *Usp2* KO MEFs respectively. **d.** Period of the PER::Luc bioluminescence rhythms. A two-way ANOVA found no significant effect of *Uchl1* knockdown, nor of *Usp2* KO. **e.** Amplitude of the PER2::Luc bioluminescence rhythms. A two-way ANOVA only found a significant main effect of *Usp2* KO. **f.** Damping rate of the PER2::Luc bioluminescence rhythms. A two-way ANOVA found no significant effect of *Uchl1* knockdown, nor of *Usp2* KO. (n = 5, * $p<0.05$, ns: >0.05).

Usp8 knockdown in Per2-Luc and Bmal1-Luc U2OS

Based on our previous results in MEFs where the knockdown of *Usp8* decreased the amplitude of bioluminescence rhythms, we wanted to assess the role of *Usp8* in a different cell line. We therefore knocked down *Usp8* in both *Bmal1*-Luc and *Per2*-Luc U2OS cells, thereby assessing the role of *Usp8* in the human cellular clock. We used three different dsiRNAs against human *Usp8* and we observed a large reduction of *Usp8* mRNA levels in all three cases (**Figure 9a**). We also transfected a pool of three dsiRNAs against *Usp2*, which led to a strong decrease in *Usp2* mRNA levels when compared to the non-specific control (**Figure 9b**).

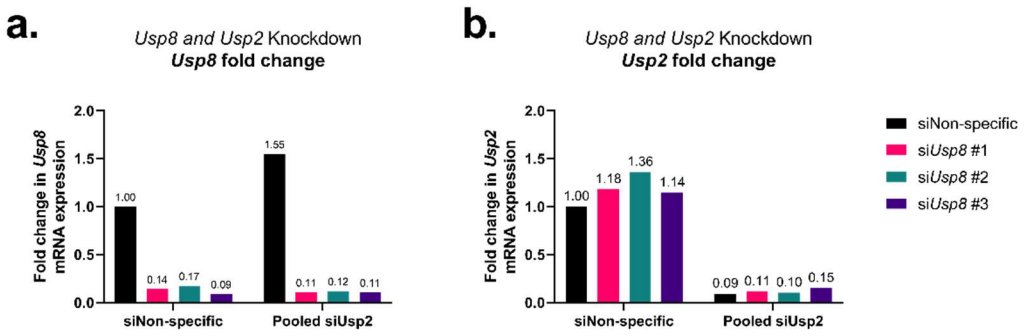


Figure 9. *Usp8* and *Usp2* knockdown efficacy in U2OS *Per2*-Luc cells. a.b. Three dsiRNAs targeting human *Usp8* were transfected with either a non-specific control or a pooled of three dsiRNAs targeting *Usp2*. A good reduction in both *Usp8* and *Usp2* was achieved. Above each bar is the exact fold changes in gene expression.

We then analyzed bioluminescence rhythms of both *Per2*-Luc and *Bmal1*-Luc U2OS cells upon *Usp8* and *Usp2* double knockdown (**Figure 10a,b and Figure 11a,b**). Similarly to knocking down *Usp8* in PER2::Luc MEFs, a two-way ANOVA found a significant interaction of *Usp8* and *Usp2* knockdown on the amplitude of *Bmal1*-Luc cells ($F(3, 62)=4.887$, $p=0.0041$) (**Figure 10d**). Pairwise comparisons revealed a significant decrease in amplitude following two dsiRNA treatments against *Usp8* when combined with a *Usp2* knockdown ($p=0.0046$ and $p<0.0001$) (**Figure 10d**). We also found a significant reduction in the amplitude of the rhythms

with one of the three *Usp8* dsiRNAs in the control background (p=0.0005) (**Figure 10d**). In *Per2*-Luc cells, we found a trend in the interaction between the two knockdowns (F(3, 63)=2.603, p=0.0597). We also found a significant main effect of *Usp8* knockdown (F(3, 63)=3.909, p=0.0126) and pairwise comparisons revealed a significant reduction in the amplitude of the rhythms following the treatment with the third dsiRNA targeting *Usp8*, which was independent of *Usp2* knockdown (p=0.0214) (**Figure 11d**). Since this reduction was only observed using a single dsiRNA, we cannot rule out the possibility that this is a non-specific

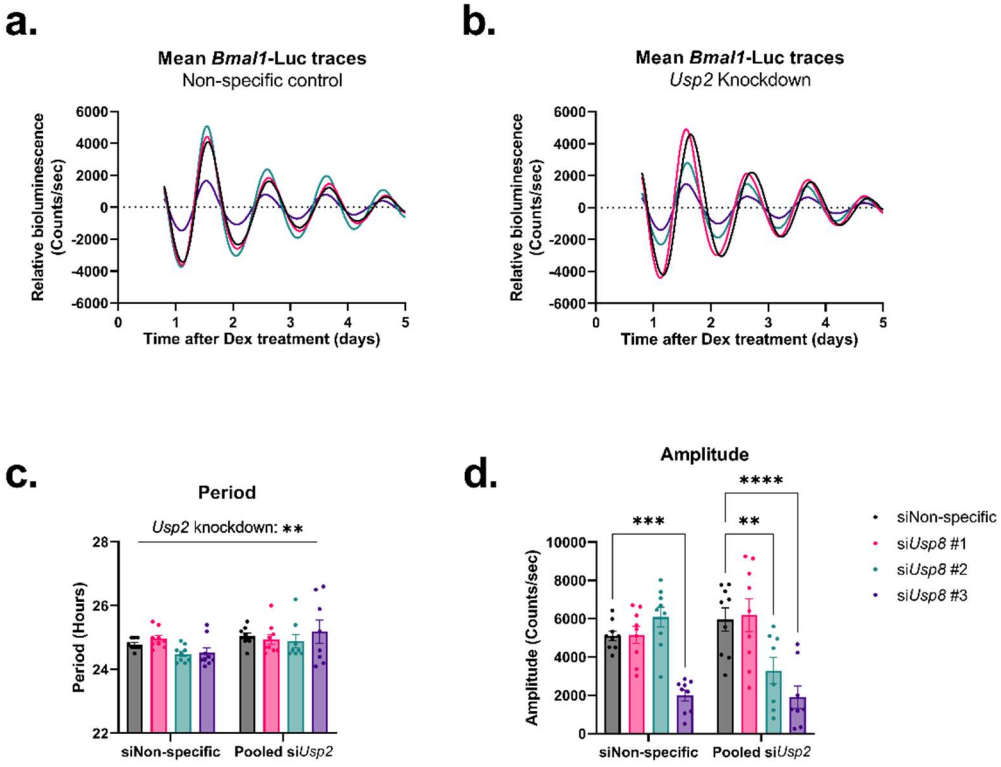


Figure 10. *Usp8* and *Usp2* double knockdown in U2OS *Bmal1*-Luc cells. a.b. Mean bioluminescence of *Bmal1*-Luc traces following the knockdown of both *Usp8* and *Usp2* **c.** Period of the *Bmal1*-Luc rhythms. A two-way ANOVA found no significant interaction between *Usp8* and *Usp2* knockdown, nor a significant effect of *Usp8* knockdown. We however found a significant increasing effect on the period of *Usp2* knocked down cells. **d.** Amplitude of the *Bmal1*-Luc rhythms. A two-way ANOVA found a significant interaction between *Usp8* and *Usp2* knockdowns. A Dunnett's multiple comparisons test was then conducted to analyze the simple effects of *Usp8* and *Usp2* knockdowns. We found that two out of the three dsiRNAs targeting *Usp8* significantly decreased the amplitude of the rhythms specifically in the *Usp2* knockdown background. This effect was only observed in one dsiRNA treatment against *Usp8* in the control background. (n = 9, **** p<0.0001, ***p<0.001, **p<0.01, ns: >0.05).

effect of the dsiRNA treatment. It is however interesting that the reduction in amplitude following *Usp8* knockdown is partly consistent across mammalian cell lines.

The effect of *Usp8* knockdown on the period of both *Bmal1*-Luc and *Per2*-Luc rhythms was not clear, as the analysis revealed to be inconsistent. Indeed, a two-way ANOVA found no interaction of both *Usp8* and *Usp2* knockdowns, but found a significant main effect of *Usp2* knockdown in *Bmal1*-Luc cells as *Usp2* deficiency slightly increase the period of cellular rhythms (Interaction: $F(3, 62)=1.425$, $p=0.2440$; si*Usp2*: $F(1, 62)=7.289$, $p=0.0089$; si*Usp8*: $F(3, 62)=0.9552$, $p=0.4196$) (**Figure 10c**). In contrast, we found a significant interaction of the double

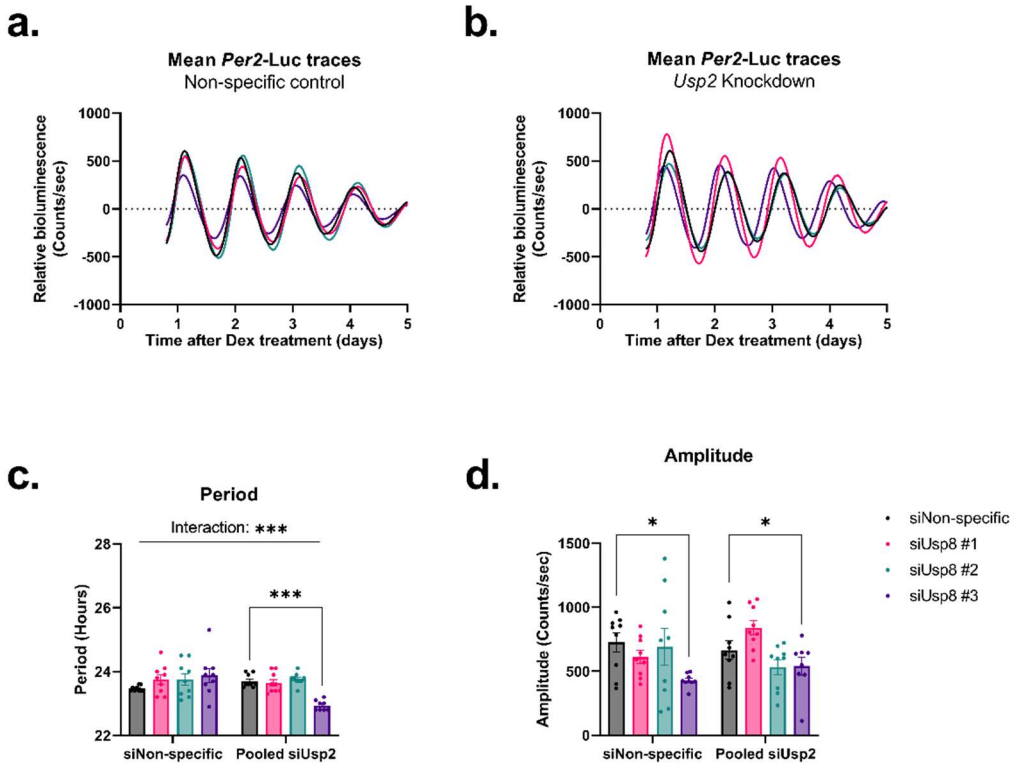


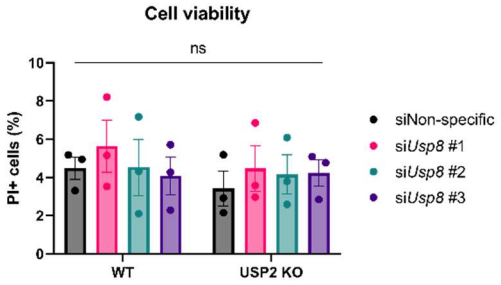
Figure 11. *Usp8* and *Usp2* double knockdown in U2OS *Per2*-Luc cells. a.b. Mean bioluminescence of *Per2*-Luc traces following the knockdown of both *Usp8* and *Usp2* **c.** Period of the *Per2*-Luc rhythms. A two-way ANOVA found a significant interaction between *Usp8* and *Usp2* knockdowns. However, a significant reduction in the period was only observed following a single dsiRNA treatment, which is not enough to state a role for *Usp8* in the clock since it could be caused by a non-specific effect of the dsiRNA treatment. **d.** Amplitude of the *Per2*-Luc rhythms. A two-way ANOVA found no significant interaction between *Usp8* and *Usp2* knockdowns. Similar to the period analysis, a single *Usp8* knockdown affected significantly the amplitude of the rhythms, independently of *Usp2* knockdown. (n = 9, *** $p<0.001$,* $p<0.05$, ns: >0.05).

knockdown of *Usp8* and *Usp2* in *Per2*-Luc cells (F(3, 63)=7.847, p=0.0002), which was mainly due to a strong decrease in the period of bioluminescence rhythms upon the treatment with the third *Usp8* dsiRNA (p=0.0003) (**Figure 11c**). Based on this assumption, we also analyzed the main effect of *Usp2* knockdown, which revealed a significant increase in the period of the rhythms (si*Usp2*: F(1,63)=4.993, p-0.0290), consistent with results in *Bmal1*-Luc cells (**Figure 11c**).

Cell viability following Usp8 knockdown in PER2::Luc MEFs

A decrease in bioluminescence amplitude could be caused by a loss of cell viability. We therefore wanted to measure the extent of cell death following *Usp8* knockdown in PER2::Luc MEFs. Using propidium iodide and flow cytometry, we were able to quantify the number of dying cells 72 hours after the treatments with dsiRNAs. A two-way ANOVA confirmed that there was no change in cell viability following treatments in both WT and *Usp2* KO cells (Interaction: F(3,16)=0.1645, p=0.9187; *Usp2* KO: F(1,16)=0.6538, p=0.4306; si*Usp8*: F(3,16)=0.3950, p=0.7583) (**Figure 12**). This suggests that cell death is not the reason for the observed reduction in bioluminescence amplitude upon *Usp8* knockdown.

Figure 12. Cellular viability following *Usp8* knockdown in WT and *Usp2* KO MEFs. Propidium Iodide (PI) positive cells were detected using flow cytometry 72 hours after transfecting dsiRNAs against *Usp8*. PI positive cells were considered dead. The percentage was drawn from the gating of single cells. A two-way ANOVA found no significant differences between conditions (n = 3, ns: >0.05).



PER2::Luc stability following Usp8 knockdown

We then wanted to assess the effect of *Usp8* knockdown on the cellular reporter itself. Since the PER2::Luc reporter is a fusion protein, it was possible that the decreased amplitude we saw in bioluminescence rhythms upon *Usp8* knockdown was due to a direct effect of *Usp8* on PER2. We thus wanted to investigate PER2::Luc stability through a CHX-mediated translation inhibition. From the degradation curves seen in **Figure 13a**, we extracted the half-life of PER2::Luc using a one phase decay analysis (**Figure 13b**). A two-way ANOVA revealed no significant difference in PER2::Luc half-life across conditions (Interaction: F(2,12)=0.3867, p=0.6874; *Usp2* KO: F(1,12)=0.4326, p=0.5231; si*Usp8*: F(2,12)=0.1344, p=0.8755). This suggest that PER2::Luc stability was not affected by either *Usp8* knockdown or *Usp2* KO.

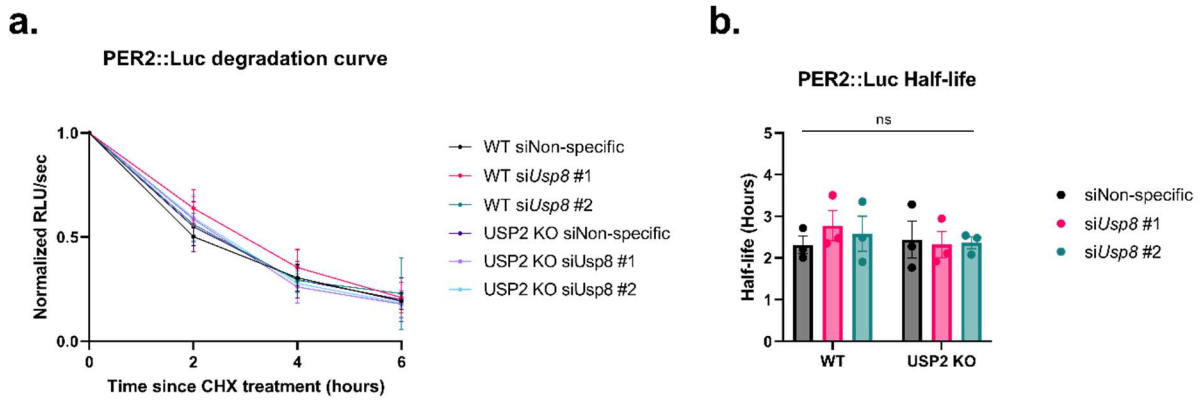


Figure 13. PER2::Luc stability following *Usp8* knockdown in WT and *Usp2* KO MEFs. a. Relative luminescence levels of WT and *Usp2* KO MEFS following the transfection with two dsiRNAs against *Usp8* and translation inhibition by CHX treatment. **b.** Half-life of PER2::Luc following a one phase decay analysis of the degradation curves. A two-way ANOVA found no significant differences between conditions (n = 3, ns: >0.05).

Clock genes expression following Usp8 knockdown in PER2::Luc MEFs

To investigate a possible role of *Usp8* in the clock regulation, we assessed its effect on the expression of clock genes *Bmal1*, *Per2* and *Rev-erbα*. Four time points over one day were chosen to assess the peak of expression of these three genes. We again used WT and *Usp2* KO MEFs to address redundancy between *Usp8* and *Usp2*. A three-way ANOVA was conducted to assess the interaction of time, *Usp8* knockdown and *Usp2* KO (**Figure 14a-c and Table 7**). We found no interaction of the three factors for any of the three transcripts (**Table 7**). As expected,

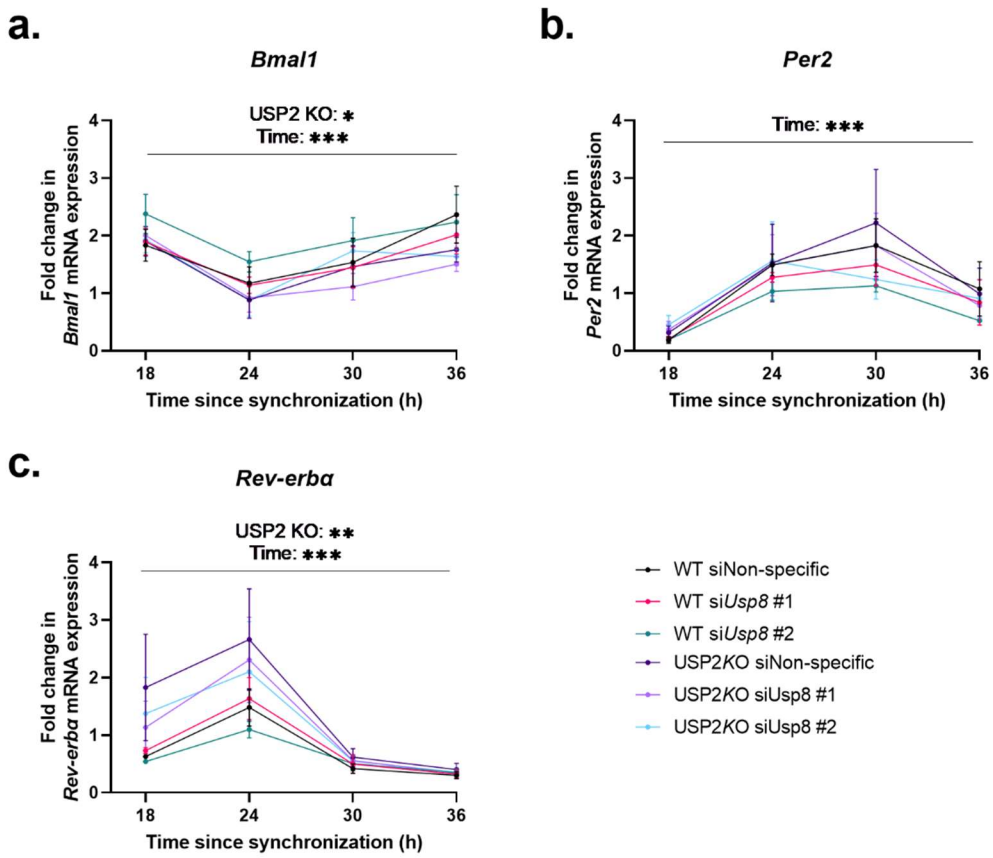


Figure 14. Clock genes expression at four time points following *Usp8* knockdown in WT and *Usp2* KO MEFs. a.b.c. Relative *Bmal1*. *Per2* and *Rev-erbα* mRNA expression 18, 24, 30 and 36 hours after synchronization of both WT and *Usp2* KO PER2::Luc MEFs treated with two distinct dsiRNAs against *Usp8*. A three-way ANOVA found no significant interaction between time, *Usp8* knockdown and *Usp2* KO. Significant main effects of time were found for all three transcripts. Significant main effects of *Usp2* KO were found for both *Bmal1* and *Rev-erbα* expression, but not for *Per2*. No significant main effects of *Usp8* knockdown were found in all three cases (n = 3, *** p<0.001, **p <0.01* p<0.05, ns: >0.05).

we found a significant effect of time for *Bmal1*, *Per2* and *Rev-erbα* expression (**Figure 14a-c and Table 7**), as all three transcripts are known to be rhythmically expressed in many tissues (Zhang et al., 2014), including primary fibroblasts (Yang et al., 2014). We further found a significant main effect of *Usp2* KO for both *Bmal1* and *Rev-erbα* expression, where the loss of *Usp2* increased *Rev-erbα* mRNA levels, while decreasing *Bmal1* levels (**Figure 14a-c**). Finally, we did not find any significant effect of *Usp8* knockdown (**Table 7**).

Although not significant, we observed a slight decrease in the peak level of *Per2* expression 30 hours after synchronization. Therefore, we wanted to replicate this assay, but using a single time point. We sampled both WT and PER2::Luc MEFs 30 hours after synchronization to assess the mRNA expression of *Per2* upon *Usp8* knockdown. A two-way ANOVA did not find any significant differences between samples, which is consistent with our previous four time point analysis (Interaction: $F(2,18)=0.01118$, $p=0.9889$; *Usp2* KO: $F(1,18)=1.311$, $p=0.2672$; si*Usp8*: $F(2,18)=0.2655$, $p=0.7698$) (**Figure 15**).

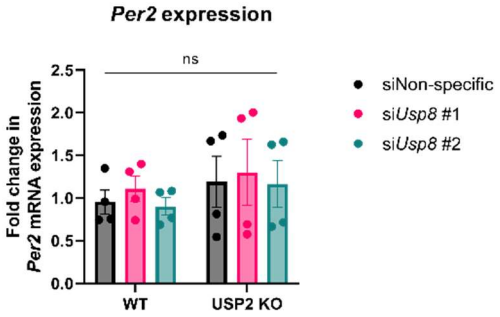


Figure 15. *Per2* expression 30 hours after synchronization. Relative *Per2* expression in in WT and *Usp2* KO MEFs following the knockdown of *Usp8* using two distinct dsiRNAs. Samples were normalized to the non-specific dsiRNA treatment. A two-way ANOVA found no significant changes in *Per2* mRNA levels between conditions (n = 4, ns: >0.05).

Table 7. Three-way ANOVA summary table.
Significant p values are displayed in bold (n = 3).

Bmal1			
Factor	**df**	**F**	**p value**
si*Usp8*	2	1.757	0.183
Usp2 KO	1	6.516	**0.014**
Time	3	11.432	**< .001**
si*Usp8* ⁎ *Usp2* KO	2	0.43	0.653
si*Usp8* ⁎ Time	6	0.392	0.88
Usp2 KO ⁎ Time	3	0.755	0.525
si*Usp8* ⁎ *Usp2* KO ⁎ Time	6	0.18	0.981
Residual	48		

Per2			
Factor	**df**	**F**	**p value**
si*Usp8*	2	1.345	0.27
Usp2 KO	1	1.555	0.218
Time	3	13.648	**< .001**
si*Usp8* ⁎ *Usp2* KO	2	0.146	0.865
si*Usp8* ⁎ Time	6	0.478	0.821
Usp2 KO ⁎ Time	3	0.078	0.972
si*Usp8* ⁎ *Usp2* KO ⁎ Time	6	0.122	0.993
Residual	48		

Rev-erb α			
Factor	**df**	**F**	**p value**
si*Usp8*	2	0.413	0.664
Usp2 KO	1	8.33	**0.006**
Time	3	17.408	**< .001**
si*Usp8* ⁎ *Usp2* KO	2	0.44	0.646
si*Usp8* ⁎ Time	6	0.223	0.967
Usp2 KO ⁎ Time	3	2.042	0.12
si*Usp8* ⁎ *Usp2* KO ⁎ Time	6	0.091	0.997
Residual	48		

siRNA screen against human DUBs

In addition to the targeted approach discussed so far, we wanted to identify more DUBs involved in the circadian clock by using a screening approach. We therefore used a siRNA library composed of 111 pooled siRNAs targeting every human DUBs (**Table 3**). We decided to use U2OS cells expressing either *Per2*-Luc or *Bmal1*-Luc circadian reporters in order to visualize and analyze bioluminescence rhythms.

1. Controls

Positive and negative controls were included in every plate of the screening experiment. The negative controls consisted of two distinct pools of two non-specific siRNAs. The rhythms obtained from transfecting these control siRNAs in both *Bmal1*-Luc and *Per2*-Luc U2OS cells were robust and were maintained for at least five days (**Figure 16a-d**). We decided to use *Cry1* knockdown as a positive control since it was previously shown to shorten the period of *Bmal1*-Luc U2OS bioluminescence rhythms as well as to decrease the amplitude (Baggs et al., 2009; Maier et al., 2009). Accordingly, our analysis of *Cry1* knockdown from the *Bmal1*-Luc screening plates resulted in a significant main effect of *Cry1* knockdown on the period of bioluminescence rhythms (**Figure 16e**) (Interaction: $F(2,12)=0.03164$, $p=0.9689$; si*Usp2*: $F(1,12)=0.7719$, $p=0.3969$; si*Cry1*: $F(2,12)=11.61$, $p=0.0016$). This reduction was further confirmed by a Tukey's multiple comparison test, where the period of *Cry1* knocked down cells was significantly reduced compared to both controls (**Figure 16e**) ($p=0.0074$ and $p=0.0019$). Furthermore, no significant difference between the period of negative controls were found (**Figure 16e**) ($p=0.7271$). However, we did not find any significant effect of *Cry1* deficiency on the amplitude of the rhythms, which probably arise from variability between screening plates (**Figure 16f**) (Interaction: $F(2,12)=0.04270$, $p=0.9583$; si*Usp2*: $F(1,12)=0.02524$, $p=0.8764$; si*Cry1*:

F(2,12)=0.8802, p=0.4398). In *Per2*-Luc cells, the bioluminescence oscillations were almost completely lost following *Cry1* knockdown and mostly resulted in arrhythmicity (**Figure 16a,b**). Taken together, these results indeed confirm the validity of our screening protocol.

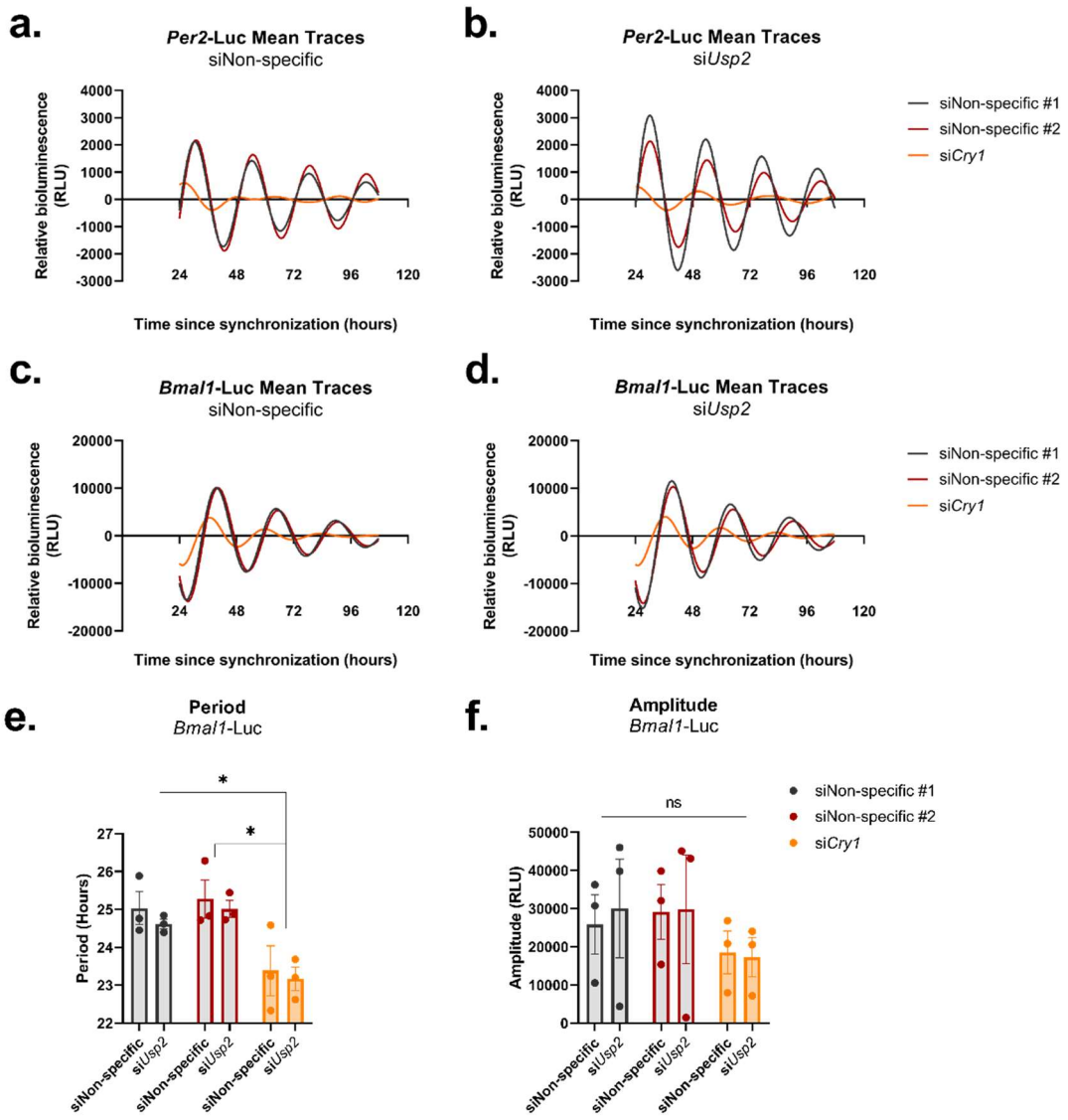


Figure 16. Positive and negative controls used in the DUB siRNA screen. a.b. U2OS *Per2*-Luc bioluminescence rhythms from the three screening plates. **c.d.** U2OS *Bmal1*-Luc bioluminescence rhythms from the three screening plates. Mean traces are shown for both pools of non-specific siRNAs and pooled siRNAs against *Cry1*. **e.** Period analysis of the *Bmal1*-Luc rhythms. A two-way ANOVA found no significant interaction between the two knockdowns. We however found a significant main effect of *Cry1* knockdown. Pair wise comparisons revealed a significant decrease of the period of the rhythms following *Cry1* knockdown when compared to both negative controls. **f.** Amplitude analysis of the *Bmal1*-Luc rhythms. A two-way ANOVA found no significant interaction, nor main effects of the two knockdowns (n = 3, * $p<0.05$, ns: $p>0.05$).

2. Period analysis

We first analyzed the period of bioluminescence rhythms following the knockdowns of DUBs. To normalize for variations between plates, we analyzed the changes in period always relative to the negative controls within the same plate. We also analyzed both *Per2*-Luc and *Bmal1*-Luc period changes, given that a DUB with a main regulatory role in the clock would be expected to have a similar effect on both circadian reporters. Therefore, the changes in period in *Bmal1*-Luc cells were plotted against the changes in period in *Per2*-Luc cells (**Figure 17a,b**). Using a cut-off value of ± 1 hour period change either in the control or in the *Usp2* knockdown background, we identified a total of 31 DUBs (**Figure 17a,b and Table 8**). Interestingly, out of these 31 DUBs, six knockdowns of DUBs resulted in an increase in the period of bioluminescence rhythms: *Usp8* was one of them, in addition to *Usp9y*, *Usp15*, *Usp17l1p*, *Usp18* and *Usp28* (**Figure 17a, 19 and Table 8**). This phenotype was only seen when they were knocked down in combination with *Usp2* (**Figure 17a and 19**). Finally, we found that the sole knockdown of *Usp50*, *Dub3* and *Otud4* shortened drastically the period of bioluminescence rhythms of both *Bmal1*-Luc and *Per2*-Luc cells (**Figure 17a and Figure 19**).

To assess the redundancy of these DUBs with USP2, we also compared the changes in the period of bioluminescence rhythms between knocking down DUBs in the control background versus in the *Usp2* knockdown background. Using the same cut-off value of ± 1 hour, we found that the knockdown of four DUBs resulted in a greater lengthening of the period when combined with *Usp2* knockdown (**Table 9**). Interestingly, the knockdown of *Usp15* alone reduced the period of bioluminescence rhythms but lengthened it when combined with *Usp2* knockdown. A similar but milder effect was also seen for *Usp9y*. In contrast, the sole knockdown of *Usp17l1p*

and *Usp18* did not change the period of bioluminescence rhythms, but drastically lengthened it in the *Usp2* knockdown background (**Figure 19** and **Table 8,9**).

Table 8. Identified DUBs following the period analysis. Identified DUB hits and their precise changes in period in both U2OS *Per2*-Luc and *Bmal1*-Luc. Thirty-one DUBs were identified as changing considerably the period of bioluminescence rhythms in either the control or the *Usp2* knockdown background. Reductions of the period are highlighted in red, while increases are highlighted in blue.

DUB	Δ Period (hours)			
	Per2-Luc		*Bmal1*-Luc	
	siNon-specific	si*Usp2*	siNon-specific	si*Usp2*
Atxn3	-0.79	-1.20	-0.42	-1.54
Atxn3l	-0.96	-0.26	-1.51	-1.44
Dub3	-1.48	-0.85	-2.49	-1.88
Josd1	-1.21	-1.00	-1.79	-2.11
Otub2	-1.07	-0.21	-1.84	-0.98
Otud4	-1.46	-0.67	-2.38	-2.26
Otud5	-1.47	-1.22	-1.13	-1.09
Otud6a	-1.14	-1.17	-1.12	-1.77
Otud7b	-0.84	-1.65	-0.57	-1.07
Parp11	-1.34	-1.29	-1.56	-1.85
Tnfaip2	-1.62	-0.89	-1.79	-1.62
Usp15	-2.39	3.74	-0.12	1.68
Usp17l1p	-0.01	2.94	0.19	1.40
Usp18	1.28	3.13	-0.11	1.24
Usp28	2.48	1.34	0.87	1.83
Usp34	-1.19	-1.14	-0.57	-1.07
Usp35	-0.97	-1.05	-1.20	-1.85
Usp37	-1.17	-0.98	-1.61	-1.23
Usp38	-1.37	-1.24	-1.77	-1.85
Usp42	-0.67	-1.47	-1.65	-1.74
Usp43	-1.05	-0.99	-1.39	-1.61
Usp44	-1.13	-1.18	-1.54	-1.83
Usp47	-1.26	-0.78	-1.21	-1.26
Usp48	-0.92	-1.04	-1.76	-1.82
Usp49	-0.70	-1.07	-1.08	-1.33
Usp50	-1.09	-1.18	-2.18	-1.92
Usp52	-1.01	-1.10	-0.89	-1.75
Usp53	-1.45	-0.42	-1.36	-1.63
Usp54	-1.10	-1.12	-1.77	-1.91
Usp8	2.05	2.64	0.35	2.65
Usp9y	-0.85	3.57	-0.56	1.19

-2.5
-2
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2.5

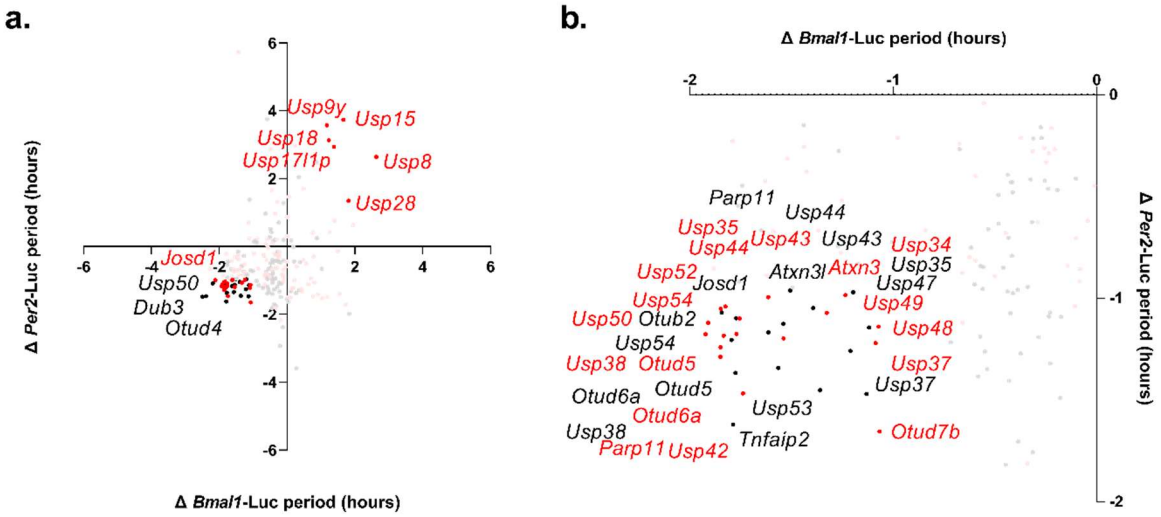


Figure 17. Period analysis of the DUB siRNA screen. a. Changes in the period of U2OS *Bmal1*-Luc and *Per2*-Luc rhythms following the knockdown of all DUBs. Changes were always calculated relative to the mean of both negative controls within each of the screening plate. Black dots represent DUB knockdowns in the control background, while red dots represent DUB knockdowns in the *Usp2* knockdown background. Darker dots represent the identified DUB hits with a cut-off value of ± 1 hour. Thirty-one DUBs were identified as changing drastically the period either in the control background or in the *Usp2* knockdown background. **b.** Magnified plot of identified DUB hits from the period analysis.

Table 9. Identified DUBs following the si*Usp2* period analysis. Precise changes in period following DUB knockdowns between the control background and the *Usp2* knockdown background in both U2OS *Per2*-Luc and *Bmal1*-Luc. A total of four DUBs were identified. Increases in the period are highlighted in blue.

DUB	Δ si*Usp2* period (hours)	
	***Per2*-Luc**	***Bmal1*-Luc**
Usp15	6.1256	1.796
Usp17l1p	2.9485	1.2127
Usp18	1.8526	1.3505
Usp9y	4.4217	1.7469

0
1
2
3
4
5

3. *Amplitude analysis*

To complement the period analysis of the DUB screen, we analyzed the amplitude of the bioluminescence rhythms. Following the DUB knockdowns, we calculated the percent changes

in amplitude relative to the negative controls from the corresponding plate. We used a ± 75% cut-off value in both *Per2*-Luc and *Bmal1*-Luc cells to identity DUB hits in either the control or the *Usp2* knockdown background.

Table 10. Identified DUBs following the amplitude analysis. Identified DUB hits and their precise percent change in amplitude in both U2OS *Per2*-Luc and *Bmal1*-Luc. Using a cut-off value of ± 75% of the control amplitude, fifteen DUBs were identified as changing the amplitude drastically in either the control or the *Usp2* knockdown background. Reductions in the amplitude are highlighted in red, while increases are highlighted in blue.

DUB	Δ Amplitude (%)			
	Per2-Luc		*Bmal1*-Luc	
	siNon-specific	si*Usp2*	siNon-specific	si*Usp2*
Josd3	0.00	23.40	10.94	16.98
Otud7a	0.00	21.95	20.06	33.58
Otud7b	13.81	50.73	19.58	76.39
Usp15	37.64	8.92	49.48	1.55
Usp17l1p	20.00	2.88	40.38	1.89
Usp18	5.65	4.47	69.08	9.52
Usp19	12.08	20.81	95.00	18.78
Usp22	30.01	16.14	51.57	4.76
Usp29	156.67	24.38	74.12	22.48
Usp30	124.82	14.31	72.84	10.35
Usp40	6.75	25.51	17.30	25.24
Usp55	0.98	3.54	9.48	36.61
Usp7	18.55	16.74	41.04	8.37
Usp8	1.49	1.68	54.26	3.66
Usp9y	116.78	5.52	88.56	13.96

0
25
50
75
100
125
150
175
200

Using this analysis, we identified a total of 15 DUBs (**Figure 18a,b and Table 10)**. The majority of these knockdowns led to a reduction in the amplitude of the rhythms (**Table 10**). *Usp8* again stood out from the group, as its knockdown largely decrease the amplitude of both *Per2*-Luc and *Bmal1*-Luc bioluminescence rhythms (**Figure 18b, Figure 19 and Table 10**). This is consistent with our previous results both in PER2::Luc MEFs and in U2OS cells (**Figures 5, 10 and 11**). Indeed, the knockdown of *Usp8* and *Usp17l1p* decreased the amplitude of the

rhythms only when achieved in the *Usp2*-deficient background (**Figure 18b, 19 and Table 10**). We also found that *Usp55*, *Otud7a* and *Josd3* knockdowns reduced drastically the amplitude of cellular rhythms (**Figure 18b, Table 10 and Figure 19**).

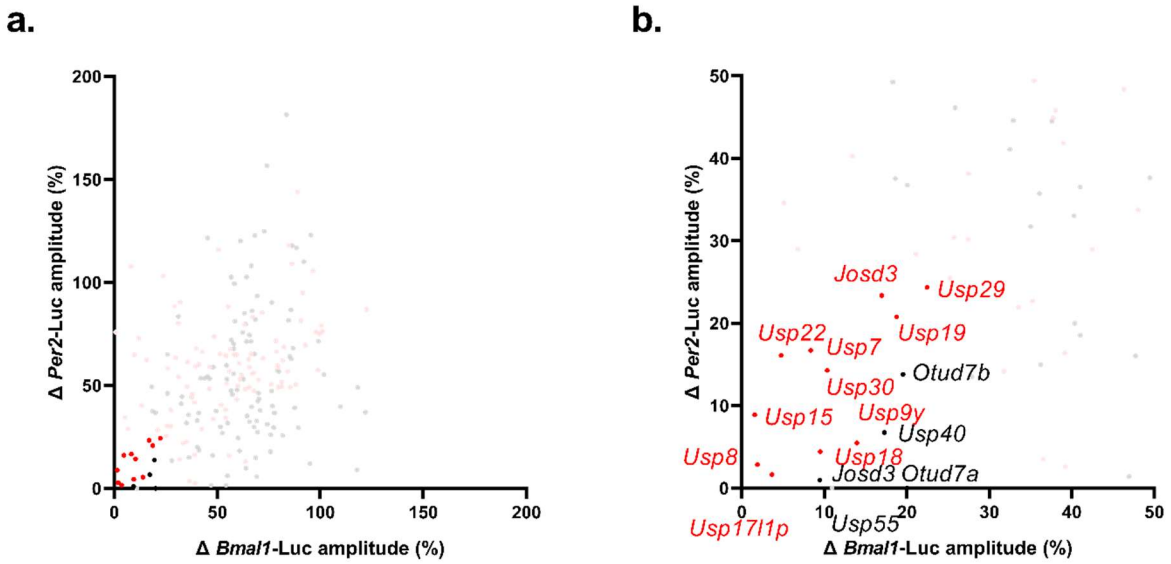


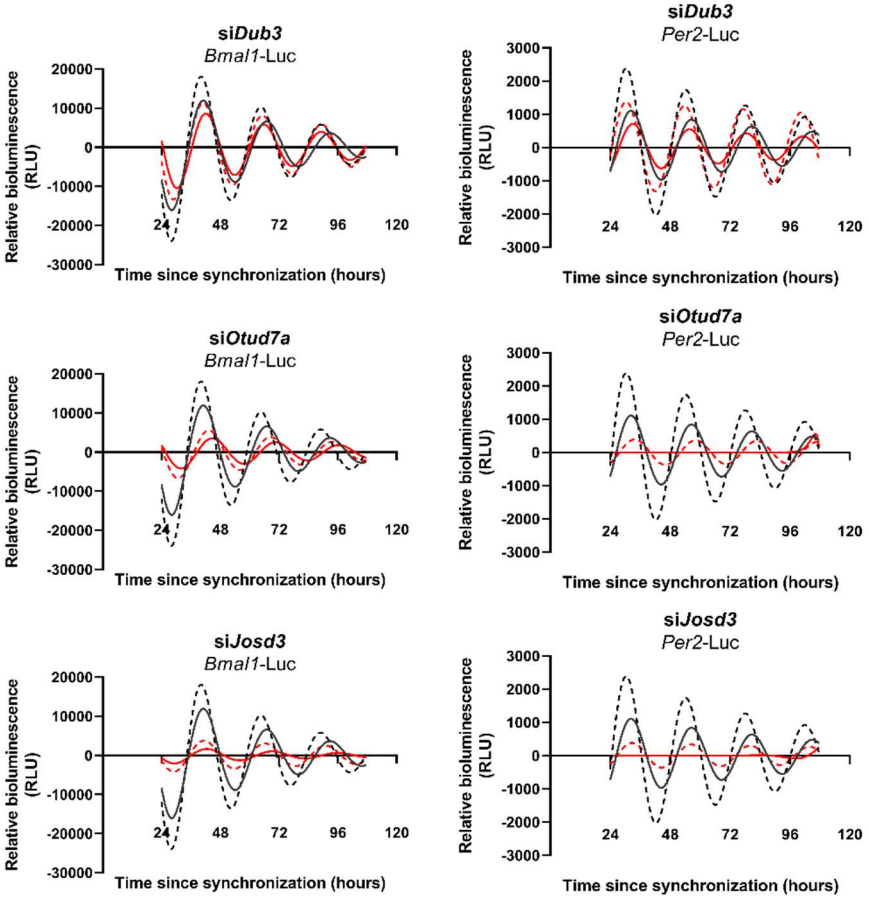
Figure 18. Amplitude analysis of the DUB siRNA screen. a. Percent changes in the amplitude of U2OS *Bmal1*-Luc and *Per2*-Luc rhythms following the knockdown of all DUBs. Changes were always calculated relative to the mean of both negative controls in each of the plate. Black dots represent DUB knockdowns in the control background, while red dots represent DUB knockdowns in the *Usp2* knockdown background. Darker dots represent the identified DUB hits using a cut-off value of ± 75%. **b.** Magnified plot of identified DUB hits from the amplitude analysis.

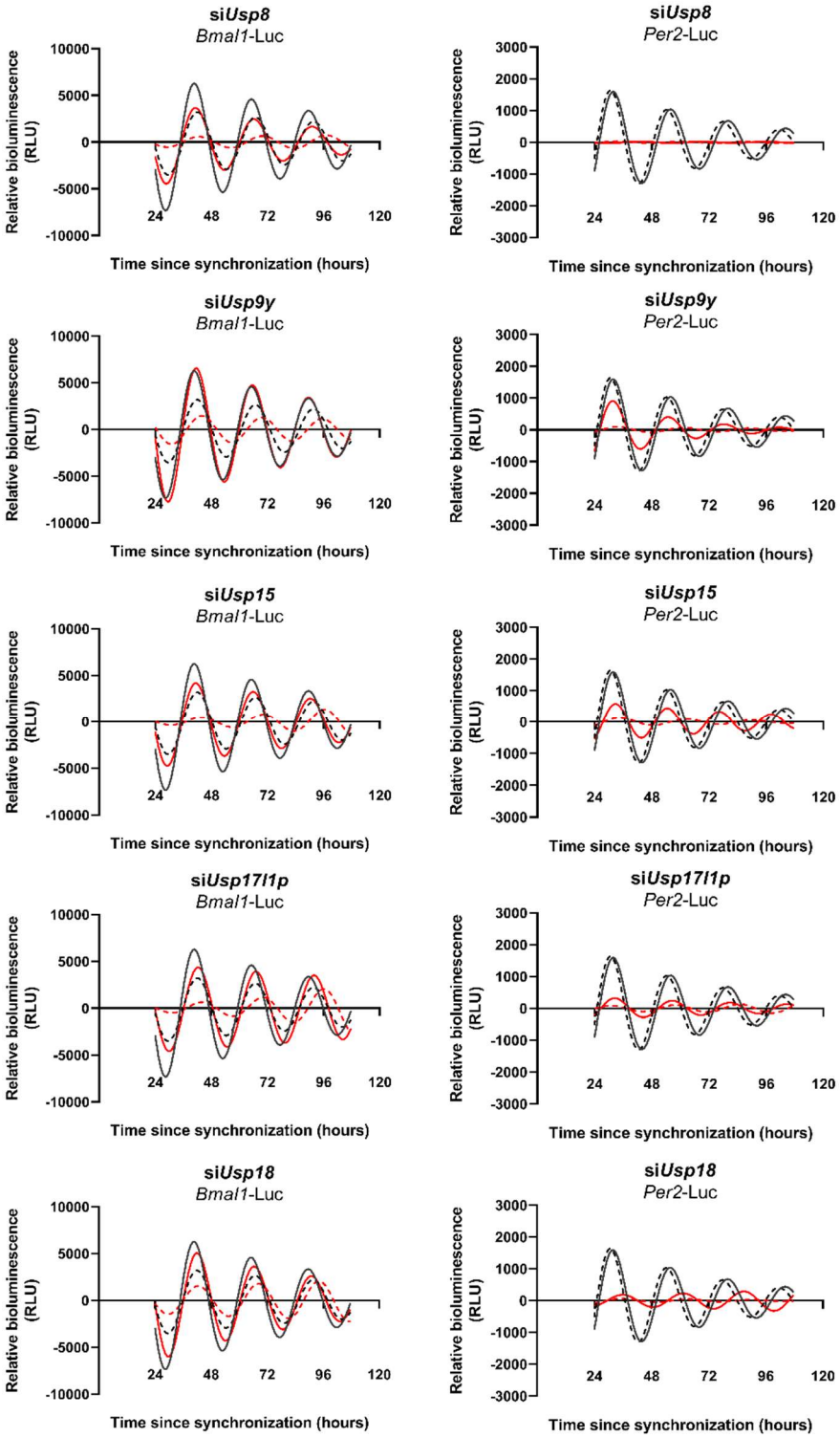
DUB	Δ si*Usp2* amplitude (%)	
	Per2-Luc	*Bmal1*-Luc
Otud7b	>200	>200
Usp15	23.70	3.14
Usp30	11.46	14.21
Usp55	>200	>200
Usp9y	4.73	15.76

0
25
50
75
100
125
150
175
200

Table 11. Identified DUBs following the si*Usp2* amplitude analysis. Identified DUBs and the precise percent changes in amplitude between their knockdown in the control background and in the *Usp2* knockdown background in both U2OS *Per2*-Luc and *Bmal1*-Luc. Using a cut-off value of ± 75%, five DUB knockdowns were found to have a more drastic effect on the amplitude when combined with *Usp2* knockdown. Reductions in the amplitude are highlighted in red, while increases are highlighted in blue.

Furthermore, we analyzed the amplitude changes following the combination of *Usp2* knockdown with the knockdown of DUBs. By comparing the amplitude levels upon DUB knockdowns in the control versus in the *Usp2* knockdown background and using the same cut-off value of ± 75%, we identified five DUBs: *Otud7b*, *Usp15*, *Usp30*, *Usp55* and *Usp9y.* Three combinations of *Usp2* and DUB deficiencies led to further decrease in the amplitude of bioluminescence rhythms. This was the case for the knockdowns of *Usp15*, *Usp30* and *Usp9y*. However, both *Otud7b* and *Usp55* knockdowns combined with *Usp2* deficiency resulted in greater amplitudes compared to their knockdown alone (**Figure 19 and Table 11**).




siUsp8
Bmal1-Luc
Relative bioluminescence (RLU)
10000
5000
0
-5000
-10000
24
48
72
96
120
Time since synchronization (hours)
siUsp8
Per2-Luc
Relative bioluminescence (RLU)
3000
2000
1000
0
-1000
-2000
-3000
24
48
72
96
120
Time since synchronization (hours)
siUsp9y
Bmal1-Luc
Relative bioluminescence (RLU)
10000
5000
0
-5000
-10000
24
48
72
96
120
Time since synchronization (hours)
siUsp9y
Per2-Luc
Relative bioluminescence (RLU)
3000
2000
1000
0
-1000
-2000
-3000
24
48
72
96
120
Time since synchronization (hours)
siUsp15
Bmal1-Luc
Relative bioluminescence (RLU)
10000
5000
0
-5000
-10000
24
48
72
96
120
Time since synchronization (hours)
siUsp15
Per2-Luc
Relative bioluminescence (RLU)
3000
2000
1000
0
-1000
-2000
-3000
24
48
72
96
120
Time since synchronization (hours)
siUsp17l1p
Bmal1-Luc
Relative bioluminescence (RLU)
10000
5000
0
-5000
-10000
24
48
72
96
120
Time since synchronization (hours)
siUsp17l1p
Per2-Luc
Relative bioluminescence (RLU)
3000
2000
1000
0
-1000
-2000
-3000
24
48
72
96
120
Time since synchronization (hours)
siUsp18
Bmal1-Luc
Relative bioluminescence (RLU)
10000
5000
0
-5000
-10000
24
48
72
96
120
Time since synchronization (hours)
siUsp18
Per2-Luc
Relative bioluminescence (RLU)
3000
2000
1000
0
-1000
-2000
-3000
24
48
72
96
120
Time since synchronization (hours)

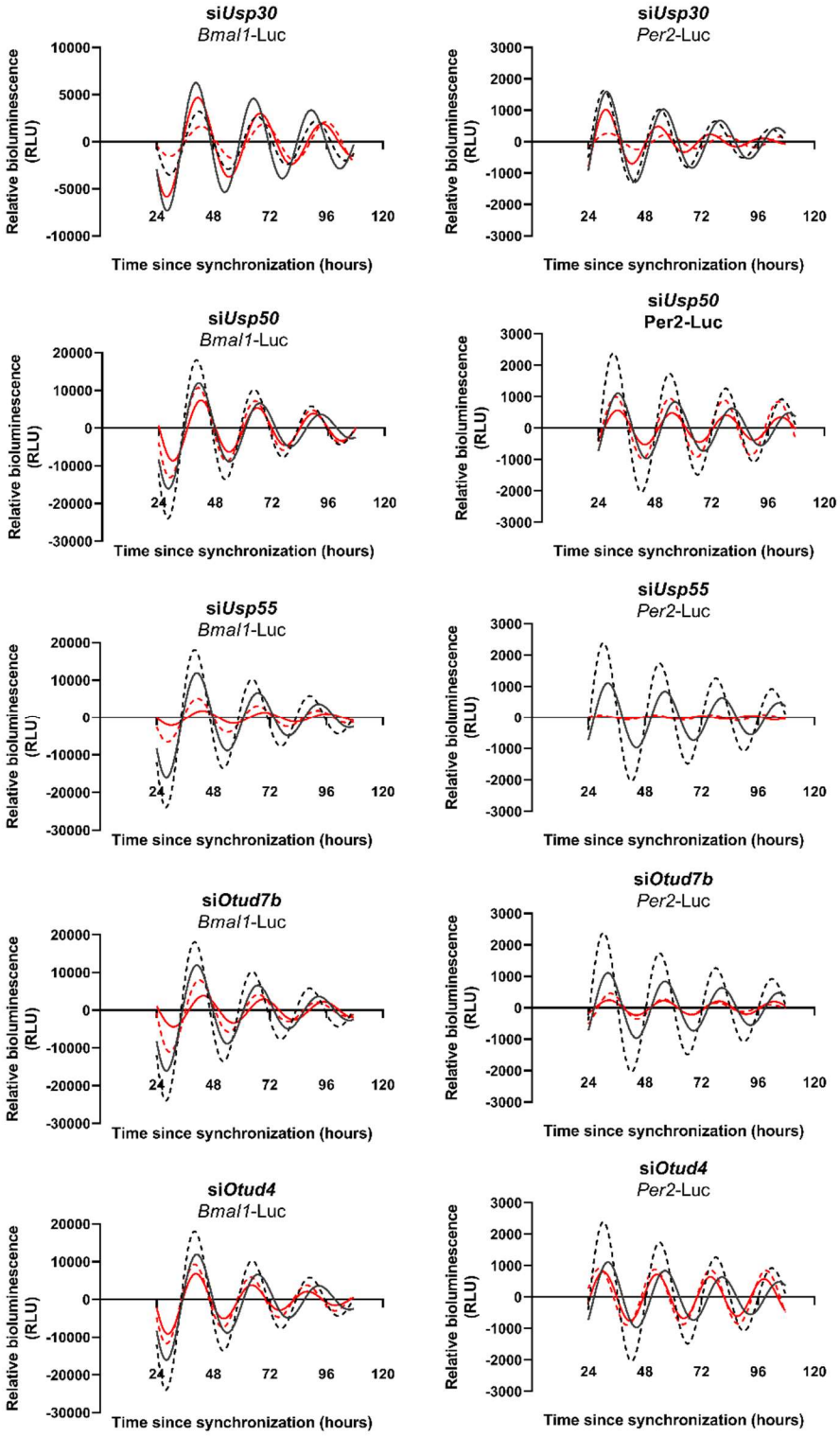


Figure 19. *Per2*-Luc and *Bmal1*-Luc bioluminescence rhythms following the knockdown of some DUBs identified in the screen. Black lines represent the negative control rhythms. Red lines represent the rhythms following DUB knockdowns. Full lines represent the rhythms in the control background. Dashed lines represent the rhythms in the *Usp2* knockdown background.

DISCUSSION

Circadian rhythms influence almost all physiological processes and their underlying mechanism is highly conserved amongst animal phyla organisms, further supporting their importance in biology. Although circadian rhythms are thought to mainly originate from the master clock in the brain, the SCN, it is now well known that other peripheral clocks are running in multiple tissues of the body (Finger and Kramer, 2021). It is thus possible to study circadian rhythms at a cellular level in order to understand globally the clock mechanism and to then apply this knowledge to understand tissue-specific clocks and physiological rhythms.

Previous studies have identified main clock components, their intrinsic relationships, as well as their implication in cellular rhythms (Partch et al., 2014). However, it becomes now necessary to understand their precise regulation. PTMs can modulate the function and stability of clock proteins. Ubiquitination, in particular, was found to play a critical role in determining the half-life of many clock proteins and therefore can control the robustness and the speed of the clock (Stojkovic et al., 2014). However, PTMs are reversible processes and deubiquitination is now also thought to be a player to consider in clock regulation (Srikanta and Cermakian, 2021). Past studies have found that USP2 can regulate the stability and cellular localization of various clock proteins (Scoma et al., 2011; Tong et al., 2012; Yang et al., 2014), but its loss in mice only led to mild circadian disruptions (Scoma et al., 2011; Yang et al., 2012). Our goal was therefore to identify other DUBs implicated in the circadian clock mechanism and to assess their possible redundancy with USP2.

Targeted approach

We first identified four candidate DUBs based on previous literature and bioinformatic analyses of transcriptomic datasets. Out of these four candidates, both USP8 and USP22 (homolog *Drosophila* NOT) had been previously investigated for their role in the circadian clock, although all studies were conducted in *Drosophila*. Interestingly, *Usp8* and *not* knockdowns specifically in clock neurons were found to lengthen the period of activity rhythms in flies (Bu et al., 2020; Luo et al., 2012; Mahesh et al., 2020), phenotypes which are similar to *Usp2* KO in mice (Yang et al., 2012). We also found that the amino acid sequences of USP8 and USP22 were similar to USP2, which suggested that they might share common substrates and similar roles. Indeed, USP8 and USP2 already have related functions in endosomal trafficking (Zhou et al., 2013). Furthermore, USP21 is the DUB the most similar to USP2 in the mammalian genome based on our alignment analysis and a previous phylogenetic study (Clague et al., 2013). It is therefore also possible that they share common substrates and functions. Finally, we chose UCHL1 as our fourth candidate mainly due to its high and rhythmic expression in the SCN (Dong et al., 2005). Although some research had already been conducted on the circadian behaviors of *Uchl1* mutant mice, its implication in the molecular mechanism of the clock remains unknown.

In contrast to data of *Drosophila* rhythmic behaviors, we did not find any effect of *Usp8 or Usp22* knockdowns on the period of cellular rhythms. The most interesting result we obtained from our targeted approach is the significant amplitude reduction of bioluminescence rhythms following *Usp8* knockdown, which was independent of whether it was conducted in WT or *Usp2* KO cells. Although the loss of clock-regulating enzymes often leads to changes in the period of bioluminescence rhythms (D'Alessandro et al., 2017; DeBruyne et al., 2015; Gossan et al., 2014;

Hirano et al., 2016b; Maier et al., 2009), previous studies on deubiquitinases and ubiquitin ligases involved in the clock have also observed a reduction in bioluminescence amplitude without a change in period (Zhang et al., 2018; Zhao et al., 2016). It is thus possible that *Usp8* is necessary for the robustness of circadian rhythms, but not for their period determination.

Usp8 knockdown-mediated dampening of bioluminescence rhythms

Following the reduction of bioluminescence rhythms upon *Usp8* knockdown in MEFs, we wanted to conduct a similar assay in another cell line. U2OS cells expressing luciferase under the control of either *Bmal1* or *Per2* promoter were already shown multiple times to produce robust and sustained bioluminescence rhythms (Baggs et al., 2009; Maier et al., 2009; Zhang et al., 2009). For both cell lines, we did not observe any consistent effects of the three siRNA treatments against *Usp8* on the period of bioluminescence rhythms, which is similar to our results in MEFs. Interestingly, in *Bmal1*-Luc cells, the combined effect of *Usp8* and *Usp2* knockdowns replicated the previously observed amplitude reduction. However, since we did not see this interaction effect between *Usp8* and *Usp2* deficiencies in *Per2*-Luc U2OS and PER2::Luc MEFs, it remains unclear as to whether *Usp8* and *Usp2* have a redundant role in mammalian circadian rhythms.

We also wanted to investigate other possible sources of this amplitude reduction. Our results suggest that this phenotype of *Usp8* knockdown is independent of cellular viability and of a direct destabilizing effect on the PER2::Luc reporter. We further investigated the role of *Usp8* in the clock by assessing the effect of its deficiency on clock genes expression at four different time points of the circadian cycle. While we did observe the expected pattern of expression for these three clock genes, we did not find any significant differences in the mRNA levels of *Per2*, *Bmal1* or *Rev-erbα.* Unfortunately, our assay might have lacked some power. Therefore, we

replicated this experiment at a single time point, when *Per2* expression was found to be the highest. However, we did not find any significant changes in *Per2* expression. As USP8 was shown to regulate the transcriptional activity of CLOCK in *Drosophila* (Luo et al., 2012), it is possible that its role is conserved in mammals. It might therefore be relevant in future experiments to look directly at the transcriptional activity of BMAL1 and CLOCK. It might also be interesting to study the expression of other CLOCK/BMAL1-controlled clock genes such as *Per1*, *Cry1* and *Cry2*. Of course, it would also be beneficial to study the interaction of USP8 with clock proteins, as it would be a more direct approach to identify post-translational roles of USP8 in the clock. Future experiments on the deubiquitinating activity of USP8 and its impact on the stability of clock proteins would also improve our understanding of its regulatory role in the clock.

There are three main limitations from our RNA interference approach. First, it is possible that the remaining levels of DUBs would be enough for them to accomplish their function in the clock. Clock proteins are thought to be expressed in very small amounts (Narumi et al., 2016) and thus, small amounts of DUBs could still be enough to target and modulate their function and stability. Recent progress in the efficacy and accessibility of the CRISPR/Cas9 genome editing technology could be useful in follow up studies to assess the effect of complete KOs of DUBs on cellular rhythms. Furthermore, specific DUB inhibitors are available and could be used to pharmacologically inhibit DUB activity (Farshi et al., 2015). This approach was previously used for studies on USP7 and USP14 (D'Alessandro et al., 2017; Hirano et al., 2016b; Papp et al., 2015). Secondly, RNA interference is based on the specificity of the RNA sequence with the targeted mRNA. However, siRNAs can bind other non-specific sequences in the cell which can lead to the degradation of unwanted targets. These non-specific effects of RNA interference are

thought to be the main drawback of these experiments, as it can lead to false positive results. The use of a non-targeting dsiRNA as a negative control can mimic the activation of the RNA interference cellular cascade, but although necessary, this control is not sufficient to account for non-specificity. Therefore, our goal in our candidate approach was to use multiple dsiRNA sequences targeting a single DUB. Accordingly, significant effects of knockdowns were only considered if at least two dsiRNA treatments showed similar phenotypes. Finally, siRNAs only maintain the knockdown of targeted proteins for a limited amount of time. Therefore, it was impossible with this approach to assess the long-term role of DUBs in circadian rhythms. In fact, DUBs could maintain the homeostasis of clock proteins without rapidly impacting their levels. Thus, a short-lasting loss of DUBs might not alter considerably clock proteins balance and might leave the pace of the clock unchanged. Complete KOs or long-lasting RNA interference against DUBs might therefore be necessary to assess the long-term influence of deubiquitination on circadian rhythms.

Global approach

With more than a hundred DUBs thought to exist in the mammalian genome (Clague et al., 2019), a screening approach was necessary to quickly assess their potential clock-regulating roles. Although it is clear that this approach could lead to false negative and false positive results due to some inevitable limitations such as low knockdown efficiencies and siRNA non-specific effects, this protocol enabled us to widely and rapidly assess the role of multiple DUBs in the cellular clock. In fact, this approach has already been used to identify kinases, micro RNAs and other modifiers of the clock (Maier et al., 2009; Zhang et al., 2009; Zhou et al., 2021). Furthermore, RNA interference screens have been extensively used to identify clock genes and clock regulators in *Drosophila* (Axelrod et al., 2015).

Following the period analysis of bioluminescence rhythms, we found six DUBs whose knockdown lengthened considerably the period of the rhythms. Interestingly, *Usp8* was amongst them. We indeed observed this phenotype when both *Usp8* and *Usp2* were knocked down together, which is not consistent with our previous U2OS and MEFs results, where no significant changes were observed. However, we did observe a strong decrease of amplitude following the knockdown of *Usp8* in our screen, which seems to be a consistent phenotype across our assays.

Otud4 knockdown resulted in the one of the most drastic reductions in period. Some literature research revealed that OTUD4 is involved in the Tumor Necrosis Factor β (TGFβ) pathway (Jaynes et al., 2020), which itself is related to the circadian clock, notably by modulating the expression of multiple clock genes (Gast et al., 2012; Sloin et al., 2018). The knockdown of *Dub3* (also known as *Usp17l2*) also led to a strong decrease in the period of bioluminescence rhythms, but we found no studies that could link it to circadian rhythms.

The knockdown of *Otud7b* led to a decrease in both the period and the amplitude of the rhythms in U2OS cells. OTUD7B, also called Cellular Zinc-Finger Anti-NF-κB (CEZANNE), is involved in the cell cycle notably by deubiquitinating the Lysine-Specific Demethylase 1A (LSD1), which regulates gene expression thought histone demethylation (Gong et al., 2021). Interestingly, LSD1 was previously shown to facilitate CLOCK/BMAL1-mediated transcription (Nam et al., 2014). It is also deubiquitinated and stabilized by another DUB, USP38 (Liu et al., 2018), whose knockdown in our screen led to a reduction in the period of bioluminescence rhythms.

The period and amplitude analyses revealed that both *Usp15* and *Usp9y* knockdowns largely impacted both parameters of the rhythms when combined with *Usp2* knockdown. Indeed, *Usp15* and *Usp9y* deficiencies in a *Usp2* knockdown background lengthened the period of

bioluminescence rhythms. Interestingly, this is similar to the period lengthening of locomotor activity rhythms previously observed in *Usp2* KO mice (Yang et al., 2012). Furthermore, we found in our alignment analysis that the protein sequence of USP15 is similar to USP2 with more than 44% amino acid identities between both DUBs. *Usp9y* is on the other hand closely related to *Usp9x* (Hall et al., 2003), a DUB already found to be involved in the clock (Zhang et al., 2018). Indeed, USP2 and USP9X interact with and regulate the stability of BMAL1 (Scoma et al., 2011; Zhang et al., 2018). It is therefore possible that both USP9Y and USP9X have redundant roles with USP2. Taken together, these results strongly suggest that USP15 and USP9Y might partially overlap with USP2 to regulate the clock.

Surprisingly, *Usp55* and *Otud7b* individual knockdowns led to a drastic decrease in amplitude, but this effect was partly reversed by its combination with *Usp2* knockdown. Perhaps, these DUBs and USP2 have counteracting roles in the clock. This type of relationship between post-translational enzymes have already been demonstrated with FBXL3 and FBXL21, where both ubiquitin ligases were shown to have opposite effects on the periodicity of circadian rhythms (Yoo et al., 2013). It is also to note that no previous studies have looked at the role of USP55 in biological processes. It was solely reported that this DUB is thought to be a highly divergent member of the USP family (Nijman et al., 2005). Whether the effect we saw were non-specific or whether *Usp55* is a novel DUB worth being investigated in the context of circadian rhythms will be confirmed by future validation of our results.

Overall, through our RNA interference screen, we were able to identify multiple interesting DUBs who have a good potential to be involved in the clock mechanism. It would however be important to validate these results and to pursue further research on their roles and their interactions in the molecular clock.

Usp2 KO and knockdown effects on cellular rhythms

Although it was not our primary objective, it is also important to note that we did find some significant effects of *Usp2* KO or knockdown on cellular circadian rhythms. In all MEF experiments, amplitude analyses revealed significant effects of *Usp2* KO, as *Usp2* loss seemed to increase the amplitude of the rhythms. A similar effect was indeed observed in a previous study, where the knockdown of *Usp2a* was shown to increase the amplitude of *Bmal1*-Luc U2OS cells (Tong et al., 2012). Moreover, we observed a significant lengthening main effect of *Usp2* knockdown on the period of *Per2*-Luc and *Bmal1*-Luc U2OS rhythms, which is consistent with behavioral data on *Usp2* KO mice (Yang et al., 2012). We however did not see this effect in PER2::Luc MEFs, which suggest that USP2 might have a tissue-specific or cell-specific role in the clock.

Redundancy of DUBs

Our main objective in this thesis was to identify DUBs with a role in the clock redundant with that of USP2. Our first approach did not identify any interaction of the knockdowns of candidate DUBs with *Usp2* KO, suggesting no redundancy between these enzymes. However, we did find some interaction between the knockdowns of *Usp8* and *Usp2* in our U2OS assays. Using our global approach, we also found a considerable change in the period and amplitude of U2OS rhythms upon the combination of *Usp8* and *Usp2* knockdowns. These discrepancies make the interpretation of redundancy between these two DUBs difficult. A complete double KO might be necessary to fully assess their redundancy in the clock. Furthermore, identifying clock protein targets of USP8 might reveal common interactions with USP2, which could suggest some overlapping functions. Finally, *in vivo* study of *Usp8*/*Usp2* double KO mice would lead to a more global understanding of the combined roles of these deubiquitinases in circadian behaviors.

It is to note however, that *Usp8* KO mice are embryonically lethal (Niendorf et al., 2007). Thus, a more targeted approach to knock out *Usp8* in mice might be necessary to study the implications of USP8 in behavioral rhythms

While the mammalian genome contains around a hundred DUBs (Clague et al., 2019), their action is balanced by more than 600 E3 ubiquitin ligases (Li et al., 2008). Thus, it seems likely that each DUB targets multiple substrates and have various roles in the cell. Indeed, DUBs were shown to recognize certain types of ubiquitin chains and positions and their specificity might originate more from ubiquitin architecture than from their substrate (Clague et al., 2019). Consistent with this principle is USP2, who has already been shown to interact and deubiquitinate BMAL1, CRY1 and PER1, although these clock proteins are structurally very different (Scoma et al., 2011; Tong et al., 2012; Yang et al., 2014). Furthermore, ubiquitination is complex in itself. Proteins can be poly-ubiquitinated at a single lysine residue or be mono-ubiquitinated at multiple sites (Sadowski and Sarcevic, 2010). Multiple DUBs could therefore interact with a single substrate by interacting with different ubiquitin moieties. Based on the ubiquitin structure specificity of DUBs mentioned above, it is likely that multiple DUBs act on a single clock protein to modulate it.

The USP family of deubiquitinases, in particular, is thought to contain multiple redundant DUBs, as many of them are evolutionally close (Zachariah and Gray, 2019). For example, USP4 and USP15 have many overlapping substrates and mainly function in similar pathways (Long et al., 2014; Song et al., 2010). Indeed, USP21 and USP2 have diverged relatively recently in evolution (Zachariah and Gray, 2019). However, we did not find any effect of *Usp21* deficiency on cellular rhythms. Perhaps, USP21 is implicated in other peripheral clocks such as the liver, where its expression is significantly rhythmic (**Figure 20**). Indeed, since our study only looked at

the role of DUBs in cancerous epithelial cells and primary fibroblasts, we could have missed DUBs involved in the clock of other cell types. A similar screening approach could be used in primary hepatocytes or neuronal cell lines with proteomes closer to that of the SCN and of the liver in order to account for this limitation.

Figure 20. *Usp21* rhythmic expression in the liver. Using the CircaDB online database (Pizarro et al., 2013b) (http://circadb.hogeneschlab.org/), *Usp21* transcript was found to be significantly rhythmic (q=0.0092) following the analysis of a mouse liver microarray dataset (Zhang et al., 2014). Tissue was collected every two hours for two days in constant darkness.

Speculative roles of DUBs in the clock

As previous studies on DUBs have identified their clock targets, it is now clear that DUBs can directly deubiquitinate clock proteins (**Figure 21b**). Additionally, it would be possible that some DUBs have more prominent roles in specific tissues. Unfortunately, our study was limited by the use of fibroblast or cancerous cell lines, and we did not assess tissue-specificity of

DUBs. Furthermore, it is now well known that most clock proteins have redundant partners. For example, NPAS2 and CLOCK were both shown to be in complex with BMAL1 and to activate E-box-mediated transcription (Bertolucci et al., 2008; Dardente and Cermakian, 2007). Other groups of partially redundant clock proteins also exist, which include BMAL1 and BMAL2 (Dardente and Cermakian, 2007; Shi et al., 2010). Therefore, DUBs might also deubiquitinate specifically one of these overlapping partners in the clock (**Figure 21a**).

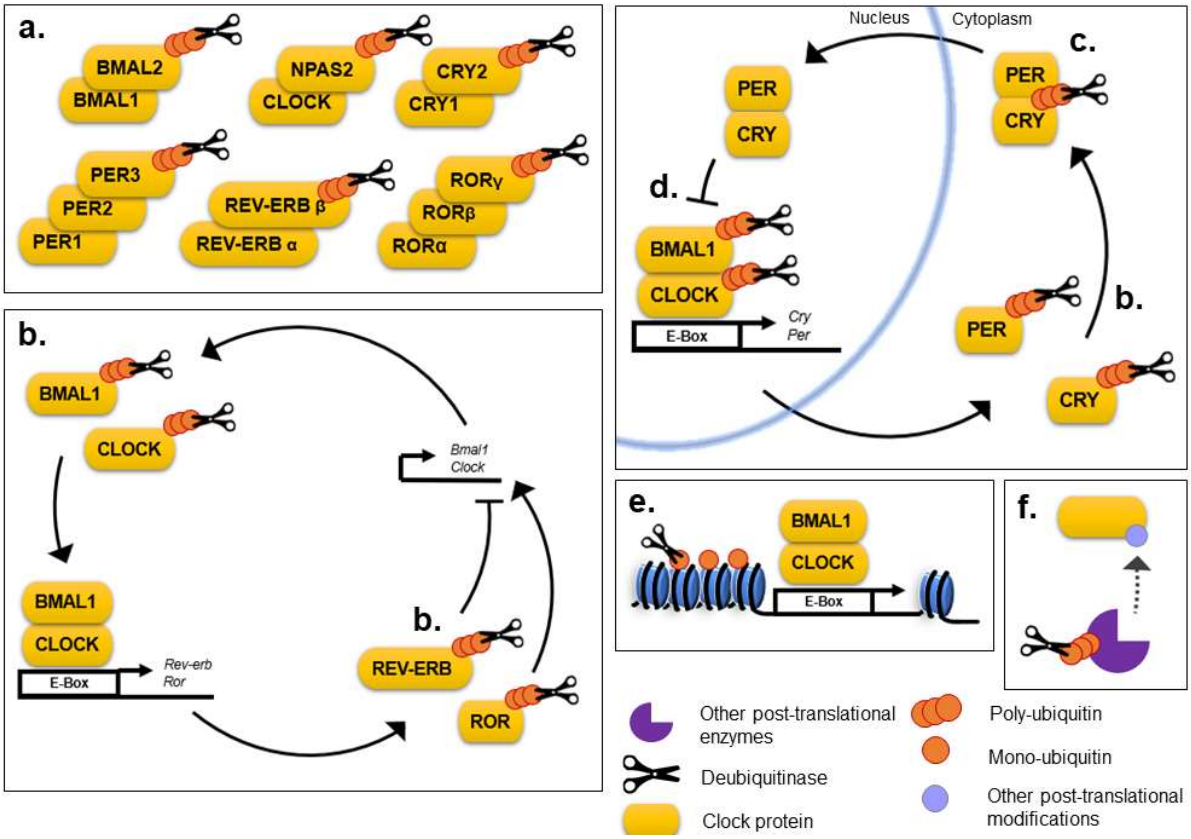


Figure 21. Speculative roles of DUBs in the circadian clock. a. Some DUBs might interact more specifically with redundant or similar clock proteins. **b.** DUBs can directly interact with clock proteins to affect their stability in every loop of the circadian clock. **c.** DUBs can deubiquitinate clock proteins to affect their nuclear translocation. DUBs could also modulate protein-protein interactions of clock proteins. **d.** DUBs can regulate the transcriptional activity of some clock proteins. **e.** DUBs can remove the mono-ubiquitination of histones in order to modulate the transcription of clock genes and clock-controlled genes. **f.** DUBs can deubiquitinate other post-translational enzymes, such as kinases, ubiquitin ligases, phosphatases and acetylases, to modulate their function and prevent or promote PTMs.

In addition to the repression of proteasomal degradation, deubiquitination can also alter the cellular trafficking and activity of clock proteins (**Figure 21c.d.**). Only one study so far has investigated the role of DUBs in the nuclear translocation of a clock protein (Yang et al., 2014), but it is likely that this kind of regulation by DUBs also takes place for other clock components, notably CRYs, REV-ERBs and RORs.

Studies in *Drosophila* have established deubiquitination of histones as an important regulation of transcription in the clock mechanism (**Figure 21e**) (Bu et al., 2020; Mahesh et al., 2020). However, although some research found evidence of a similar mechanism in mammals (Tamayo et al., 2015), this regulatory role of DUBs is still poorly understood.

Furthermore, DUBs probably affect the activity of other clock-regulating post-translational enzymes (**Figure 21f**). In fact, it is the case for USP4-mediated deubiquitination of the ubiquitin ligase TNF Receptor-Associated Factor 2 (TRAF2), which is involved in the repression of BMAL1 activity (Chen et al., 2018a; Xiao et al., 2012), although this effect was not yet linked to the circadian clock. Ubiquitin can also itself be modified at a post-translational level. Notably, it can be phosphorylated and acetylated, which can further impact the function of targeted proteins (Song and Luo, 2019). These chains of post-translational events suggest that the regulation of the molecular clock is probably more complex than we initially thought. In fact, post-translational modifying enzymes can also interact together to precisely modulate the function of their substrates. For example, SUMOylation of BMAL1 was shown to precede its ubiquitination and consequent degradation (Lee et al., 2008). BMAL1 phosphorylation by Protein Kinase Cγ (PKCγ) was inversely shown to promote its deubiquitination and stability (Zhang et al., 2012).

Finally, it was suggested that some enzymes can read a precise “ubiquitin code” created by different linkages and configurations of ubiquitin signals (Stojkovic et al., 2014). As DUBs are able to modify this code by changing and modulating ubiquitin architecture, it becomes clear that they are key regulators of protein functions in a multitude of cellular processes, which most probably includes circadian rhythms.

CONCLUSION

This research on deubiquitination is essential as it will improve our fundamental knowledge of the molecular clock through the identification of new deubiquitinases involved in its regulation. In previous years, most studies have focused on identifying kinases and ubiquitin ligases involved in the clock. However, up to now, only a few clock-regulating deubiquitinases have been identified and no other study has screened all DUBs for their role in circadian rhythms. Consequently, this work will further establish deubiquitination as a cornerstone of clock mechanism.

Out of the four DUBs chosen through databases analyses and published literature, only *Usp8* knockdown resulted in a significant cellular phenotype, which indicates a possible role for USP8 in the mammalian circadian clock. Indeed, we found that *Usp8* knockdown decreased the amplitude of PER2::Luc and *Per2*-Luc bioluminescence rhythms in MEFs and U2OS cells respectively and that the combination of both *Usp8* and *Usp2* knockdowns also resulted in a reduction in amplitude of U2OS *Bmal1*-Luc cells.

To complement this specific approach, we assessed the clock-regulating role of all known human DUBs using an RNA interference screen. This approach led us to identify 40 DUBs whose deficiency either changed the period, the amplitude or both parameters of bioluminescence rhythms. Furthermore, we identified some potential USP2-rendundant DUBs, since their combined knockdowns were shown to amplify cellular rhythms disruptions. Although it is still early to state any involvement of these DUBs in the circadian clock, we hope future research will confirm that some of these DUBs are key regulators of circadian rhythms.

Circadian disruptions were shown to be risk factors for multiple diseases (Touitou et al., 2017) and other disorders, such as schizophrenia, were found to impact circadian behaviors

(Delorme et al., 2020). A better understanding of clock regulatory mechanisms such as deubiquitination is thus necessary to develop novel treatments and prevention strategies. In fact, DUB inhibitors are already a promising field in the development of treatments against cancer (Farshi et al., 2015; Schauer et al., 2020). Therefore, the identification of novel clock-regulating DUBs will expand potential drug targets aiming to modulate circadian rhythms and reduce adverse health effects of circadian disruption.

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