# Aged Stem Cells Reprogram Their Daily Rhythmic Functions to Adapt to Tissue-Specific Stress

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# Aged Stem Cells Reprogram Their Daily Rhythmic Functions to Adapt to Tissue-Specific Stress

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“Twenty years from now you will be more disappointed by the things you didn't do than by the things you did do. So throw off the bowlines. Sail away from the safe harbour. Catch the trade winds in your sails. Explore. Dream. Discover.”

– H. Jackson Brown Jr.

Aos meus pais e à minha irmã por me apoiarem incondicionalmente e me ajudarem a tornar possível cada um dos meus sonhos sem nunca perder o rumo.

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## *Abstract*

The correct timing of adult stem cell function is essential for tissue homeostasis. Our group was among the first to show that circadian rhythms segregate important stem cell functions along the day. For instance, in mouse epidermal stem cells this mechanism ensures that their proliferation occurs at night and not during day, when maximal oxidation takes place. Likewise, circadian rhythms separate DNA replication from the time when human epidermal stem cells are exposed to UV radiation. Importantly, circadian control of stem cells is a general mechanism operating in hematopoietic, mesenchymal, muscle, neural, and intestinal stem cells, as shown by others. A current dogma in the field is that circadian rhythms dampen during ageing, and that this dampening is in fact a major cause of many traits associated to ageing. With this in mind we decided to ask: is the timing of stem cell function altered during ageing? If so, how is it perturbed? Are the changes common, or stem cell-specific? To study these questions, we have performed a large-scale analysis of the circadian transcriptome of FACS-sorted stem cells from adult and aged mice, which has provided us with an unprecedented understanding of how their physiology changes during ageing. We chose two types of stem cells for our study, those from the epidermis and the skeletal muscle, since they represent two populations with very different behaviours (the former being highly proliferative, while the latter remaining predominantly quiescent throughout the lifetime of the mouse).

Unexpectedly, we find that aged mice remain behaviourally circadian, and that their epidermal and muscle stem cells retain a robustly rhythmic core circadian machinery. However, the oscillating transcriptome is extensively reprogrammed in aged stem cells, switching from genes involved in homeostasis to those involved in tissue-specific stresses, such as DNA damage or inefficient autophagy. Importantly, deletion of circadian clock components (Bmal1, or Period-1 and Period-2) did not reproduce the hallmarks of this reprogramming, underscoring that rewiring, rather than arrhythmia, is associated with physiological ageing. Normal homeostatic functions of adult stem cells have rhythmic daily oscillations that are believed to become arrhythmic during ageing.

Age-associated rewiring of the oscillatory diurnal transcriptome is significantly prevented by long-term caloric restriction in aged mice. This striking effect further highlights the anti-ageing benefits of this type of diet. Conversely, although a high-fat diet strongly reprograms the circadian output of both stem cells, there is little overlap with the age-related rewired oscillating transcriptome. Thus, stem cells rewire their diurnal timed functions to adapt to metabolic cues and to tissue-specific age-related traits.

## *Resumen*

El control temporal de las funciones de las células madre es esencial para la homeostasis de los tejidos. Nuestro grupo de investigación fue uno de los primeros en mostrar como los ritmos circadianos coordinan distintas funciones celulares importantes a lo largo del día en las células madre epidérmicas de ratón. Además, tal y como han demostrado otros grupos, el control circadiano de las células madre, constituye un mecanismo de vital importancia en el caso de las células madre hematopoyéticas, mesenquimales, musculares, neurales e intestinales. Actualmente, en este campo de investigación, el dogma prevalente es la existencia de un desajuste progresivo de los ritmos circadianos durante el envejecimiento. Este desajuste se cree que puede ser el causante de muchas de las manifestaciones asociadas con el envejecimiento. Considerando todos estos aspectos, nos cuestionamos ¿está el control temporal de la función de las células madre alterado durante el envejecimiento? Y en caso de estar afectado, ¿de qué forma está perturbado?, ¿Existen cambios comunes durante el envejecimiento o estos cambios son específicos de cada tejido? Para resolver estas incógnitas hemos realizado un análisis a gran escala del transcriptoma circadiano de células madre de epidermis y músculo esquelético de ratones adultos y de edad avanzada. Estas células se obtuvieron a través de FACS (*Fluorescence-Activated Cell Sorting*) y los resultados obtenidos de este transcriptoma nos han permitido entender los cambios fisiológicos que ocurren durante el envejecimiento en estos tipos celulares tan dispares: la epidermis es altamente proliferativa mientras que en el músculo esquelético las células madres permanecen quiescentes durante la vida del ratón.

Sorprendentemente, observamos que los ratones de edad más avanzada permanecen con un comportamiento circadiano, y que sus células madre epidérmicas y de músculo esquelético presentan una maquinaria circadiana robusta. A pesar de ello, el transcriptoma oscilatorio está reprogramado de una forma extensa en las células madres de más edad, cambiando los genes involucrados en homeostasis por aquellos asociados a estrés específico de tejido, como el daño celular o la autofagia ineficiente. Cabe destacar que la deleción de componentes del reloj circadiano (BmaI1 o Period-1 i Period-2) no reproduce las marcas distintivas de esta reprogramación sino que causa arritmia circadiana, que está asociada con envejecimiento fisiológico. Así pues, las funciones homeostáticas de las células madre adultas tienen unas oscilaciones rítmicas diarias alteradas - aunque no perdidas - durante el envejecimiento.

La reprogramación del transcriptoma oscilatorio diurno asociada con la edad está significativamente reducida en los ratones sometidos a una restricción calórica a largo

plazo. Este efecto tan dramático resalta los beneficios antienvejecimiento de este tipo de dieta. En cambio, a pesar de que una dieta rica en grasas reprograma el ritmo circadiano de ambas células madre (epidérmicas y de músculo esquelético), hay poca superposición con la reprogramación debida al envejecimiento. Así pues, las células madre reprograman sus funciones diurnas para adaptarse a las señales metabólicas y a las características específicas de tejido asociadas con el envejecimiento.

## *List of abbreviations*

SC - stem cell

ESC - embryonic stem cell

iPSC - induced pluripotent stem cell

ECM - extracellular matrix

EpSC - epidermal stem cell

MuSC - muscle stem cell

MRF - myogenic regulatory factor

H3K27me3 - histone H3 lysine 27 trimethylation mark

H3K4me3 - histone H3 lysine 4 trimethylation mark

HF - hair follicle

IFE - interfollicular epidermis

TA - transiently amplifying

EPU - epidermal proliferative unit

CP - committed progenitor

EDU - epidermal differentiation unit

MCSP - melanoma chondroitin sulphate proteoglycan

EGFR - epidermal growth factor receptor

SCN - suprachiasmitc nucleus

ZT - zeitgeber (“time giver”)

TRF - time restricted feeding

HFD - high fat diet

CR - caloric restriction

SIRT - NAD+-dependent deacetylase Sirtuin

Per1/Per2dKO - Per1 and Per2 double-mutant mice

WT - wild type

ROS - reactive oxygen species

mtDNA - mitochondrial DNA

SOD2 - superoxide dismutase 2

NHEJ - non-homologous end joining

BER - base excision repair

mTORC1 - mammalian TOR complex 1

L:D - cycles of 12h of light followed by 12h of darkness housing conditions

GO - Gene Ontology

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## *Introduction*

“It is remarkable that after a seemingly miraculous feat of morphogenesis a complex metazoan should be unable to perform the much simpler task of merely maintaining what is already formed.”

- G.C. Williams (1957)

## *Stem cells*

The term ‘stem cell’ (Stammzelle) was first created in 1868 by Ernst Haeckel, a firm advocate of Darwin’s theory of evolution, while describing the ancestral unicellular organism that evolved and originated all multicellular organisms (Haeckel, 1968). Later on, around 1900, Artur Pappenheim and his colleagues made use of the term ‘stem cell’ as the designation of the “common progenitor” concept they supported (Pappenheim, 1896). Since then, this concept has been thoroughly accepted and has become the basis of embryonic development, organ maintenance and tissue regeneration studies.

Nowadays, stem cells (SCs) are defined according to their behaviour, more than by the particular genes that they may express. These are cells capable of self-renewing and differentiating into more specialised cell types. In the case of embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC), these cells can give rise to all three embryonic lineages and, as a result, to all cell types in the mammalian body. Therefore, such cells have been designated as pluripotent (Evans and Kaufman, 1981; Martin, 1981; Takahashi and Yamanaka, 2006). On the other hand, adult SCs, which remain present in the body during adulthood, are only capable of differentiating into a specific set of cell types (multipotent and unipotent SCs). Nonetheless, throughout life, adult SCs are responsible for the regeneration and replenishment of their respective tissue either upon injury or simply during tissue homeostasis (Fuchs and Chen, 2013).

Due to such characteristics, SCs have been the central focus of regenerative medicine. Either by using endogenous SCs or exogenous replacements originated from progenitor or SCs, SCs seem to hold the key for tissue rejuvenation and repair. For organs lacking a SC pool, iPSCs represent a viable and promising strategy.

Due to SCs’ long lasting nature and their established proliferative capacity, it has been hypothesised that these may be accumulating sufficient mutations to, for instance, develop certain types of cancer. For example, skin tumours such as sebaceous adenomas, basal cell carcinomas, squamous cell carcinomas, and different types of hair tumours, display multilineage differentiation, the main characteristic of multipotent SCs (Ambler and Määttä, 2009; Locke et al., 2005). Therefore, it is of high interest to further expand the understanding we have on what factors regulate adult SC function and how those may become deregulated later in life. Finally, the clinical success achieved with SC therapy highlights the crucial importance of this field.

## *Adult stem cells*

During embryonic development, cell division ensures the emergence of new cell types and the continuous increase of the embryo's cell number. After birth, cell proliferation is instead mostly responsible for maintaining a constant number of cells in each tissue both after injury and cell death. Since most cells in adult tissues are terminally differentiated and not long-lived, tissue replenishment is driven by adult SCs and the highly proliferative progenitors that they originate. Several reservoirs of adult SCs can be found in the human body, each one of them with the capacity to self-renew ensuring their own long-term maintenance, and differentiate into particular cell types repairing possible tissue injury. Certain progenitor cells are also known to self-renew and contribute to tissue cell number. However, contrary to such progenitors, adult SCs retain this self-renewing capacity over most of the organism's life (He et al., 2009). This does not imply an unlimited proliferative potential nor that adult SCs undergo constant self-renewal. Instead, this means that the proliferation rate of each SC within their own tissue is fine-tuned, according to the needs of the tissue and in order to preserve the SC population throughout life. Consequently, different adult SCs populations exhibit remarkably different self-renewal and proliferative frequency and potentials. These distinct cell populations can be found in the stomach, mammary gland, prostate, muscle, intestine, blood and skin, among others (Barker et al., 2007; Bjerknes and Cheng, 2002; Blanpain and Fuchs, 2006; Huang et al., 2015; Karthaus et al., 2014; Keller and Snodgrass, 1990; Relaix and Zammit, 2012; Shackleton et al., 2006; Stingl et al., 2006). It has also become clear in recent years how strongly the tissue microenvironment and systemic cues impact on SC function. Extracellular signals can dictate whether a SC stays quiescent or becomes active, whether it proliferates and how often, and what cell lineages it differentiates into. The tissue microenvironment, also often defined as niche, is constituted by the extracellular matrix (ECM) and any cell or tissue in close proximity to a given SC population. The niche communicates to the SC both the state of the local tissue but also of the whole organism, and it does so through stromal cells, immune cells, vasculature, neuronal fibres, and the ECM. Hormones, growth factors, immune-derived and other signalling molecules travel through the vasculature from any organ and either affect directly SC function or first modulate the local niche. These signals may indirectly influence SC function by changing the nature and the number of resident immune cells, or even their secretome (Neves et al., 2017).

Adult SCs may be categorised based on the resident tissue turnover and, hence, demand for repopulation. Tissues such as the intestine, the hematopoietic system and the skin are characterised by a very high turnover and require constant fuelling from the SC compartment. In other tissues, adult SCs are only activated upon very specific stimuli. In the

brain adult SCs become active during the learning process when new neural circuits must form. In the skeletal muscle, adult SCs play a minimal role in muscle maintenance but vigorously engage in regeneration after injury. Notwithstanding, the proper function of these different populations of adult SCs is required throughout life and their decline is often associated with tissue malfunction and ageing (Goodell and Rando, 2015).

In the present study, we have decided to focus on epidermal SCs (EpSCs) and muscle SCs of the skeletal muscle (MuSCs, or satellite cells). In spite of sharing an aged-associated functional decay, these SC types are characterised by their contrasting proliferative behaviour. Therefore, these represent two extreme SC populations that could potentially age through very distinct mechanisms.

MuSCs were first identified by electron microscopy by their anatomical localisation and unique morphology in the 60's by Alexander Mauro and Bernard Katz. They were defined as mononucleated dormant cells, ready to repair damaged muscle fibres when needed (Katz, 1961; Mauro, 1961). Since then, numerous studies have confirmed these as the skeletal muscle SCs. They give rise to myoblasts contributing to muscle growth, repair and homeostasis (Kuang et al., 2008; Scharner and Zammit, 2011). However, *Myod*$^{Cre}$ and *Pax7*$^{CreERTM}$ based studies have almost unequivocally demonstrated the unipotency of adult satellite SCs, since most if not all seem to give rise only to the myogenic lineage (Lepper et al., 2009; Starkey et al., 2011).

EpSCs were first described in the early 70s as slow cycling and long-lived cells. They have the capability of differentiating and renewing into all the cell lineages and layers of the epidermis (Cotsarelis et al., 1990). Due to their easy accessibility and well-studied developmental stages, the skin and, more specifically, the epidermal SCs became one of the most frequently used models for the study of adult SCs. However, the degree of complexity of this model has become more obvious over the years. It is now evident that more than one SC compartment resides within the epidermis. Such different SC niches are believed to almost separately maintain the interfollicular epidermis, the hair follicles and the glands. Nevertheless, the cross-talk between these niches has been consistently demonstrated, for instance in the context of wounding. So far, most efforts in understanding the cellular and molecular dynamics of epidermal stem cells have been placed on SCs of the hair follicle. However, and since the purpose of this thesis was to study the age-related functional changes of SCs in homeostatic conditions, we have uniquely investigated interfollicular SCs that are the utmost important population for the skin barrier maintenance (Page et al., 2013; Plikus et al., 2012).

## *The muscle and satellite stem cells*

The skeletal muscle represents over 30% of an average woman's body weight and around 38% of an average man's. Its main function is contraction, which allows the body to create force and movement. The skeletal muscle is both rich in connective tissue and highly vascularised to ensure the necessary nutrients reach the tissue. It is constituted of bundles of myofibres wrapped in a basal membrane. Each individual myofibre, as a syncytial cell, can be controlled by hundreds of myonuclei in vertebrates, and contains a high density of tubular myofibrils within its plasma membrane. These myofibrils are composed of repetitive sections of sarcomeres, units of myosin and actin filaments. After neuronal activation, the sarcomeres contract and generate force through a "sliding mechanism" of each myosin-rich thick filament over actin-rich thin filaments **(Fig. 1)** (Scharner and Zammit, 2011).

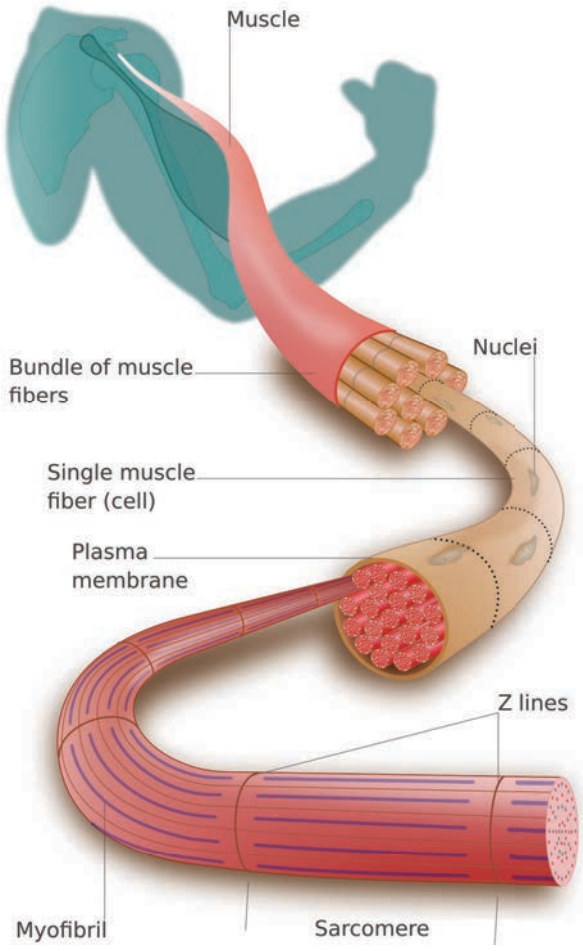



**Figure 1 - The architecture of the skeletal muscle.**

The skeletal muscle is formed by bundles of muscle fibres wrapped in a basal membrane. A single myofibre is densely packed with myofibrils and may be controlled by hundreds of myonuclei. Each myofibril is composed of myosin and actin filaments that are repeated in units designated sarcomeres. Neuronal activation drives the contraction of the sarcomeres that generate force through a "sliding mechanism" of each myosin-rich thick filament over the actin-rich thin filaments.

In mammals the nuclei of myofibres are post mitotic, hence, when needed, satellite cells function as a supply of new myonuclei. During muscle tissue homeostasis, satellite SCs contribution to myofibre maintenance is minimal. Surprisingly, it has been shown that even after 6 months of satellite cell depletion from muscle, the tissue displays no evident changes or strong signs of atrophy. Satellite cells are, nevertheless, essential for muscle regeneration upon injury (Cheung et al., 2012; Lepper et al., 2009; 2011). They reside on the surface of myofibres, between their plasma membrane and the basal membrane that wraps up the fibres along their length, where they sit in a quiescent state while scanning the muscle fibres for damage (Scharner and Zammit, 2011). Satellite cells display higher nuclear-to-cytoplasmic ratio, lower organelle content, and a smaller nucleus size with increased amounts of heterochromatin in comparison with myonuclei. Such characteristics reflect the mitotically dormant state of these cells. Once activated, they enter cell cycle and give rise to myogenic precursor cells that then undergo several rounds of cell division before fusing with existing or new myofibres (Schultz, 1976; Schultz et al., 1978).

Due to the distinct morphology and specific localisation of muscle satellite cells, it has been possible to unequivocally identify them by electron microscopy. As consequence, accurate markers have also been identified and such markers have been the key to isolate the satellite cells. The paired box containing transcription factor Pax7 has been shown to mark both quiescent and activated satellite cells (Seale et al., 2000). Pax7(+) satellite cells are capable of self-renewing and differentiating upon muscle fibre damage. During muscle repair, a subpopulation of Pax7(+) cells returns to quiescence to maintain the satellite cell pool (Abou-Khalil and Brack, 2010). While quiescent, Pax7(+) satellite cells also express *Myf5*, a gene of the family of myogenic regulatory factors (MRFs) that are essential during developmental myogenesis. Upon satellite cell activation, MyoD, another MRF, starts being expressed and once terminal differentiation is reached the expression of the MRF Myogenin follows (Rudnicki et al., 1993). Attachment and adhesion proteins such as CD34 (Beauchamp et al., 2000), integrin- $\alpha_7$ (Burkin and Kaufman, 1999; Gnocchi et al., 2009) and M-cadherin (Irintchev et al., 1994); cell-surface receptors such as CXCR4 (Ratajczak et al., 2003), HGF receptor/c-Met (Allen et al., 1995) and calcitonin receptor (Fukada et al., 2007); nuclear envelope proteins emerin and lamin A/C (Gnocchi et al., 2009); and the heparin sulfate proteoglycans syndecan-3 and -4 (Cornelison et al., 2001), have all been described to be present in quiescent satellite cells and used to isolate them **(Table 1)**. Nonetheless, some of these markers – such as CD34 and integrin- $\alpha_7$ - are also expressed by other skeletal muscle cell types, requiring the use of additional differentiation markers.

The satellite SC self-renewal capacity and myogenic differentiation has been unequivocally demonstrated by transplantation studies (Collins et al., 2005; Montarras et al., 2005). Transplantation of single myofibres with their resident *Myf5*$^{lacZ}$(+) satellite cells into

| Table 1 - Satellite cell markers. | |
|---|---|
| Transcription factors | MyoD, Pax7, Pax3, Myf5 |
| Membrane proteins | cMet, Cav1, Notch, Ncam1, Vcam1, CalcR, Itga7, Itgb1, Synd3/4, CD34, CXCR4, Fzd7, Mcad, Lamin A/C (nuclear envelope), Emerin (nuclear envelope) |

radiation-ablated muscles demonstrated how these cells can generate numerous new myofibres containing thousands of myonuclei. *Myf5*$^{lacZ}$(+) satellite cells were also able to repopulate the host muscle with new satellite cells (Collins et al., 2005). Not long after, another study proved the self-renew and regenerative capacity of satellite cells at the single cell level. The authors isolated CD34 and integrin- $\alpha_7$ positive cells (and negative for blood markers CD45 and CD11b, mesenchymal marker Sca1 and endothelial marker CD31) that constitutively expressed Pax7. Transplantation of a single one of these cells validated this population as the muscle SC pool (Sacco et al., 2008). These cells were then shown to have a lower metabolic rate and to be capable of originating committed progenitors after several rounds of transplantation (Rocheteau et al., 2012).

The self-renewal capacity of satellite SCs implies the need of specialised mechanisms that balance SC maintenance and SC differentiation. Perturbation of such balance would lead either to SC depletion or to uncontrolled numbers of SCs potentially resulting in tumourigenesis. Therefore, growth factor signalling ought to be tightly regulated. P38/MAPK pathway, for instance, has been shown to function as a reversible molecular switch of the quiescent state of satellite SCs. Its inhibition was linked to exit from the cell cycle, and prevention of differentiation (Jones et al., 2005). Upon injury, Sprouty1, a receptor tyrosine kinase signalling inhibitor highly expressed by quiescent Pax7+ satellite cells, becomes downregulated in proliferating myogenic cells. More importantly, Sprouty1's expression is reinduced as Pax7+ cells re-enter quiescence and is required to restore back the pool of muscle SCs (Shea et al., 2010). Myostatin, on the other hand, is a growth factor from the Tgf-beta family shown to negatively regulate G1 to S progression, inhibiting satellite cell activation and thus promoting their quiescence (McCroskery et al., 2003). Furthermore, myostatin is also able to regulate Pax7 expression (McFarlane et al., 2008). Notch signalling leads to the activation of satellite SCs and expansion of myogenic precursors but inhibits myogenic differentiation (Conboy and Rando, 2002; Sun et al., 2007). Notch-3, however, was found to negatively regulate the number of self-renewing Pax7-positive satellite cells (Kitamoto and Hanaoka, 2010).

Homeostasis has also been proposed to be achieved through satellite SC asymmetric division that gives rise to a daughter cell primed to self-renew and a second one primed to differentiate. An alternative mechanism is the stochastic self-renewal of satellite SCs where two identical daughter cells are generated by symmetric division and subsequently adopt stochastic self-renewal or differentiating fates. Finally, it is also conceivable that a subset of SCs solely originate self-renewing daughters while others give rise only to differentiating daughters **(Fig. 2)**. BrdU pulse-chasing experiments revealed the importance of the asymmetric division mechanism: upon SC division the newly produced DNA strand is passed on to the daughter cell prone to differentiate, whereas older DNA strand is passed on to the daughter cell with preserved self-renewing capacity (Conboy et al., 2007; Shinin et al., 2006) **(Fig. 3)**. This capacity was shown to be characteristic of a subpopulation of satellite cells

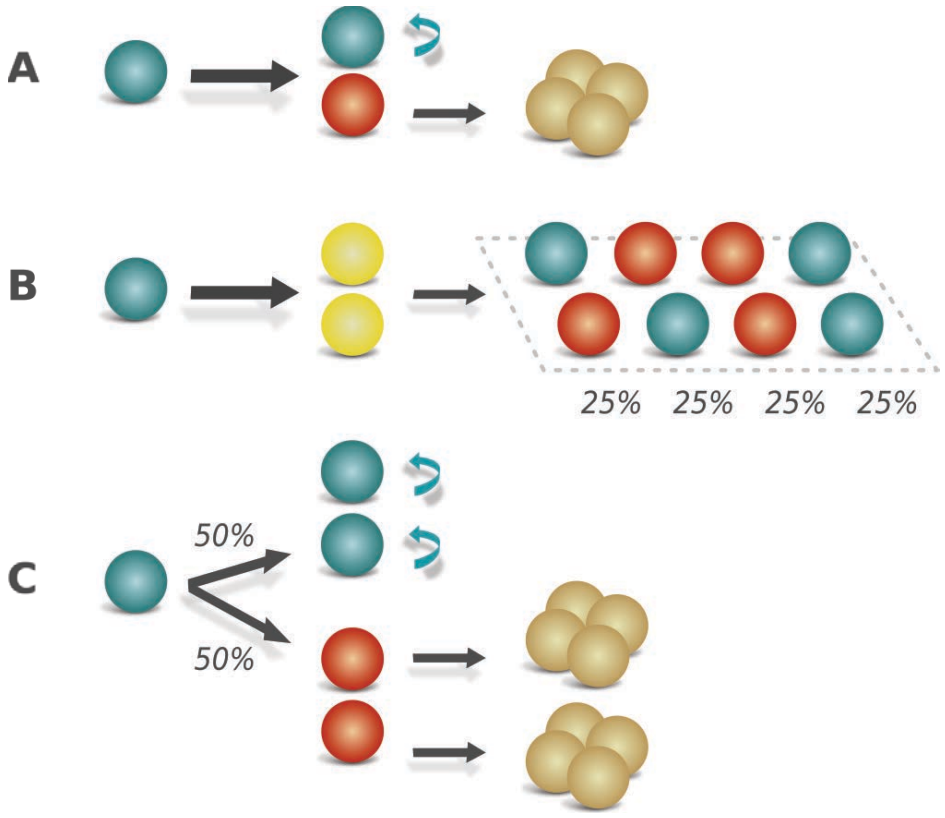



**Figure 2 - Stem cell renewal mechanisms.**

A) Asymmetric cell division originates two daughter cells with different self-renewal capacity and asymmetrically primed for differentiation.

B) Symmetric cell division results in two naive daughter cells that later follow a stochastic fate determination process that randomly leads to a self-renewing or differentiating fate.

C) Symmetric cell division originates two daughter cells with the same fate: either both are self-renewing cells or primed for differentiation.

Blue - stem cells; red - cells committed to differentiation; tan - differentiated cells; yellow - fate-undetermined cells; curved blue arrows - self-renewal capacity.

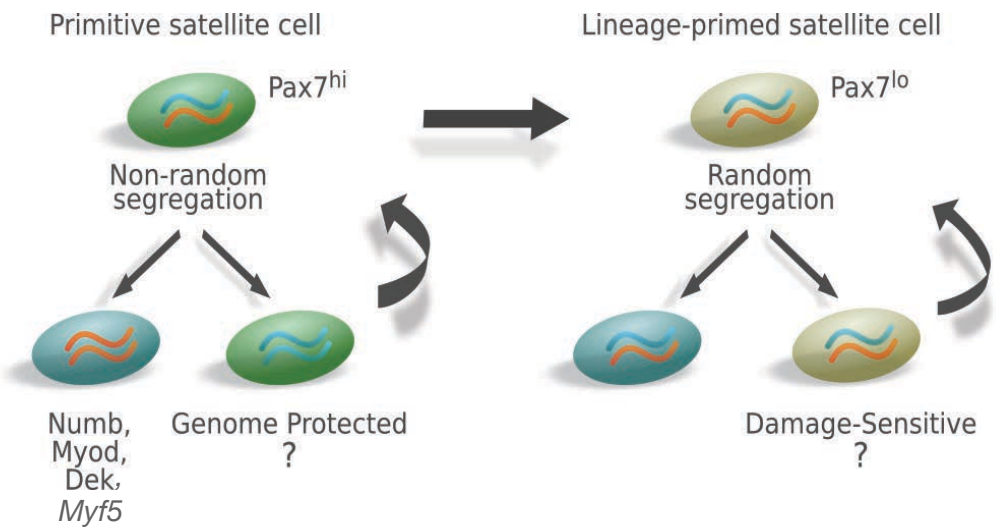



**Figure 3 - Non-random chromosome segregation and asymmetric fate determination in MuSCs.**

The pool of MuSCs is composed of more primitive satellite cells that express high levels of Pax7 (Pax7hi) and more committed lineage-primed satellite cells that express lower levels of Pax7 (Pax7lo). Upon cell division, one of the daughter cells of Pax7hi MuSCs will inherit, through a non- random segregation process, the old DNA template, which likely contains fewer lesions, and preserve their self-renewal capability. The low metabolic rate of Pax7hi might also protect their DNA from oxidative stress. The other daughter will inherit the new DNA strand, which may have suffered from DNA-replication errors, and will be primed for differentiation. Asymmetric distribution of the proteins Numb, Myod, Dek and expression of *Myf5* has also been linked to a differentiating fate. Pax7lo satellite cells, on the other hand, segregate their DNA in a random manner potentially giving rise to damage-sensitive progeny with a lower self-renewal capability.

(Pax7-nGFP high) that are less metabolically active and remain dormant. Upon activation, Pax7-nGFP high cells segregate their DNA in a nonrandom manner wherein the original DNA template is passed on to the daughter cell with SC potential. Myoprecursors (Pax7-nGFP low satellite cells), on the other hand, segregate sister chromatids randomly (Rocheteau et al., 2012). This could be an effective strategy for the SCs to avoid the accumulation of errors arising from the consecutive rounds of DNA replication. The niche seems to play a central role for the asymmetric division of DNA strands since this capacity was lost with time *in vitro* (Conboy et al., 2007; Shinin et al., 2006). The Notch inhibitory protein Numb was also found to asymmetrically segregate between two daughter cells by localising to one pole, perpendicular to the cell division plane. As a result, one daughter cell inherits Numb and consequently becomes primed for differentiation while the other does not

and so remains undifferentiated (Conboy and Rando, 2002). Upon satellite SC activation, MyoD is also asymmetrically distributed and mainly expressed in cells prone to differentiate (Zammit et al., 2004). The microRNA miR-489, a suppressor of the oncogene Dek, was found to be essential for satellite cell quiescence and quickly downregulated once satellite cells become activated. Furthermore, once expressed, Dek localises asymmetrically to the division plane and promotes transient proliferative expansion of the respective progenitors (Cheung et al., 2012). Finally, asymmetric satellite cell division has been mentioned in the context of *Myf5* gene expression. Satellite SCs, which do not express *Myf5* (Pax7(+)/ Myf5(-)), were found to divide asymmetrically and give rise to another Pax7(+)/Myf5(-) satellite SC and a satellite cell that starts expressing *Myf5* (Pax7(+)/Myf5(+)). The authors did not observe asymmetric segregation of any specific protein. Nonetheless, it became clear how a plane of division perpendicular to the axis of the myofibers more frequently generates daughter cells with distinct fates than cell divisions with a parallel plane (Kuang et al., 2007). However, only a subset of satellite cells follows asymmetric division, posing the question whether these may constitute a pool of more primitive SCs responsible for the homeostasis of the SC niche. Indeed, whether satellite cells were true SCs or rather committed progenitors, or dedifferentiated myoblasts remained unclear for a long time. The known heterogeneity of the satellite cell compartment has only made this task even more challenging (Collins et al., 2005; Kuang et al., 2007). The biological significance of such satellite cell heterogeneity is not yet fully understood. For instance, it is still poorly understood if there are different developmental origins, hierarchical lineage relationships or intrinsic self-renewing capacities among the satellite cell subpopulations. Within this context, the niche most likely plays a central role. Aside from the muscle fibres, satellite cells also reside in proximity to other cell types that may send paracrine or even juxtacrine signals. These include endothelial cells, wherein angiopoietin-1 signalling promotes satellite cell self-renewal (Abou-Khalil et al., 2009; Christov et al., 2007); fibro-adipogenic progenitors that upon muscle damage expand and propagate pro-differentiation signals facilitating myogenesis (Joe et al., 2010); and TCF4+ fibroblasts, which contribute to the regulation of satellite cell differentiation, thus playing a crucial role during muscle regeneration (Murphy et al., 2011). In addition, the basal lamina also contributes to SC identity and maintenance as it serves as an anchoring surface for the satellite SCs. It is mainly constituted of laminin, collagen, and proteoglycans, and is known for being a major component of the ECM not only of the muscle SC niche (Fuchs et al., 2004).

Finally, studies shedding light on the epigenetic features that contribute to muscle SC regulation will be key to better define the different subpopulations and their respective characteristics. It became clear very early on that satellite cells suffered DNA modifications upon activation: electron micrographs showed changes in chromatin organisation associated

with the switch from quiescence to a proliferative state (Church, 1969). Since then, the efforts into characterising the epigenetic mechanisms that control myogenesis have been extensive. However, such analyses often require large numbers of cells, therefore, highly limiting the advances with respect to satellite SCs in particular. Nevertheless, it has been demonstrated that in quiescent satellite cells only a small subset of genes is marked with the repressive histone H3 lysine 27 trimethylation mark (H3K27me3). The permissive histone H3 lysine 4 trimethylation mark (H3K4me3), on the other hand, is present on half of the annotated genes. During satellite cells activation, a large number of genes becomes repressed due to *de novo* H3K27me3 (Liu et al., 2013). Additionally, Pax7 associates with the Wdr5-Ash2L-MLL2 histone methyltransferase complex, which directs methylation of H3K4, leading to H3K4me3 at the *Myf5* locus (McKinnell et al., 2008). This strongly suggests that Pax7 may regulate the myogenic program through chromatin modifications.

Future studies should lead to the development of novel strategies to control the different molecular mechanisms that regulate the self-renewal, proliferation and differentiation of muscle satellite SCs.

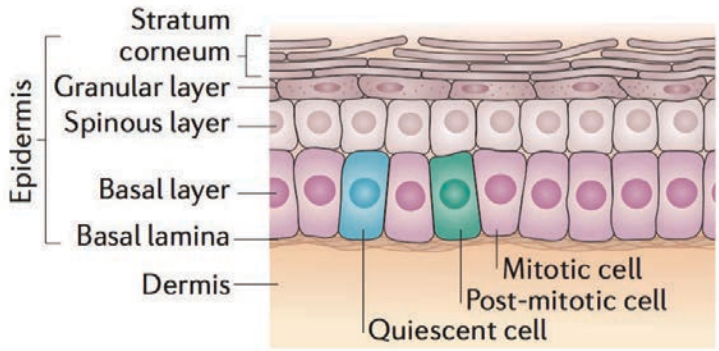
## *The skin and epidermal stem cells*

Vertebrates are covered by skin, which protects them from external aggressions such as pathogens, radiation and water loss, and helps regulate body temperature. The skin also plays a central role in social and reproductive interactions, contributing to the exchange of important information about the species. Animals explore the surrounding environment also through the skin's tactile function, and a great number of species resort to skin camouflage to escape from predators (Blanpain and Fuchs, 2009).

The skin, the largest mammalian organ, is composed of a superficial layer mainly constituted of keratinocytes – epidermis – and a subjacent layer of mostly fibroblasts – dermis **(Fig. 4)**. The epidermis is a non-vascular stratified epithelium, formed of four different strata: the basal layer, the spinous layer, the granular layer and the stratum corneum. Virtually all mammals are covered in hair follicles (HF) that follow a cycle of growth (anagen), cell death (catagen) and rest (telogen). The stretches of epidermis found in between are designated as interfollicular epidermis (IFE). The blood and lymph vessels necessary for nourishing the epidermis, and the sebaceous and sweat glands, which lubricate and protect the hair and skin surface, are all located in the dermis (Gilbert, 2000).

The skin epidermis has a nigh turnover rate due to the continuous shedding of cells from the uppermost cornified cells. Consequently, niches of multipotent adult SCs localised in the epidermis are responsible for skin postnatal homeostasis. These SC populations reside in

**A)**




**B)**

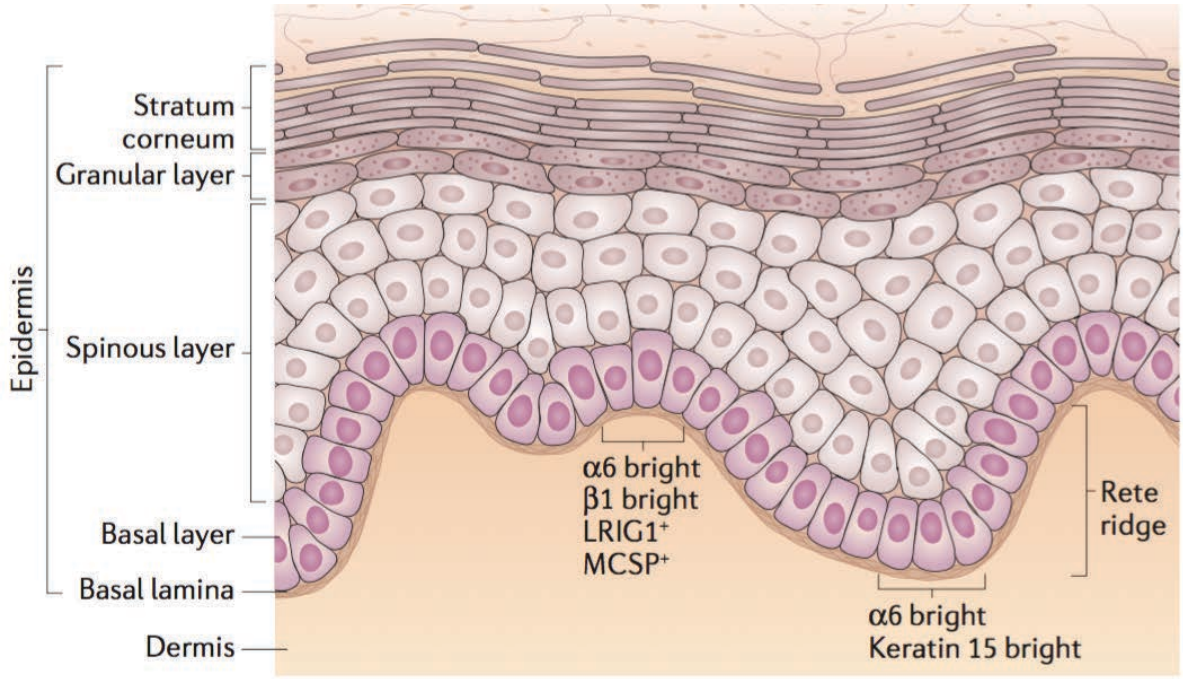



**Figure 4 - The architecture of the epidermis.**

(A) EpSCs are located in the basal layer of the mouse interfollicular epidermis, where they sit on a basal lamina that directly contacts with the dermis. As the EpSCs proliferate and give rise to more differentiated progeny, they move upwards into the spinous layer, then into the granular layer and finally into the stratum corneum from where they eventually shed. (B) In the human interfollicular epidermis, the EpSCs express high levels of α6 and β1 integrins, as well as MCSP and LRIG1. It remains unclear whether quiescent EpSCs locate mostly at the bottom of rete ridges (adapted from Solanas and Benitah, 2013).

the basal layer of the epidermis. On the other hand, structures like the HFs and sweat glands rely mostly on their respective SC niches. Located beneath the sebaceous glands, protruding near the base of the HF, is a SC niche – the bulge – that supplies each hair cycle. These are label-retaining cells and they give rise to transiently amplifying (TA) cells that then proliferate in the hair bulb, and terminally differentiate into a new inner root sheath

and hair shaft (Cotsarelis et al., 1990). HF bulge SCs do not contribute to the IFE except upon damage (Braun et al., 2003; Levy et al., 2007; Taylor et al., 2000; Tumbar, 2004). More recently other SCs niches located above the HF bulge have been identified, each one with different regenerative potentials (Jensen et al., 2009; 2008; Snippert et al., 2010).

The human epidermis is considerably thicker than the one of rodents, with epidermal cavities – rete ridges – anchoring the epidermis to the dermis. These structures have been proposed to function as individual epidermal proliferative units (EPUs): located in each one of these EPUs, a putative SC of origin undergoes asymmetric cell division and gives rise to TA progenitors (Allen and Potten, 1974; Potten, 1975; Smart, 1970). Indeed, slow-cycling IFE cells were found at the bottom of rete ridges, presumably the IFE's most protective area (Lavker and Sun, 1982). Slow-cycling SCs were also found in mouse epidermis (Mascre et al., 2012; Sada et al., 2016). Similarly to MuSCs, it was first proposed that IFE SCs divide mostly asymmetrically by orienting the mitotic spindle parallel to the basal layer. As a result, one daughter cell moves away from the IFE SC compartment. This mechanism leads to the unequal distribution of cell fate and growth factors, such as specific RNAs and polarity proteins. The asymmetric division may also occur in a plane parallel to the basal membrane, in which case both daughter cells remain momentarily in the basal layer. While one daughter cell may receive signals to remain a SC, the other will quickly activate a differentiation program and move upwards into the spinous layer (Lechler and Fuchs, 2005). The TA progeny of the IFE SCs goes through a number of cell divisions and finally differentiates into postmitotic keratinocytes, compensating for the high epidermal turnover (Potten, 1975).

Despite the fact that the stem-TA-cell model had been widely accepted, Clayton and her colleagues have later on challenged it. According to Clayton's proposed model, unipotent committed progenitors (CPs) constitute the proliferating basal cell compartment rather than a SC compartment specific to the IFE. The model proposed that CPs stochastically adopt one of three fates upon division: two progenitor cells, two differentiated cells, or follow asymmetric division. In contrast with the stem-TA-cell model, this model did not include a specific role for a IFE SC population in homeostatic conditions, but did not exclude a possible contribution during growth of wound healing (Clayton et al., 2007). In 2012, a dual lineage-tracing study by Mascre *et al.* suggested the existence of two distinct populations of epidermal progenitors: a slow-cycling SC population that gives rise to a second and more proliferative CP one. Interestingly, the authors reported that both populations undergo asymmetric self-renewal where the balance between a proliferative or differentiated fate is stochastically established, as described by the CP model. Nonetheless, while highly proliferative CPs were described as the main contributors to epidermal homeostasis, dormant SCs quickly became active upon injury. During epidermal wound repair, the SC compartment was observed to almost entirely contribute to the tissue regeneration process

(Mascré et al., 2012). More recently however, it has been demonstrated by an *in vivo* pulse/chase system for single-cell genetic label retention that murine EpSCs display stochastic and equal potential to divide or directly differentiate in homeostatic conditions, excluding the SC hierarchy or asymmetric SC division hypothesis supported by previous static clonal models. This study indicated instead that EpSCs differentiate by moving upwards into preexisting spatial units, and that fate and lifetime of EpSCs might be coupled with spatial localisation. These vertical columns into which EpSCs differentiate and that inspired the EPU hypothesis, were defined as epidermal differentiation units (EDUs) (Rompolas et al., 2016).

Keratinocytes of the basal layer express Keratin 14 in both HF and IFE. EpSCs have also been shown to express higher levels of *α6*- and *β1-integrins*, and lower levels of *Notch3*, *Grhl3*, among others, in comparison with their more differentiated (Mascré et al., 2012; Rangarajan et al., 2001; Ting et al., 2005). *In vitro* studies with human keratinocytes identified the cell surface receptor β1-integrin as a marker for a more proliferative potential. Highest expression of β1-integrin was also correlated with melanoma chondroitin sulphate proteoglycan (MCSP) expression. MCSP was shown to be essential for proper cadherin-mediated cell-cell adhesion and maintenance of cortical actin cytoskeleton in high β1-integrin-expressing cells. *In vitro* proliferating human keratinocytes with slower adhesion capacity to ECM proteins display a lower SC status, differentiating after only one to five rounds of division (Jones and Watt, 1993). Therefore, it is likely that *in vivo* high β1-integrin and MCSP expression is required for IFE SC interactions with the ECM and clustering at EDUs (Legg et al., 2003). *In vivo*, β1-integrin-bright cells were identified in clusters in the basal layer. Nevertheless, depending on the location of the human body, patches of β1-integrin-bright cells can be found not only at the tip of rete ridges (Jones et al., 1995). On the other hand, human IFE SCs expressing high levels of α6-integrin also express Keratin15, a protein mainly expressed at the bottom of rete ridges (Li et al., 1998; Schlüter et al., 2011; Webb et al., 2004). Finally, a lineage-tracing study with human xenografts suggested that, regardless of their location within the basal layer, all IFE SCs contribute to epidermal replenishment (Ghazizadeh and Taichman, 2005). The exact location of human EpSCs remains elusive to date. However, these studies have established a clear relationship between the ability to adhere to the ECM and SC features like clonogenic potential. More importantly, they have allowed for partial histological characterisation and efficient isolation of IFE SCs. Meanwhile, other markers of IFE SCs have been further identified: LGR-6 (Snippert et al., 2010), LRIG1 (Jensen and Watt, 2006), the polycomb protein CBX4 (Luis et al., 2011), and Delta1 (Lowell et al., 2000). On the other hand, in order to remain dormant, IFE SCs require low levels of the transferrin receptor CD71 (Li et al., 1998), DSG3 (Wan et al., 2003) and of the epidermal growth factor receptor (EGFR) (Fortunel et al., 2003; Jensen and Watt, 2006). Nowadays, the identification of all these SC markers and the development

of good antibodies has allowed for the efficient isolation and segregation of the murine IFE and bulge SC populations. While both express high levels of α6-integrin, only bulge SCs are also positive for CD34 (Jensen et al., 2010).

The importance of the microenvironment for proper epidermal SC function has been unequivocally demonstrated by a number of studies. Although the vast majority of these studies has been focused on bulge SCs, it became evident that cues originating from the dermal papilla (a condensate of fibroblasts that sits below the bulge), muscle, nerve, melanocytes, adipose tissue and other systemic signals contribute to the microenvironment of the epidermis in a vital manner (Solanas and Benitah, 2013). Moreover, the basement membrane can provide polarisation signals for the orientation of the SCs during mitosis (Lechler and Fuchs, 2005). Nonetheless, it will be crucial to characterise the impact of the above-mentioned signals on IFE SCs and how they might change during or contribute to ageing of such SC population.

## *Circadian Rhythms*

During the past two decades, the work of many has contributed to the increasing understanding on what circadian rhythms are and how they control body functions. Circadian biology is known to influence a vast number of physiological processes such as sleep-awake cycles, body temperature, hormonal oscillations, metabolism, feeding rhythms, and cell cycle. Circadian rhythms have been observed in organisms ranging from photosynthetic prokaryotes to higher eukaryotes. They are believed to have evolved in mammals to allow the organism to anticipate and adapt to the daily environmental changes in temperature, food availability, predator avoidance, and cycles of light exposure in order to improve survival rate (Harmer et al., 2001). Located in our brain, more precisely in the hypothalamus, there is a structure often called the “master clock”. This designation refers to the suprachiasmatic nucleus (SCN), which consists of 15-20,000 neurons whose activity oscillates following a 24h pattern. The SCN functions as a central pacemaker by coordinating the “peripheral clocks” in virtually all other organs (Ralph et al., 1990; Silver et al., 1996). Day-night oscillation of genes important for tissue homeostasis has already been confirmed in epidermis, muscle, myoblasts, adipocyte progenitors, chondrocytes, neural progenitors, neurons and glia, lung epithelium, liver and retina (Ali et al., 2015; Andrews et al., 2010; Bouchard-Cannon et al., 2013; Chatterjee et al., 2013; Costa et al., 2011; Dudek et al., 2016; Fu et al., 2005; Gibbs et al., 2009; Gossan et al., 2013a; Janich et al., 2011; 2013; Lamia et al., 2008; McCarthy et al., 2007; Musiek et al., 2013; Samsa et al., 2016; Storch et al., 2007).

The clock in every cell is both self-sustained and cell-autonomous, meaning that without any external input these oscillations still stick to an interval of roughly 24 hours. However, in response to certain external cues – zeitgebers (ZTs), or time givers - the clock may be reset. There are a few factors known to entrain our circadian rhythms in different ways. The light, a well-studied entrainment factor, is captured by our retinas that in turn communicate with the SCN through the retino-hypothalamic tract and reset/adjust the clock according to the time of the day (Asher and Sassone-Corsi, 2015; Saini et al., 2011). Metabolism has also been tightly linked with circadian rhythms. Many genes central in a number of metabolic pathways are in fact under circadian regulation (see section below). Moreover, changes in feeding schedules have been shown to impact both on metabolism and circadian oscillations. Night time restricted feeding (TRF) leads to a 50% decrease in triglyceride content in the liver, even though the total daily caloric intake remained unchanged (Adamovich et al., 2014). Mice on high fat diet (HFD) usually develop obesity, hyperinsulinemia, hepatic steatosis, and inflammation. However, when HFD is fed following a TRF schedule, even though they consume an equal amount of calories, they become protected against all these pathologies (Hatori et al., 2012; Sherman et al., 2012). Curiously, obesity resistance was achieved in these studies even though the TRF schedule was set for completely different times of the day: while in one study mice were fed with HFD for 8h during the dark phase, in the second one HFD chow was only available for 4h during the light phase. Another study from Panda and colleagues demonstrated that even when followed for only 5 days a week, TRF remains effective due to an after effect following cessation. The authors also demonstrated the efficacy of TRF against metabolic disorders developed by other nutritional challenges such as HFD, high-fructose, HFD combined with high-fructose (Chaix et al., 2014). TRF regimens enhance pathways such as CREB, mTOR, and AMPK, while improving the oscillations of circadian core clock and output genes (Hatori et al., 2012; Sherman et al., 2012). Furthermore, mice subjected to at least one week of TRF during the rest phase (light phase) show antiphasic oscillations of core clock genes in the liver, kidney, heart and pancreas, but not in the SCN (Damiola et al., 2000). This highlights the strong impact feeding schedules have on peripheral tissues and how changes in metabolism may even uncouple the peripheral clocks from the central pacemaker. On the other hand, HFD fed ad libitum results in perturbed feeding-fasting cycles (mice tend to eat more during the rest phase) and disrupts the circadian rhythmicity (Kohsaka et al., 2007). Therefore, diet composition also seems to influence the circadian oscillations in peripheral tissues and perturb feeding-fasting cycles that normally act as strong ZTs. Conversely to TRF, caloric restriction (CR) entrains the clock in the SCN and is able to reset it when mice are fed during the rest phase (Challet et al., 1998; 2003; Mendoza et al., 2005; Resuehr and Olcese, 2005). CR consists in a reduction in calories (around 30% reduction) without malnutrition. It has been reported in

species from yeast to monkeys that CR is able to extend lifespan and delay the onset of most of age-associated pathologies (Colman et al., 2009; Froy and Miskin, 2010). However, the exact mechanisms through which CR operates remain largely unclear. Transcriptome studies have shown that the nutritional, metabolic and epigenetic state of a cell or tissue may contribute for the distinct circadian outputs found across tissues. Importantly, such studies have also revealed that a striking 10% of the genome may in fact be under clock regulation in a tissue-dependent manner (Akhtar et al., 2002; Panda et al., 2002).

## *The circadian clock*

The molecular mechanism responsible for circadian oscillations is identical between the neurons located in the SCN and any other somatic cell. In both cases, the circadian rhythms are created and maintained by interlocked negative feedback loops in gene expression **(Fig. 5)**. The main loop consists of heterodimers of the PAS domain helix-loop-helix transcription factors BMAL1 and CLOCK. The complex BMAL1:CLOCK drives the expression of a large subset of genes by binding to E-boxes located in their promoter regions. Among those genes are three period genes, *Per1*, *Per2* and *Per3*, and two cryptochrome genes, *Cry1* and *Cry2*. These two families of genes can form heterodimers and act as repressors of the BMAL1:CLOCK-driven transcription. Therefore, the levels of PER and CRY molecules in the nucleus determine whether the BMAL1:CLOCK complex binds or not to target genes. As such, during the day while BMAL1:CLOCK bind to the chromatin, PER and CRY proteins slowly accumulate. By night-time, once their levels reach critical protein levels, PERs and CRYs dimerize and translocate to the nucleus where they start to repress their own expression. PERs and CRYs also suffer post-translational modifications that lead to their controlled degradation. As a result of PER:CRY transcriptional repression and gradual protein degradation, the inhibition of BMAL1:CLOCK binding to the DNA is slowly alleviated and gene expression restored by the start of the day (Asher and Sassone-Corsi, 2015; Crane and Young, 2014; Saini et al., 2011). The retinoic acid-related orphan nuclear receptors ROR (RORα, RORβ and RORϒ) and the orphan nuclear receptors REV-ERB (REV-ERBα and REV-ERBβ) create an additional layer of circadian regulation. Both RORs and REV-ERBs recognise and bind to two ROR-binding elements (RREs) within the *Bmal1* promoter. While ROR activates the expression of *Bmal1*, REV-ERB represses it (Preitner et al., 2002). Moreover, post-translational modifications of clock proteins, such as phosphorylation, acetylation, ubiquitination, poly(ADP)-ribosylated, and SUMOylation, add further complexity to the system and allow for tight regulation of the core clock machinery (Asher and Sassone-Corsi, 2015; Cardone et al., 2005; Mohawk et al., 2012; Reischl et al.,

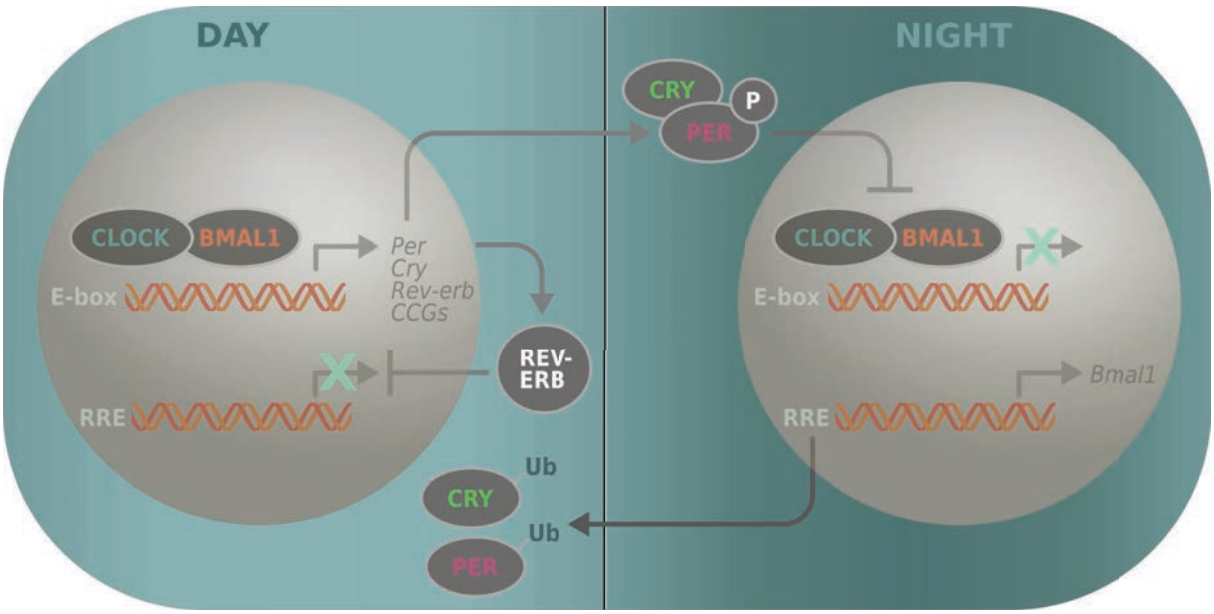



**Figure 5 - The core clock machinery.**

The transcriptional activators BMAL1 and CLOCK dimerise and bind to specific promoter elements in the DNA (E-boxes). By doing so, they drive the expression of numerous genes including the *Period* (Per) and *Cryptochromes* (Cry) gene families. PERs and CRYs translocate to the nucleus as hetero-dimers and slowly accumulate. When finally at night they reach sufficiently high levels, they start to repress the CLOCK:BMAL1-mediated transcription. PERs and CRYs are continuously undergoing a number of post-translational modifications that induce their degradation, required to start off a new circadian cycle. An auxiliary circadian loop includes the proteins REV-ERBα/β, whose levels increase during the day, and bind to promoter elements (RRE elements) also found in the promoter of *Bmal1*, hence inhibiting *Bmal1* transcription. At night, REV-ERBα protein levels are low, thus allowing *Bmal1* transcription to occur.

2007; Virshup et al., 2007). For instance, PER2 and BMAL1 can be deacetylated in a circadian manner by the $NAD^+$-dependent deacetylase Sirtuin 1 (SIRT1), involved in cellular metabolism, inflammation and ageing, creating a very strong link between the circadian clock and metabolism (Asher et al., 2008; Nakahata et al., 2008). Furthermore, CLOCK:BMAL1 regulates the expression of *Nampt* (nicotinamide phosphorybosil transferase), the gene that encodes for the rate-limiting enzyme in the $NAD^+$ biosynthetic pathway (Nakahata et al., 2009; Ramsey et al., 2009). As a result, the clock regulates in a cyclic manner the synthesis of $NAD^+$ and potentially the cyclic activity of $NAD^+$ -consuming enzymes such as the SIRTs (Guarente, 2011). The $NAD^+$-dependent ADP-ribosyltransferase PARP-1 was also shown to oscillate in a daily manner in response to feeding, and to bind to and poly(ADP-ribosyl)ate CLOCK. By doing so, PARP-1 enhances the binding of CLOCK:BMAL1 to the DNA and participates in the phase entrainment of the clock according to feeding rhythms in the liver (Asher et al., 2010).

A number of mouse models have been used aiming to further unveil the importance of the molecular clock for physiology and homeostasis. In particular, *Clock* mutant mice develop obesity and a metabolic syndrome associated with strongly attenuated diurnal feeding rhythms (Turek et al., 2005). *Rev-Erb-α* KO mice, on the other hand, show alterations in lipid utilization in the muscle, where normally fatty acids are used as the main source of energy (Delezie et al., 2012). This phenotype was later linked to the reduction in mitochondria and concomitant impaired oxidative function that these mice have, causing a metabolic switch to an energy richer substrate (Woldt et al., 2013). MyoD was also found to be under circadian regulation in skeletal muscle and *Clock*(Δ19) and *Bmal1* KO mutants exhibit a significant decrease in normalized maximal force (Andrews et al., 2010). Additionally, BMAL1 binds to promoters of the canonical Wnt pathway genes and drives their expression is an oscillatory manner, positively regulating myogenesis (Chatterjee et al., 2013). Complete deletion of *Bmal1* in mice results in the premature development of many age-related pathologies, such as low body weight, arthritis, brittle bones, corneal degeneration, diabetes, intestinal permeability and inflammation, skin ageing and neurodegeneration. Ultimately, this array of pathologies leads to a severely shortened lifespan (Bouchard-Cannon et al., 2013; Gossan et al., 2013b; Janich et al., 2011; Kondratov et al., 2006; Lin et al., 2009; Marcheva et al., 2010; Musiek et al., 2013; Samsa et al., 2016; Storch et al., 2007; Summa et al., 2013; Yang et al., 2016).

There is vast evidence supporting the idea that SCs are highly regulated by circadian rhythms. In fact, circadian rhythms are capable of dictating the active versus dormant state of certain SC populations. In the case of bulge SCs, previous work performed by our group suggests that certain subsets within a single population may be prone to react to circadian stimuli in different time frames. This could in part explain the heterogeneity found in bulge SCs (Greco et al., 2009; Zhang et al., 2009a). Remarkably, the BMAL1:CLOCK complex was found to recognize and bind to the promoter regions of a number of key epidermal SC genes, including modulators of Wnt (which promote growth), TGF-β (which inhibit proliferation and differentiation), Bmp and Notch signaling. The expression of those genes was then shown to be perturbed by conditional deletion of *Bmal1*, possibly accounting for the thicker cornified layer, indicating higher keratinocyte differentiation, that these mice display. Moreover, the expression of some of those genes oscillated within a 12h period in purified bulge SCs from WT mice. Bulge SCs from *Bmal1*-deletion mice, on the other hand, did not display similar patterns of expression of such key epidermal SC genes: they were more responsive to TGFβ signaling as well as maintained higher expression levels of TGFβrelated genes while displayed reduced expression of Wntrelated. Accordingly, Per1 and Per2 double-mutant mice (Per1/Per2dKO) (in which BMAL1 activity is no longer inhibited at night) showed increased proliferation levels of bulge SCs (Janich et al., 2011). These studies have

linked the clock machinery to the direct regulation of epidermal signaling pathways and the consequent predisposition of epidermal SCs to become active or remain dormant. A second study from our group has further demonstrated the importance of circadian gene regulation in EpSCs. Cultured and synchronized human EpSCs display successive peaks of gene expression that establish concatenated temporal intervals along the 24h day period and correlate with expression peaks of the core clock genes. The transcripts peaking at each one of those intervals were related to specific segregated biological functions, such as DNA replication in the evening, proliferation at night and differentiation in the morning. By doing so, human EpSCs are able to respond to different cues along the day and perform functions accordingly, avoiding for instance replicating their DNA at the time of maximal UV-exposure (Janich et al., 2013). In mice it has been proposed that the circadian clock helps uncouple functions like DNA replication and oxidative phosphorylation to avoid genotoxic stress and DNA exposure to UV light. As observed by our group, this study showed a peak of DNA replication at night while oxidative phosphorylation was mostly observed during the light phase of the day (Geyfman et al., 2012). This suggests that although mice and humans display opposite phases of activity (mice are nocturnal while humans are diurnal animals), certain physiological functions are still coordinated by the clock in a similar manner. Accordingly, mice are more susceptible to developing skin cancer if exposed to UV light during the dark phase (Gaddameedhi et al., 2011a). Finally, in skeletal muscle the number of satellite cells is significantly lower in *Bmal1* KO mice. Upon injury the induction of Pax7 is nearly abolished and satellite cells fail to expand in order to regenerate the damaged tissue in comparison with the wild type (WT) counterparts (Chatterjee et al., 2015). Nonetheless, the precise circadian regulatory mechanisms that contribute to proper SC function in each individual tissue are still far from well understood.

## *Ageing*

As the average human life expectancy rises, new age-associated diseases have appeared as causes of mortality. Hence, it has become of great interest both the study of the evolutionary reason why we age, and the physiological and molecular mechanisms behind how we age. The holy grail of the new millennium has become the concept of healthy ageing and the development of therapeutic strategies that may increase organismal resiliency into old age.

Ageing can be defined as the progressive and generalised malfunction of the organism, leading to a decrease in fitness and growing vulnerability to disease and death. It is a highly complex process since almost every phenotypic characteristic of an organism suffers

gradual changes through ageing. It is clear that genetics plays a central role in this process and so far, a list of genes that either contribute or shorten life expectancy have been identified for instance in *Caenorhabditis elegans*. Nevertheless, ageing-related features may significantly vary even between monozygotic human twins (Kirkwood, 2005). Different theories resulted from numerous studies, aiming to define and explain the pivotal cause of ageing. Carlos Lopez-Otin, Maria A. Blasco, Linda Partridge, Manuel Serrano and Guido Kroemer have carefully defined and extensively described nine hallmarks of ageing: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, SC exhaustion, and altered intercellular communication (López-Otín et al., 2013). It is expected that each different tissue or even cell type exhibits a combinations of two or more of these hallmarks during the process of ageing. Adult SCs are no exception and numerous efforts are being focused on determining exactly what factors characterise and contribute to the course of ageing of each different SC population. The ultimate goal in SC and ageing research would be the identification or design of strategies that could either prevent the loss or restore traits of youthful SCs in aged ones as a way to promote healthy tissue ageing. Below I describe studies, in the context of some of the nine hallmarks of ageing, that are relevant for the understanding of the work later explained in this thesis.

### *Genome instability*

Among the possible causes for adult SC ageing is genomic instability. The genomic instability theory suggests the accumulation of mutations and other types of DNA damage in somatic cells as the main cause for ageing. Genome instability may increase due to lesions in nuclear DNA, mitochondrial DNA, telomere shortening, and also defects in nuclear architecture (Blackburn et al., 2006; Kazak et al., 2012; Worman, 2012). The stability and integrity of the DNA are constantly being exposed both to extrinsic chemical, physical and biological threats, and intrinsic challenges such as DNA replication errors, reactive oxygen species (ROS), and spontaneous hydrolytic reactions (Hoeijmakers, 2009). Among the DNA lesions resulting from both exogenous and endogenous challenges, are chromosomal gains and losses, point mutations, translocations, telomere shortening, and gene disruption. In order to cope with these types of damage, species have evolved and developed specialised molecular mechanisms to efficiently detect and correct genetic lesions. Somatic mutations are known to accumulate in cells from aged individuals as well as in model organisms (Moskalev et al., 2013). Indeed, many premature ageing diseases are the result of excessive DNA damage accumulation due to defects in DNA repair mechanisms. These include progeroid syndromes, such as Bloom syndrome, trichothiodystrophy, xeroderma

pigmentosum, Werner syndrome, Cockayne syndrome, and Seckel syndrome. These studies in humans and in mouse models mimicking such syndromes have unequivocally demonstrated the link between somatic mutations and ageing (Burtner and Kennedy, 2010; Gregg et al., 2012; Hoeijmakers, 2009; Murga et al., 2009). However, such progeroid syndromes only display a subset of the main characteristics of normal ageing, therefore the relevance of these cases to the study of ageing is not yet fully clear. On the other hand, chromosomal aneuploidies, copy number variations, and increased clonal mosaicism for large chromosomal anomalies have also been associated to ageing (Faggioli et al., 2012; Forsberg et al., 2012; Jacobs et al., 2012; Laurie et al., 2012). For instance, the BubR1 transgenic mice, which are more protected against aneuploidy and cancer, display an extended lifespan due to the overexpression of this mitotic checkpoint component that ensures proper segregation of chromosomes (Baker et al., 2013).

In the case of the skin, UV exposure is the main driver of mutations. It has been reported that normal skin, as a consequence of sun exposure, exhibits marked clonal expansion associated with specific mutations such as *TP53* and *NOTCH1*. Life-long skin exposure to sun is very likely to cause most of DNA lesions that accumulate with age (Martincorena et al., 2015). The authors reported that, in spite of the high frequency of clones carrying cancer-related mutations, the actual number of individuals that developed skin cancer was in fact low. This further highlights how fined-tuned the mechanisms for malignancy-avoidance are. Nevertheless, it remains to be investigated whether other age-related diseases or aspects, such as the decline of the skin barrier function, correlate with the described mutant clone expansion.

Most likely due to the fast turnover of HF cells, HF SCs resort more often to high non-homologous end joining (NHEJ) activity. This DNA damage pathway can be activated in any phase of the cell cycle but is know to be error-prone (Sotiropoulou et al., 2010). Furthermore, over time, HF SCs also start to loose their DNA repair capacity. Consequently, aged-HF SCs accumulate significant amounts of DNA damage, including double-strand breaks that may result in chromatin rearrangements. In spite of this, the physiological ageing process does not cause HF SCs to undergo cellular senesce nor apoptosis (Schuler and Rübe, 2013). Importantly, EpSCs are also protected from genotoxic stress by the circadian clock that dictates and temporally segregates DNA replication from the light phase of the day, when maximal oxidative phosphorylation occurs (Gaddameedhi et al., 2011b; Geyfman et al., 2012; Stringari et al., 2015).

***Telomere shortening***

The telomere loss theory suggests a decline in cellular division rate with age associated with the progressive shortening of telomeres – the protective ends of chromosomes. This

shortening is due to the absence of the enzyme telomerase, only expressed in germ cells and specific adult SCs. It has been suggested that telomeres function as a count-down mechanism of the number of cell divisions, an evolutionary strategy to avoid uncontrolled proliferation as in cancer but with ageing at cost (Campisi, 2005). It has been demonstrated that stress, particularly oxidative stress, greatly contributes to telomere loss since oxidative damage is less well repaired in telomeric DNA (Zglinicki, 2002). Additionally, upon 12-*O*-tetradecanoylphorbol 13-acetate (TPA) treatment (a potent stimulator of cell proliferation), telomerase-deficient mice exhibit decreased hair growth, inhibited mobilization of HF SCs out of their niche and decreased cell proliferation *in vitro* (Flores et al., 2005).

***Epigenetic changes***

Throughout life, all cells and tissues are affected by epigenetic changes including DNA methylation patterns, histone posttranslational modifications and chromatin remodeling. Enzymes like histone acetylases, deacetylases, DNA methyltransferases, methylases, and demethylases, are responsible for maintaining or modifying the chromatin. It has been reported in monozygotic twin pairs that epigenetic alterations indeed appear from early adulthood to old age (Talens et al., 2012). Furthermore, studies have suggested an aged-associated changes in histone H3K27 trimethylation, H3K9 methylation, H4K20 trimethylation, H4K16 acetylation, or H3K4 trimethylation (Fraga and Esteller, 2007; Han and Brunet, 2012). For example, the repressive H3K27 mark was found increased in both aged MuSCs and HSCs (Liu et al., 2013; Sun et al., 2014).

The family of $NAD^+$-dependent deacetylases sirtuins (SIRT) has been identified as promising anti-ageing therapeutic targets. The different members of the family (SIRT1 to SIRT7) act in different cellular compartments by signaling nutrient scarcity and catabolism. By deacetylating histones and several transcriptional regulators in the nucleus (e.g. SIRT1, SIRT6) and also proteins in the cytoplasm and mitochondria (e.g. SIRT3), they regulate energy levels, cell metabolism and, thus, ageing. Some members were found to be protective against some age-related diseases like cancer, neurodegeneration, and cardiovascular disease (Houtkooper et al., 2012; Sebastián et al., 2012; Wang et al., 2008). In mouse embryonic SCs SIRT1 relocalizes to sites of DNA damage where it promotes repair and, consequently, alters transcription by relieving the repression of the genes where it was previously locating to (Oberdoerffer et al., 2008). SIRT1 transgenic mice that only mildly overexpress *Sirt1*, for instance, do not show an increased longevity but exhibit protectiveness against cancer and lower levels of DNA damage (Herranz et al., 2010). SIRT6, on the other hand, functions as a master regulator of metabolic homeostasis by repressing Hif1alpha activity (important regulator of nutrient stress response), and by protecting against the consequences of diet-induced obesity (Kanfi et al., 2010; Zhong et al.,

2010). Furthermore, SIRT6 was found to also participate in base excision repair (BER) and suppress genomic instability, while SIRT6-deficient mice age develop severe pathologies and die at a very early age (Mostoslavsky et al., 2006). Finally, SIRT6 transgenic male mice show reduced serum levels of insulin-like growth factor 1 and live longer than control animals (Kanfi et al., 2012). SIRT3 regulates energy levels through the deacetylation of mitochondrial proteins in response to CR, seemingly mediating some of the beneficial effects of this diet (Someya et al., 2010). SIRT3 activity was found to decrease with age in HSCs. However, its activity is essential at an old age and SIRT3 upregulation in aged HSCs is capable of promoting their regenerative capacity (Brown et al., 2013).

***Deregulated nutrient sensing and growth factor signalling***

The insulin and insulin-like growth factor 1 signaling pathway is the most evolutionarily conserved pathway also implicated in ageing. Downstream of this signaling pathway are the mTOR complexes (mTORC) that are also tightly involved in the process of ageing. Mammalian TOR kinase is part of two multiprotein complexes, mTORC1 and mTORC2, and is responsible for the sensing of high amino acid concentrations (Barzilai et al., 2012; Fontana et al., 2010; Laplante and Sabatini, 2012). In lower organisms, downregulation of mTORC1 extends lifespan and has a synergistic effect together with CR, indicating that mTOR inhibition mimics CR (Johnson et al., 2013). Additionally, genetically modified mice expressing low levels of mTORC1 as well as mice deficient for S6K1 – an important mTORC1 substrate – display extended lifespans (Lamming et al., 2012; Selman et al., 2009). Conversely, mTOR activity has been shown to increase with age for instance in mouse hypothalamic neurons and HSCs (Chen et al., 2009; Yang et al., 2012). Administration of rapamycin, a mTOCR1 inhibitor also known to improve proteostasis, to aged mice increases their life span (Harrison et al., 2009). This treatment also restores the self-renewal capacity of HSCs, therefore improving the immune system's function (Chen et al., 2009). Rapamycin also reverts the SC loss effect and alopecia caused by Wnt1 overexpression in the HF SCs (Castilho et al., 2009). In spite of the fact that TOR inhibition has an obvious beneficial impact during ageing, it also causes harmful side effects such as testicular degeneration and cataracts (Wilkinson et al., 2012).
This means that it will be essential to further dissect such metabolic pathways in order to design therapies capable of manipulating specific age related aspects without causing undesired secondary effects. Along these lines, CR has also been shown to enhance MuSC availability and activity both in adult and aged mice, as well as increasing their regenerative capacity (Cerletti et al., 2012).

During ageing, the muscle fibers in close proximity with MuSCs start expressing *Fgf2* at much higher levels than muscle fibers from adult mice. FGF2 is a well-characterized growth

factor with a mitogenic effect and the capacity to drive aged MuSCs out of quiescence. As a result, FGF signaling can ultimately compromise the regenerative potential and lead to the depletion in the number of aged MuSCs (Chakkalakal et al., 2012). The remaining MuSCs become unable to respond to FGF2 and to the need to expand or self-renew, resulting in the impairment of the regenerative potential of the muscle. This is due to the fact that aged MuSCs undergo age-associated changes in adhesion and ECM interactions that interfere with their response to different signals. For instance, less efficient fibronectin signaling – normally induced during regeneration - in aged muscle contributes to the FGF2 unresponsiveness described above. Accordingly, activators of integrin signaling rescue the MuSC responsiveness to FGF2 and the proliferative and self-renewal dysfunction in aged mice (Lukjanenko et al., 2016; Rozo et al., 2016).

***Mitochondrial malfunction***

Mitochondrial DNA (mtDNA) has long been considered a very likely target of mutations during ageing. MtDNA is subjected to the mitochondrial oxidative environment and, aside from lacking protective histone, the mtDNA repair mechanisms are very limited when comparing to those of nuclear DNA. Nonetheless, ultra-deep sequencing of mtDNA led to the observation that most mtDNA mutations are in fact caused by replication errors during development, instead of a consequence of oxidative damage accumulation. Upon polyclonal expansion, which occurred with time, these mutations may lead to dysfunction of the respiratory chain (Ameur et al., 2011). Further proof that mtDNA mutations could promote ageing was obtained from studies with mice deficient for mtDNA polymerase γ that accumulate random mtDNA point mutations and deletions and age prematurely and have a shortened lifespan (Kujoth et al., 2005; Trifunovic et al., 2004; Vermulst et al., 2008). Finally, multisystem disorders caused by mtDNA mutations were found to caused age-associated diseases in human patients (Wallace, 2005).

Mitochondrial malfunction and deterioration is strongly associated with changes in the balance of ROS production. In recent years new studies have shed light on the intracellular function of ROS and led to the re-evaluation of the concept that ROS were purely a source of oxidative stress and a cause of ageing (Hekimi et al., 2011; Van Raamsdonk and Hekimi, 2009; Zhang et al., 2009b). Therefore, it has been proposed that, similarly to $NAD^+$ and AMP, ROS could in fact act as a stress-induced survival signal. As the organism gradually ages, accumulation of different types of damage and cellular stress may result in increasing levels of ROS as a survival response. Nonetheless, it is likely that beyond a particular threshold, the levels of ROS become harmful betraying the purpose ROS have in a homeostatic setting (Hekimi et al., 2011). The mitochondrial deacetylase SIRT3 negatively regulates the levels of

ROS by deacetylating the superoxide dismutase 2 (SOD2) enzyme and, hence, promoting its antioxidative activity (Qiu et al., 2010).

***Loss of proteostasis***

Protein homeostasis, or proteostasis, involves the mechanisms necessary for the stabilization of properly folded proteins or degradation of misfolded proteins by the lysosome or the proteasome. These mechanisms are essential to prevent the accumulation of misfolded or damaged proteins and ensure the sustained renewal of intracellular peptides. Certain age-associated diseases like cataracts, Parkinson's disease, and Alzheimer's disease are in fact the result of chronic expression of misfolded, unfolded or aggregated peptides (Koga et al., 2011; Powers et al., 2009). There is also strong evidence for the decline of lysosomal and proteasomal activities with ageing (Rubinsztein et al., 2011; Tomaru et al., 2012).

Recently, it has been demonstrated how basal autophagy is essential to maintain the MuSC quiescent state in mice. Not only do autophagy levels decrease in aged mice, leading MuSCs into an irreversible senescent state, but also genetic impairment of autophagy in young cells leads them into senescence. This loss in proteostasis is also accompanied by increased mitochondrial dysfunction and oxidative stress that ultimately results in a decline of function and number of MuSCs. Finally, autophagy levels are also lower in human geriatric satellite cells (García-Prat et al., 2016).

***Ageing of adult stem cells***

During ageing the accumulation of all the above-mentioned types of DNA alterations and deregulated signaling may gradually contribute to the altered expression of vital genes and the activity of key signaling pathways. As result, tissues accumulate dysfunctional cells that, if not depleted by apoptosis, senescence, or other mechanisms, may compromise the homeostasis of the tissue and organism. The aged-associated SC loss of function may result from a drop in the activation/proliferation potential or in decreased self-renewal capability (Liu and Rando, 2011). The study of the ageing process of the different SC types is particularly relevant since, when damaged, tissue regeneration and renewal is at stake.

The current knowledge on the causes underlying SC ageing is still very limited. It has been hypothesized that a decrease over time on SCs function may majorly contribute to the overall changes perceived as ageing. Adult SCs have characteristics that may protect them against age-associated threats, including a tightly controlled turnover rate and a highly specialized niche. Nevertheless, adult SC function declines with ageing and lesions may be passed on to their progeny. As a consequence, the regenerative potential of the respective tissues significantly decreases with age (Liu and Rando, 2011). Regarding the skin, previous

studies found no detectable differences between adult and aged epidermal SCs (Giangreco et al., 2008; Stern and Bickenbach, 2007). However, not long after, it was reported an increased number of bulge SCs accompanied by decreased function and lower tolerance to stress conditions in association to skin ageing. Interestingly, in these cells, Jak-Stat signaling - implicated in molecular reprogramming (Yang et al., 2010), was also found to be altered with ageing (Doles et al., 2012). Stat3 skin-specific KO aged mice suffer from ulcers and seldom hair formation (Sano et al., 1999). Notwithstanding, while EpSCs have been intensively studied in the context of homeostasis and malignancy, there is a general lack of knowledge concerning the mechanisms that govern EpSC ageing. In fact, there is still very little known about what characterizes an aged EpSC.

Aged MuSCs, on the other hand, apart from lower levels of basal autophagy, show a tendency to convert from a myogenic to either a fibrogenic or adipogenic lineage upon proliferation (Brack et al., 2007; Taylor-Jones et al., 2002). The conversion into a fibrogenic lineage is the result of the activation of the canonical Wnt signaling pathway (Brack et al., 2007). Changes in Notch and TGF-β signaling have also been reported in aged MuSCs interfering with their regenerative capacity (Carlson et al., 2008; Conboy et al., 2005). The overall number of MuSCs is also known to decrease with age although there has been some discrepancy between studies. Their ability to respond to activation cues and proliferate, as well as differentiate and fuse into myotubes is also severely compromised with age. Furthermore, recent studies have demonstrated the importance of extrinsic factors for SC function by transplanting MuSCs from young mice into progeroid mice. As a result, progeroid mice displayed extended lifespan and improvement of generic age-associated degenerative changes even in tissues where donor cells were not detected (Lavasani et al., 2012). This indicates that the therapeutic benefits of young tissue transplantation may also arise from systemic effects derived from secreted factors. Accordingly, parabiosis experiments lead to improvement of MuSC function in aged mice, further underscoring the importance of humoral factors for tissue regeneration and SC potential(Conboy and Rando, 2012; Conboy et al., 2005).

Recent evidence suggests that in physiologically-aged mice both the daily electrical activity and the expression of the core clock machinery are dampened in their SCN (Bonaconsa et al., 2014; Chang and Guarente, 2013; Farajnia et al., 2012; Nakamura et al., 2011). Accordingly, humans show a decline in the robustness of their sleep and wakefulness cycles during ageing (Roenneberg et al., 2007). Concomitantly, it has been hypothesised that overall behaviour and tissue function become arrhythmic during ageing. Nonetheless, to date no studies have addressed if and how these rhythms are altered in aged stem cells.

The process of SC ageing is extremely complex and thus has raised the attention of experts from various fields. It is conceivable and very likely that, according to the demands and idiosyncrasies of their respective tissue, each SC type will develop a combination of the above-mentioned hallmarks of ageing. Interestingly though, all the so far identified hallmarks of ageing might at some level either be under circadian regulation, or impact on cellular functions that are indeed rhythmic. Future studies will determine to what extent this is the case, and the physiological relevance of circadian regulation of such hallmarks during the ageing process of SCs.

## *Objectives*

Tissue function is subject to daily fluctuations, and the activity of adult SCs and their progenitors is consequently under robust circadian control. The core circadian machinery is predominantly responsible for establishing most day-night rhythmic oscillation of genes important for tissue homeostasis in numerous tissues.

Due to the observation that the rhythmic activity of the SCN decreases with age, it has been assumed that most peripheral tissues, if not all, suffer dampening of their rhythmic functions as a consequence of ageing. However, to our knowledge, there is no formal proof to date that confirms this hypothesis and, hence, several important questions remain unanswered, particularly when considering the proven functional decline of most stem cell functions with ageing. With this in mind, we have decided to explore the following objectives in the context of two very distinct types of adult SCs - EpSCs and MuSCs:

A) Understand if and how the clock is perturbed during ageing of epidermal and muscle stem cells.

B) Depict the impact of the newly rhythmic program on the affected downstream pathways and its contribution to physiological ageing.

C) Determine whether changes in diet may prevent or mimic the newly-described age-related traits.

D) Identify novel factors or interactions that may contribute to such age-related traits.

E) Compare the age-related rhythmic reprogramming across different tissues and identify a putative stem cell rhythmic signature and a tissue-independent signature.

## *Materials and Methods*

## *Experimental models and subject details*

### *Animal models*

*Bmal1* KO, *Per1/Per2* double KO (Zheng et al., 2001) and wild-type mice were bred and aged at the animal facilities of the Barcelona Science Park. All mice were of C57Bl/6 background, except for *Per1/Per2* double KO that is a mixed B6:129S background. For the experiments specified below, C57Bl/6J mice were purchased from Charles River, in strict accordance with the Spanish and European Union regulations. The Catalan Government approved the work protocols, in accordance with applicable legislation. Both male and female mice were used in each experiment unless stated otherwise. All mice were fed with the standard chow used at the animal facilities of the Barcelona Science Park, composed of 12.2%/w protein, 2.27%/w fatty acids and 82.08%/w carbohydrates, with 12.22 Kcal/g, unless stated otherwise.

For the transcriptomic study of rhythmic oscillations in adult and aged mice, C57Bl/6J female mice purchased from Charles River. We used adult (8-week-old) and aged adult (between 102–116-week-old) mice. Four mice were used per each of the six time points (ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20) and per age group.

For the transcriptomic study of daily rhythmic oscillations in calorie-restricted aged mice, C57Bl/6 aged and non-aged adult mice were fed either *ad libitum* a control diet (Harlan TD.120685) or with a 30% food reduction as compared to the *ad libitum* group intake of calorie-restricted diet (Harlan TD.120686) (Harlan Industries, Indianapolis, IN, USA). The control diet was composed of 18.8%/w protein, 7.3% %/w fatty acids and 55.1%/w carbohydrates, with 3.6 Kcal/g. The restricted diet was composed of 32.9%/w protein, 12.7%/w fatty acids and 31.9%/w carbohydrates, with 3.7Kcal/g. Mice were weighed every two weeks. Calorie-restricted mice were housed individually to prevent food competition with cage mates.

The experiment lasted for 25 weeks and started after an adaption period of 3 weeks, during which calorie-restricted animals were subjected to a 10% food reduction per week. The age groups were composed of 19–29-week-old and 55–69-week-old mice at the start of the adaption period. Four mice were used per each of the 6 time points (ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20) and per diet group. For sample collection, one mouse per diet group was sacrificed per day, all within the same time point. Both muSCs and EpSCs were collected for transcriptome analyses.

For the transcriptomic study in adult mice fed a high-fat diet, eight-week-old female C57Bl/6J mice were purchased from Charles River and fed *ad libitum* either a high-fat diet (Harlan TD. 06414) or a control diet (Harlan TD.120455). The control diet was composed of 18.8%/w protein, 7.3%/w fatty acids and 55.1%/w carbohydrates, with 3.6Kcal/g. The high-fat diet was composed of 23.5%/w protein, 34.3%/w fatty acids and 27.3%/w carbohydrates, with 5.1 Kcal/g. Mice were weighed every two weeks.

The experiment lasted for 7 weeks, and four mice were used for each of the six time points (ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20) and for each diet group. For sample collection, four mice per diet group were sacrificed each day (all of which belonged to the same time point).

For physiological activity monitoring, mouse activity was measured in IntelliCage™ cages (Lipp et al., 2005). This method allows measurement of basal physiological activity in a social environment. Microchips were inserted in the back of 10 adult (13-week-old) and 10 old (83-week-old) C57Bl/6J to monitor individual visits to the drinking corners. Only females were used in order to avoid fighting due to hierarchies established by male mice when pooled during adulthood. Adult and aged mice were caged in separate IntelliCage™ cages to avoid interference of activity between groups. Mice had continuous access to food and water and were adapted for a week to the cages before the experiment started. Mice were subjected to a 12hr light:12hr dark photoperiod for the first week to use as reference, then they were switched to constant darkness for two weeks, followed by two weeks of 12hr light: 12hr dark photoperiod. The results for each mouse and each week were analysed

separately. In order to avoid interference of the difference in total activity between adult and aged mice, the assessment of the rhythmicity of the activity was performed using the proportion of visits during each 4hr-bin divided by the total number of visits. To identify rhythmic behaviour, the values of activity during each interval for each week were given to the Jonckheere-Terpstra-Kendall (JTK) algorithm (Hughes et al., 2010). Two of the aged mice had to be removed of the study due to health issues.

To study the aged-related features in *Bmal1* KO and *Per1/Per2* double KO, wild-type, *Bmal1* KO and *Per1/Per2* double KO mice were fed control diet (Harlan TD.120685) composed of 18.8%/w protein, 7.3%/w fatty acids and 55.1%/w carbohydrates, with 3.6 Kcal/g. For *Bmal1* KO, four 18-week-old mice were used per genotype; for *Per1/Per2* double KO, four 27-39-week-old mice were used per genotype. All mice were sacrificed at ZT16.

## *Method Details*

### *FACS sorting and analysis of epidermal and muscle stem cells*

After sacrifice, 1cm$^2$ skin samples were collected for histology. Skin samples were scraped with a scalpel to remove the hypodermis and then floated on trypsin 0,25% (Gibco) dissolved in PBS for approximately 50 min at 37ºC, allowing the epidermis to be separated from the dermis. Trypsin was inactivated with 15% chelated foetal bovine serum (Gibco) diluted in EMEM medium without calcium (Lonza). Epidermis was mechanically disaggregated and dissociated and sequentially filtered through a 100-m and a 40-m filter. Anti-mouse CD34-biotin (1:50, eBioscience 13-0341-85), streptavidin-APC (1:400, BD Pharmingen 554067) and anti-human CD49f-RPE (1:200, AbD Serotec MCA699PE) were used for sorting and FACS analysis of CD34 negative/CD49f bright interfollicular stem cells,

Muscles were mechanically disaggregated and dissociated in Ham's F10 media containing collagenase II 0.2 % (Sigma) with $CaCl_2$ (2.5 mM) at 37 °C for 30 min. Tissue

solution was then centrifuged and further dissociated in a collagenase D/Dispase solution (Roche, 1.5 U/ml and 2.4 U/ml respectively) at 37 °C for 60 min and then filtered through a 40-m filter. Cells were incubated in lysing buffer (BD Pharm Lyse) for 10 min on ice, re-suspended in PBS with 2.5% goat serum and counted with a haemocytometer. FITC-conjugated anti-CD45 (1:300, Biolegend 103113/14), Alexa 647 anti-F4/80 (1:200, AbD Serotec MCA497A647), APC anti-CD31 (1:300, Biolegend 102418), Pe-Cy7 anti-Sca-1 (1:300, Biolegend 108113/14) and PE-conjugated anti-α7-integrin (1:200, Ablab AB10STMW215) were used to separate macrophages, endothelial cells, mesenchymal cells and muSCs populations, respectively. Sorting gates were strictly defined based on isotype control (fluorescence minus one) stains. To assess viability, cells were stained with

DAPI (1 µg ml–1) immediately before sorting. Sorted muSCs were used for RNA extraction, microarray analysis, or cytospin for immunostaining. MuSCs were confirmed as a homogenous population of Pax7+ cells (data not shown).

***Microarrays***

EpSCs RNA was extracted using TRIzol (Sigma-Aldrich). cDNA library preparation and amplification were performed from 25 ng total RNA using WTA2 (Sigma-Aldrich), with 17 cycles of amplification.

MuSCs were lysed for 15 minutes at 65°C in a TrisHCl, SDS, proteinase K and DTT solution (final concentrations were 10mM, 0.5%, 1 g/l and 20 mM, respectively). RNA was purified using magnetic beads (RNAClean XP beads, Agencourt). All purified RNA was used for library preparation and amplification. Amplified cDNA libraries were prepared according to manufacturer's (Sigma-Aldrich) recommendations for WTA2. In order to monitor the amplification, SYBR Green was added to the reaction, which was was stopped at 24 cycles when SYBR Green signal had reached a plateau. cDNA was purified using Purelink (Invitrogen).

cDNA (8 µg) from both EpSCs and muSCs was subsequently fragmented by DNaseI and biotinylated by terminal transferase obtained from GeneChip Mapping 10Kv2 Assay Kit

(Affymetrix). Hybridisation mixtures were prepared according to the Gene Atlas protocol (Affymetrix). Each sample target was hybridised to a Mouse Genome 430 PM array. After hybridisation for 16 h at 45°C, samples were washed and stained in the GeneAtlas Fluidics Station (Affymetrix). Arrays were scanned in a GeneAtlas Imaging Station (Affymetrix). All processing was performed according to manufacturer's recommendations. CEL files were generated from DAT files using Affymetrix Command Console software. Microarrays processing was performed at IRB Barcelona Functional Genomics Core Facility.

Processing of microarray samples was carried out separately for each dataset using R (http://www.R-project.org/)(RDevelopment, 2008) and Bioconductor (Gentleman et al., 2004). Raw CEL files were normalised using RMA background correction and summarisation (Irizarry et al., 2003). Standard quality controls were performed in order to identify abnormal samples (Davis, 2007) regarding: a) spatial artefacts in the hybridisation process (scan images and pseudo-images from probe level models); b) intensity dependences of differences between chips (MvA plots); c) RNA quality (RNA digest plot); d) global intensity levels (boxplot of perfect match log-intensity distributions before and after normalisation and RLE plots); and e) anomalous intensity profile compared to the rest of samples (NUSE plots, Principal Component Analyses). A total of 15 samples were excluded across all datasets due to quality issues (from the skin HFD array samples Skin_Control.CT4.4, Skin_Control.CT8.3, Skin_Control.CT8.4, Skin_HFD.CT8.3, Skin_HFD.CT8.4, Skin_Control.CT8.3 Skin_HFD.CT0.4 and Skin_Control.CT12.4; from the skin CR array samples Skin_ND_CT8.4, Skin_CR_CT12.5, Skin_CR_CT4.4 and Skin_Adult_ND_CT8.4; from the muscle CR array samples SC_Adult_ND_CT20.2, SC_Adult_ND_CT20.3, SC_Adult_ND_CT20.4 and SC_Aged_CR_CT20.5). Probesets were annotated using information available on the Affymetrix webpage (https://www.affymetrix.com/analysis/index.affx). Prior to downstream analyses, expression values were corrected for amplification and scanning batches using a linear model in which gender, time point, diet, age and the pairwise interaction terms between the three last were included as covariates. Correction by metrics (Eklund and Szallasi, 2008) was then carried out for the muSCs

calorie restriction dataset, and for both muSCs and EpSCs high fat diet datasets. Additionally, correction for extraction date was performed for the EpSCs high fat diet dataset. Microarrays were analysed at IRB Barcelona Biostatistics/Bioinformatics Core Facility.

***Immunofluorescence and immunohistochemistry***

Primary antibodies used are rabbit polyclonal anti-RPA32/RPA2 (phospho Ser4, Ser8) (1:100, Abcam ab87277), mouse monoclonal anti-phospho-histone H2A.X (Ser139) (1:200, Millipore 05-636), mouse monoclonal anti-8-hydroxyguanosine (1:2000, Abcam ab62623), mouse monoclonal anti-PCNA (PC10) (1:200, Santa Cruz sc-56), rabbit polyclonal anti-MCM4 (1:500, kindly provided by Dr. Juan Mendez, CNIO, Madrid, Spain (Búa et al., 2015)), rabbit polyclonal anti-caspase 3 (1:500, Cell Signaling Technology, 9661S), rabbit polyclonal anti-phosphohistone-3 (Ser10) (1:500, 06-570 EMD, Millipore), mouse monoclonal anti-Pax7 (1:20, DSHB), rat polyclonal anti-LAMP-1 (1:200, Santa Cruz Biotechnology sc-19992), rabbit polyclonal anti-LC3 (1:100, Novus Biologicals NB100-2331) and rabbit polyclonal anti-53BP1 (1:200, Abcam ab21083).

Mouse back skin was fixed in 10% NBF for 3 h at room temperature and then processed for embedding in paraffin blocks. Antigen retrieval was performed for 20 min at 97ºC with citrate (pH 6) on 3-micron tissue sections. Sections were permeabilised for 20 min with a 0.05% Triton X-100 in PBS solution, and blocked with a 10% goat serum in PBS solution for 1 h at room temperature. Primary antibody incubation was done overnight at 4ºC. Secondary antibody incubation was done at room temperature for 2 h. Secondary antibodies used are anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor 647 (1:500, Molecular Probes). Nuclei were stained with DAPI (Invitrogen). All washes were with PBS except when staining for phospho-histone H2A.X and RPA32/RPA2, in which case washes were done with PBS-T 0.4% BSA. To stain with MCM4, phosphohistone-3 or caspase3, endogenous peroxidase was quenched after antigen retrieval with a 10-min incubation with peroxidase-blocking solution (Dako REAL S2023). After primary antibody incubation (for 60, 90 and 120 min, respectively) at room temperature, a BrightVision poly-HRP anti-rabbit IgG

biotin-free (Immunologic, DPVR-110HRP) was used. Antigen–antibody complexes were revealed with 3-3-diaminobenzidine (K3468, Dako). Sections were counterstained with hematoxylin (Dako, S202084).

*Tibialis anterior* muscles were frozen in isopentane cooled with liquid nitrogen and stored at –80 °C until analysis, at which point 10-µm sections were cut and immunostained. Cryosections were rinsed once with PBS and fixed in

2–4% paraformaldehyde for 10 min at room temperature. Sections were rinsed three times for 5 min with PBS, permeabilised with 0.5% Triton X-100 in PBS for

10 min at room temperature followed again by rinsing them three times with PBS.

Sections were blocked in PBS supplemented with 5% goat serum, 5 % BSA and 1:40 mouse on mouse blocking reagent (Vector labs) for 1 h at room temperature. Incubation with primary antibodies was carried out overnight at 4 °C. The next day, sections were rinsed three times with PBS followed by incubation with secondary antibodies coupled to Alexa-488, Alexa-568 or Alexa-647 fluorochromes (1:500, Molecular Probes) for 1 h at room temperature. Sections were rinsed again with PBS and nuclei were counterstained with DAPI (1 µg ml–1) in PBS before mounting with Fluoromount (Sigma). Slides were stored at 4 °C until analysis. For all the stainings, at least four biological replicates were used.

***Microscopy and image analysis***

Fluorescence pictures from four biological replicates were acquired using either a Leica TCS SP5 or a Zeiss LSM780 confocal microscope (63/1.40 oil objective at 512  512 or 1024  1024 pixel resolution) and processed using the Fiji v2.0.0-rc-14/1.49g software (ImageJ) (Schindelin et al., 2012).

PCNA-stained sections (from the calorie restriction study) and MCM4-stained sections were imaged using a NanoZoomer 2.0HT (Hamamatsu, Japan) (20 objective at 0.46m/pixel). Caspase 3–stained sections were imaged with a upright Nikon E800 microscope (60/1.4 oil objective at 2070  1548 pixel resolution).

### *RT-qPCR*

RNA was extracted using TRIzol and converted into cDNA by reverse transcriptase using the high-capacity cDNA reverse transcription kit (AppliedBiosystems). Gene expression was quantified by quantitative real-time PCR using TaqMan Master Mix and the following TaqMan probes (ThermoFisher Scientific): *E2f1* (Mm00432939_m1), *Chek1* mouse (Mm01176757_m1), *Mcm2* mouse (Mm00484815_m1), *Mcm6* mouse (Mm00484848_m1) and *Pola2* mouse (Mm00447142_m1), and (for normalisation) *B2m* mouse (Mm00437762_m1). A LightCycler® 480 instrument (Roche) was used. Four biological replicates were used in each assay.

### *Gene ontology*

Gene Ontology (GO) analyses was done using Genomatix (https://www.genomatix.de/), using the Gene Ranker package, with a *p*-value of 0.01. The categories belonging to “biological processes” (BP) were used to perform GO comparisons.

Heatmaps of the GO genes were performed by extracting the genes from the corresponding GO terms, along with the corresponding expression values from the arrays. For each gene, the mean expression value of all replicates per time point was calculated and normalised on a [–1,1] scale. Then, genes were ordered according to their lag obtained from the JTK output file. Heatmaps were plotted using R version 3.2.4.

## *Quantification and statistical details*

### *Number of replicates used*

The number of biological and/or technical replicates for each experiment is stated in the “Methods details” section and the figure legends.

***Quantification***

PCNA and phosphohistone 3–positive nuclei from epidermis of adult and aged mice were manually counted. MuSC LC3–positive autophagosomes, and LAMP1-positive lysosomes were manually counted, and the co-localisation of both vesicles was determined on digital images with respect to the total cellular area (background was reduced with Photoshop CS5 [Adobe], with brightness and contrast adjustments applied to the entire image). Phospho-histone H2A.X, RPA32/RPA2 and 8-hydroxyguanosine signal intensity levels were determined using Fiji v2.0.0-rc-14/1.49g software (ImageJ). PCNA-stained sections (from the calorie restriction study) and MCM4-stained sections were quantified using TMARKER v2.142 software (NEXUS Personalised Health Technologies).

Distance between hair follicles and size of the cornified layer were quantified by measuring these parameters on calibrated images on Fiji v2.0.0-rc-14/1.49g software (ImageJ).

To compare the muscle SC abundance between mice, we calculated the number of SCs per mg of tissue by multiplying the SC proportion generated by FACS to the total number of cells counted following digestion over the initial weight of isolated skeletal muscle (to normalise the efficiency of the enzymatic digestion). Muscle SC proportion was calculated over the living cells (gated with DAPI) as shown in gating scheme. Total number of mononuclear cells after tissue enzymatic digestion was counted using a haemocytometer.

***Statistical significance tests***

Statistical analysis was performed using Prism 6 software (Pad Software, Inc.). Experimental groups were compared by unpaired t-test with Welch's correction. The ZT16 time point of the rhythmic genes in the different conditions was compared by two-sided t-test (scipy.stats package). *, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$, ****, $p \leq 0.0001$. Error bars represent ± standard error of the mean (S.E.M.), unless otherwise stated in the figure legends.

### *Identification of rhythmic genes*

To identify genes with a daily rhythmic expression from the different sets of gene expression data, the Jonckheere-Terpstra-Kendall (JTK) algorithm was used (Hughes et al., 2010). For all array sets except for the calorie restriction dataset, a permutation-based *p*-value of less than 0.05 was considered significant. Applying a 0.05 threshold to the calorie restriction array identified non-rhythmic genes as rhythmic. Therefore, the threshold was adjusted to the average *p*-value of the known rhythmic genes—in this case, 0.005.

The expression levels for all rhythmic genes at each time point were calculated as the average expression of the biological replicates. The error bars represent standard deviation.

Amplitude and phase estimations of oscillating genes were extracted from the JTK algorithm. Kernel-density estimations (KDE) were computed and plotted for the oscillation amplitudes from each array using Python Seaborn library. Phase estimations were performed by computing the corresponding histogram from each array, and by fitting the parametric distribution to the data, using the Python Seaborn library.

For the gene length graph, early and late phase genes were identified according to their phase estimated by JTK. Early phase genes were defined as those phasing between ZT20 and ZT4, and late phase genes, those between ZT8 and ZT16. The gene lengths were extracted from the UCSC annotation, and a KDE for each category was computed, along with the estimation for the whole mouse gene set. Plotting was done with the Seaborn library (version 0.7.1) in Python (2.7.11 within Anaconda 4.0.0; 64-bit).

## *Data and software availability*

All array expression data files have been uploaded to the NCBI GEO database (GSE84580).

## *Results*

# *Chapter 1 –*

# *Aged MuSCs and EpSCs undergo rhythmic reprogramming specific to tissue stress*

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***The core clock machinery is robustly rhythmic, but the oscillatory output is reprogrammed in aged adult stem cells.***

Aiming to unveil possible disturbances or the disruption of the molecular clock and associated rhythmic functions occurring during physiological ageing of both EpSCs and MuSCs, we first designed an experiment to obtain and analyse their oscillatory transcriptome. We FACS-sorted integrin 6$^{bright}$/CD34– EpSCs, and integrin 7$^{bright}$/CD34+ MuSCs from adult and aged mice **(Fig. 6a and b)**. Four adult and four aged mice were

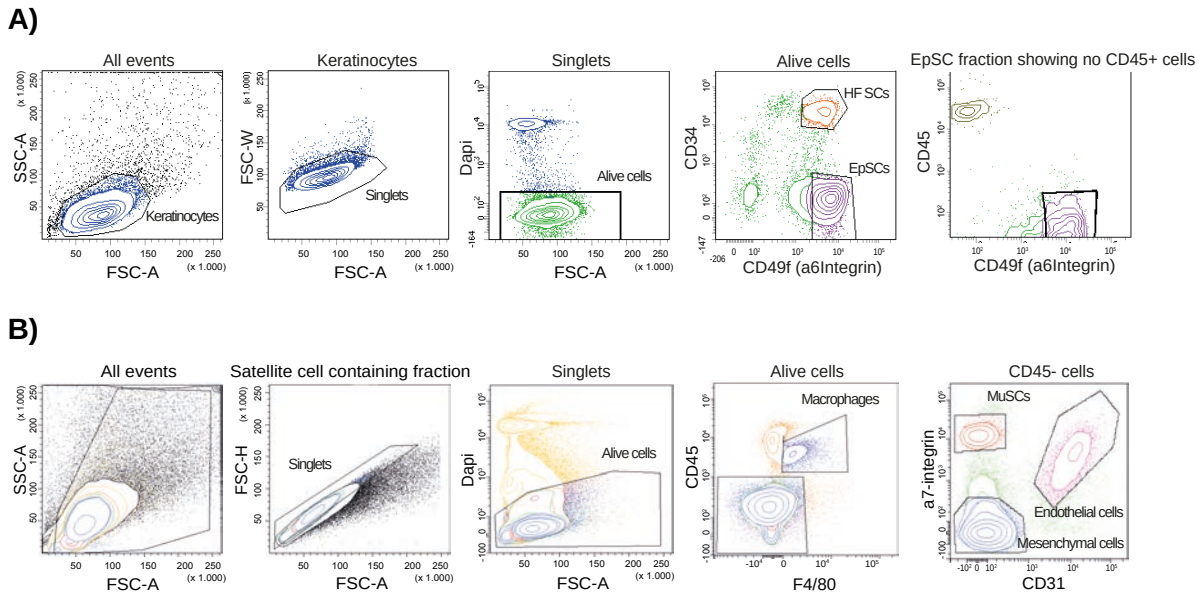



**Figure 6 - FACS Isolation Strategy.**

(A) Representative examples are shown of the FACS strategy and gating scheme for EpSC and hair follicle SC isolation from mice; and FACS analysis of EpSCs verifying that no CD49f-positive cells were also CD45-positive in epidermal preparations. (B) Representative example of the FACS strategy and gating scheme for MuSC isolation from mice. All cellular populations present in skeletal muscle are indicated.

sacrificed at each one of six time points over a day (four hour interval between time points). Time points were defined using the zeitgeber times of ZT0 (defined as lights on), ZT4, ZT8, ZT12 (defined as lights off), ZT16 and ZT20 **(Fig. 7a)**. We then analysed the whole transcriptome with gene expression arrays, and used the JTK algorithm **(Methods)** to identify the transcripts that are rhythmically expressed in a diurnal manner. Transcripts that oscillate in a diurnal manner are defined as genes which expression follows a periodicity of approximately 24 hours, oscillating with a 12-hour phase (the peak and trough of expression are 12-hour apart).

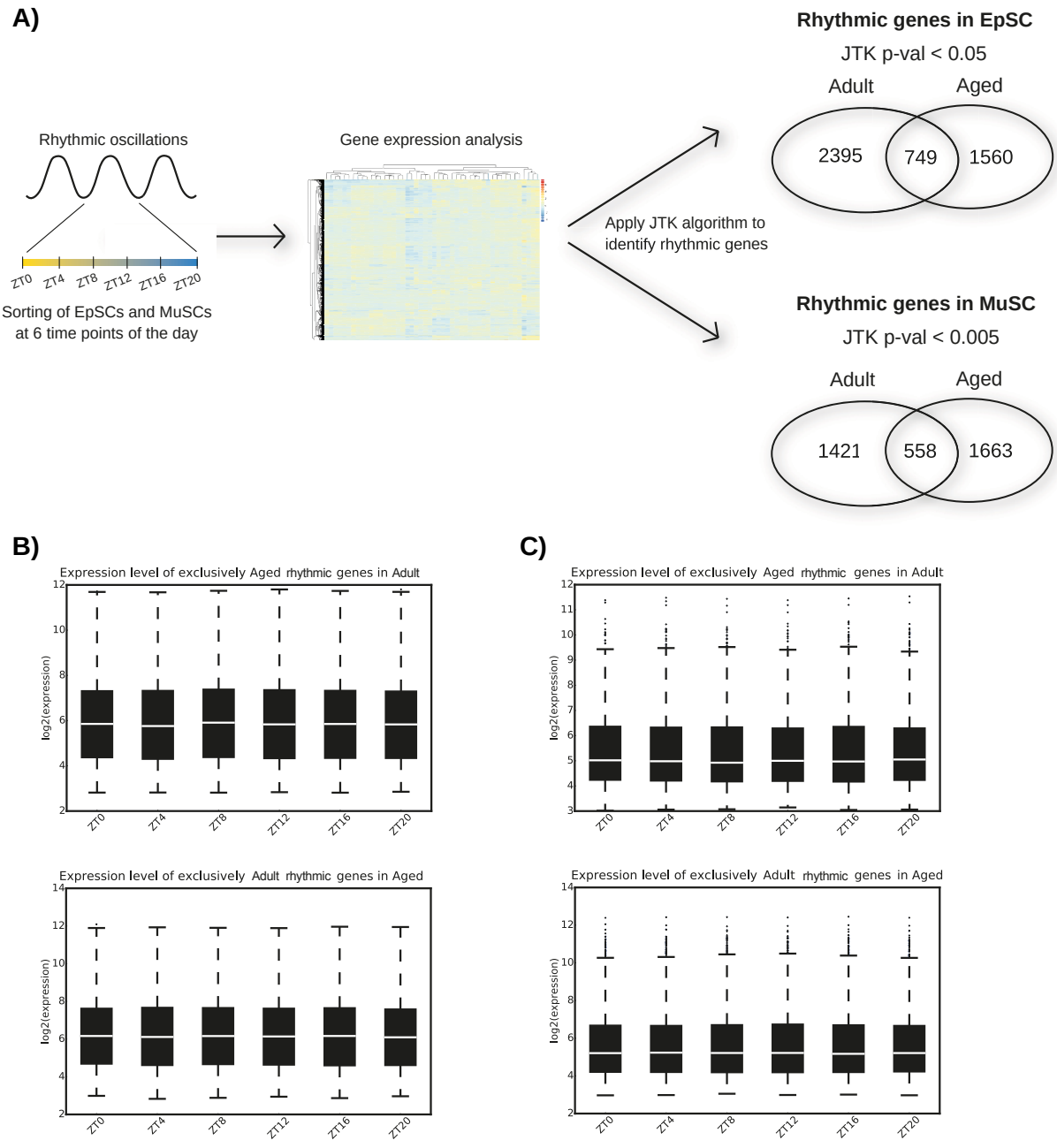



**Figure 7 - Reprogramming of the Rhythmic Transcriptome in Aged EpSCs and MuSCs.**
(A) Experimental setup and number of rhythmic genes in adult (3144 genes) and aged (2309 genes) EpSCs, and in adult (1979 genes) and aged (2221 genes) MuSCs. SCs were sorted every 4 hr, at time points ZT0 (lights on), ZT4, ZT8, ZT12, ZT16, and ZT20 (with ZT given in hours). Extracted RNA was processed for microarrays, and the JTK algorithm was used to determine daily rhythmic genes ($p \leq 0.05$). (B) Expression levels in adult EpSCs of genes exclusively rhythmic in aged EpSCs (above) and expression levels in aged EpSCs of genes exclusively rhythmic in adult EpSCs (below). (C) Expression levels in adult MuSCs of genes exclusively rhythmic in aged MuSCs (above) and expression levels in aged MuSCs of genes exclusively rhythmic in adult MuSCs (below).

We detected robust rhythmic expression in 3,144 transcripts from adult EpSCs, and 1,979 transcripts from adult MuSCs, of which 76% and 72%, respectively, ceased to be oscillatory

in aged mice **(Fig. 7a)**. Although both types of aged SCs retained a similar percentage (23–28%) of transcripts that remained rhythmic, they both expressed a new cohort of oscillatory genes that was not present in adult counterparts. Note that the average level of expression of genes oscillatory only in adult SCs remained the same over time in aged mice and conversely, indicating that the loss or gain of rhythmically expressed genes in aged SCs is not explained by the downregulation or upregulation of their expression respectively **(Fig. 7b, and c)**.

Since it has been previously described that the circadian clock is dampened in the SCN of aged mice, we wondered whether the reprogramming of the oscillatory output we observed could be directly caused by the deregulation of core clock gene expression in EpSCs or MuSCs. We, therefore, analysed the core clock genes and compared their expression patterns between adult and aged SCs. In both types of aged SCs, all core clock genes oscillated with equal amplitude and period than in adult SCs, including those that are either transcriptionally regulated directly by Bmal1/Clock **(Figs. 8a and b)**, or by the secondary circadian loop ROR/Rev-Erb (data not shown). In addition, the average amplitude of oscillations of the daily rhythmic transcriptome between adult and aged SCs was identical **(Figs. 8c and d)**.

Finally, we wondered if the rhythmic reprogramming observed in aged EpSCs and MuSCs could be related to age-related alterations of their circadian nocturnal behaviour. We measured the activity of ten adult and ten aged mice by placing them in intelliCages. Activity was measured by monitoring the number of individual visits to the drinking corners **(Methods)**. Visits were first recorded for one week in a L:D photoperiod (12hr of light followed by 12hr of darkness housing conditions), then for two consecutive weeks in D:D (constant darkness housing conditions), and lastly mice were placed back in a L:D schedule for two more weeks **(Fig. 9a)**. Overall, the total number of visits of aged mice throughout the 24hr of the day was significantly lower – reflecting less activity – than in adult mice **(Fig. 9b)**. By applying the JTK algorithm we found that their physiological activity, however, remained robustly circadian. During L:D conditions and after two weeks of constant darkness, both adult and aged mice were more active at ZTs correspondent to the “night” phase with comparable amplitude and p-value **(Figs 9c, d and e, and Methods)**.

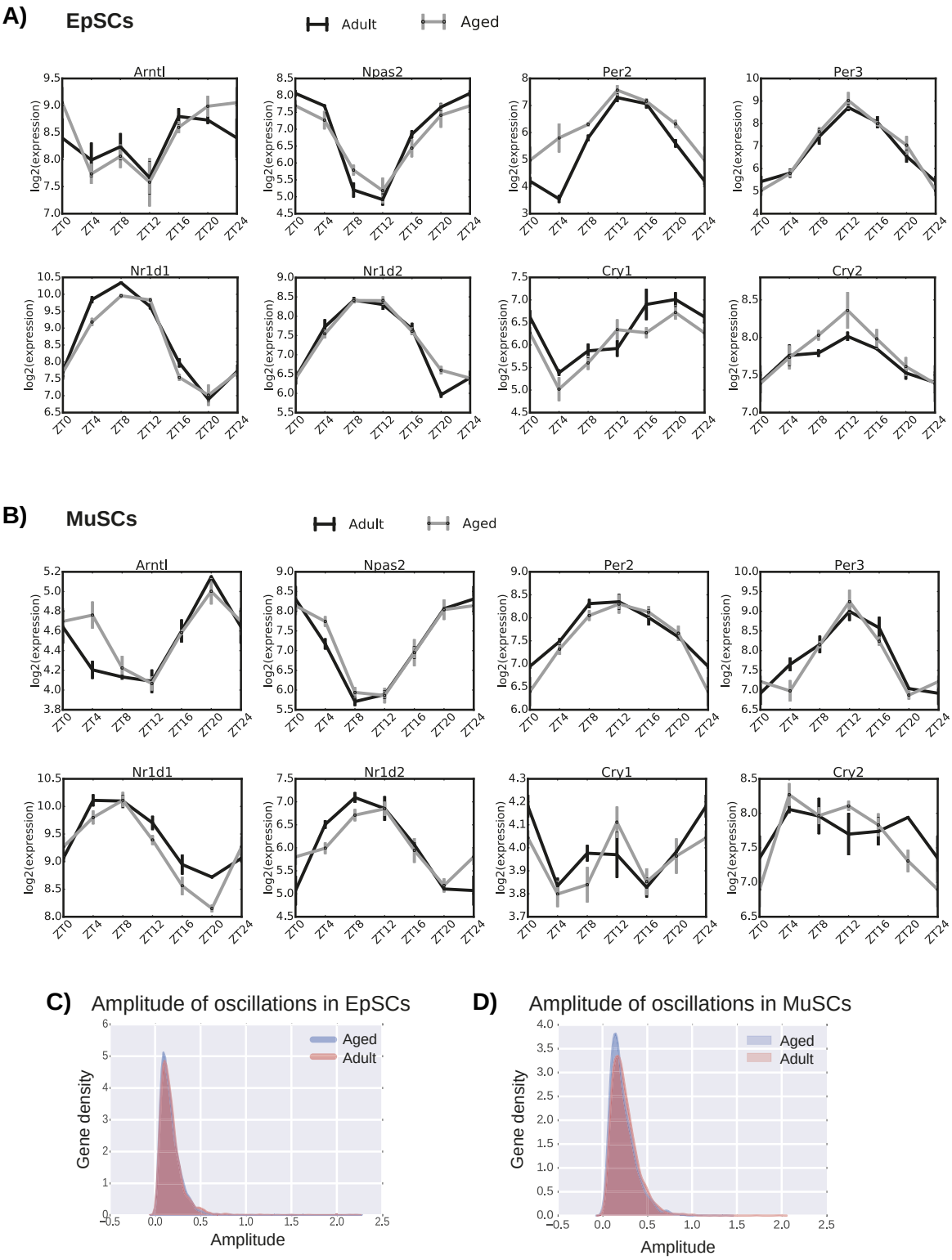



**Figure 8 - The Core Clock Machinery remains robustly Circadian in Aged EpSCs and MuSCs.** (A, B) Expression levels over time of core clock circadian genes (*Arntl*, *Npas2*, *Per2*, *Per3*, *Nr1d1*, *Nr1d2*, *Cry1,* and *Cry2*) in adult and aged EpSCs (A) and MuSCs (B). Error bars represent SD. ZT24 = ZT0. (C, D) Distribution of oscillation amplitude of the adult and aged sets of rhythmic genes in EpSCs (C) and MuSCs (D), normalised for their total number of rhythmic genes. Results are presented as gene density.

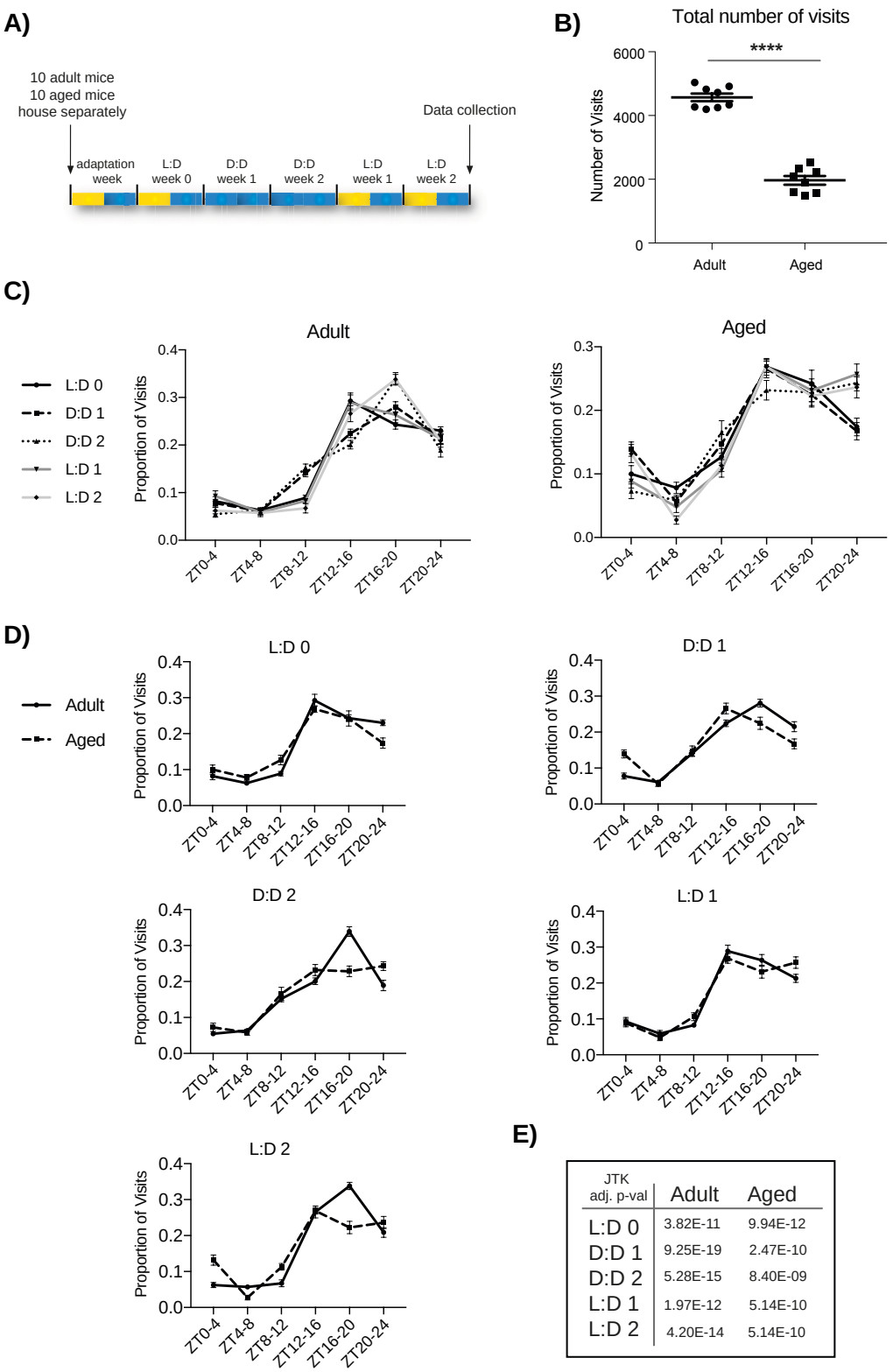



**Figure 9 - Aged Mice Retain Circadian Physiological Activity.**

(B) Total number of visits to the drinking corners of adult and aged mice during experiment (5 weeks). (C) Proportion of visits for each week of experiment comparing adult and aged mice. (D) Proportion of visits for adult and aged mice during each week of the experiment. Bins of 4 hr were accumulated and plotted together. Error bars represent ±S.E.M. (E) Adjusted p-values obtained when applying the JTK algorithm on the values of number of visits for every week and age group. L:D, 12hr light:12hr dark photoperiod. D:D, constant darkness. **** $p \leq 0.0001$.

### *Aged EpSCs lose their diurnal epidermal homeostasis, but establish a de novo oscillatory output predominantly associated to DNA damage*

In order to start understanding the functional consequences of this rhythmic reprogramming in aged SCs, we first performed GO analysis of the oscillatory genes. Transcripts that were oscillatory only in adult EpSCs predominantly regulated homeostatic functions, such as *keratinocyte differentiation*, *wound healing,* and *epidermis development.* Such transcripts ceased to oscillate in a diurnal manner in aged EpSCs as further shown by the respective heatmaps **(Fig. 10a)**. However, transcripts that became newly rhythmic in aged EpSCs were no longer related to homeostatic functions but rather to stress conditions: mainly inflammation (*cellular response to IL6*) and DNA damage (*mismatch repair*, *mitotic DNA damage checkpoint*, *double strand break repair* and *replication fork processing*). The respective heatmaps corroborate the rhythmic expression of these transcripts in aged EpSCs only **(Fig. 10b)**. The cohort of genes that remained rhythmic upon ageing belonged to three main categories: *control of circadian rhythms*, *DNA replication*, and *mitosis*. The respective heatmaps further underline how the oscillatory behaviour of these transcripts is maintained during ageing **(Fig. 10c)**.

**A)**

| **Adult only** rhythmic genes | P-value |
|---|---|
| secretion | 5.89E-08 |
| wound healing | 8.72E-06 |
| response to oxygen levels | 3.89E-05 |
| autophagy | 7.80E-04 |
| epidermis development | 1.06E-03 |
| keratinocyte differentiation | 1.45E-03 |

**B)**

| **Aged only** rhythmic genes | P-value |
|---|---|
| DNA repair | 4.50E-12 |
| double strand break repair | 5.10E-06 |
| mitotic DNA damage checkpoint | 1.28E-05 |
| recombinational repair | 1.12E-04 |
| cellular response to IL-6 | 3.00E-04 |
| mismatch repair | 4.18E-04 |
| replication fork processing | 8.72E-03 |

**C)**

| **Common** rhythmic genes | P-value |
|---|---|
| circadian regulation of gene expression | 3.02E-08 |
| DNA repair | 1.44E-06 |
| cell cycle DNA replication | 2.99E-04 |
| double strand break repair | 1.81E-03 |
| mitotic M phase | 2.80E-03 |

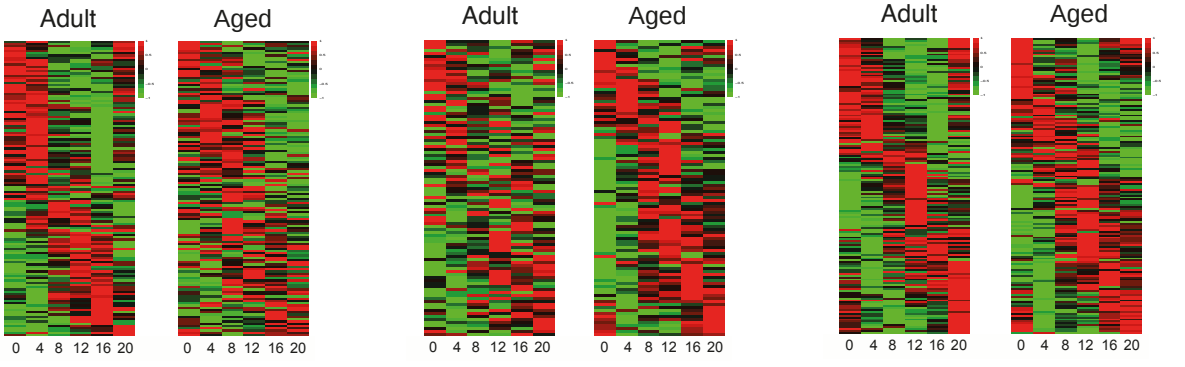
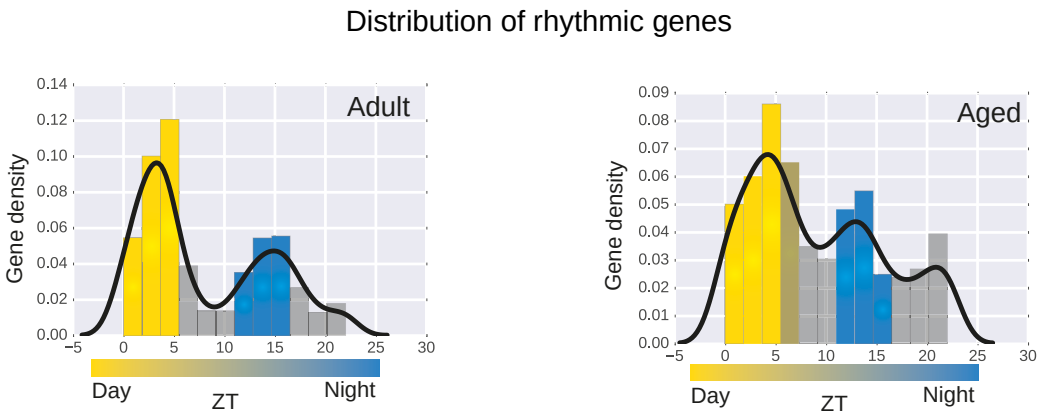



**Figure 10 - Biological Functions associated to Rhythmic Genes in Adult and Aged EpSCs.** (A, B) GO analysis of genes exclusively rhythmic in adult EpSCs (A) or aged EpSCs (B). (C) GO analysis of common rhythmic genes; heatmaps show their expression levels in adult EpSCs (left) and aged EpSCs (right).

To better dissect which functions EpSCs perform when mice are active (at night) and at rest (during the day), and to determine if these functions change during ageing, we first determined the distribution of rhythmic genes along the day according to their peak of expression **(Fig. 11a)**. We observed a bimodal distribution of genes rhythmic in adult EpSCs, characterised by a group of “diurnal” genes (peaking during ZT0–ZT6) and another group of

**A)**




**B)**

| **Adult “day”** rhythmic genes (**phase 0-6**) | P-value |
|---|---|
| carbohydrate metabolic process | 1.79E-05 |
| cytokinesis | 2.41E-05 |
| oxidation-reduction process | 4.17E-04 |
| regulation of BMP signaling pathway | 1.76E-03 |
| response to oxidative stress | 5.49E-03 |
| wound healing | 6.68E-03 |
| mitochondrion organization | 7.73E-03 |

**D)**

| **Aged “day”** rhythmic genes (**phase 0-6**) | P-value |
|---|---|
| cytokinesis | 1.73E-07 |
| mitochondrial genome maintenance | 1.11E-05 |
| mitochondrion organization | 2.19E-03 |
| negative regulation of G0 to G1 transition | 6.69E-03 |

**C)**

| **Adult “night”** rhythmic genes (**phase 12-18**) | P-value |
|---|---|
| DNA replication | 1.41E-12 |
| DNA repair | 6.82E-07 |
| ER unfolded protein response | 2.16E-06 |
| rhythmic process | 4.22E-06 |
| response to hypoxia | 3.13E-05 |
| epidermal cell differentiation | 2.40E-05 |
| keratinocyte differentiation | 7.49E-05 |
| keratinization | 2.11E-04 |
| regulation of translation | 2.81E-04 |
| response to transforming growth factor beta | 7.13E-03 |

**E)**

| **Aged “night”** rhythmic genes (**phase 12-18**) | P-value |
|---|---|
| DNA replication | 9.00E-40 |
| DNA repair | 7.79E-30 |
| recombinational repair | 4.08E-12 |
| DNA damage checkpoint | 2.10E-09 |
| replication fork processing | 1.52E-07 |
| nucleotide-excision repair | 2.78E-04 |
| base-excision repair | 2.61E-04 |
| rhythmic process | 4.63E-04 |
| DNA replication checkpoint | 6.24E-04 |
| TOR signaling | 6.50E-04 |
| non-recombinational repair | 9.32E-04 |
| mismatch repair | 1.45E-03 |

**Figure 11 - Distribution and Biological Function of “Day” and “Night” Rhythmic Genes in Adult and Aged EpSCs.**

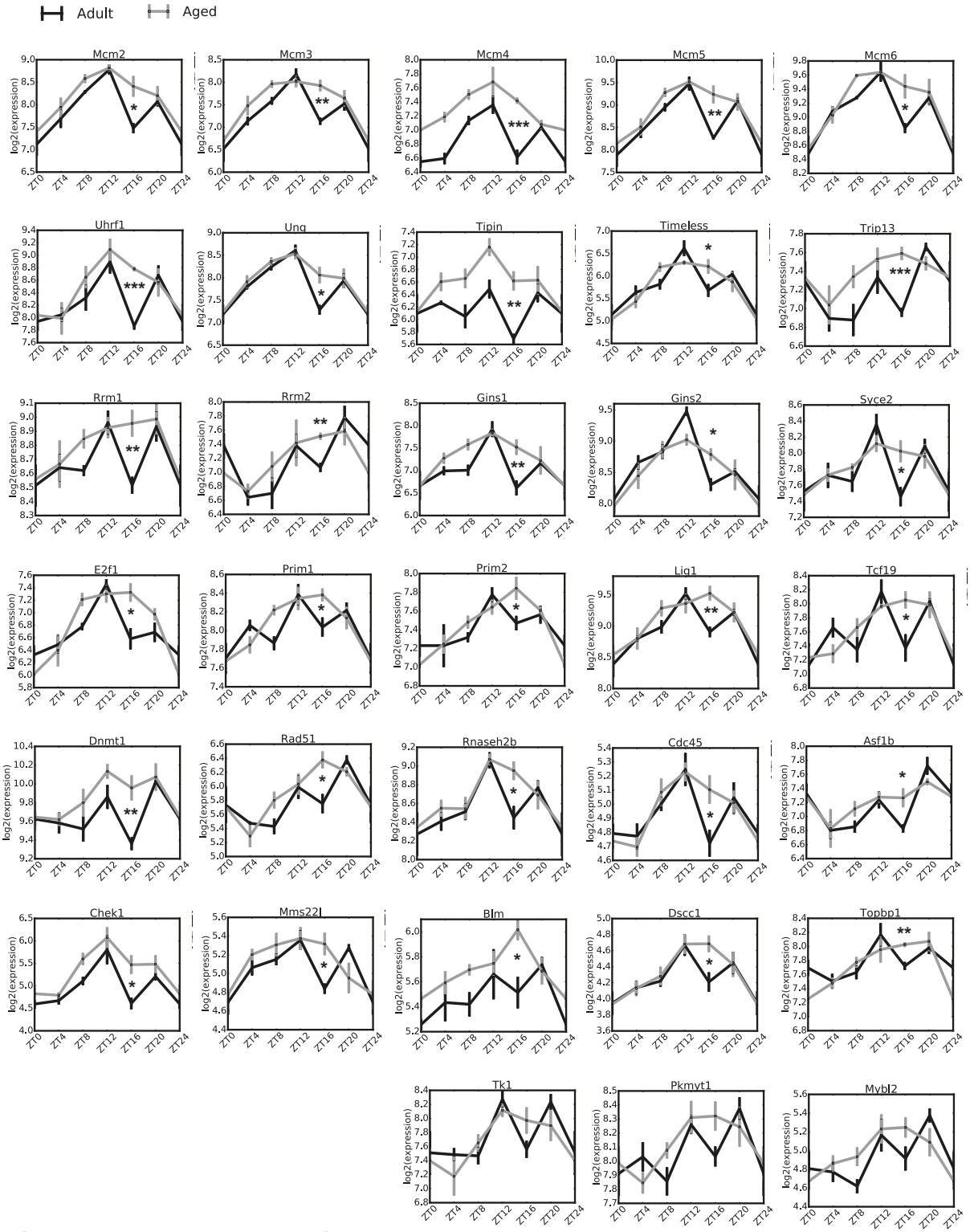
(A) Phase distribution of adult and aged rhythmic genes in EpSCs, and selection of rhythmic genes with phase ZT0–ZT6 (“day” genes) and ZT12–ZT18 (“night” genes). (B, C) GO analysis performed separately on rhythmic genes with phase ZT0–ZT6 (“day” set) in adult (B) or aged (C) EpSCs. (D, E) GO analysis performed separately on rhythmic genes with phase ZT12–ZT18 (“night” set) in adult (D) or aged (E) EpSCs.

“nocturnal” genes (peaking during ZT12–ZT18) of the day. This bimodal distribution was perturbed in rhythmic genes from aged EpSCs.

GO analyses revealed, among the adult “day” gene set, enrichment for biological functions related to certain metabolic processes: *carbohydrate metabolic process*, *oxidation-reduction process*, *response to oxidative stress*. Furthermore, genes associated to specific epidermal homeostatic categories (*regulation of BMP signalling pathway* and *wound healing*) mostly peaked during the light period **(Fig. 11b)**. On the other hand, the “night” genes were significantly involved in distinct epidermal homeostatic categories, such as *response to TGFbeta* and *keratinocyte differentiation*. Among the “night” categories, we also observed the GO categories *DNA replication* and *DNA repair*, with the expression of the majority of the DNA replication machinery peaking at night **(Fig. 11c)**. These genes included those encoding the MCM proteins, *Cdc45*, *Rrm1*, *Rrm2*, *Gins1*, *Gins2*, *Lig1* and *E2f1*, many of which are essential for the initiation of DNA replication as highlighted by GO analysis of this particular group of genes **(Fig. 12a and b)**.
Conversely, aged EpSCs no longer showed enrichment for the metabolic or for the homeostatic GO categories observed for the “day” gene set in adult EpSCs **(Fig. 11d)**. Furthermore, the “night” homeostatic functions related to epidermal differentiation observed for the adult EpSCs were absent in aged EpSCs as well **(Fig. 11e)**. Nonetheless, the DNA replication genes remained oscillatory in aged EpSCs and peaked at night **(Fig. 11e and Fig. 12a)**. However, their expression in aged EpSCs, but not in adult EpSCs, was accompanied by the rhythmic expression of many genes involved in different types of DNA damage/repair (such as *NER*, *BER* and *Mismatch Repair*), including those related to replicative stress such as *Brca2* or *Tipin* (*replication fork protection*) **(Fig. 11e)**.

The oscillatory expression of the DNA replication and replicative fork processing genes shared a very particular pattern. In adult EpSCs, all presented a statistically significant drop precisely at ZT16, whilst in aged EpSCs the transcript levels did not decrease at ZT16 **(Fig. 12a)**. We confirmed this difference in expression at the protein level for MCM4, a protein crucial to the replication fork assembly and progression **(Fig. 12c)**.
Aiming to understand if these differences in the oscillatory pattern of DNA replication genes correlated with putative alterations of the timing of replication, we next stained along all six time points for PCNA, a protein directly involved in the control of DNA replication through the increase of the polymerases processibility during elongation of the leading strand. In adult EpSCs, we observed the highest number of PCNA positive cells precisely at ZT16 (night) and dropped around ZT4 (day), indicating that S-phase predominantly occurs at this time of the day **(Fig. 13a)**. Accordingly, the total mean intensities of phospho-RPA32/RPA2 foci,

**A)**




**B)**

| **ZT16 subset** rhythmic genes | P-value |
|---|---|
| DNA replication | 8.89E-28 |
| cell cycle | 2.57E-19 |
| cell division | 8.31E-10 |
| DNA repair | 1.06E-09 |
| double strand break repair | 1.72E-08 |
| DNA conformation change | 1.07E-07 |
| DNA damage checkpoint | 1.43E-07 |
| replication fork processing | 9.99E-05 |

**C)**

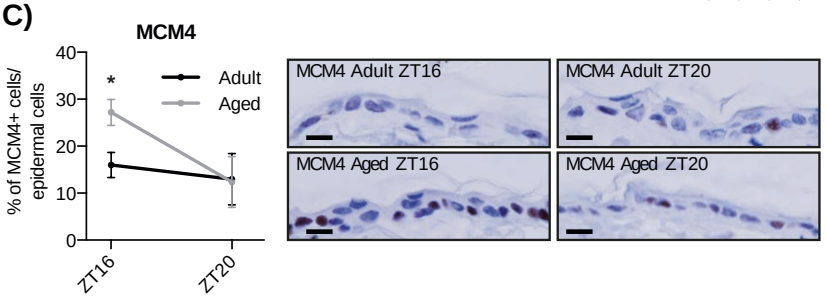


**Figure 12 - ZT16 subset of Rhythmic Genes are involved in DNA Replication and their Expression becomes altered during Ageing of EpSCs.**

(A) Expression levels across time points of ZT16-subset genes in adult and aged EpSCs. Error bars represent SD. ZT24 = ZT0. (B) GO analysis performed separately on ZT16-subset genes. (C) Quantification of MCM4-positive adult and aged basal cells at the time points ZT16 and ZT20, and representative images. Scale bar, 10 m. Error bars represent ±S.E.M. *, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$.

which marks single-stranded DNA, and of γH2Ax foci, which marks replicating as well as damaged DNA, were slightly elevated at ZT16 as compared to ZT4, further indicating that DNA replication (which exposes single-stranded DNA) took place at this time in the adult epidermis **(Fig. 13b and c)**. We next measured whether aged EpSCs showed any changes in DNA replication as compared to their adult counterparts. In contrast, the percentage of aged EpSCs in S-phase was 4-fold lower at ZT16 than that of adult EpSCs **(Fig. 13a)**. Yet, a similar percentage of aged EpSCs underwent maximal S-phase 8-12 hours later (ZT4) than adult EpSCs at ZT16. High levels of pRPA/γH2Ax in aged EpSCs were also observable at ZT4, while those of adult EpSCs had returned to background levels in the absence of DNA replication **(Fig. 13b and c)**. Finally, we observed that, in aged EpSCs, the longest genes were expressed at lower levels during the night hours, when the delay in DNA replication is strongest **(Fig. 13d)**.

The rhythmic GO analysis confirmed that the expression of genes involved in *oxidation-reduction process*, *mitochondrion organisation* and *response to oxidative stress* peaked during the day (ZT0–ZT6) in adult EpSCs **(Fig. 11a and b)**. We found that, even though “night” genes from aged epidermis did not enrich for these GO categories, the oscillatory profile (i.e. phase and amplitude) of several genes important for these processes was maintained in aged EpSCs **(Fig. 14a)**. Moreover, aged EpSCs presented persistently high levels of DNA, as measured by 8oxodG, a marker of oxidised guanosine residues that constitute the major product of DNA oxidation **(Fig. 14b)**.

Maximal M-phase occurred at ZT20 in both adult and aged epidermis, 4hr after the S-phase peak in adult EpSCs, as shown by phosphorylated histone 3 staining – a marker of M-phase - along the six time points, and the shared rhythmic GO category *mitotic M phase* **(Fig. 10c, Fig.15a)**. No additional mitosis peak was observed in adult or in aged EpSCs. Accordingly, both adult and aged EpSCs expressed numerous genes involved in the last step of cell division (GO category *cytokinesis*) in the early hours of the day (ZT0–ZT6) **(Fig. 11b and d).**

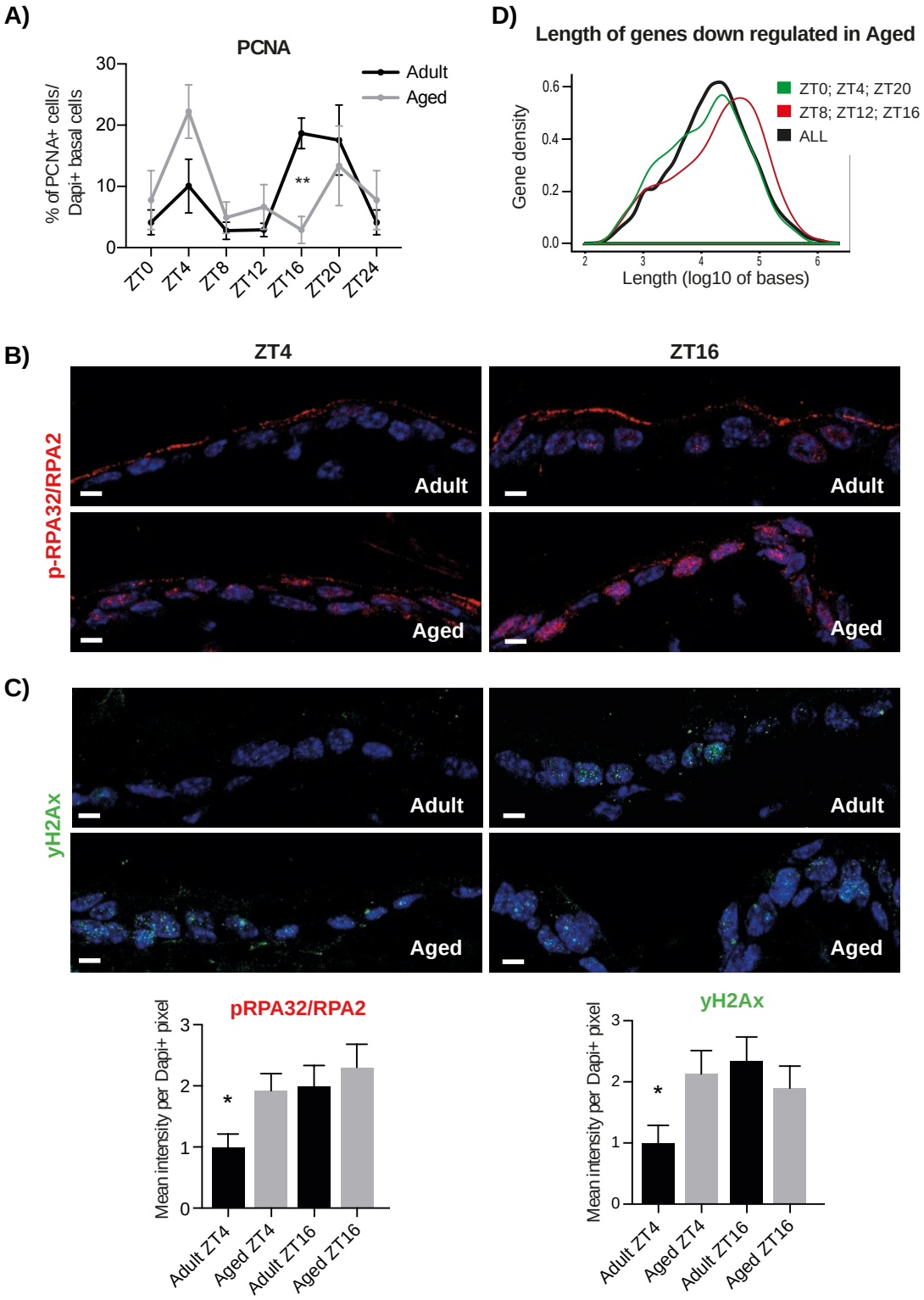



**Figure 13 - Aged EpSCs Delay the Timing of DNA Replication and Show Signs of Replicative Stress.**

(A) Quantification of PCNA-positive adult and aged EpSCs every 4 hr from ZT0. (B, C) Representative images and quantification of intensity levels of p-RPA (B) and γH2Ax (C) at ZT4 and ZT16 in adult and aged EpSCs (normalised to adult EpSCs at ZT4). Scale bar, 5 μm. (D) Length of early phase (ZT20, ZT0, ZT4) and late phase (ZT8, ZT12, ZT16) genes that were downregulated in aged EpSCs, and of all genes (ALL) in the mouse genome. *p ≤ 0.05, **p ≤ 0.01. Error bars represent ±S.E.M. ZT24 = ZT0.

Notwithstanding, at ZT20, approximately 50% fewer aged EpSCs than adult EpSCs entered M-phase. We then confirmed by caspase 3 staining that this decrease in the number of mitotic cells did not correlate with an increase in apoptotic cells **(Fig. 15b)**.

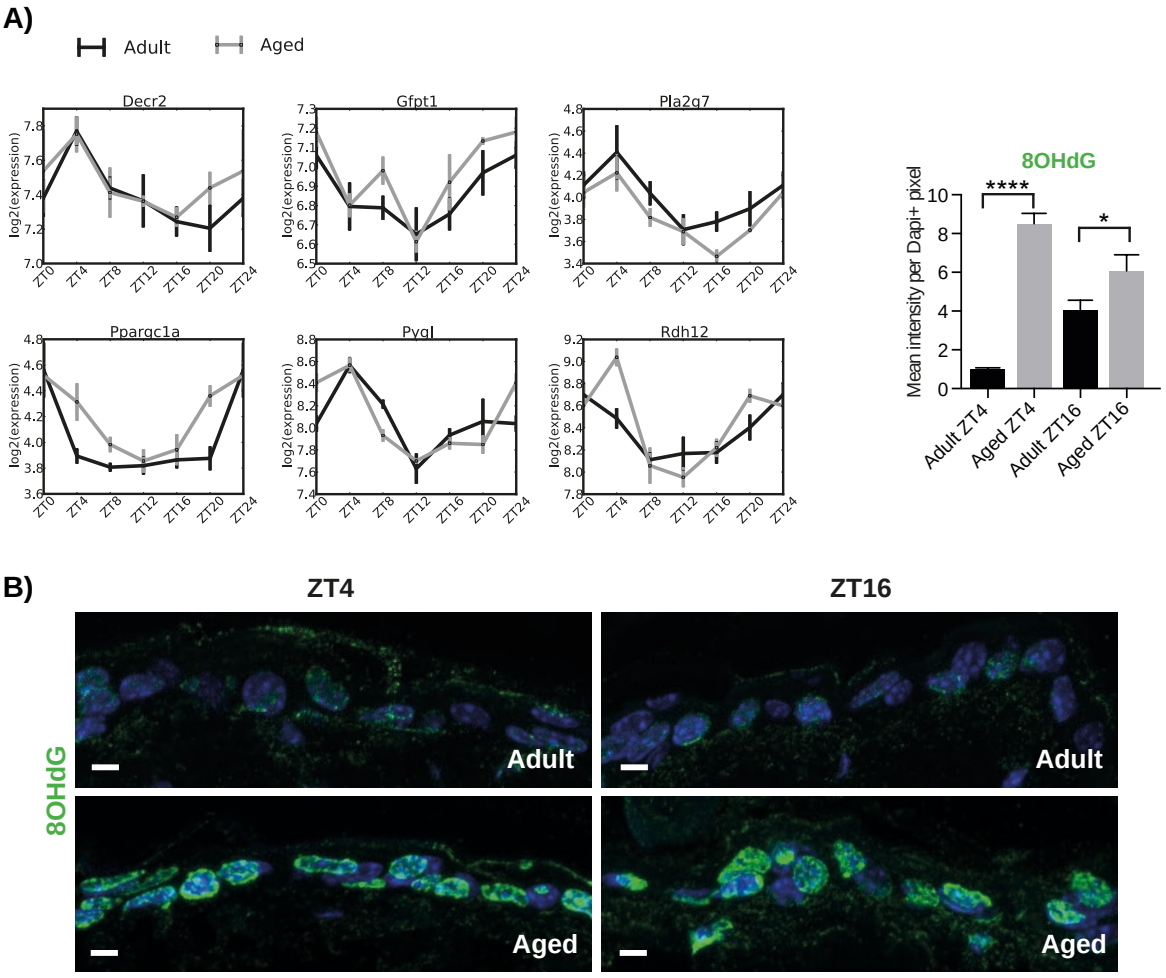



**Figure 14 - Oxidative Metabolism Timing and accumulated Oxidised DNA in Aged EpSCs.** (A) Expression levels over time of oxidative metabolism representative genes in adult and aged EpSCs. Error bars represent SD. (B) Representative images and quantification of intensity levels of oxidised DNA at ZT4 and ZT16 in adult and aged EpSCs (normalised to adult EpSCs at ZT4). Scale bar, 5 μm. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Error bars represent ±S.E.M. ZT24 = ZT0.

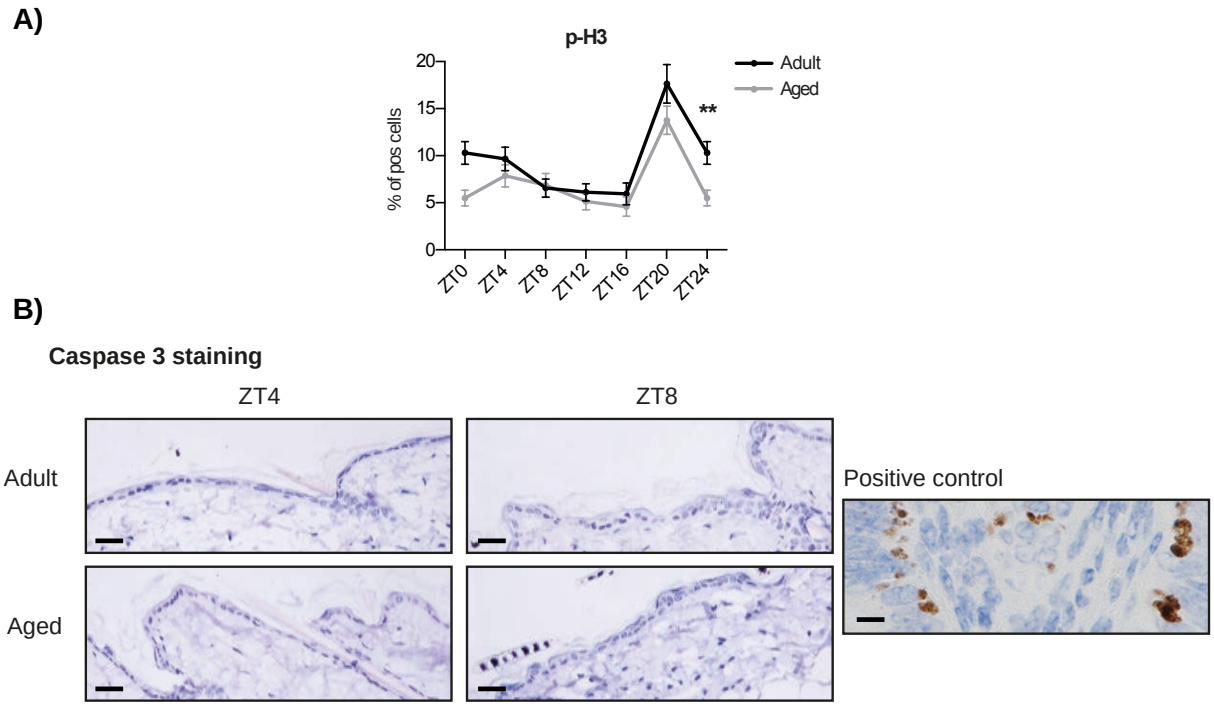



**Figure 15 - Timing of Mitosis in Adult and Aged EpSCs and absence of Apoptosis in Skin Sections.**

(A) Quantification of phosphohistone H3 (p-H3)-positive adult and aged basal cells across time points ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20. **, $p \leq 0.01$. Error bars represent ±S.E.M. ZT24 = ZT0. (B) Representative images of Caspase 3 staining in adult and aged EpSCs, at ZT4 and ZT8, and C57Bl/6J mouse ovary tissue as a positive control. Scale bar, 15 µm.

### *Rewiring of rhythmic functions in aged quiescent MuSCs associates with loss of basal autophagy but not DNA damage*

MuSCs, in contrast with EpSCs, remain mostly quiescent throughout life, becoming activated in response to injury. We did not observe any signs of unsolicited activation in aged MuSCs, which, as their young counterparts, remained in a quiescent state, as demonstrated by the absence of expression of proliferative markers (MyoD or Ki67) (Data not shown). We, therefore, wondered about the functional meaning and consequences of the rhythmic reprogramming observed in ageing in a stem cell population with such a distinct proliferative rate. We first performed GO analysis of the oscillatory genes and checked whether genes involved in DNA replication were expressed in a rhythmic manner in adult or aged MuSCs. None of the GO categories were related to DNA replication **(Fig. 16a-c)**. On the other hand, adult MuSCs did express in an oscillatory manner transcripts involved in double-strand break repair, (*cellular response to DNA damage stimulus*), such as *Ercc4* and *Xpa* **(Fig. 16a)**

**A)**

| **Adult only** rhythmic genes | P-value |
|---|---|
| cell proliferation | 4.11E-13 |
| skeletal muscle tissue development | 1.95E-05 |
| myotube differentiation | 7.50E-04 |
| cellular response to DNA damage stimulus | 4.74E-03 |
| response to insulin | 1.45E-03 |

**B)**

| **Aged only** rhythmic genes | P-value |
|---|---|
| inflammatory response | 6.70E-03 |
| mitochondrial DNA repair | 2.40E-03 |
| cytokine production | 1.30E-03 |

**C)**

| **Common** rhythmic genes | P-value |
|---|---|
| circadian rhythm | 1.94E-10 |
| cell-substrate adhesion | 5.47E-05 |
| actin cytoskeleton organization | 5.21E-05 |
| response to redox state | 5.39E-04 |

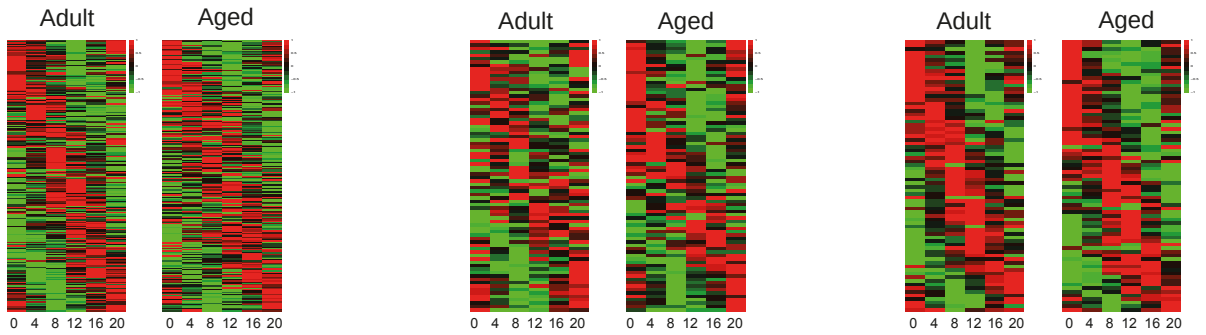



**Figure 16 - Biological Functions associated to Rhythmic Genes in Adult and Aged MuSCs.** (A, B) GO analysis of genes exclusively rhythmic in adult MuSCs (A) or aged MuSCs (B). (C) GO analysis of common rhythmic genes; heatmaps show their expression levels in adult MuSCs (left) and aged MuSCs (right).

(Vahidi Ferdousi et al., 2014). The expression of the majority of these genes peaked during the night, suggesting that this activity is strongest in MuSCs when mice are active. Importantly, these genes ceased to be circadian in aged MuSCs. Nonetheless, aged MuSCs did not show any signs of replicative stress as we observed no accumulation of yH2Ax foci nor of 53BP1 bodies **(Fig. 17b, c)**.

Adult quiescent MuSCs expressed many transcripts required for their homeostasis in a rhythmic manner (e.g., *myotube differentiation* and *cell proliferation*) (note that both GO terms represented genes pertaining to the TGF/Bmp and Fgf pathways, among others, which were previously shown to regulate MuSC quiescence maintenance and readiness for activation, rather than cell division or myogenic differentiation genes *per se*) **(Fig. 16a)** (Bernet et al., 2014; Chakkalakal et al., 2012; Chakkalakal and Brack, 2012; McCroskery et al., 2003). Aged MuSCs mostly lost their regulated timing of these homeostatic functions as further shown by the respective heatmaps. Instead, they gained a new rhythmic transcriptional program involved in *inflammatory response*, *cytokine production* and *mitochondrial DNA repair* **(Fig. 16b)**. The majority of the rhythmic genes common to adult and aged MuSCs were predominantly involved in *cell-substrate adhesion* and *cytoskeletal organisation* **(Fig. 15c)**, and included genes encoding *paxillin*, *vinculin, calponin*, and *CSF1r.* We next determined the distribution of rhythmic genes along the day according to their peak of expression **(Fig. 18a and b)**. Adult MuSCs presented a “day” and “night” gene distribution

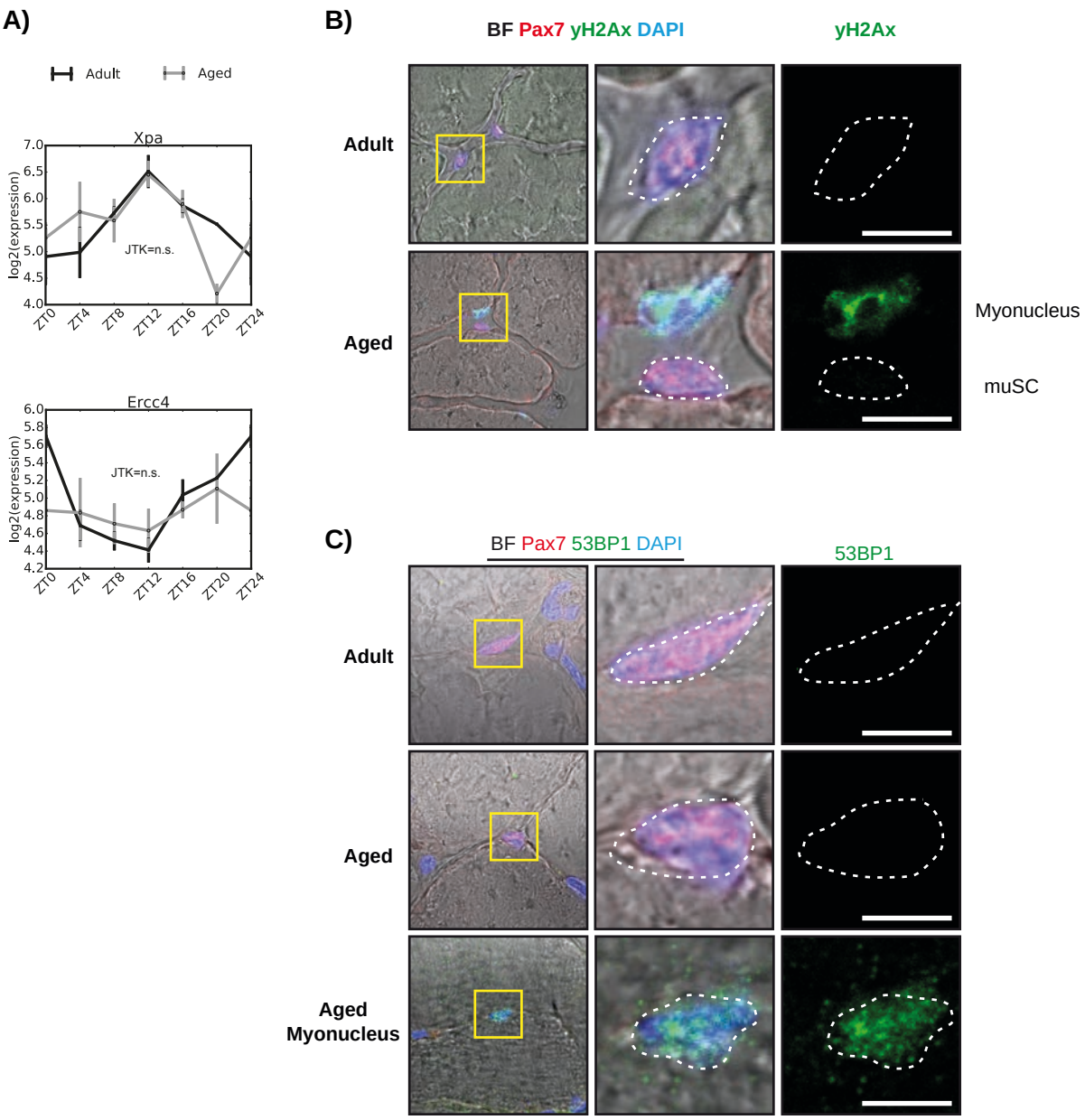
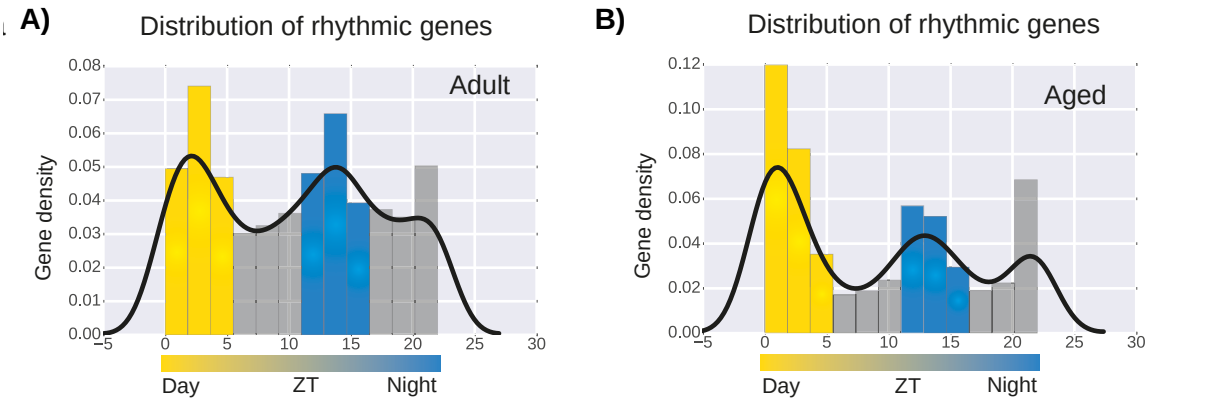



**Figure 17 - Aged MuSCs show no signs of DNA Damage.**

(A) Expression levels of *Xpa* and *Ercc4* genes over time in adult and aged MuSCs. *Xpa* and *Ercc4* were no longer detected as rhythmic in aged MuSCs. Error bars represent SD.

(B, C) Representative images of yH2Ax (B) and 53BP1 (C) levels in MuSCs (Pax7-positive), with myonucleus as a positive control. MuSC boundaries are indicated (dotted lines). Bright field (BF). Scale bar, 5 µm.

different from what we observed for EpSCs but that remained unchanged during ageing. The GO analysis of the “day” and “night” genes, however, revealed that very different biological functions were timely controlled between adult and aged MuSCs. For instance, adult MuSCs activated the *Wnt signaling pathway* during the day and repressed it at night (*negative*




| **Adult "day"** rhythmic genes (**phase 0-6**) | P-value |
|---|---|
| cell migration | 9.88E-06 |
| regulation of transcription, DNA templated | 2.09E-05 |
| cellular lipid metabolic process | 2.21E-05 |
| skeletal muscle tissue regeneration | 9.37E-05 |
| activation of NF-kappaB-inducing kinase activity | 9.10E-04 |
| Wnt signaling pathway | 5.39E-03 |
| circadian regulation of gene expression | 5.58E-03 |
| response to nutrient levels | 5.76E-03 |

| **Aged "day"** rhythmic genes (**phase 0-6**) | P-value |
|---|---|
| locomotion | 1.04E-08 |
| small GTPase mediated signal transduction | 4.66E-05 |
| regulation of immune effector process | 4.90E-05 |
| wound healing | 7.42E-05 |
| circadian rhythm | 4.70E-04 |
| response to transforming growth factor beta | 5.04E-04 |
| mitochondrion organization | 8.36E-04 |
| mitochondrial DNA repair | 1.31E-03 |
| response to nutrient levels | 1.35E-03 |
| regulation of small GTPase mediated signal transduction | 2.28E-03 |
| negative regulation of cellular response to hypoxia | 3.44E-03 |

| **Adult "night"** rhythmic genes (**phase 12-18**) | P-value |
|---|---|
| small GTPase mediated signal transduction | 1.97E-08 |
| transcription, DNA-templated | 5.67E-08 |
| negative regulation of Wnt signaling pathway | 3.44E-05 |
| locomotion | 3.58E-04 |
| lipid metabolic process | 3.44E-03 |
| regulation of mitochondrion organization | 4.03E-03 |
| skeletal muscle organ development | 4.48E-03 |
| negative regulation of TOR signaling | 6.03E-03 |
| immune system process | 6.86E-03 |
| cellular response to insulin stimulus | 7.26E-03 |
| positive regulation of autophagy | 7.58E-03 |

| **Aged "night"** rhythmic genes (**phase 12-18**) | P-value |
|---|---|
| transcription, DNA-templated | 3.01E-07 |
| Ras protein signal transduction | 2.87E-05 |
| locomotion | 4.17E-05 |
| small GTPase mediated signal transduction | 2.34E-04 |
| cytokine production | 3.66E-04 |
| oxidation-reduction process | 8.63E-04 |
| lipid metabolic process | 4.14E-03 |

**Figure 18 - Distribution and Biological Function of "Day" and "Night" Rhythmic Genes in Adult and Aged MuSCs.**

Phase distribution of rhythmic genes and GO analysis performed separately on rhythmic genes with phase ZT0-ZT6 ("day" genes) or those with phase ZT12-ZT18 ("night" genes) in MuSCs from adult (A) and aged (B) mice.

*regulation of Wnt signalling pathway*) **(Fig. 17a)**. We did not observe this pathway to be rhythmically regulated in aged SCs. By contrast, aged MuSCs expressed in an oscillatory manner genes associated with the GO term *response to transforming growth factor beta* during the day and genes associated with the GO term *Ras protein signal transduction* by night **(Fig. 18b)**. Furthermore, adult MuSCs rhythmically expressed genes involved in the *negative regulation of TOR signalling* and *positive regulation of autophagy* during the night

**(Fig. 18a)**. Key autophagy genes, including *Becn1*, *Flcn*, *Atg13* and *Svip*, peaked late at night or early in the morning in adult MuSCs. We then confirmed by co-immunostaining of Pax7, a positive marker for quiescent MuSCs, and LC3 and LAMP1, two autophagy/ lysosomal markers, that adult MuSCs have higher levels of autophagy during the day than at night **(Fig. 19)**. Aged MuSCs, on the other hand, in addition to no longer showing autophagy as an enriched GO term in the “day” or “night” gene set, exhibited significantly lower levels of autophagy which persisted throughout the 24-h day **(Fig. 18b and Fig. 19)**.

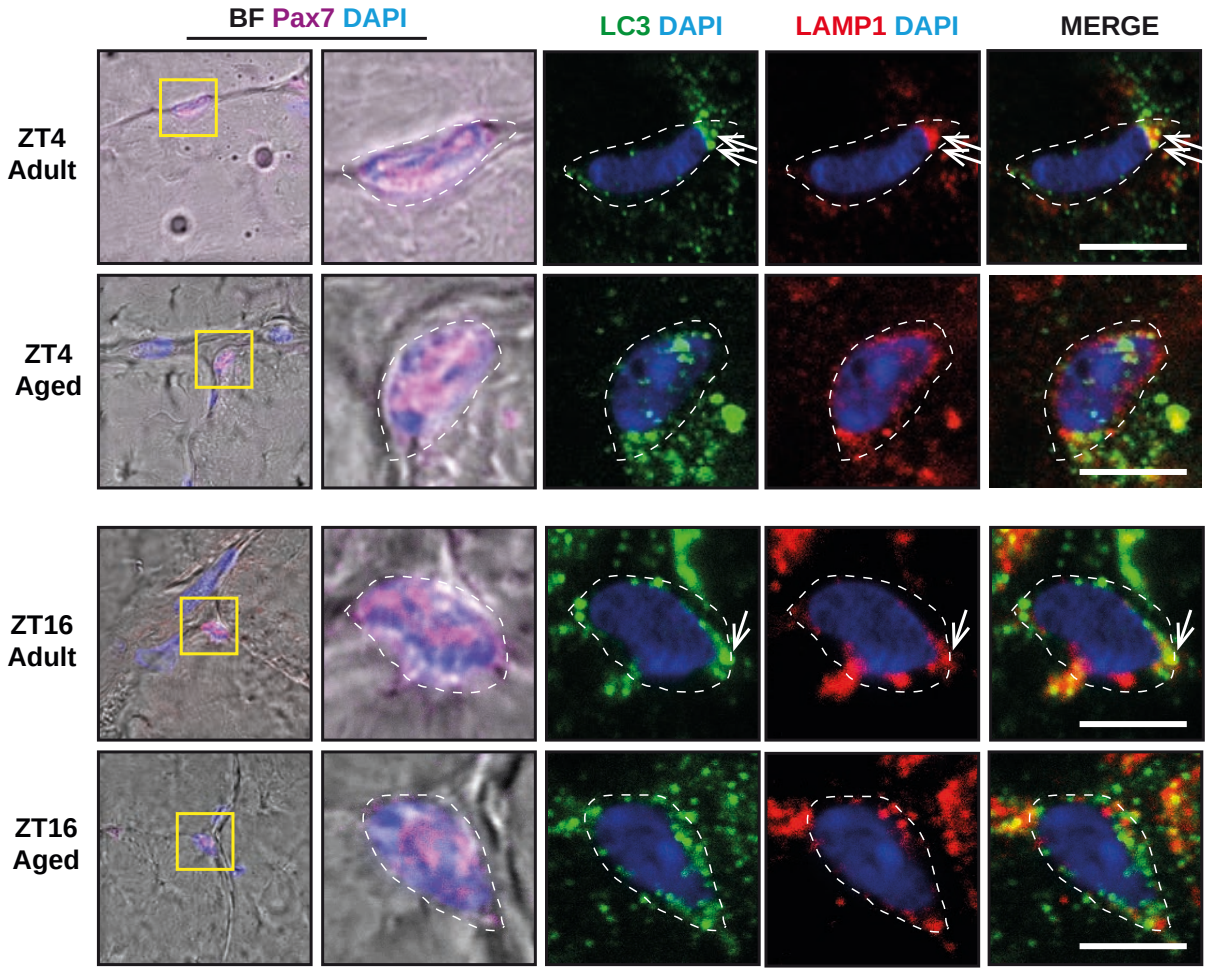
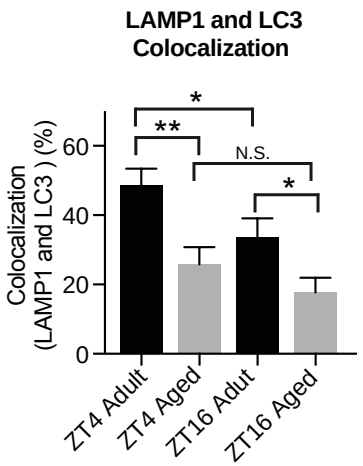



**Figure 19 - Autophagy levels in Adult and Aged MuSCs.** Autophagy levels. Percentage of colocalisation of LAMP1 and LC3 puncta in adult and aged MuSCs at ZT4 and ZT16. Representative images of LC3 and LAMP1 levels and their colocalisation in adult and aged MuSCs (Pax7-positive), at ZT4 and ZT16. MuSCs boundaries are indicated (dotted lines). Bright field (BF). Scale bar, 5 µm.Error bars represent ±S.E.M. *p ≤ 0.05; **p ≤ 0.01



## *Chapter 2 –*

# *Stem cells rewire their diurnal timed functions to adapt to metabolic cues*

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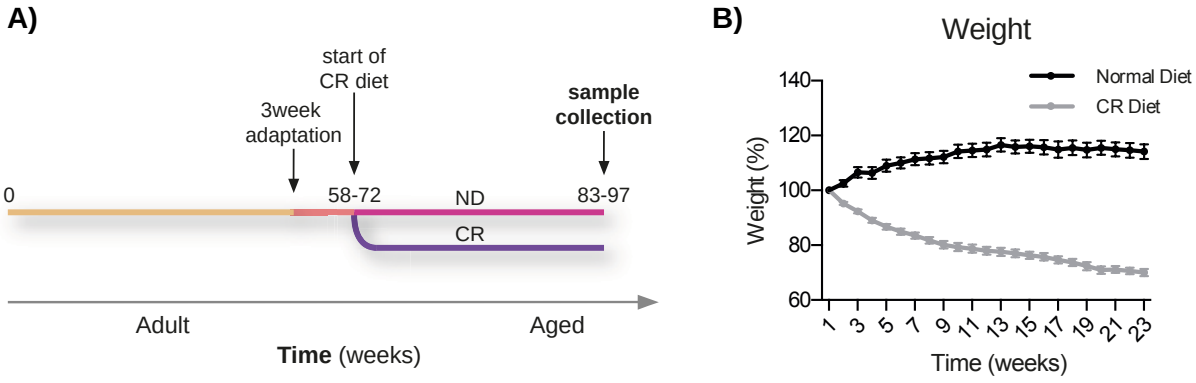
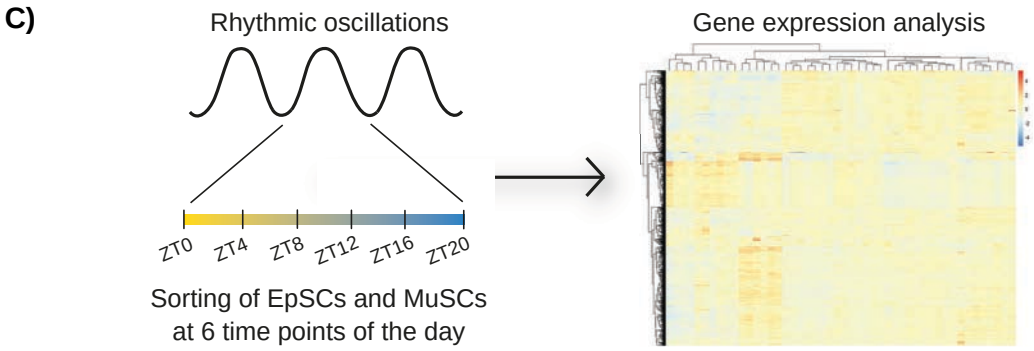
### *Caloric restriction prevents the age-dependent reprogramming of diurnal oscillations of aged stem cells*

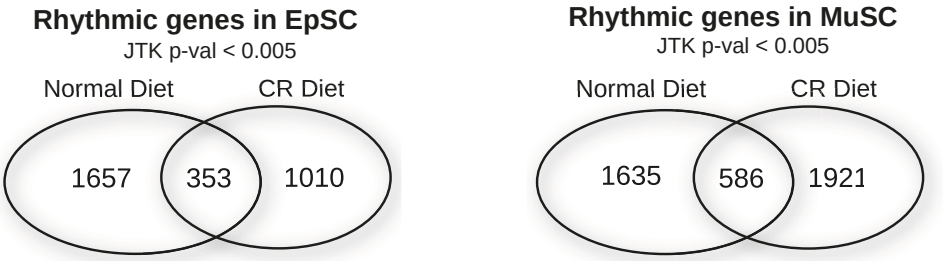
In order to study the effect of CR on aged EpSCs and MuSCs and determine whether this type of diet could prevent the reprogramming of their rhythmic functions, we fed a large cohort of 60-week-old mice (C57Bl6 pure background) with either a 30% CR diet or a control diet *ad libitum* for 25 weeks **(Fig. 20a)**. Accordingly, mice under CR conditions lost weight in comparison with the control group **(Fig. 20b)**. Thereafter, as previously described, we FACS-sorted EpSCs and MuSCs at six time points over 24hr and then analysed whole transcriptomes by gene expression arrays **(Fig. 20c)**. Aged mice subjected to CR showed a significant amelioration of ageing-associated traits both in the epidermis and the muscle, such as a decrease in the thickness of the cornified envelope, a thicker fur, and a higher number of MuSCs **(Fig. 20d-f)**.

In addition to physical signs of improvement, CR induced a strong reprogramming of the rhythmic transcriptome of aged EpSCs and MuSCs **(Figs 20c)**. While a large number of genes ceased to oscillate (approximately 82% and 73% in EpSCs and MuSCs respectively), 1010 in EpSCs and 1921 genes in MuSCs became newly rhythmic in response to the CR diet. The average level of expression of the genes that gained or lost the oscillatory behaviour in SCs isolated from CR-fed mice remained the same over time, indicating that the changes in rhythmicity were not due to loss of, or *de novo*, expression upon exposure to distinct nutritional diets **(Figs. 21a and b)**. The amplitude and period of the core clock genes was unaffected by CR, but we observed a 4-h advance in their zenith likely due to a food anticipation effect **(Fig. 22a and b)**.

GO analysis of the genes that were uniquely rhythmic in aged EpSCs from control-diet-fed mice revealed very similar categories as in the previous cohort of aged mice, including DNA damage, inflammation and a predominant lack of epidermal homeostatic categories **(Fig. 23a)**. Noteworthy, however, daily oscillatory homeostatic functions previously observed in adult epidermis were recovered in aged EpSCs from CR-fed mice (*keratinocyte development*, *wound healing* and *epithelial cell proliferation*) **(Fig. 23b)**. These also included genes involved in Bmp and TGFbeta signalling, as well those for EGFR, VDR, or Jarid2. Aged EpSCs from control-diet and CR-diet fed mice shared a common rhythmic transcriptome that was predominantly associated with the core clock machinery and DNA replication **(Fig. 23c)**. Among common GO terms we still found terms associated with DNA damage or replicative stress (*DSB repair*, *Base-excision repair, gap-filling* and *DNA damage*


A)
start of
CR diet
3week
adaptation
sample
collection
0
58-72
ND
83-97
CR
Adult
Aged
Time (weeks)
B)
Weight
Normal Diet
CR Diet
Weight (%)
140
120
100
80
60
1
3
5
7
9
11
13
15
17
19
21
23
Time (weeks)


C)
Rhythmic oscillations
ZT0
ZT4
ZT8
ZT12
ZT16
ZT20
Sorting of EpSCs and MuSCs
at 6 time points of the day
Gene expression analysis


Rhythmic genes in EpSC
JTK p-val < 0.005
Normal Diet
CR Diet
1657
353
1010
Rhythmic genes in MuSC
JTK p-val < 0.005
Normal Diet
CR Diet
1635
586
1921

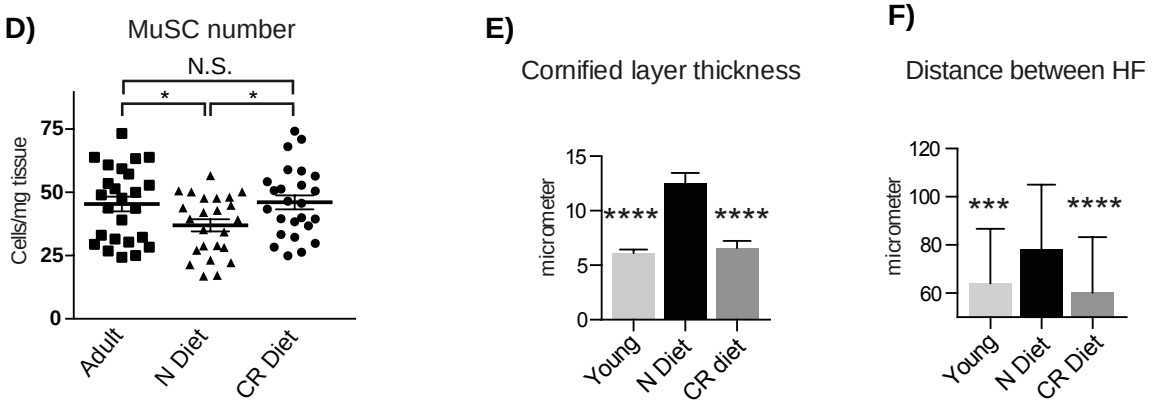

D)
MuSC number
N.S.
*
*
Cells/mg tissue
75
50
25
0
Adult
N Diet
CR Diet
E)
Cornified layer thickness
micrometer
15
10
5
0
****
****
Young
N Diet
CR diet
F)
Distance between HF
micrometer
120
100
80
60
***
****
Young
N Diet
CR Diet

**Figure 20 - Caloric Restriction reprograms the Rhythmic Transcriptome of Aged EpSCs and MuSCs.**

(A) Experimental setup. Middle age mice were fed either with caloric restriction (CR) or a control diet (normal diet, ND) for 50 weeks, after which MuSCs and EpSCs were collected. (B) Percentage of weight relative to week 1 of mice fed either a ND or a CR diet. (C) SCs were sorted every 4 hrs from ZT0. Extracted RNA was processed for microarrays, and the JTK algorithm was used to determine daily rhythmic genes ($p \leq 0.005$). Number of rhythmic genes in adult (3144 genes) and aged (2309 genes) EpSCs, and in adult (1979 genes) and aged (2221 genes) MuSCs. (D) Number of MuSCs per mg of muscle tissue in ND adult MuSCs ("adult"), ND aged MuSCs ("N diet"), and CR aged MuSCs ("CR diet"). (E, F) Quantification of the cornified layer thickness and distance between hair follicles from adult mice (fed a normal diet) or aged mice fed a normal (N) or CR diet. Error bars represent ±S.E.M.

*checkpoint*). However, in aged EpSCs from CR-fed mice (but not those from control-fed mice), replicative stress levels were significantly reduced as demonstrated by the decrease in yH2Ax and phospho-RPA32/RPA2 signal **(Fig. 23d)**. Next we investigated the expression pattern of all the DNA replication machinery genes that we previously found to be altered

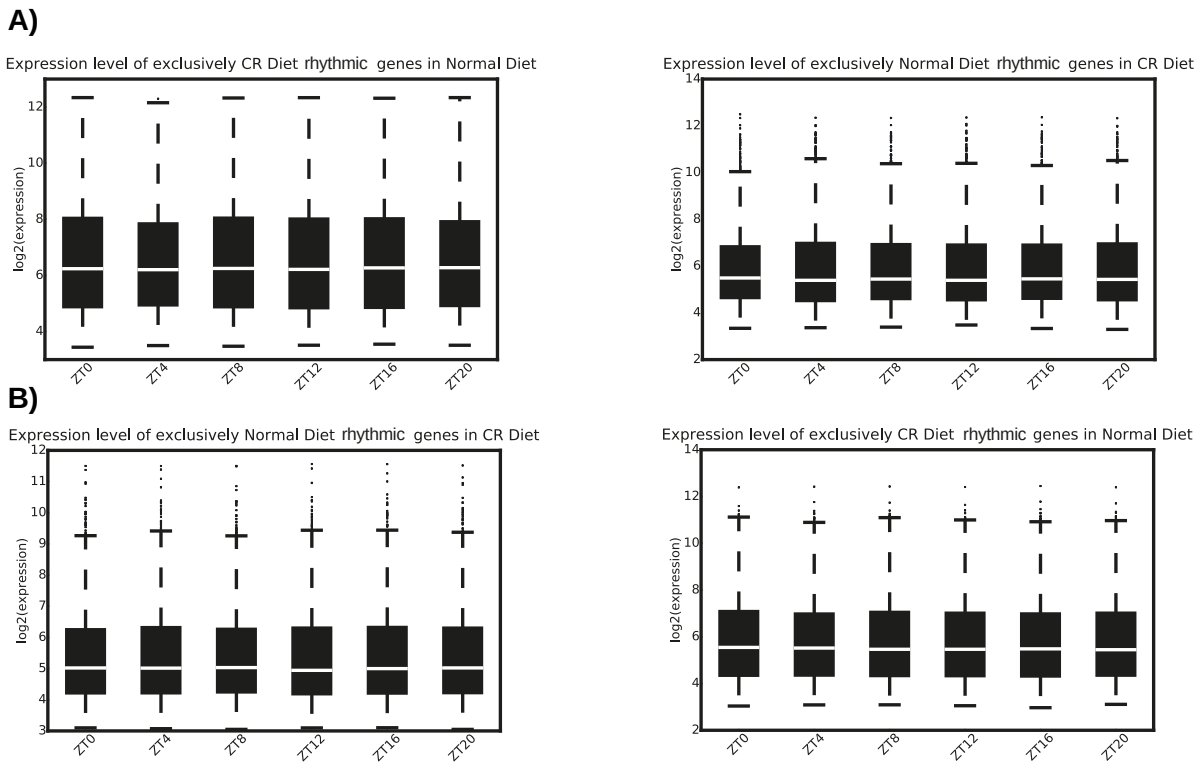



**Figure 21 - Gene Expression Controls for the Caloric Restriction Protocol**.

(A, B) Gene expression levels in normal-diet, aged EpSCs (A) and MuSCs (B) showing genes exclusively rhythmic in CR-diet, aged EpSCs (A) and MuSCs (B); and in CR-diet, aged EpSCs (A) and MuSCs (B) showing genes exclusively rhythmic in normal-diet, aged EpSCs (A) and MuSCs (B).

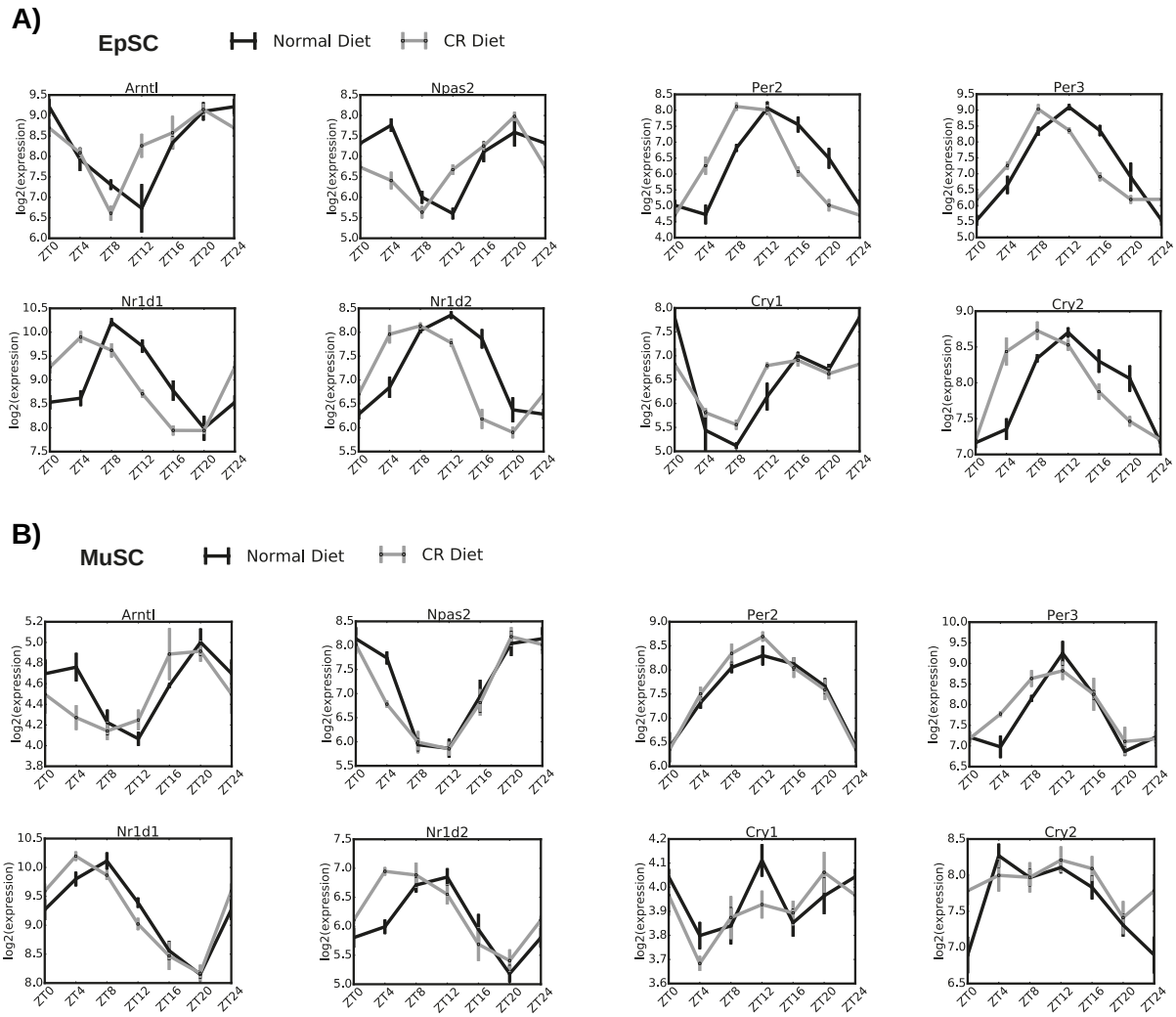



**Figure 22 - The Amplitude and Period of the Core Clock Genes is unaffected by CR in Aged EpSCs and MuSCs.**

(A, B) Expression levels across time points of core clock genes (*Arntl*, *Npas2*, *Per2*, *Per3*, *Nr1d1*, *Cry1* and *Cry2*) in aged EpSCs (A) and in aged MuSCs (B) from mice fed a ND or a CR diet. Error bars represent SD.

during ageing. Surprisingly, in EpSCs from CR-fed mice the expression pattern of virtually all genes was found to be identical to the adult EpSCs pattern **(Fig. 24a)**. Moreover, the nocturnal S-phase peak observed in adult EpSCs was restored, now peaking at ZT12 in agreement with the food anticipation effect that we also observed for the core clock machinery **(Fig. 24b)**. Finally, the accumulation of oxidised DNA was significantly prevented by the CR-diet as indicated by the low levels of 8oxodG signal **(Fig. 24c)**.

As observed for aged EpSCs from control-diet-fed mice, aged MuSCs from control-diet-fed mice also displayed categories identical to what we observed in the previous cohort of aged mice (*inflammatory response*, *cytokine production* and *mitochondrial DNA repair*) **(Fig. 25a)**.

**A)**

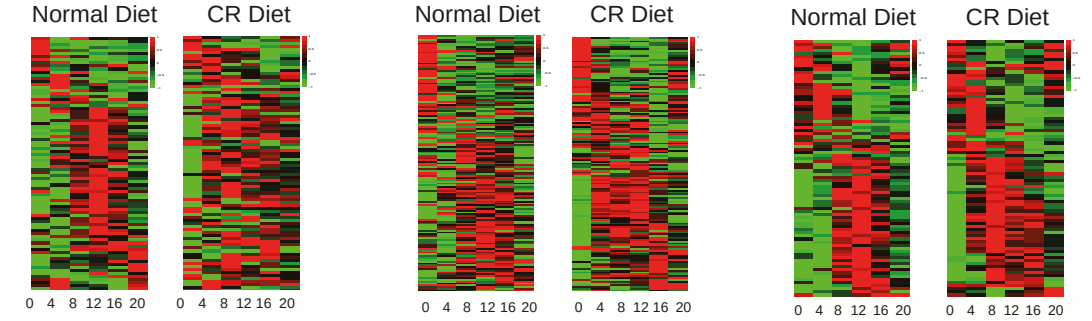
| **Normal Diet** rhythmic genes | P-value |
|---|---|
| DNA repair | 1.92E-10 |
| intra-S DNA damage checkpoint | 9.21E-04 |
| response to ER stress | 1.24E-03 |
| nucleotide-excision repair | 1.08E-03 |
| response to superoxide | 3.33E-03 |
| TNF superfamily cytokine production | 5.47E-03 |

**B)**

| **CR Diet** rhythmic genes | P-value |
|---|---|
| translation | 5.14E-08 |
| oxidation-reduction process | 4.98E-05 |
| epithelial cell proliferation | 6.93E-05 |
| keratinocyte development | 6.00E-04 |
| BMP signaling pathway | 2.91E-04 |
| regulation of Tgf beta1 production | 1.16E-03 |
| positive regulation of TOR signaling cascade | 1.84E-03 |
| wound healing | 3.93E-03 |

**C)**

| **Common** rhythmic genes | P-value |
|---|---|
| cell cycle | 1.57E-15 |
| DNA replication | 7.32E-14 |
| cell cycle checkpoint | 6.67E-06 |
| circadian rhythm | 1.84E-04 |
| DSB repair | 1.63E-04 |
| DNA integrity checkpoint | 1.45E-03 |
| Base-excision repair, gap-filling | 3.00E-03 |
| DNA damage checkpoint | 5.43E-03 |



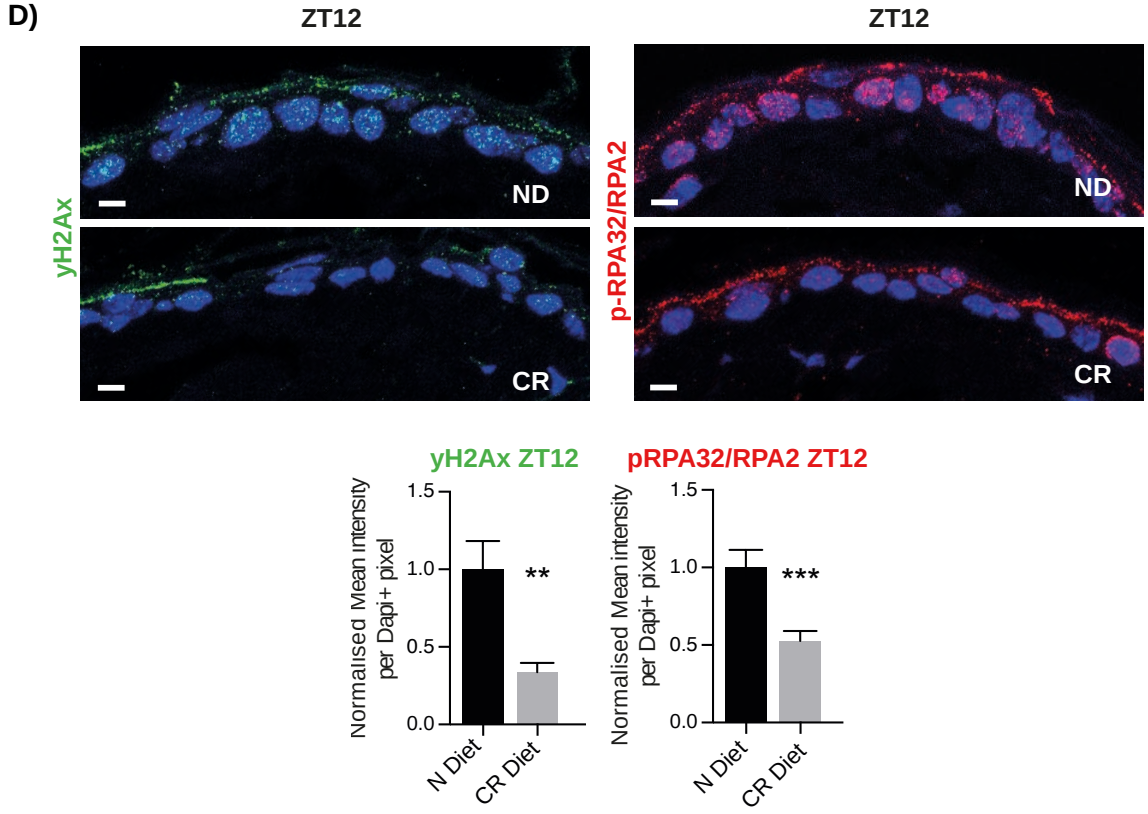
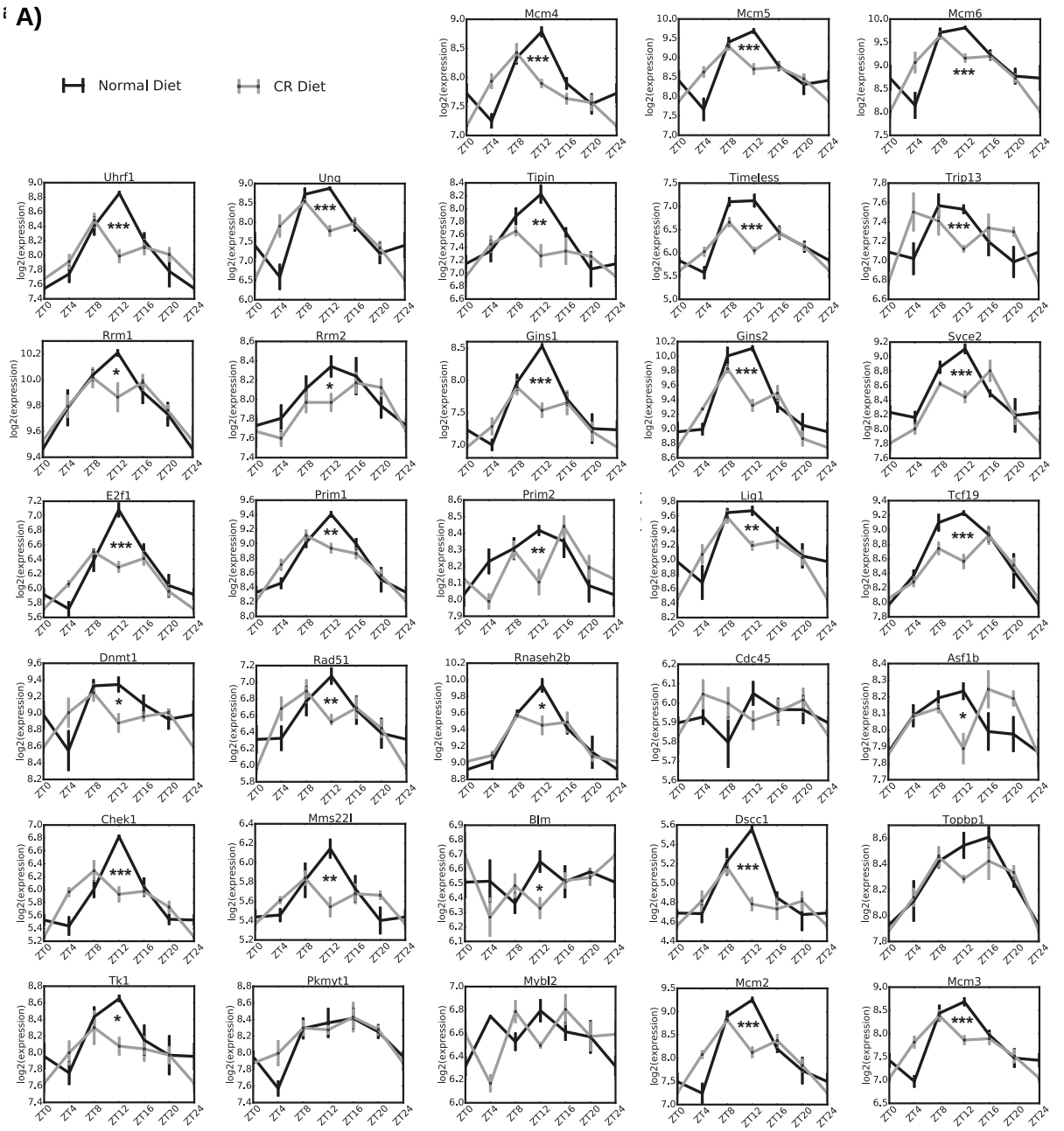
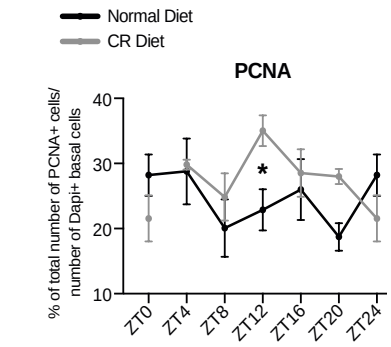




**Figure 23 - Caloric Restriction Prevents the Reprogramming of the Rhythmic Transcriptome of Aged EpSCs.**

(A, B) GO analysis of genes exclusively rhythmic to ND (A) or CR diet (B). (C) GO analysis of common rhythmic genes; heatmaps show their expression levels in ND EpSCs (left) and CR EpSCs (right). (D) Representative images and quantification of intensity levels of p-RPA and H2ax at ZT12 in normal diet and CR basal cells (normalised to normal-diet cells at ZT12). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Error bars represent ±S.E.M. Scale bar, 5 μm.


A)
Normal Diet
CR Diet
Mcm4
Mcm5
Mcm6
Uhrf1
Ung
Tipin
Timeless
Trip13
Rrm1
Rrm2
Gins1
Gins2
Syce2
E2f1
Prim1
Prim2
Lig1
Tcf19
Dnmt1
Rad51
Rnaseh2b
Cdc45
Asf1b
Chek1
Mms22l
Blm
Dscc1
Topbp1
Tk1
Pkmyt1
MybI2
Mcm2
Mcm3
log2(expression)
ZT0 ZT4 ZT8 ZT12 ZT16 ZT20 ZT24


B)
Normal Diet
CR Diet
PCNA
% of total number of PCNA+ cells/ number of Dapi+ basal cells
ZT0 ZT4 ZT8 ZT12 ZT16 ZT20 ZT24

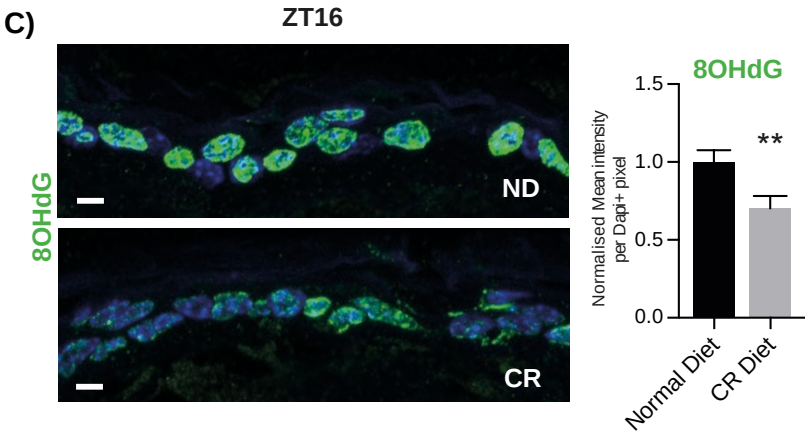

C)
ZT16
8OHdG
ND
CR
8OHdG
Normalised Mean intensity per Dapi+ pixel
Normal Diet
CR Diet

**Figure 24 - Caloric Restriction Prevents the Age-associated Delay in S-phase and the accumulation of Oxidised DNA.**

(A) Expression levels across time points of ZT16-subset genes in aged EpSCs from mice fed either a normal or a CR diet. Error bars represent SD. (B) Quantification of PCNA-positive normal-diet and CR EpSCs analysed every 4 hrs from ZT0. (C) Representative images and quantification of intensity levels of oxidised DNA at ZT16, are given for normal-diet and CR EpSCs (normalised to normal-diet cells at ZT16). Error bars represent ±S.E.M. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. ZT24 = ZT0.

Aged MuSCs from CR-fed mice also underwent a dramatic reprogramming of their rhythmic transcriptome and daily biological functions **(Fig. 20c and Fig. 25a-c)**. For instance, MuSCs from CR-fed mice no longer expressed genes involved in inflammation or mitochondrial DNA repair in a rhythmic manner, but instead expressed an oscillating homeostatic transcriptome program (with GO terms of *regulation of myotube differentiation*, *myoblast proliferation* and *skeletal muscle regeneration*) **(Fig. 25a, b)**. Of note, the CR-diet was also responsible for restoring the oscillatory expression of genes involved in autophagy, including *Tmem59*, *Pycard*, *Ulk3*, *Park2* and *Dap1*. Importantly, we confirmed by LC3/LAMP1 staining that this re-established their ability to undergo rhythmic autophagy during the day, as seen in adult mice **(Fig. 25b, d)**. Expression of core timed functions related to circadian rhythms and cellular adhesion that remained unaltered in aged MuSCs remained unaffected by CR **(Fig. 25b, c)**. Analysis of the “day” and “night” GO terms revealed potential CR-associated rhythmic chromatin modifications with categories such as *histone H4-K16 acetylation*, *regulation of histone methylation* and *chromatin modification* popping up during the day, and *chromatin assembly* at night **(Fig. 26)**.

**A)**

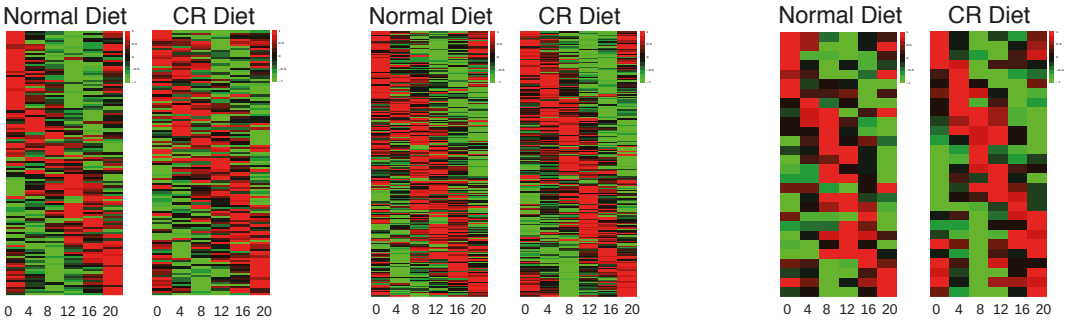
| **Normal Diet only** rhythmic genes | P-value |
|---|---|
| mitochondrial DNA repair | 2.20E-03 |
| ROS metabolic process | 2.98E-03 |
| response to hypoxia | 3.99E-03 |
| inflammatory response | 6.52E-03 |
| cytokine production | 8.84E-03 |
| response to nutrient levels | 9.04E-03 |

**B)**

| **CR Diet only** rhythmic genes | P-value |
|---|---|
| translation | 5.78E-09 |
| regulation of cell cycle | 2.48E-07 |
| ribosome biogenesis | 9.99E-04 |
| myoblast proliferation | 1.87E-03 |
| skeletal muscle tissue regeneration | 1.97E-03 |
| response to oxidative stress | 4.11E-03 |
| regulation of autophagy | 5.98E-03 |
| TOR signaling | 7.46E-03 |
| regulation of myotube differentiation | 8.15E-03 |
| oxidative phosphorylation | 8.68E-03 |

**C)**

| **Common** rhythmic genes | P-value |
|---|---|
| adherens junction assembly | 1.61E-07 |
| circadian rhythm | 1.19E-06 |
| response to redox state | 7.00E-04 |
| skeletal muscle satellite cell differentiation | 8.06E-03 |



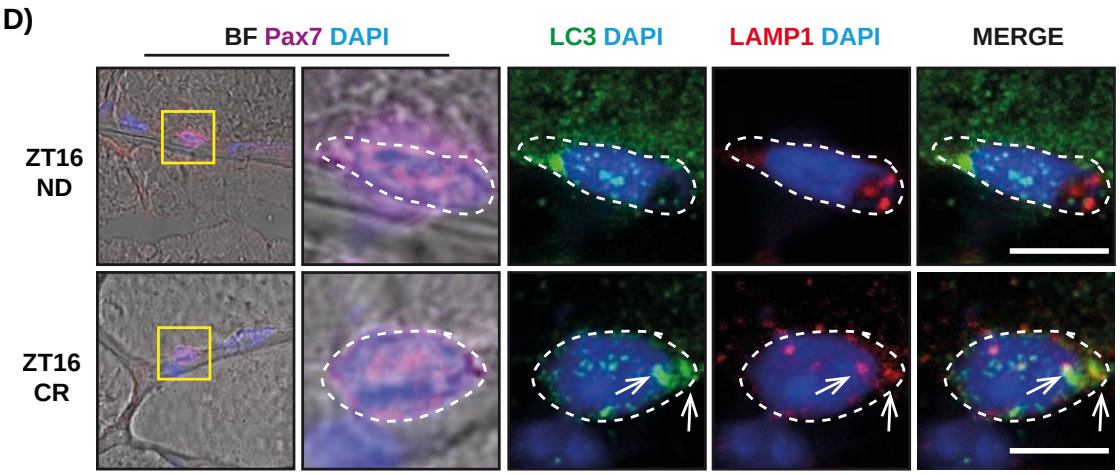
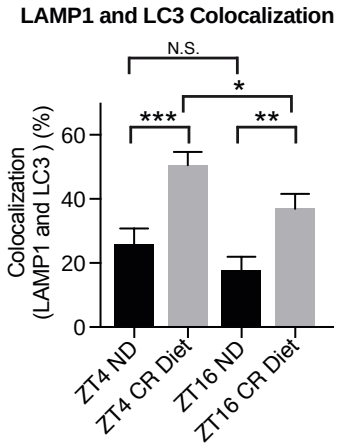
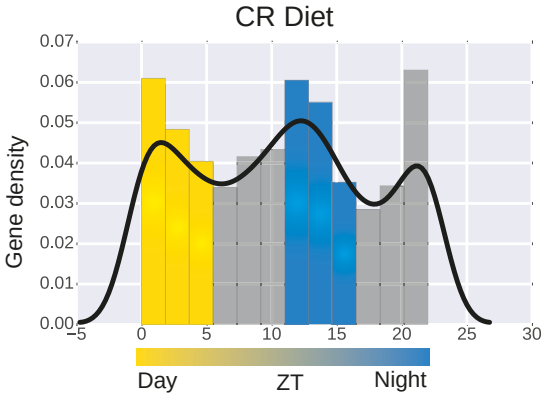




**Figure 25 - Caloric Restriction Prevents Reprogramming of the Daily Rhythmic Transcriptome of Aged MuSCs.**

(A, B) GO analysis of genes exclusively rhythmic in aged normal-diet MuSCs (A) or CR-diet aged MuSCs (B). (C) GO analysis of common rhythmic genes; heatmaps show their expression levels in ND MuSCs (left) and CR MuSCs (right). (D) Autophagy levels. Representative images and percentage of colocalisation of LAMP1 and LC3 puncta in aged muSCs from normal-diet or CR mice, at ZT4 and ZT16. Note that LAMP1 and LC3 puncta are present in all interstitial muscle cells and in muscle fibres. MuSCs boundaries are indicated (dotted line). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Error bars represent ±S.E.M. N.S., not significant. Scale bar, 5 µm.






| **CR Diet** rhythmic genes with **phase 0-6** | P-value |
|---|---|
| histone H4-K16 acetylation | 2.18E-05 |
| regulation of histone methylation | 6.53E-05 |
| circadian rhythm | 1.75E-04 |
| immune system development | 6.37E-04 |
| locomotion | 6.72E-04 |
| lipid biosynthetic process | 7.68E-04 |
| chromatin modification | 9.32E-04 |
| negative regulation of interleukin-10 production | 1.85E-03 |
| negative regulation of translation | 2.42E-03 |
| TOR signaling | 3.97E-03 |

| **CR Diet** rhythmic genes with **phase 12-18** | P-value |
|---|---|
| locomotion | 1.73E-06 |
| NADP metabolic process | 5.97E-04 |
| insulin receptor signaling pathway | 7.96E-04 |
| immune system process | 1.97E-03 |
| chromatin assembly | 3.51E-03 |
| response to hypoxia | 4.11E-03 |
| small GTPase mediated signal transduction | 4.37E-03 |
| response to decreased oxygen levels | 4.99E-03 |
| intracellular lipid transport | 6.00E-03 |
| negative regulation of mitochondrion organization | 8.18E-03 |
| response to DNA integrity checkpoint signaling | 9.84E-03 |

**Figure 26 - Distribution and Biological Function of “Day” and “Night” Rhythmic Genes in Caloric Restricted Aged MuSCs.**

Phase distribution of rhythmic genes in MuSCs from aged CR-fed mice. GO analysis performed separately on rhythmic genes with phase ZT0-ZT6 (“day” genes) or those with phase ZT12-ZT18 (“night” genes) in CR aged MuSCs.

### *High-fat diet induced circadian rewiring in adult stem cells does not significantly overlap with the circadian reprogramming of aged stem cells*

We next investigated whether a short-term high-fat diet (HFD) would reprogram the rhythmic transcriptome of adult SCs similar to physiological ageing. We *ad libitum* fed adult (8-week-old) C57Bl6 mice a HFD or a control diet **(Methods)** for 7 weeks, at which point the HFD-fed mice had not yet become obese **(Fig. 27a and b)**, and analysed the oscillatory transcriptome of EpSCs and MuSCs (as described above). HFD induced a very potent reprogramming of the rhythmic daily transcriptome of both adult EpSCs and MuSCs, which was not due to major gains or losses in gene expression **(Figs 27c, b, and 28a, b)**. In both

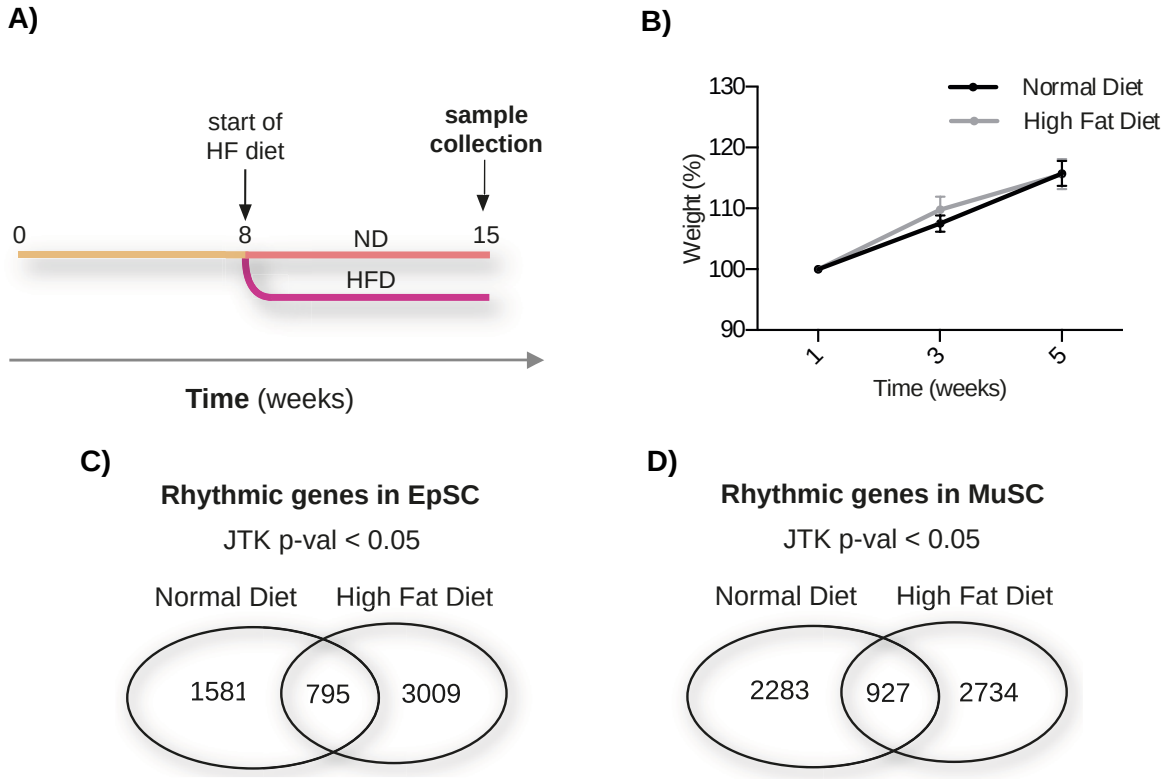



**Figure 27 - HFD Induces Rhythmic Transcriptome Reprogramming in Adult SCs.**
(A) Experimental setup. Eight week-old mice were fed either with a high fat diet (HFD) or a control diet (normal diet, ND) for 7 weeks, after which MuSCs and EpSCs were collected. (B)Percentage of weight relative to week 1 of adult mice fed either a ND or a HFD. (C, D) SCs were sorted every 4 hrs from ZT0. Extracted RNA was processed for microarrays, and the JTK algorithm was used to determine daily rhythmic genes ($p \leq 0.05$). Number of rhythmic genes in ND (2376 genes) and HFD (3804 genes) EpSCs, and in ND (3210 genes) and HFD (3661 genes) MuSCs.

SC types, many genes involved in this oscillatory transcriptome rewiring were related to *fatty acid oxidation*, *response to oxidative stress* and *mitochondrion organisation* **(Figs 29a, b)**.

To determine to what extent the HFD had reprogrammed each SC type to an aged-like onset, we then compared and overlapped the HFD oscillatory dataset with the oscillatory dataset from aged animals. In spite of the significant rewiring caused by the diet, only a 24% in EpSCs and a 20% in MuSCs of the aged rhythmic output overlapped with the HFD rhythmic output. Moreover, HFD-fed mice retained the non-aged–associated rhythmic expression of genes involved in the homeostatic function of EpSCs and MuSCs **(Fig. 30a and b)**. These included *Keratinocyte proliferation* and *skin development* in HFD EpSCS, and *autophagy* and *skeletal muscle tissue development* in HFD MuSCs. EpSCs from HFD-diet mice did share some DNA damage associated categories with the aged mice (*double strand break* repair, *postreplication* repair, *DNA replication* checkpoint) **(Fig. 30c)**. However, unlike

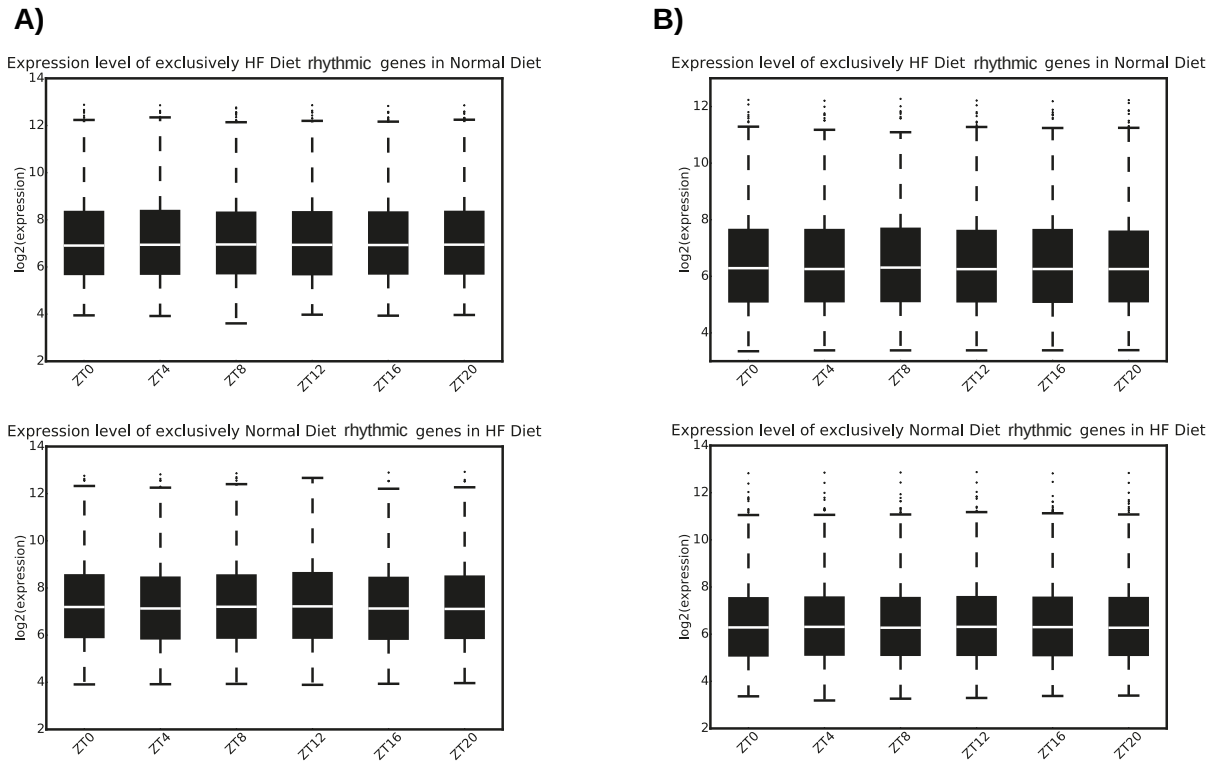



**Figure 28 - Gene Expression Controls for the High Fat Diet Protocol.**

(A, B) Gene expression levels in normal-diet, aged EpSCs (A) and MuSCs (B) showing genes exclusively rhythmic in HF-diet, aged EpSCs (A) and MuSCs (B); and in HF-diet, aged EpSCs (A) and MuSCs (B) showing genes exclusively rhythmic in normal-diet, aged EpSCs (A) and MuSCs (B).

ageing, HFD did not lead to increased levels of replicative stress (yH2Ax and phospho-RPA), or the amount of oxidised DNA in EpSCs, at ZT16 **(Figs 31)**. In MuSCs, the oscillatory levels of autophagy also remained identical despite the diet **(Figs 32a)**. On the other hand, the overlap between the rhythmic transcriptome in MuSCs from HFD-fed mice with those from aged mice revealed a significant enrichment for genes involved in inflammation (*regulation of inflammatory response*) **(Fig. 30d)**. Finally, this short 7-week interval of HFD led to a significant reduction in the number of MuSCs **(Fig. 32b)**.

**A)**

**EpSC**

| **High Fat Diet** rhythmic genes | P-value |
|---|---|
| fatty acid oxidation | 1.37E-05 |
| response to oxidative stress | 1.23E-05 |
| mitochondrion organization | 9.79E-05 |
| regulation of translation | 2.43E-04 |

| **Common** rhythmic genes | P-value |
|---|---|
| DNA replication | 6.87E-26 |
| chromosome segregation | 3.50E-17 |
| DNA repair | 8.30E-15 |
| circadian rhythm | 8.68E-15 |
| DNA integrity checkpoint | 1.25E-05 |
| epithelium development | 4.73E-03 |

**B)**

**MuSC**

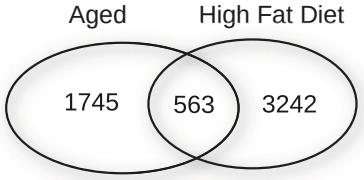
| **High Fat Diet** rhythmic genes | P-value |
|---|---|
| mitochondrion organization | 1.17E-09 |
| response to toxic stress | 2.93E-05 |
| cellular response to oxidative stress | 6.29E-05 |
| oxidative phosphorylation | 7.58E-04 |
| ribosome biogenesis | 1.33E-03 |
| response to ROS | 2.57E-03 |
| autophagy | 3.39E-03 |
| interferon-gamma production | 4.24E-03 |
| TOR signaling | 6.83E-03 |

| **Common** rhythmic genes | P-value |
|---|---|
| circadian rhythm | 1.57E-06 |
| skeletal muscle tissue development | 2.32E-06 |
| fatty acid metabolic process | 6.88E-05 |
| regulation of inflammatory response | 1.17E-04 |
| triglyceride biosynthetic process | 2.18E-03 |
| wound healing | 3.94E-03 |
| regulation of DNA damage checkpoint | 5.67E-03 |

**Figure 29 - Gene Ontology Analysis of the High Fat Diet-Induced rhythmically reprogrammed genes.**

(A) GO analysis is shown for genes exclusively rhythmic in HF-diet, adult EpSCs (top), or for rhythmic genes common to both normal-diet and HF-diet adult EpSCs. (B) GO analyses are shown for genes exclusively oscillating in HF-diet, adult MuSCs (top), and rhythmic genes common between normal-diet and HF-diet, adult MuSCs.

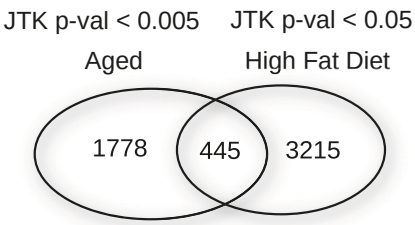
**A)**




| **Aged** rhythmic genes | P-value |
|---|---|
| DNA repair | 4.77E-06 |
| DNA damage checkpoint | 5.45E-05 |
| mitotic DNA damage checkpoint | 2.64E-04 |
| double strand break repair | 4.10E-04 |
| cellular response to IL-6 | 6.72E-04 |

| **High Fat Diet** rhythmic genes | P-value |
|---|---|
| oxidation-reduction process | 9.31E-10 |
| fatty acid metabolic process | 3.72E-07 |
| response to oxidative stress | 8.86E-06 |
| skin development | 5.45E-05 |
| wound healing | 9.63E-04 |
| mitochondrial transport | 1.05E-03 |
| epidermal cell differentiation | 2.12E-03 |
| ribosome biogenesis | 3.21E-03 |
| mismatch repair | 4.68E-03 |
| response to hypoxia | 5.50E-03 |
| keratinocyte proliferation | 6.32E-03 |

**B)**




| **Aged** rhythmic genes | P-value |
|---|---|
| cytokine production | 2.92E-03 |
| mitochondrial DNA repair | 1.17E-03 |
| immune response | 7.59E-03 |

| **High Fat Diet** rhythmic genes | P-value |
|---|---|
| ribosome biogenesis | 1.04E-05 |
| response to oxidative stress | 4.57E-06 |
| regulation of muscle cell differentiation | 2.02E-05 |
| autophagy | 6.63E-05 |
| fatty acid metabolic process | 1.85E-04 |
| skeletal muscle tissue development | 2.53E-04 |
| DNA damage checkpoint | 5.48E-04 |
| regulation of mitochondrial organization | 1.83E-03 |
| response to ROS | 2.55E-03 |
| regulation of fatty acid beta-oxidation | 7.15E-03 |
| respiratory electron transport chain | 8.29E-03 |
| TOR signaling | 9.46E-03 |
| regulation of DNA repair | 9.46E-03 |

**C)**

| **Common** rhythmic genes | P-value |
|---|---|
| DNA repair | 3.49E-16 |
| DNA replication initiation | 1.18E-15 |
| rhythmic process | 3.03E-06 |
| double strand break repair | 3.85E-06 |
| postreplication repair | 2.48E-05 |
| recombinational repair | 5.40E-05 |
| DNA replication checkpoint | 3.44E-04 |
| hexose metabolic process | 4.79E-04 |

**D)**

| **Common** rhythmic genes | P-value |
|---|---|
| circadian rhythm | 1.94E-08 |
| regulation of inflammatory response | 1.91E-03 |
| cytokine biosynthetic process | 8.86E-03 |

**Figure 30 - High Fat Diet Induces Rhythmic Transcriptome Reprogramming in Adult SCs That Is Distinct From Ageing-Reprogramming.**

(A, B) Overlap between aged and HF-diet rhythmic genes in EpSCs (A) and MuSCs (B). GO analyses are shown for rhythmic genes exclusive to aged SCs (top) or SCs from HFD adult mice (bottom). Note that many genes involved in homeostatic functions remained rhythmic (included in GO categories such as *keratinocyte proliferation*, *epidermal cell differentiation*, and *skin development*, in HFD EpSCs [A], and *skeletal muscle tissue development, regulation of muscle cell differentiation*, and *autophagy* in HFD MuSCs [B]). (C, D) GO analysis showing oscillatory genes common between aged mice and adult HFD mice in EpSCs (C) and MuSCs (D).

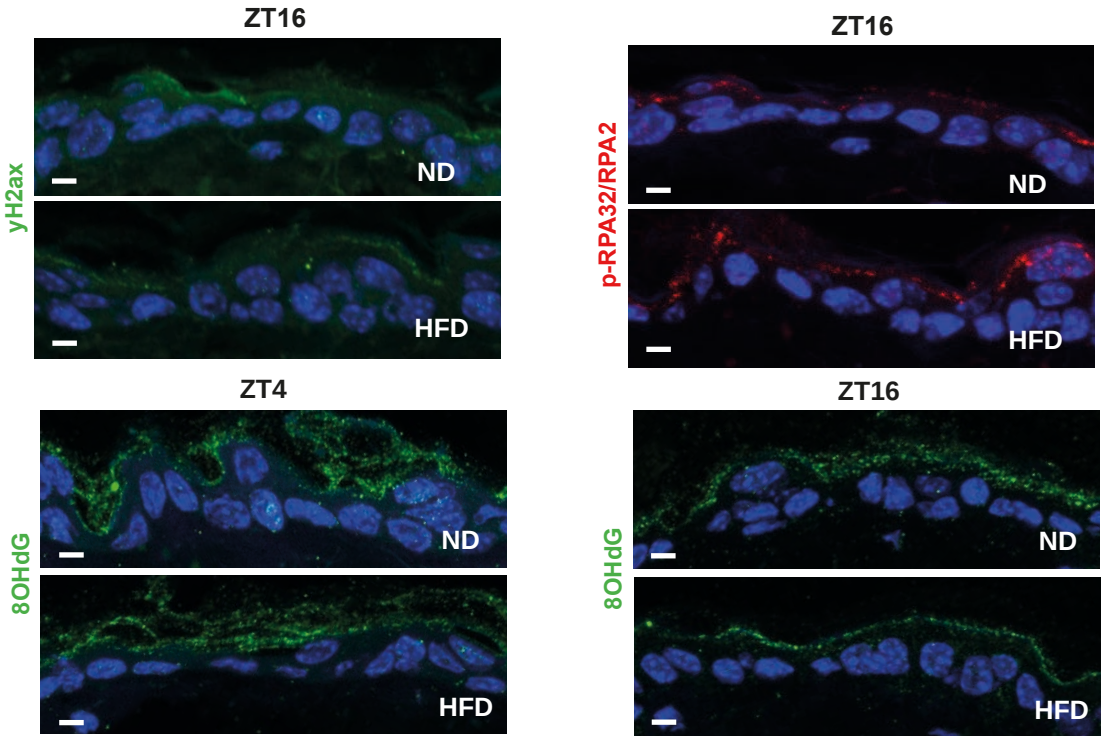
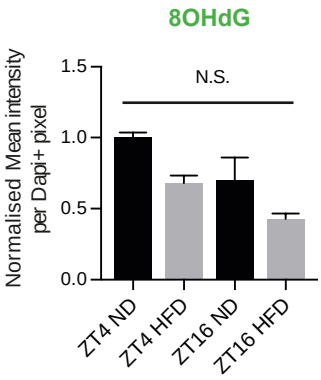



**Figure 31 - EpSCs from High Fat Diet Adult mice show no Signs of Replicative Stress nor DNA Damage.**

Representative images of intensity levels of yH2ax and p-RPA, at ZT16, and oxidised DNA at ZT4 and ZT16 from adult mice fed with a normal diet or HF diet. ) Quantification of intensity levels of oxidised DNA at ZT4 and ZT16 (normalised to normal-diet EpSCs at ZT4). Scale bar, 5 µm. Error bars represent ±S.E.M.; N.S., not significant.



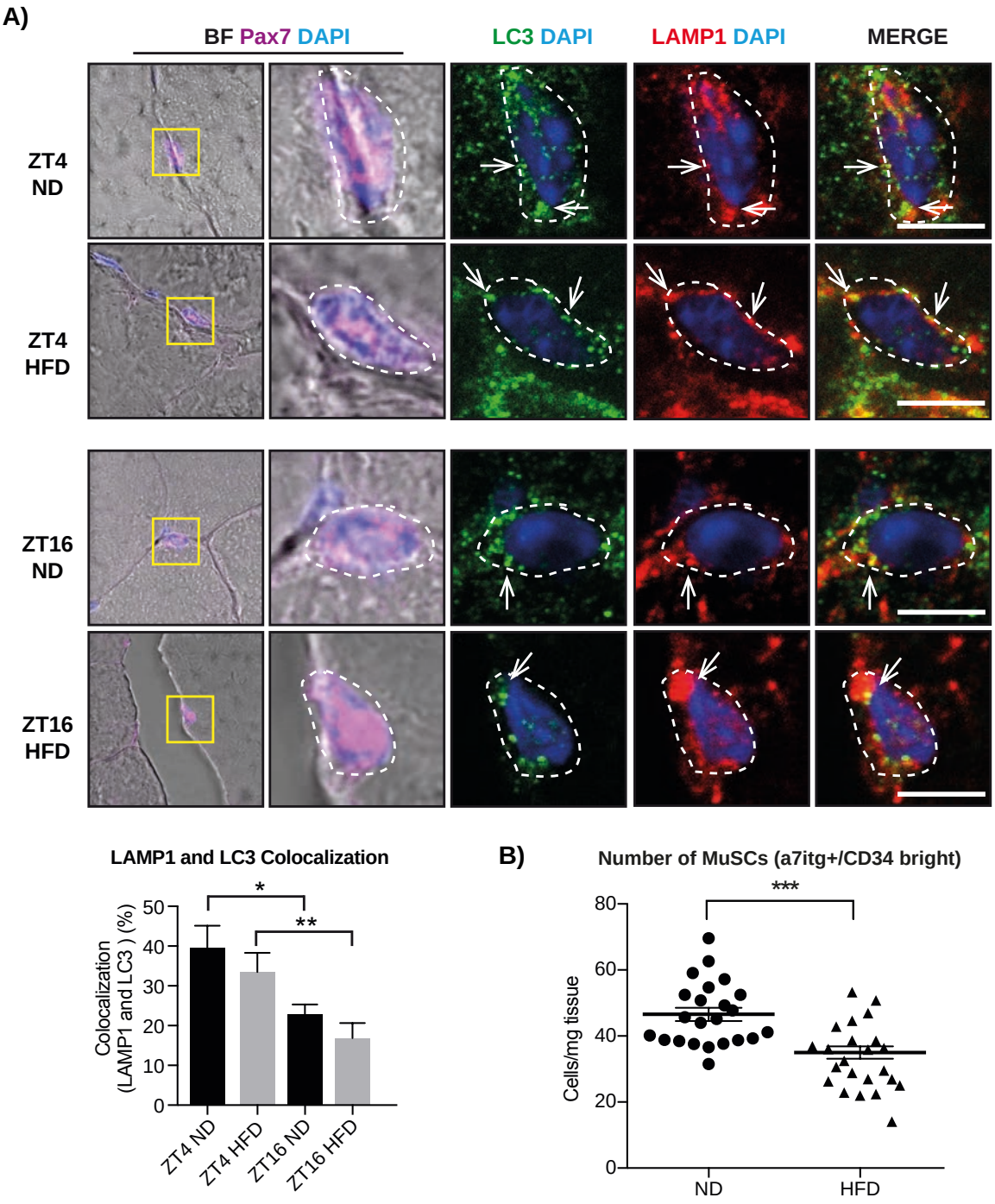



**Figure 32 - MuSCs from High Fat Diet Adult mice show unaltered levels of autophagy but a reduced number of MuSCs.**

(A) Autophagy levels.Representative images and quantification of percentage of colocalisation of LAMP1 and LC3 puncta in normal diet and High Fat (HF) diet MuSCs at ZT4 and ZT16. Scale bar, 5 µm. (B) Number of MuSCs per mg of muscle tissue in adult MuSCs from normal-diet or HFD mice. Error bars represent ±S.E.M.; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

## *Chapter 3 –*

# *Deletion of circadian clock components does not recapitulate the age-associated circadian rewiring of physiologically aged stem cells*

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### *Clock disruption does not recapitulate the diurnal oscillation reprogramming of physiologically aged stem cells*

We used *Bmal1* KO, and *Per1/Per2* double KO mice to analyse if clock disruption could recapitulate any of the features of age-associated reprogramming of the rhythmic function of stem cells.

First we checked for signs of replicative stress in 18-week-old *Bmal1* KO mice and found no significant accumulation of yH2Ax or phosphor-RPA foci, nor higher levels of oxidised DNA than in the wild-type controls **(Fig. 33a and b)**. Moreover, at ZT16 (when the peak of S-phase takes place) we saw no change in the mRNA levels of 5 of the "ZT16 genes

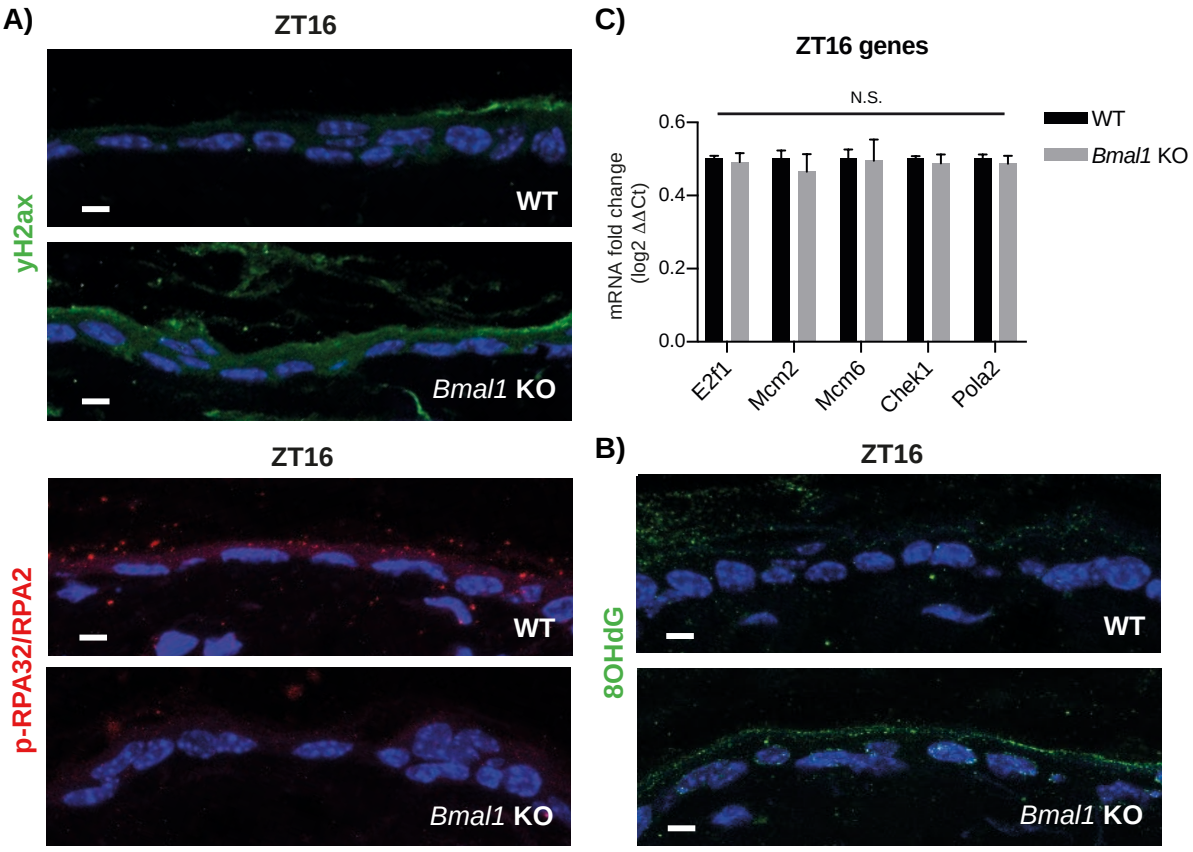
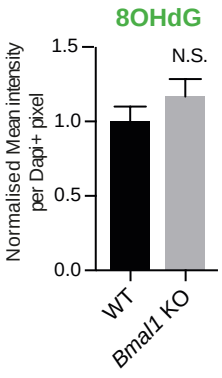



**Figure 33 - EpSCs from *Bmal1* KO Mice show no Signs of Replicative Stress nor DNA Damage.**

(A) Representative images of intensity levels of H2ax and p-RPA at ZT16, in 18-week-old wild-type and *Bmal1* KO EpSCs. (B) Representative images and quantification of intensity levels of oxidised DNA at ZT16 in wild-type and *Bmal1*-KO EpSCs (normalised to wild-type ZT16). (C) Fold-change of mRNA expression levels of the ZT16 subset genes *E2f1*, *Mcm2*, *Mcm6*, *Chek1*, and *Pola2*, normalised against *B2m* at ZT16, in wild-type or *Bmal1* KO. Scale bar, 5 m. Error bars represent ±S.E.M.; N.S., not significant.



subset" (*E2f1, Mcm2, Mcm6, Chek1, Pola2*), which we previously found to be increased upon physiological ageing **(Results, Chapter 1) (Fig. 33c)**.

Epidermal SCs from 27-39-week-old *Per1/Per2* double KO mice (*Per1/2* dKO) showed no increase in replicative stress levels during the active phase of the day **(Fig. 34a)**. Furthermore, the expression levels of the "ZT16 genes" *E2f1, Mcm2, Mcm6, Chek1* and *Pola2* remained identical to those of the controls **(Fig. 34b)**.

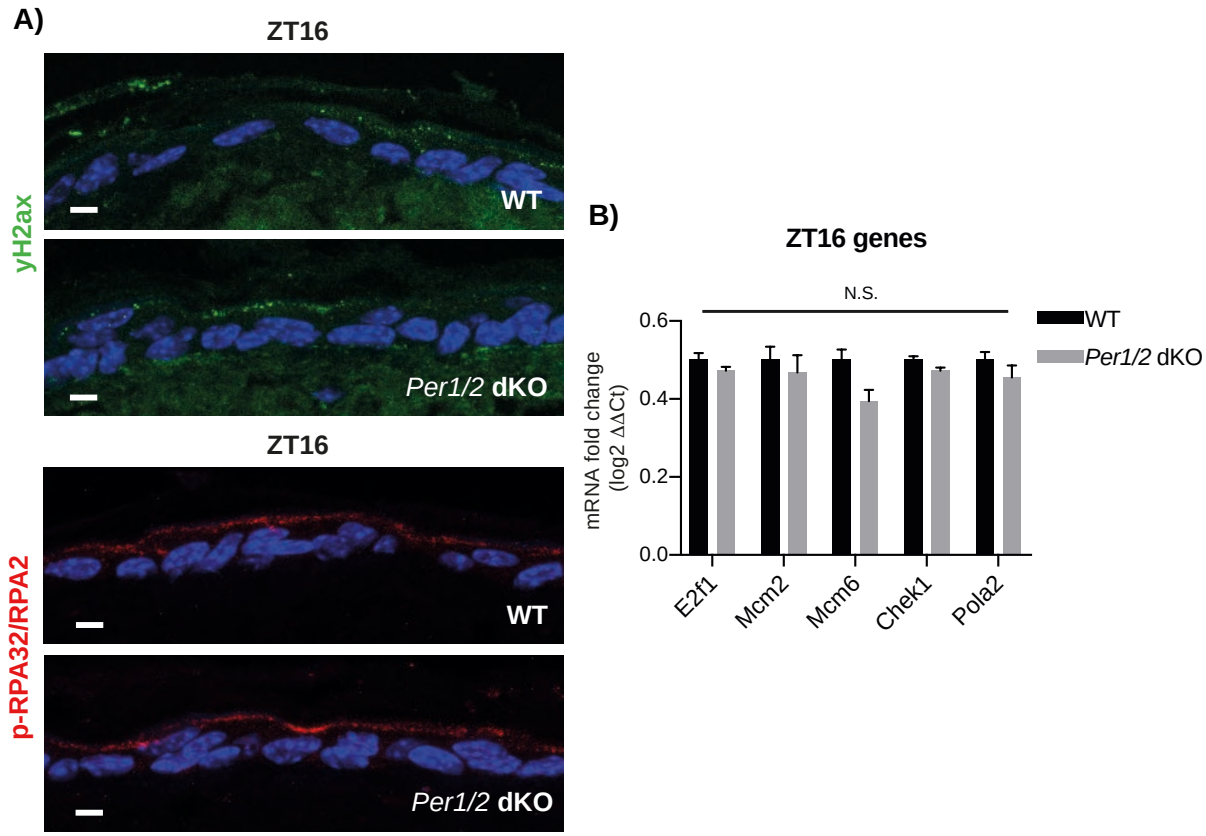



**Figure 34 - EpSCs from *Per1/2* dKO Mice show no Signs of Replicative Stress nor DNA Damage.**

(A) Representative images of intensity levels of H2ax and p-RPA at ZT16, in 27-to-39-week-old wild-type and *Per1/2* dKO EpSCs. (B) Fold-change of mRNA expression levels of the ZT16 subset genes *E2f1*, *Mcm2*, *Mcm6*, *Chek1*, and *Pola2*, normalised against *B2m* at ZT16, in wild-type or *Per1/2* dKO. Scale bar, 5 m. Error bars represent ±S.E.M.; N.S., not significant.

Finally, we confirmed by LC3/LAMP1 co-immunostaining that MuSCs from *Bmal1* KO mice did not show altered levels of autophagy at ZT16 **(Fig. 35)**.

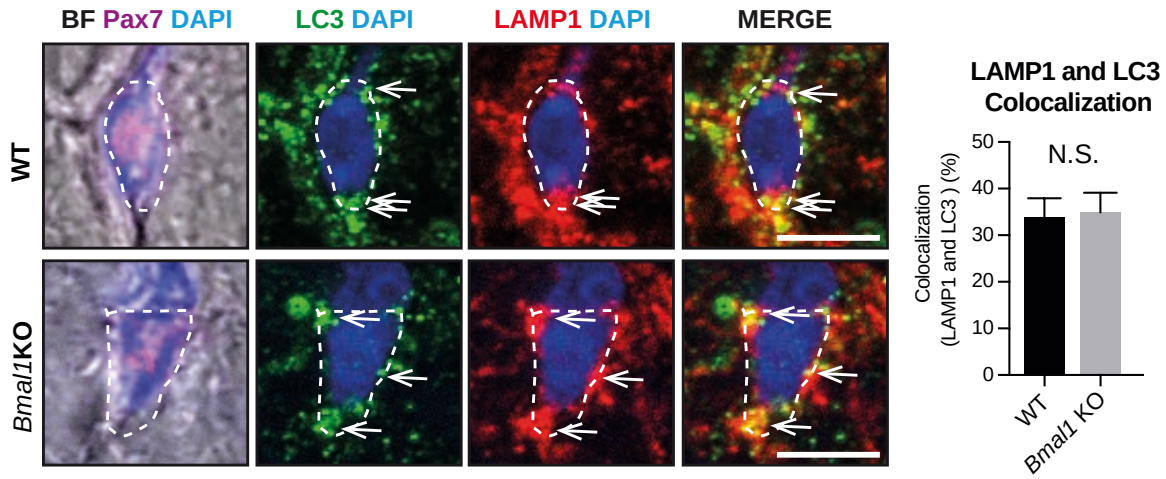



**Figure 35 - MuSCs from *Bmal1* KO Mice show no Changes in Autophagy Levels.**

Autophagy levels. Representative images and percentage of colocalisation of LAMP1 and LC3 puncta in wild-type and *Bmal1* KO MuSCs at ZT16. Note that LAMP1 and LC3 puncta are present in all interstitial muscle cells and in muscle fibres. MuSCs boundaries are indicated (dotted line). Scale bar, 5 m. Error bars represent ±S.E.M.; N.S., not significant.

## *Chapter 4 –*

# *Tissue comparison of oscillatory functions in liver, MuSCs and EpSCs from adult, aged and caloric restricted-fed mice*

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### *Circadian Liver Transcriptome Reveals Ageing-Induced Reprogramming*

Several hallmarks of ageing are tightly linked with disruption of metabolic disruption. Moreover, the connection between the circadian clock and metabolic pathways is indisputable. Therefore, to unravel the metabolic pathways that underlie the interplay between circadian rhythms and ageing, we have decided to analyse the circadian transcriptome of one of the most metabolic tissues in the entire body - the liver.

We collected livers from adult mice and aged mice (≥18 months) at six time points over a day, using the zeitgeber times (ZT; time in 12h light-12h dark cycles) of ZT0 (defined as lights on), ZT4, ZT8, ZT12, ZT16, and ZT20. We then analysed the whole transcriptome with gene expression arrays, and used the JTK algorithm to identify transcripts that are rhythmically expressed in a diurnal manner (as described before). We detected robust rhythmic expression in 3226 transcripts from adult liver, and 2422 transcripts from aged liver **(Fig. 36a)**. As a consequence of ageing, 44.8% of the rhythmic genes in adult ceased to oscillate. However, a large set of genes (1319) became newly rhythmic upon ageing as previously observed for EpSCs and MuSCs. On the other hand, we observed that both the amplitude of oscillations and the phase distribution of the rhythmic genes along the day remained identical between ages **(Fig. 36b and c)**

To address whether CR can reorganise rhythmic gene expression in the liver during ageing, we fed a large cohort of 60-week-old mice with either a 30% CR diet or a control diet *ad libitum* for 25 weeks, as previously explained **(Fig. 20a)**. As mentioned, mice lost the expected amount of weight **(Fig. 20b)**. Then, as previously described, we analysed the transcriptome by gene expression arrays from liver samples collected at six time points over a 24-hr period. CR induced a strong reprogramming of the rhythmic genes found in livers from aged mice **(Fig. 37)**. While, 3439 genes became newly oscillatory in response to the diet, only 1095 genes (21.8%) remained commonly oscillatory regardless of the diet.

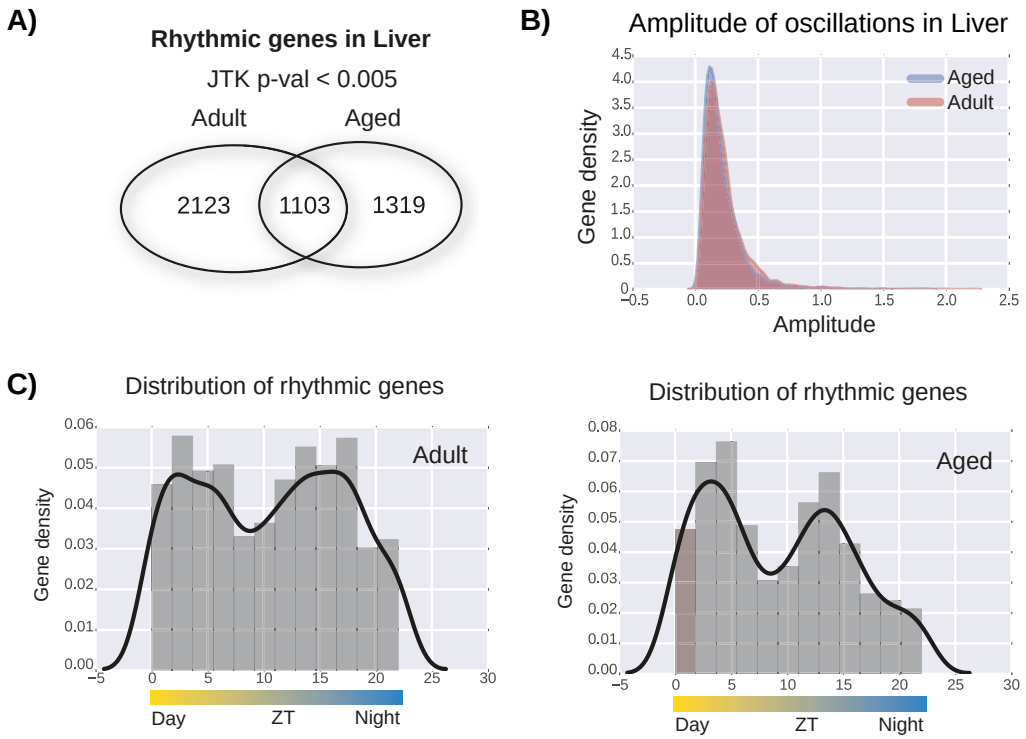



**Figure 36 - Reprogramming of the Rhythmic Transcriptome in Aged Liver.**

(A) Number of rhythmic genes in adult (3226 genes) and aged (2422 genes) liver. Liver were collected every 4 hr, at time points ZT0 (lights on), ZT4, ZT8, ZT12, ZT16, and ZT20 (with ZT given in hours). Extracted RNA was processed for microarrays, and the JTK algorithm was used to determine daily rhythmic genes ($p \leq 0.005$). (B) Distribution of oscillation amplitude of the adult and aged sets of rhythmic genes, normalised for their total number of rhythmic genes. Results are presented as gene density. (C) Phase distribution of adult and aged rhythmic genes in Liver.

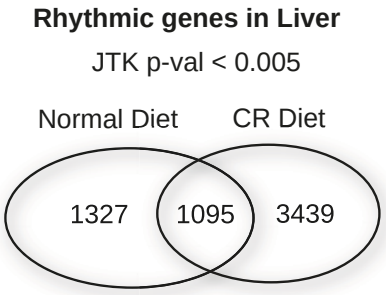



**Figure 37 - Caloric Restriction induces Reprogramming of Rhythmic Genes in Aged Liver.**

(A) Number of rhythmic genes in liver samples from normal diet-fed aged mice (2422 genes) and CR-fed aged mice (4534 genes). Liver were collected every 4 hr, at time points ZT0 (lights on), ZT4, ZT8, ZT12, ZT16, and ZT20 (with ZT given in hours). Extracted RNA was processed for microarrays, and the JTK algorithm was used to determine daily rhythmic genes ($p \leq 0.005$).

### *Inter-Tissue comparison of rhythmic functions*

Since we observed a similar reprogramming of the rhythmic output as a consequence of ageing for EpSCs, MuSCs and liver, we wondered if we could identify biological rhythmic functions specific to SCs only and biological rhythmic functions common to the three types of tissues. We, therefore, overlapped the rhythmic genes found in adult, aged and aged CR-fed mice and performed GO analysis on the genes exclusively overlapping between SCs but not with liver - “stem cell rhythmic signature”-, and on the genes that overlapped between all three tissues - “tissue-independent rhythmic signature” **(Fig. 38)**.

In adult mice **(Fig. 39)**, the “stem cell rhythmic signature” was enriched for GO terms such as *hippo signalling, Wnt signalling pathway* and *stem cell development.* The “tissue-independent rhythmic signature” shared genes also related to the Wnt path way (*regulation of canonical Wnt signalling pathway*) but also SMAD signalling (*SMAD protein import into the nucleus*). In aged mice **(Fig. 39)**, the “stem cell rhythmic signature” was characterised by some epigenetic changes - *chromatin modification* and *histone acetylation* -, as well as *dNTP biosynthetic process.* The aged “tissue-independent rhythmic signature”, on the other hand, was composed of GO terms related to metabolic dysfunction and metabolism in general (*response to dietary excess* and *TOR signalling*) and inflammation (*regulation of inflammatory response*). Finally, in CR-fed aged mice **(Fig. 39)**, the “stem cell rhythmic signature” was remarkably enriched for epigenetic-related GO terms (*histone modification, histone 4 acetylation, covalent chromatin modification,* etc.). Interestingly, the *stem cell*

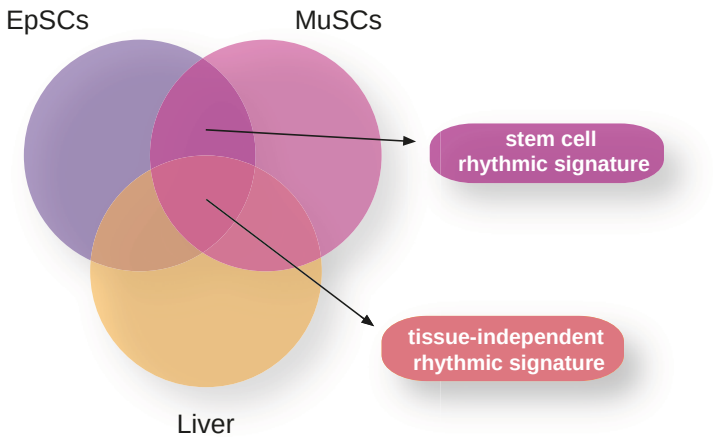
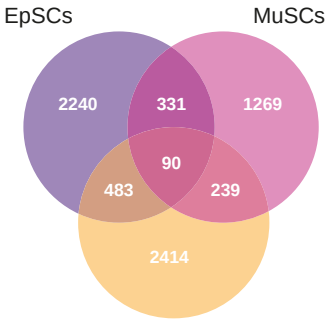



**Figure 38 - Inter-Tissue comparison of rhythmic functions.**
Comparison between rhythmic genes in liver, EpSCs, and MuSCs. The “Stem cell rhythmic signature” was defined by the oscillatory genes exclusively overlapping between EpSCs and MuSCs. The “Tissue-independent rhythmic signature” was defined by the oscillatory genes overlapping between liver, EpSCs, and MuSCs.

**Adult**



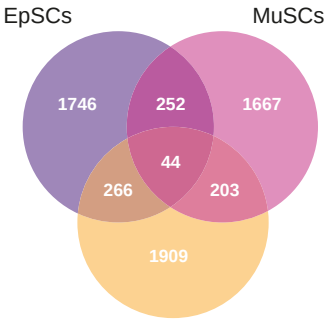

stem cell rhythmic signature

| (331 rhythmic genes) | P-value |
|---|---|
| regulation of epithelial cell proliferation | 4.69E-05 |
| response to stress | 8.05E-05 |
| hippo signaling | 4.75E-04 |
| response to wounding | 6.30E-04 |
| lipid storage | 1.28E-03 |
| positive regulation of interleukin-4 production | 2.45E-03 |
| positive regulation of interleukin-5 production | 2.71E-03 |
| Wnt signaling pathway | 5.57E-03 |
| stem cell development | 7.35E-03 |

tissue-independent rhythmic signature

| (90 rhythmic genes) | P-value |
|---|---|
| response to redox state | 3.68E-04 |
| regulation of DNA repair | 1.70E-03 |
| regulation of canonical Wnt signaling pathway | 2.89E-03 |
| SMAD protein import into nucleus | 2.94E-03 |

**Aged**



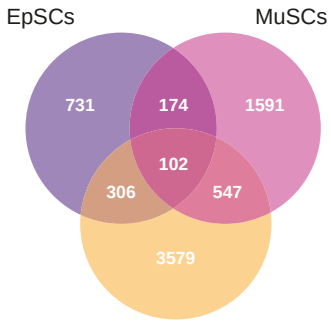

stem cell rhythmic signature

| (252 rhythmic genes) | P-value |
|---|---|
| protein lipidation | 1.55E-04 |
| chromatin modification | 1.32E-03 |
| histone acetylation | 1.99E-03 |
| dNTP biosynthetic process | 6.05E-03 |

tissue-independent rhythmic signature

| (44 rhythmic genes) | P-value |
|---|---|
| response to redox state | 3.21E-07 |
| hexose metabolic process | 1.69E-06 |
| response to glucose | 4.96E-06 |
| response to carbohydrate | 7.74E-06 |
| regulation of lipid metabolic process | 1.08E-05 |
| regulation of inflammatory response | 7.74E-05 |
| gamma-delta T cell activation | 2.96E-04 |
| response to dietary excess | 9.63E-04 |
| defense response | 1.42E-03 |
| ageing | 2.08E-03 |
| eating behavior | 2.34E-03 |
| L-alpha-amino acid transmembrane transport | 3.97E-03 |
| TOR signaling | 8.19E-03 |

**Aged CR**




stem cell rhythmic signature

| (174 rhythmic genes) | P-value |
|---|---|
| covalent chromatin modification | 4.01E-06 |
| histone modification | 1.59E-05 |
| histone 4 acetylation | 8.02E-04 |
| histone H3-K4 demethylation | 1.01E-03 |
| histone H2B ubiquitination | 2.14E-03 |
| insulin receptor signaling pathway | 4.11E-03 |
| tRNA modification | 4.72E-03 |
| stem cell development | 4.78E-03 |
| histone H4-K16 acetylation | 7.02E-03 |

tissue-independent rhythmic signature

| (102 rhythmic genes) | P-value |
|---|---|
| regulation of insulin secretion | 4.39E-03 |
| negative regulation of TOR signaling | 5.47E-03 |
| negative regulation of IRE1-mediated unfolded protein response | 8.17E-03 |

**Figure 39 - Gene Ontology Analysis of Rhythmic Functions from the Inter-Tissue Comparison in Adult, Aged and Aged Caloric Restriction.**

GO analysis of the rhythmic genes that compose the "Stem cell rhythmic signature" and the "Tissue-independent rhythmic signature" in adult (top), aged (centre), and CR-fed aged mice (bottom).

*development* GO term, only observed in the adult "stem cell rhythmic signature", was preserved by the CR diet. The "tissue-independent rhythmic signature" in CR-fed aged mice was mostly associated with *negative regulation of TOR signalling* and *regulation of insulin secretion.*

## *Discussion*

***Chapter 1***

Studies focused on the circadian oscillations of the SCN have revealed that ageing has a dampening effect on the circadian machinery (Bonaconsa et al., 2014; Chang and Guarente, 2013; Farajnia et al., 2012; Nakamura et al., 2011). The SCN has been defined as the “central pacemaker” of the body, sending out signals responsible for coordinating physiological functions and adjusting the timing of the peripheral clocks located in other tissues according to the light schedule. Since the circadian oscillations in the SCN were observed to become dampened with age, it was assumed that age could have the same effect on all other tissues. However, to date, such assumption has not been formally proven and it is still unknown how the clock in peripheral tissues responds to ageing. Perhaps more importantly, the physiological impact of such putative clock disruption remained wildly unexplored.

We first aimed to confirm whether peripheral tissues suffered the hypothesised dampening of the circadian clock upon ageing. We chose to investigate two distinct types of adult SCs, those from the epidermis – EpSCs-, and those from the skeletal muscle – MuSCs. These not only come from very different tissues in the body, they also majorly differ in terms of proliferative rate: while EpSCs constantly proliferate to generate high numbers of epidermal cells and compensate for the continuous shedding of keratinocytes; MuSCs are almost only required to proliferate upon muscle injury and, therefore, remain quiescent throughout most of their lifetime. Given that these two types of stem cells have evolved to respond to the distinct idiosyncrasies of their own tissue, we wondered if their circadian clock would age differently. And, if so, we were interested in determining in what ways that could impinge on the different functions of these SCs and, concomitantly, on the homeostasis of the skin and muscle.

In order to start understanding the impact of ageing on the diurnal functional rhythms of SCs, we performed the first large-scale analysis of the circadian transcriptome from skin and muscle SCs. We purified these SCs around the clock (every 4h) to be able to dissect simple changes in gene expression from differences in oscillatory gene expression. Unexpectedly, we found robust rhythmic gene expression in SCs both from adult and aged mice. However, a 72-76% of the rhythmic genes in adult SCs ceased to oscillate in aged mice, while a large set of genes became newly rhythmic in both aged EpSCs and MuSCs. We next confirmed that the loss of oscillatory genes in aged SCs was not a consequence of their overall downregulation, and that the gain of oscillatory genes in aged SCs was not due to their upregulation in comparison with the adult counterparts. We also observed identical overall amplitude of gene oscillations between adult and aged mice. Additionally, we found that the core clock machinery oscillates in an identical manner between adult and aged

mice, further underscoring that aged EpSCs and MuSCs do not become arrhythmic. Curiously, the timing of each core clock gene was also approximately the same between EpSCs and MuSCs, suggesting that the “physiological timing” perceived by both tissues is virtually the same. These observations led us to one major conclusion: EpSCs and MuSCs do not become arrhythmic with age; rather, their oscillatory transcriptome is drastically reprogrammed during physiological ageing. We believe that this observation will most likely have strong repercussions in the circadian and SC fields as it breaks the current dogma of age-associated dampening of rhythmic functions.

We next investigated whether dampened oscillations in the SCN could be translated into changes in diurnal behaviour and could somehow account for the newly oscillatory genes found in both types of aged SCs. Adult and aged mice showed significant changes in the frequency of movement (measured by the number of visits to drinking corners). However, although aged mice were significantly less active than adult mice throughout the 24-hr day, their physiological activity remained robustly circadian. Both adult and aged mice were more active during the dark phase of the photoperiod (ZT12-ZT24), in agreement with their nocturnal nature, even when the light input – one of the strongest entrainment factors – was removed for two weeks. Thus, two important conclusions can be drawn from these results: first, aged mice do not become behaviourally arrhythmic and the core clock machinery remains robustly circadian in their stem cells; second, the reprogramming of the oscillatory transcriptome in aged stem cells cannot be attributed to changes in the physiological circadian behavioural activity.

As mentioned above, even though the core clock machinery remained robustly rhythmic during ageing, there was still a striking reprogramming of the rhythmic output. We performed GO analysis, a bioinformatics and literature-based approach, to compare and identify biological functions associated to the rhythmic genes in adult and aged EpSCs and MuSCs.

In adult EpSCs we found mostly GO terms related to the homeostasis of the skin such as *epidermis development* and *keratinocyte differentiation*. On the other hand, the genes that oscillated only in aged EpSCs were mainly associated with stress functions, including inflammation (*cellular response to IL-6*) and DNA damage/repair (i.e. *double strand break repair*). The group of genes that remained rhythmic upon ageing was associated with three main categories: *control of circadian rhythms* (further underscoring that aged EpSCs do not lose their ability to remain rhythmic), *DNA replication* and *mitotic M phase*. This analysis yielded the first indication that perhaps EpSCs reprogram their homeostatic rhythmic output during ageing as an attempt to cope with specific types of stress. It is also conceivable that

the common rhythmic genes represent a group of housekeeping functions whose oscillatory behaviour is vital even during ageing.

Previous studies from our group had shown that adult EpSCs are more predisposed to replicate in the evening and differentiate late at night as a potential mechanism to protect their DNA from UV-exposure (Janich et al., 2011; 2013). Therefore, we also looked at the phase distribution of rhythmic genes along the day and performed GO analysis separately on “night” and “day” genes. This analysis gave us further insight regarding the timing of specific rhythmic functions. Adult EpSCs displayed a clear bimodal distribution of rhythmic genes, with a group of genes with phase ZT0-6 (“day” genes) and another with phase ZT12-18 (“night” genes). Indeed, among the “night” gene set, we observed a significant enrichment for GO categories related to differentiating cues such as *response to TGFbeta* and *keratinocyte differentiation* (Janich et al., 2013). Among the “day” GO terms, we found epidermal homeostatic functions such as *regulation of BMP signalling pathway*. BMP signalling is known to promote EpSC quiescence in opposition to TGFbeta signalling (Genander et al., 2014; Oshimori and Fuchs, 2012). Thus, BMP signalling during the day is likely to help prevent undesired activation of proliferation and consequent exposure of single-stranded DNA to UV-light; while at night, TGFbeta signalling induces proliferation and differentiation of EpSCs. By contrast, upon ageing, not only did the phase distribution of rhythmic genes changed, the newly rhythmic genes were related to very different biological functions. The “night” rhythmic functions were mostly associated with DNA damage/repair and no longer related to epidermal differentiation. Moreover, we did not observe any BMP signaling GO terms enriched by the “day” rhythmic genes in aged EpSCs.

Even though aged EpSCs showed evident signs of stress and impairment of homeostatic processes, the timing of basic functions such as the core clock machinery and cell cycle regulation seems to remain tightly controlled with age: *mitotic M phase* is a common rhythmic category; *DNA replication* is a “night” category and *cytokinesis* is a “day” category in both adult and aged EpSCs. Accordingly, and as depicted by the GO analysis, the majority of the DNA replication machinery was rhythmic in adult but also aged SCs, with its expression peaking at night. We also confirmed by PCNA immunostaining (a protein that acts as a processivity factor for DNA polymerase during DNA replication), that maximal DNA replication occurred at ZT16 (night) and dropped to low levels by ZT4 (day) in adult EpSCs, as previously shown (Geyfman et al., 2012; Stringari et al., 2015). However, the percentage of aged EpSCs in S-phase was 4-fold lower at ZT16 than that of adult EpSCs. Yet, intriguingly, a similar percentage of aged EpSCs underwent maximal S-phase 8-12 hours later (i.e. ZT4) than adult EpSCs at ZT16. Additionally, the GO terms unique to aged SCs also included a significant number of DNA damage/repair categories (such as *NER*, *BER* and *Mismatch Repair*), including those related to replicative stress such as *Brca2* or *Tipin*

(*replication fork protection*). This suggests that DNA replication might be delayed or stalled, indicative of replicative stress. As further proof, we observed high levels in the evening (ZT16) (when S-phase starts) and in the morning (ZT4) (when S-phase should be finished) of phospho-RPA (a protein that binds to and protects single-stranded DNA during replication) and yH2ax (a general marker of DNA damage) in aged EpSCs. Adult EpSCs, as expected, only showed increased levels of phosphor-RPA and yH2ax during the timing of DNA replication and, by morning, these two markers were back to basal levels.

We also found that at least 33 genes essential for DNA replication presented a different pattern of expression between ages. These genes included those encoding the MCM proteins, *Cdc45*, *Rrm1*, *Rrm2*, *Gins1*, *Gins2*, *Lig1* and *E2f1.* In adult EpSCs, all presented a statistically significant drop precisely at ZT16, coinciding with the time of maximum DNA replication in the epidermis of adult mice. Intriguingly, this drop was no longer present in aged EpSCs. We hypothesised that this might reflect a regulatory loop that prevents the DNA replication machinery from unnecessarily re-initiating DNA replication during the night. In aged EpSCs, since S-phase is delayed, these genes would be actively transcribed for a while longer to help finish S-phase and/or to perform a protective effect in replicative stress conditions (Ibarra et al., 2008).

Finally, since transcription-stalling lesions generally occur stochastically, longer genes are more likely to accumulate such lesions and their expression is more likely challenged (Vermeij et al., 2016). Indeed we also observed that, in aged EpSCs, the longest genes were expressed at lower levels during the night hours, when the delay in DNA replication is strongest.

Importantly, previous reports have shown that EpSCs replicate their DNA at night also as a strategy to avoid the interval of maximum cellular oxidative phosphorylation that occurs during the day, and thereby minimising genotoxic stress (Geyfman et al., 2012; Stringari et al., 2015). Accordingly, the rhythmic GO analysis confirmed that the expression of genes involved in processes such as *oxidation-reduction process*, *mitochondrion organisation* and *response to oxidative stress* peaked during the day (ZT0–ZT6) in both adult and aged EpSCs. Additionally, the oscillatory profile (i.e. phase and amplitude) of these genes was maintained in aged EpSCs. However, aged EpSCs were less likely to enter S-phase at ZT16 (the physiological time at which DNA replication occurs in adult EpSCs), and the peak of DNA replication was extended to well within day hours (i.e. ZT4). This timing likely exposes unwound DNA to genotoxic stress, as it occurs when oxidative phosphorylation takes place (Geyfman et al., 2012; Stringari et al., 2015). Strikingly, we found that aged EpSCs presented high levels of DNA oxidation, as measured by 8oxodG immunostaing (an antibody that recognises the 8-Hydroxydeoxyguanosine base that occurs in DNA due to attack by hydroxyl radicals), which persisted throughout the day. Thus, the concomitant peaks of DNA

replication and oxidative stress likely contribute to the accumulation of oxidised DNA in aged EpSCs.

As aged EpSCs extended their DNA replication into the day (ZT4) as a consequence of replicative stress, and accumulated high amounts of DNA damage, we checked whether mitosis was also delayed. Even though about 50% fewer aged EpSCs entered M-phase than adult EpSCs, maximal M-phase occurred at ZT20 in both adult and aged epidermis (4h after the S-phase peak in adult EpSCs). Since no additional mitosis peak was observed after ZT4 in aged EpSCs, they might either be stalled until ZT20, as occurs in yeast with replicative stress undergoing mitosis (Amaral et al., 2016), or be cleared away by a yet-unidentified mechanism, independent of apoptosis. The observation that these aged EpSCs, burdened with damaged DNA, still underwent mitosis at the correct time correlates well with the large set of genes involved in *mitotic M-phase* that remained rhythmic in aged EpSCs.

These data strongly support the idea that upon ageing, even if homeostasis is somehow compromised and SCs have to cope with increasing levels of replicative stress and DNA damage, EpSCs are still carefully programmed to follow the correct timing of the cell cycle. This is likely explained by the high and constant demand from EpSCs to fuel the epidermis with new progeny, even if at the expense of more damaged daughter cells. On the other hand, these data also highlight the crucial role of circadian rhythms in segregating rhythmic functions and preventing the undesired overlap of certain cellular processes. In the case of EpSCs, incorrect segregation of DNA replication and oxidative phosphorylation may lead to the progressive accumulation of oxidised DNA.

MuSCs also suffered a strong reprogramming of their rhythmic output as a consequence of ageing. However, consistent with the normally quiescent state of MuSCs, genes involved in DNA replication were not expressed in a rhythmic manner in adult or aged MuSCs, and aged MuSCs did not show any signs of replicative stress. Nonetheless, adult MuSCs expressed several transcripts in a oscillatory manner that may be involved in double-strand break repair, (*cellular response to DNA damage stimulus*), such as *Rad23a*, *Ercc4* and *Xpa*, in agreement with previous observations that quiescent MuSCs are more predisposed to repairing this type of damage than differentiated muscle cells (Vahidi Ferdousi et al., 2014). Interestingly, the majority of the rhythmic genes common to adult and aged MuSCs were predominantly involved in *cell-substrate adhesion* and *cytoskeletal organisation*, and included genes encoding *paxillin*, *vinculin, calponin*, and *CSF1r*, which have previously been associated with MuSCs interactions with the local environment (Goetsch et al., 2014; Ishii and Lo, 2001; Segawa et al., 2008; Shibukawa et al., 2013). This is consistent with the well-accepted notion that the resting MuSC is ready to respond with the appropriate receptors and signalling pathways, but protects its quiescence by

mechanisms that include immobilisation of ligands by extracellular matrix (ECM) components, and synthesis of inhibitors for intracellular signalling pathways (Montarras et al., 2013). Thus, rhythmically expressed genes allowing muscle stem cell interaction with the ECM and surrounding niche components may be required for preservation of the *bona fide* quiescence state of MuSCs throughout life (Bentzinger et al., 2013; Lukjanenko et al., 2016; Rozo et al., 2016; Tierney et al., 2016). Loss of quiescence by anticipated proliferation or senescence entry can affect stemness and regenerative functions in MuSCs (Chakkalakal et al., 2012; Sousa-Victor et al., 2014). However, we did not observe any signs of unsolicited activation in aged MuSCs, which, as their young counterparts, remained in a quiescent state, as demonstrated by the absence of expression of proliferative markers MyoD or Ki67 (data not shown).

Maintenance of a constitutive autophagy flux (a process that eliminates dysfunctional cellular components in the lysosome (He and Klionsky, 2009)) in resting MuSCs has been shown to be fundamental for preservation of stemness by preventing intracellular proteotoxicity. A progressive decline in basal autophagy in quiescent MuSCs has been recently shown to underlie the loss of fitness and functionality in old mice (García-Prat et al., 2016). Importantly, we found that the levels of basal autophagy oscillate during the day and are higher during the rest phase in mice (ZT4). The same observation has been reported for whole skeletal muscle samples, liver, kidney and heart (Ma et al., 2011). In aged MuSCs however, autophagy was significantly reduced and became flat throughout the 24-h day, reinforcing the notion that basal autophagy decline is a hallmark of circadian rewiring in these quiescent SCs with ageing.

Thus, altogether our results indicate that aged muscle stem cells still retain their ability to remain quiescent, and to rhythmically regulate their interaction with their niche. However, they significantly lose their capacity to rhythmically recycle the damaged components that are likely generated on a daily basis and may become more prone to undergo senescence once they reach advanced geriatric age (García-Prat et al., 2016). At variance with this, and very importantly, our results identified a link between basal autophagy decline in quiescent MuSCs and rhythmic rewiring during ageing. In this context, the inability of aged quiescent MuSCs to sustain a rhythmic autophagy may be at the basis of their failure to sustain proteostasis.

In all likelihood, healthy SCs segregate their predisposition to perform different functions along the 24-hr day to maximise energetic efficiency and performance efficacy, while minimising exposure to potentially harmful situations. Hence, the loss of the oscillatory transcriptome of SCs as they age can progressively carry dire consequences for their functions. Thus, aged human EpSCs that have lost their ability to separate the timing of DNA

replication from that of maximal UV exposure or that of their oxidative daily metabolism, have a significantly higher risk of accumulating DNA damage than their younger homeostatic counterparts (Janich et al., 2013; Stringari et al., 2015). Nonetheless, some cellular functions remain strongly rhythmic in aged SCs. For instance, aged EpSCs still undergo daily rhythmic cell divisions, despite having oxidised and damaged DNA. We hypothesise that rhythmic functions that are essential for each subset of adult SCs are those that are more likely to be retained during ageing. Thus, in tissues with a high demand of cellular replenishment, like the epidermis, the clock machinery appears to play a dominant role in ensuring a daily input of new cells at the right time of the day, irrespective of the presence of damaged DNA. In contrast, in MuSCs, the genes most resilient to loss of rhythmic control during ageing were those involved in the physical interaction with the surrounding niche (myofibre, stroma, and niche cells)—which is essential for protection of the key quiescent state in resting muscle, and hence for preservation of stemness.

Intriguingly, the rhythmic functions of aged SCs are rewired such that they adapt to the new conditions of stress associated with the aged environment. This reprogramming is likely generally associated with ageing, as we also observed it for the liver (Results, Chapter 4).

***Chapter 2***

A CR diet is known to extend the lifespan of many organisms, including rodents (Froy and Miskin, 2010)(Colman et al., 2009; Froy and Miskin, 2010). CR also enhances the function of adult stem cells, including the regenerative capacity of MuSCs of old age (Cerletti et al., 2012; Mihaylova et al., 2014), through yet uncharacterised mechanisms. CR is also capable of entraining the clock in the SCN (Challet et al., 1998; 2003; Mendoza et al., 2005; Resuehr and Olcese, 2005). However, whether CR regulates the rhythmic behaviour of EpSCs and MuSCs during ageing was unknown. We were interesting in understanding if the well known beneficial effect of CR could also be due to the prevention of the age-associated reprogramming we reported for EpSCs and MuSCs. Hence, we started the diet once the mice were fully developed and half way through their lifetime expectancy. After 6 months of CR, when the animals had reached an advanced aged, we collected the samples and checked the rhythmic transcriptome for the age-related traits that we characterised in each tissue. Strikingly, we found that CR induced a dramatic change in the rhythmic transcriptome of both SC types. While approximately 82% and 73% of the genes ceased to oscillate in aged EpSCs and MuSCs respectively, 1010 in EpSCs and 1921 genes in MuSCs became newly oscillatory in response to the CR diet. Apart from changes at the molecular level, CR prevented the decrease in fur density and the increase in cornified layer thickness in the skin, as well as the decrease in the MuSC number, all age-associated features. This clearly

highlights the impact that diet and metabolism have on the physiology and ageing process of every tissue, including adult SCs.

The amplitude and period of the core clock genes was unaffected by CR, but we observed a 4-h advance in their zenith due to a food anticipation effect, which was strongest for the EpSCs and has been previously described for CR diets. Interestingly, however, daily oscillatory homeostatic functions were recovered in aged EpSCs from CR-fed mice (*keratinocyte development* and *epithelial cell proliferation*). These included genes involved in BMP and TGFbeta signalling, as well those for EGFR, VDR, or Jarid2, all of which regulate EpSC activation or differentiation (Solanas and Benitah, 2013). CR did not prevent the age-related rhythmic expression of certain genes involved in double-strand break repair or base-excision repair, suggesting that some age-related DNA damage persists in aged EpSCs even in CR-fed mice. Nonetheless, CR was able to prevent the accumulation of both yH2ax and phosphor-RPA as well as the S-phase delay into light hours of the day. Note that also the peak of S-phase suffered a 4-h advance in comparison to the adult EpSCs. Accordingly, the group of “ZT16 genes” essential of DNA replication was 4-h advance in their zenith. This shows how tightly linked the circadian clock and the cell cycle are in EpSCs since the circadian and the cell cycle-related genes were the only ones we found advanced in response to diet. Moreover, at the precise timing of S-phase (now ZT12), due to CR the “ZT16 genes” showed a drop in expression identical to what we had observed in adult EpSCs. Finally, likely due to the fact that S-phase was no longer occurring during the peak of oxidative phosphorylation, the levels of oxidised DNA was drastically reduced in response to the diet.

CR also induced a strong reprogramming of the rhythmic functions in MuSCs. Importantly, genes involved in autophagy such as *Tmem59*, *Pycard*, *Ulk3*, *Park2*, and *Dap1* recovered their oscillatory expression as depicted by the GO category *regulation of autophagy*. We further confirmed this by co-immunostaining of LAMP1 and LC3: autophagy remained oscillatory with higher levels during the rest phase, as seen in healthy adult mice (Boada-Romero et al., 2013; Durcan and Fon, 2015; Koren et al., 2010; Suzuki et al., 2007; Young et al., 2009) (Figure 5C). These results could provide a possible mechanistic explanation to the previously reported functional rejuvenating effect of CR on aged quiescent MuSCs (Cerletti et al., 2012). Additionally, aged MuSCs from CR-fed mice no longer expressed genes linked to inflammation or mitochondrial DNA repair in a rhythmic manner. Instead, their rhythmic transcriptome associated with GO terms of *regulation of myotube differentiation*, *myoblast proliferation*, and *skeletal muscle regeneration*. Expression of housekeeping rhythymic functions related to circadian rhythms and cellular adhesion were unaffected by CR.

In contrast to CR, long-term exposure to HFD accelerates the development of many ageing-related pathologies, including chronic inflammation, cardiovascular disease, diabetes, as well as higher predisposition to develop obesity, hyperinsulinemia, hepatic steatosis, and cancer (López-Otín et al., 2013)(Hatori et al., 2012; Sherman et al., 2012). HFD also results in deregulated feeding cycles and disrupts circadian rhythms (Kohsaka et al., 2007). We know from previous literature that short-term HFD is capable of rewiring the circadian transcriptome and metabolome of the liver, in an obesity-independent manner (Eckel-Mahan et al., 2013). Therefore, in order to dissect the age-associated pathologies from the obesity-induced ones, we decided to test whether short-term HFD was sufficient to induce reprogramming of the rhythmic transcriptome also in adult SCs. And if so, whether such reprogramming mimicked any of the age-related rhythmic traits.

We fed 8-week-old mice with HFD for 7 weeks and confirmed that the mice did not become obese. As previously observed for the liver, HFD induced a very potent reprogramming of the rhythmic daily transcriptome of adult EpSCs and MuSCs. In this case, this diet resulted in strong metabolic changes since both SC types many genes involved in this oscillatory transcriptome rewiring were related to *fatty acid oxidation*, *response to oxidative stress*, and *mitochondrial organisation*. However, after we compared the transcriptome of HFD-fed mice with physiologically aged mice, we observed that the diet did not interfere with the non-aged–associated rhythmic expression of genes involved in the homeostatic function of EpSCs and MuSCs. Moreover, HFD did not induce any signs of replicative stress in EpSCs nor did it led to the accumulation of oxidised DNA. Finally, the levels and oscillatory behaviour of basal autophagy were unperturbed by this type of diet. In spite of that, intriguingly, this short 7-week interval of HFD led to the rhythmic expression of genes related to inflammation, and a reduction in the number of MuSCs, a hallmark of skeletal muscle ageing. Thus, HFD strongly rewires the rhythmic output of adult stem cells, most likely to allow them to cope with fatty acid metabolism, yet - unlike physiological ageing - it does not prevent adult SCs from expressing their homeostatic transcriptome in an oscillatory daily manner.

Overall, our data indicates that CR is significantly capable of preserving the correct timing of adult stem cell functions lost during ageing. The ageing-related rhythmic reprogramming in SCs seems to heavily rely on a metabolic entrainment. Indeed, we have found that CR has a profound protective effect on many of the rhythmic changes that occur during physiological ageing, which is consistent with the beneficial effects of CR on the function of aged stem cells (Cerletti et al., 2012). Circadian rewiring has previously been shown to occur in the liver of mice either fed a HFD or with systemic signals secreted by

lung-tumour cells, allowing the liver to adapt to the metabolic changes required in both scenarios (Eckel-Mahan et al., 2013; Masri et al., 2016). Thus, SCs might be evolutionarily prepared to quickly reprogram their timed functions in response to different contexts of metabolic changes and/or tissue-specific stresses. Importantly, this reprogramming ability of SCs may underlie the previously observed beneficial effects of CR on SC function. Future studies will be required to identify which components are responsible for the ageing-related rewiring of the daily fluctuating functions of SCs, and to determine whether they could be therapeutically targeted to maintain the proper timing of SC function during ageing in humans.

***Chapter 3***

In mice, deletion of the core clock transcription factor *Bmal1* leads to the development of numerous ageing-like pathologies. These include low body weight, arthritis, brittle bones, corneal degeneration, diabetes, intestinal permeability and inflammation, skin aging, and neurodegeneration, and they severely reduce the lifespan of these mice (Ali et al., 2015; Bouchard-Cannon et al., 2013; Gossan et al., 2013; Janich et al., 2011; Kondratov et al., 2006; Lin et al., 2009; Marcheva et al., 2010; Musiek et al., 2013; Samsa et al., 2016; Storch et al., 2007; Summa et al., 2013; Yang et al., 2016). Therefore, this mouse model has been used as a model to study physiological ageing. However, our results show that physiological ageing is associated with reprogramming, rather than with arrhythmia. In order to further substantiate this, we analysed whether clock disruption could recapitulate any features of the age-associated reprogramming of the rhythmic function we described for SCs. We used *Bmal1* KO, and *Per1/Per2* double KO mice (in which BMAL1 activity is no longer inhibited at night) and found that, albeit *Bmal1* KO SCs display very strong symptoms of ageing, these changes do not recapitulate the rhythmic changes observed during physiological ageing in either of the mouse models. We found no signs of replicative stress neither of accumulation of oxidised DNA. Additionally, the expression levels of 5 of the “ZT16 genes” at ZT16 (moment when adult EpSCs, but not aged EpSCs, show a drop in expression) were identical between genotypes. The levels of basal autophagy were also unaffected by the deletion of *Bmal1*. We therefore conclude that the reprogramming of the rhythmic functions observed during physiological ageing cannot be attributable to an arrhythmic function of the circadian clock.

Mechanistically, we do not know the driving forces that change the rhythmic transcriptome of adult SCs as they age. However, we show that this is unlikely to be related to direct disruption of the major clock components, as aged SCs still have a clock machinery

as robustly rhythmic as adult SCs, and neither *Bmal1* KO nor *Per1/2* dKO mice develop the ageing features that we characterised in EpSCs and MuSCs. Nonetheless, even though we observed robust rhythmic RNA expression of the clock genes, it is still highly likely that their activity is post-transcriptionally regulated.

***Chapter 4***

Ageing has been tightly linked with perturbed metabolic homeostasis that include deregulated nutrient sensing, mitochondrial dysfunction, reprogramming of signalling pathways and insulin-resistance (Fontana and Partridge, 2015; Ocampo et al., 2016). Our results support the idea that each tissue reprograms their rhythmic functions in a distinct manner in order to cope with the type of stress it faces during ageing. We have also demonstrated the remarkable impact that diet and metabolic cues have on the rhythms of adult SCs. The reprogramming potential of diet has also been demonstrated in liver from young mice (Asher and Sassone-Corsi, 2015; Eckel-Mahan et al., 2013). Thus, we wondered whether the liver also underwent rhythmic reprogramming as a consequence of ageing. Not only did we find a very similar age-induced reprogramming of rhythmic functions in the liver, we also observed a strong CR-induced effect.

Since we observed a similar reprogramming of the rhythmic output as a consequence of ageing for EpSCs, MuSCs and liver, we wondered if we could identify biological rhythmic functions specific to SCs only and biological rhythmic functions common to the three types of tissues. We, therefore, overlapped the rhythmic genes found in adult, aged and aged CR-fed mice and performed GO analysis on the genes exclusively overlapping between SCs but not with liver - “stem cell rhythmic signature”-, and on the genes that overlapped between all three tissues - “tissue-independent rhythmic signature”

We decided to compare the rhythmic output of EpSCs, MuSCs and liver from adult, aged and CR-fed aged mice.

# *Conclusions*

- During ageing, the transcriptome of EpSCs and MuSCs remains robustly rhythmic.

- The transcription of the core clock genes is virtually unaffected upon ageing, and both types of aged SCs present a tissue-specific rhythmic output.

- The behaviour of aged mice, albeit significantly lower than that of their young counterparts, is as circadian as in young mice. On that account, the reprogramming of the oscillatory transcriptome in aged stem cells cannot be attributed to changes in the physiological circadian behavioural activity.

- Aged SCs show a dramatic reprogramming of their rhythmic output. That is, most circadian genes that regulate the healthy homeostasis of adult stem cells cease to oscillate. Instead, aged SCs display a new cohort of oscillating genes involved in different types of stress.

- Aged EpSCs show signs of replicative stress (persistent high levels of phosphor-RPA and yH2ax) and delayed replication (assessed by the number of PCNA positive cells every 4h of the day). Moreover, longer genes normally transcribed by night, are downregulated in aged EpSCs, likely as a consequence of DNA lesions and stalled forks.

- The delayed peak of S-phase in aged EpSCs overlaps with the previously described timing of oxidative phosphorylation. Associated with this, aged EpSCs accumulate high levels of oxidised DNA that persist unresolved throughout the day.

- Regardless of the accumulation of DNA damage and replicative stress, aged EpSCs still undergo mitosis and cytokinesis at the correct timing.

- In agreement with their quiescent nature, MuSCs do not accumulate DNA damage nor show signs of replicative stress.

- The newly rhythmic output in aged MuSCs mostly associates with inflammation rather than ECM-interacting functions.

- Adult MuSCs display oscillatory levels of basal autophagy.

- During ageing, MuSCs lose the rhythmicity of basal autophagy in addition to decreasing the overall levels of this pathway along the day.

- Although ageing has a strong systemic component, the age-related circadian reprogramming is tissue-specific and a consequence of the idiosyncrasies of each tissue.

- Age-associated circadian reprogramming is significantly prevented by a long-term CR diet. This striking effect further highlights the anti-ageing benefits of this type of diet.

- CR prevents the age-associated decrease in MuSC number, as well as prevents the increase in thickness of the cornified layer of the skin and the decrease in fur density.

- CR prevented the age-associated increase in replicative stress levels, the delay in S-phase and accumulation of oxidised DNA in aged EpSCs.

- In aged MuSCs from CR-fed mice, the levels of basal autophagy remain oscillatory and at much higher levels than those from control diet mice.

- Although a HF diet strongly reprograms the circadian output of both SCs in adult mice, there is little overlap with the age-related rewired oscillating transcriptome.

- HF diet does not lead to changes in replicative stress levels nor in levels of oxidised DNA in adult EpSCs.

- HF diet does not induce changes in basal autophagy but leads to decreased numbers of MuSCs in adult mice.

- Even though Bmal1 knockout stem cells are functionally impaired, they do not show the predominant changes that we have characterised in both types of physiologically aged SCs.

- Per1/2 double knockout mice do not show signs of increased replicative stress and, therefore, do not seem to reflect the changes that we have characterised in physiologically aged SCs.

- The age-associated rewiring of rhythmic functions seems to be a general coping mechanism since the liver, a highly metabolic tissue, also suffers rhythmic reprogramming during ageing.

- The comparison of the rhythmic output in MuSCs, EpSCs and liver allowed us to identify a “stem cell rhythmic signature” and a “tissue-independent rhythmic signature”. The “tissue-independent rhythmic signature” revealed that the three tissues share many oscillatory metabolic pathways that change during ageing. On the other hand, in response to CR, the “stem cell rhythmic signature” shifts drastically towards epigenetics functions that may lie upstream of many of the beneficial effects that this diet has on SCs.

- As a general and final conclusion of the results, we believe that when speaking of ageing, there might be no general hallmarks; instead, different tissues show specific (i.e. and often not common) age-related alterations.

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