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YAP as a Regulator of DNA Replication Timing

Rodrigo Meléndez García

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YAP as a regulator of DNA replication timing

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"The job of a scientist is to listen carefully to nature,
not to tell nature how to behave"

Richard Feynman

RÉSUMÉ DÉTAILLÉ

Avant la division cellulaire, l'ADN doit être entièrement et précisément dupliqué pour être transmis aux cellules filles. Ceci est particulièrement important dans les cellules souches qui se renouvellent continuellement et produisent de nouvelles cellules nécessaires à la croissance ou au renouvellement cellulaire des organes. Dans la plupart des cellules métazoaires, la réplication commence au niveau de plusieurs milliers de sites assez spécifiques appelés origines de réplication et ce fait d'une manière hautement orchestrée dans le temps et l'espace bien qu'aucune séquence d'ADN consensus strict n'a été identifié jusqu'à présent. En mitose tardive et en phase G1, les protéines du complexe pré-réplicatif (pré-RC) se fixent sur les origines de réplication. Il s'agit des six protéines ORC (origin recognition complex), puis de Cdc6 (cell-division-cycle 6) et Cdt1, et enfin du MCM (mini-chromosome maintenance) qui comprend six protéines (Mcm2-7). Le pré-RC est ensuite activé pendant la phase S par les kinases Cdc7 (cell division cycle 7) et CDK (cyclin-dependent kinase), ce qui conduit au recrutement de nombreux autres facteurs, au déroulement de l'ADN et au début de la synthèse de l'ADN au niveau de chaque fourche de réplication. Chez les eucaryotes, des segments de chromosomes se répliquent de manière organisée en temps opportun tout au long de la phase S, certaines régions étant répliquées en début, d'autres en milieu et d'autres en fin de phase S. Deux régions majeures d'activation peuvent être visualisées par des expériences de marquage par impulsions utilisant des analogues nucléotidiques. Au cours de la première moitié de la phase S, la chromatine à réplication précoce, principalement transcriptionnellement active, est localisée dans les régions centrales du noyau, tandis que la chromatine à réplication tardive est spatialement située à la périphérie du noyau. Les récents développements de méthodes de capture de la chromatine à haute résolution (Hi-C) ont confirmé qu'il existe une relation entre l'architecture 3D du génome et le « timing » de réplication: les domaines de réplication précoce et tardive présentent une très bonne corrélation avec les compartiments sous-nucléaires d'euchromatine et d'hétérochromatine. Ce modèle spatio-temporel de réplication de l'ADN, appelé programme temporel de réplication (RT) de l'ADN, s'est avéré stable, somatiquement héréditaire et spécifique du type de cellule.

L'ensemble de ces caractéristiques rend le programme RT compatible avec la définition d'une marque épigénétique, et fournit une signature spécifique associée à l'état de la cellule. Cette signature est en effet considérablement modifiée lors des changements d'état cellulaire et la dérégulation du programme RT est associée à de nombreuses maladies, y compris le cancer. Malgré les progrès technologiques majeurs et la richesse des protocoles visant à étudier la réplication de l'ADN, les mécanismes de régulation impliqués dans le contrôle temporel de la réplication ne sont pas encore élucidés et par conséquent la pertinence biologique du programme RT reste méconnue.

Il a été démontré que très peu de mutants génétiques déclenchent des altérations du programme RT. Jusqu'à présent, le Rap1-interacting factor 1 (RIF1) est l'un des très rares facteurs *trans* dont la perte de fonction s'est avérée entraîner des modifications majeures du programme RT. Par ailleurs, mon laboratoire avait mis en évidence un nouveau rôle pour YAP, l'effecteur de la voie de signalisation Hippo, dans le contrôle du programme RT. La voie Hippo est bien connue pour son rôle dans la croissance des organes et le contrôle de la prolifération des cellules souches mais son implication dans la réplication n'avait jamais été envisagée auparavant. Mon équipe d'accueil a cependant constaté que la perte fonction de YAP dans les cellules souches neurales de la rétine du xénope conduit à un programme RT altéré (augmentation de la réplication précoce aux dépens de la réplication tardive), associé à un fort raccourcissement de la phase S. L'implication directe de YAP dans la régulation du programme RT restait cependant à démontrer. C'est cette question qui a fait l'objet de mon projet de recherche de Thèse.

L'objectif de mon projet de Thèse visait donc à mettre en évidence le mécanisme moléculaire sous-tendant le rôle de YAP dans le contrôle de la réplication de l'ADN et d'identifier des partenaires potentiels dans cette fonction. L'ambition de ce travail consistait donc à évaluer si YAP pouvait constituer un nouveau facteur de régulation du programme spatio-temporel de la réplication. Pour ce faire, et en collaboration avec l'équipe de Kathrin Marheineke (I2BC, Gif-sur-Yvette), j'ai mis à profit le modèle d'extraits d'œufs de xénope, un système acellulaire qui récapitule les transitions nucléaires clés du cycle cellulaire eucaryote *in vitro*. Ce système est particulièrement adapté à l'étude des mécanismes et de la dynamique de la réplication de l'ADN. Nous avons

constaté que YAP est recruté à la chromatine pendant la réplication d'une manière dépendante de la formation du pré-RC.

Nous avons ensuite évalué le taux de réplication et le nombre d'origines actives, avec ou sans YAP, *via* l'incorporation de dCTP radioactif dans l'ADN ou en utilisant la technique du peignage moléculaire. Cette dernière permet de visualiser directement les origines de réplication actives et d'étudier la cinétique de réplication sur les molécules d'ADN peignées. Nos résultats suggèrent que YAP régule la dynamique de réplication de l'ADN en limitant à la fois l'activation des origines de réplication et la vitesse globale de réplication de l'ADN.

Afin de savoir si le rôle de YAP dans la réplication de l'ADN a également lieu *in vivo*, nous avons tiré parti des premières divisions embryonnaires du xénope, qui constitue un système simplifié d'analyse du cycle cellulaire. En effet, au cours du développement précoce, avant la transition mi-blastuléenne (MBT), les cellules se divisent très rapidement et présentent une structure de cycle cellulaire sans phase G (donc en absence de transcription). Par conséquent, les variations du nombre de cellules pendant cette période de développement reflètent une modification du temps passé dans les 2 phases restantes (S ou M). Comme la protéine YAP est exprimée par l'ovocyte, nous avons mis au point chez le xénope la technique «Trim-away», afin de provoquer la dégradation de la protéine YAP *in vivo*, et l'avons combinée à des injections de Morpholinos, pour bloquer la traduction et ainsi empêcher la synthèse de protéines *de novo*. Nos résultats montrent que l'absence de YAP entraîne une augmentation de la vitesse des divisions cellulaires. Nous avons obtenu le même phénotype après la déplétion de RIF1. Si l'on considère la fonction bien connue de RIF1 dans la réplication de l'ADN et la présence des seules phases S et M dans les embryons pré-MBT, ces données suggèrent fortement que le taux accru de divisions cellulaires en l'absence de RIF1 résulte de l'accélération de la réplication de l'ADN et du raccourcissement de la longueur de la phase S. De manière comparable, nous proposons donc que YAP est également impliqué dans le contrôle de la dynamique de réplication de l'ADN *in vivo* dans les embryons pré-MBT. Comme les divisons pré-MBT ont lieu en absence de transcription, cette fonction de YAP est nécessairement indépendante de son rôle en tant que co-facteur transcriptionnel.

Afin d'identifier des partenaires de YAP dans cette fonction régulatrice de la réplication, nous avons immunoprécipité YAP dans des extraits d'œufs de xénope, puis nous avons identifié par spectrométrie de masse les protéines ainsi enrichies. Parmi les protéines identifiées, nous nous sommes particulièrement intéressés à RIF1. En effet, comme mentionné plus haut, RIF1 est un des rares facteurs connus pour contrôler le programme RT. Deux mécanismes d'action ont été proposés : (1) RIF1 pourrait réguler le programme RT *via* son rôle dans l'organisation 3D du génome, (2) il pourrait empêcher l'activation des origines tardives de réplication *via* son interaction avec la phosphatase PP1 qui promeut la déphosphorylation du complexe MCM. Afin de valider l'existence d'une interaction physique entre YAP et RIF1, nous avons effectué des co-immunoprécipitations de YAP dans deux systèmes, les extraits d'œufs de xénope et les extraits protéiques de cellules HEK293 préalablement transfectées avec des plasmides exprimant les deux protéines YAP et RIF1. Nos données soutiennent l'hypothèse de l'existence d'un complexe YAP/RIF1.

Comme il a récemment été démontré que RIF1 fonctionne de manière tissu-spécifique, nous avons étudié son expression et sa fonction dans la rétine post-embryonnaire du xénope et comparé les résultats avec les données obtenues précédemment par mon laboratoire sur YAP dans cet organe. Nos résultats montrent que RIF1 est spécifiquement exprimé, tout comme YAP, dans les cellules souches et les cellules progénitrices précoces de la rétine. Nous avons ensuite entrepris une approche *in vivo* de perte de fonction de RIF1 à l'aide de Morpholinos. Le phénotype observé est similaire à celui obtenu chez les morphants *Yap,* avec en particulier la modification de la distribution des foyers de réplication (diminution de la proportion des cellules présentant un profil de phase S tardif). Ces données montrent que RIF1, tout comme YAP, est essentiel à l'établissement du programme RT dans les cellules souches/précurseurs précoces de la rétine.

Dans l'ensemble, nos résultats révèlent l'implication de YAP, indépendante de son activité de régulateur transcriptionnel, dans le contrôle de la dynamique de réplication et identifient RIF1 comme un nouveau partenaire. Nous proposons que YAP, comme RIF1, agit comme un frein lors de la réplication, pour contrôler la vitesse de synthèse de l'ADN.

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INTRODUCTION

1. STEM CELLS

In this chapter, I would like to start with a general introduction on stem cells. For this, I thought it was important to begin with the definition of stem cells and their classification, not a trivial issue as we will see. Then, I will focus on neural stem cells, and more specifically, on retinal stem cells of the frog *Xenopus laevis*. Finally, I will move on to the main topic of my PhD project, the proliferative properties of stem cells and their peculiar cell cycle structure that is different from non-stem proliferative cells.

1.1 Definition

Even if it is quite difficult to know exactly when or by whom “stem cells” were first discovered, the consensus is that they were first rigorously defined in the mouse hematopoietic system in the early 1960 (Becker *et al*., 1963). Since then, efforts in research have been made to know more about their characteristics and use in medicine. For instance, important breakthroughs in stem cell research include differentiation studies (to convert stem cells into a desired cell type), reprogramming a somatic cell into a pluripotent state or the discovery of extrinsic/intrinsic factors that determine stemness and stem cell fate, only to mention a few.

Stem cells are generally defined as unspecialized cells that can self-renew and differentiate into multiple cell types of an organism (Zakrzewski *et al*., 2019). More precisely, stem cells can generate daughter cells identical to their mother (self-renewal), as well as produce progeny with more restricted potential (differentiated cells). To maintain the stem cell population and preserve the homeostasis of a tissue, stem cells can divide asymmetrically or symmetrically (**Figure 1**).

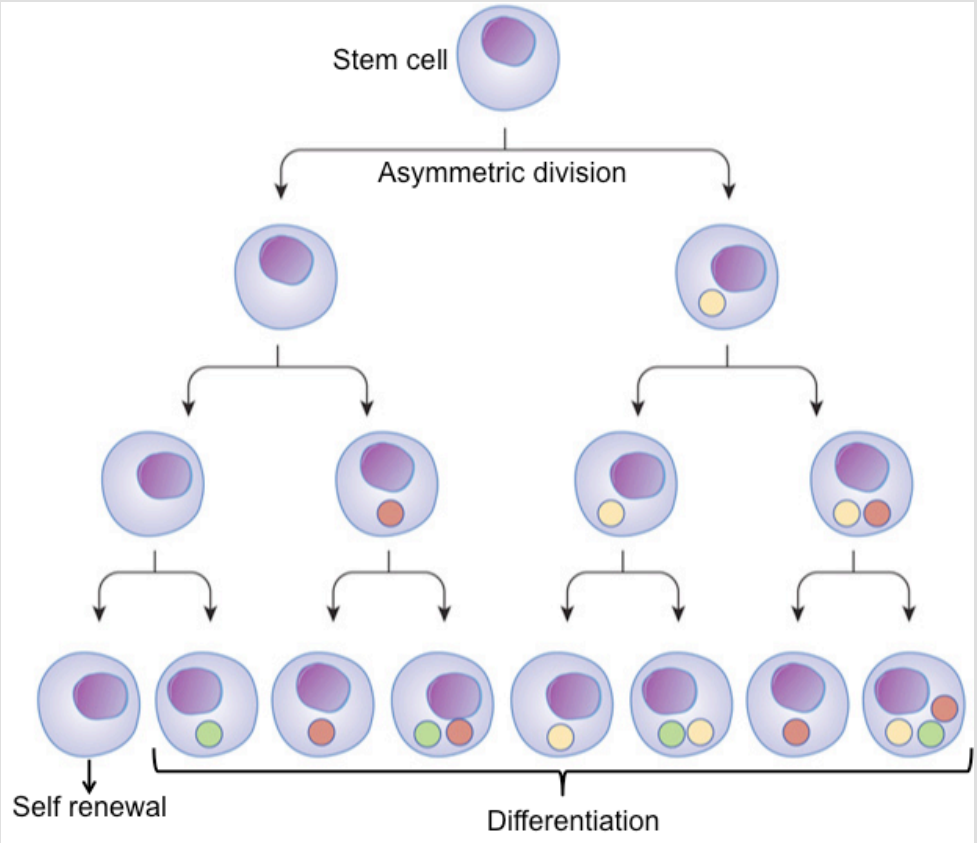


Figure 1. Stem cell division. With asymmetric division, each of the two resulting daughter cells has a different destiny. In this case, one of the daughter cells has a finite capacity for cell division and begins to differentiate (colored balls), whereas the other daughter cell remains a stem cell with unlimited proliferative ability (self-renewal). Adapted from (Sablowski, 2010).

Understanding the mechanisms governing whether stem cells self-renew or decide to differentiate in an exquisite balance to avoid aberrant growth or tissue degeneration has been a challenging task in stem cell biology. Even if investigation in the field attribute that stem cell fate could be stochastically defined, they also remark the importance of molecular signals and space limitations from their environmental surroundings (Garcia-Gomez *et al*., 2020; Simons and Clevers, 2011).

1.2 Classification

Traditionally, classification of stem cells can be done based on their functionality, for example according to their differentiation potency; molecular properties, depending on their gene expression; by phenotype, studying their cell surface markers; or by origin, either obtained in the embryo or in the adult;

among other possible classifications. However, current knowledge in the field has shown that these classifications fail to accurately host all types of stem cells.

The problem of the previously cited classifications is that stem cells display different behaviours according to the tissue they reside, the stage of development of the organism and whether the stemness is tested *in vivo* or *in vitro*. Additionally, recent advances in science showed that stemness is a property that can be obtained by differentiated cells.

1.2.1 Plasticity of stem cells makes their classification confusing

In vitro, it is accepted that by genetic manipulation differentiated cells can become again stem cells. It was in 2006 and 2007, when Takahashi and Yamanaka induced somatic fibroblasts from murine and human to become pluripotent (the ability of a cell to differentiate in any of the three germ layers) (Takahashi *et al*., 2007; Takahashi and Yamanaka, 2006). They used retroviral transduction to make these cells express *Oct4*, *Sox2*, *c-Myc* and *Klf4*, factors allowing a reprogramming process sufficient to generate induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006).

In vivo, this process depends on the context. For instance, the plasticity in plants exists (Gaillochet and Lohmann, 2015). To test reprogramming in plants, scientists challenged stem cells using laser ablation to entirely deplete the stem cell niche (specialized microenvironment of stem cells *in vivo*). Surprisingly, surrounding cells were able to *de novo* form a stem cell system, showing the extraordinarily developmental plasticity of the vegetal kingdom (Reinhardt *et al*., 2003). In animals, dedifferentiation is also found in certain species capable to regenerate, such as salamanders. Studies of limb regeneration showed that the myofiber of newt could dedifferentiate acquiring stem cell markers and eventually enter the cell cycle to produce a new limb (Wang and Simon, 2016). In other species, like humans, this process is hard to prove. However, there are studies in which epithelial cells were capable to dedifferentiate notably in a context of injury (Blanpain and Fuchs, 2014; Donati and Watt, 2015).

Nowadays, scientists utilize two strategies to kill resident stem cells, either by laser ablation or through diphtheria toxin expression. It was first discovered in

Drosophila germ stem cells (Kai and Spradling, 2003, 2004), and now it is well established in mammalian epithelial, that when stem cell are removed, the niches trigger differentiated cells to activate their proliferation and to go back to a stem cell state. One example occurs with the committed secretory cells of the lung (Rawlins *et al*., 2009), which after ablation of the basal stem cells, can respond to the injury by dedifferentiation and ultimately repair the tissue converting into stable and functional stem cells *in vivo* (**Figure 2**).

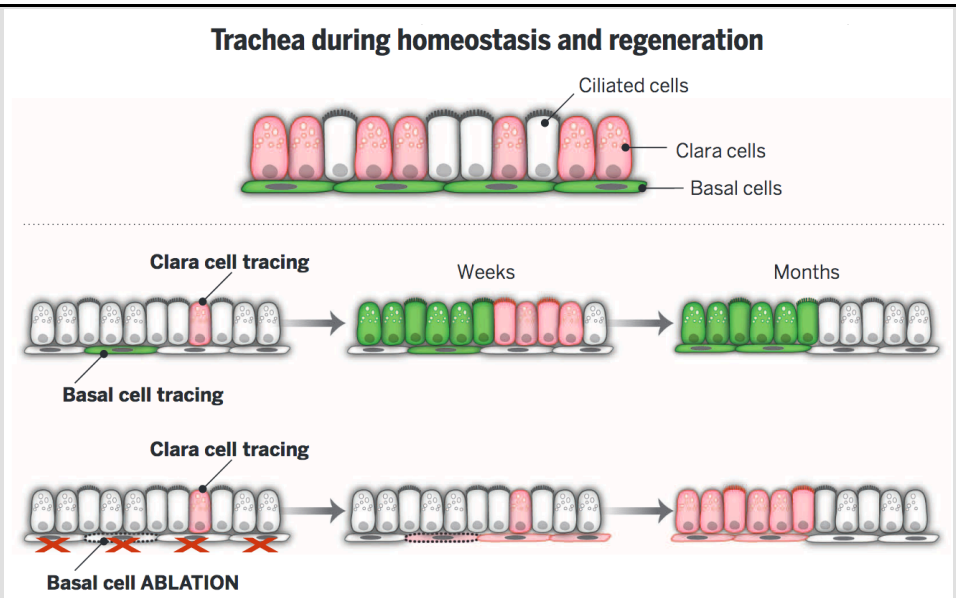


Figure 2. Plasticity of differentiated cells into stem cells during tracheal regeneration. During tracheal homeostasis, basal cells (green) give rise to TA Clara cells (pink) and terminally differentiated ciliated cells (white). Lineage ablation of basal cells (red X's) induces the interconversion and/or ciliated cells into basal stem cells (Tata *et al*., 2013).

Thus, the line of classification between stem cells (unspecialized cells) and other type of cells may seem blurry (Laplane and Solary, 2019). Together, this is to show that common concepts of stem cell biology need to be updated, first for scientists to change the view of designing, making and interpreting experiments and also to innovate the way in which knowledge of stem cells could be applied to health.

1.2.2 Philosophy brings a new classification of stem cells

Recent discoveries showed that the definition of stem cell can be either an entity in a classical view following the two main strict properties discussed before (self-renewal and differentiation), or it can be a cell state, that is indeed

changing according to the context (Clevers and Watt, 2018). Traditional views of stem cells have focused on the classification to find molecular markers to distinguish and sort stem cells, however this could not be entirely helpful because it has been demonstrated that stem cells showed plasticity in cell fate depending on the tissue.

Recently, Laplane and Solary (Laplane and Solary, 2017, 2019) attempted to provide a philosophical analysis based on metaphysics of the term stemness, in order to find a modern classification for stem cells that could explain why the current treatments against cancer have limitations. For them, stemness encloses four distinct properties, two intrinsic and two extrinsic:

- Categorical: intrinsic property of stem cells that is environmental-independent. This traditional point of view is insufficient to categorize the vast diversity of stem cells, since science unveiled that it only applies to certain types of cancer cells. An example is seen with oncogenic mutations, such as in one of the genes of the Ras pathway. This alteration makes them insensitive of their microenvironment (Emanuel *et al*., 1991).
- Dispositional: intrinsic property of stem cells that arises only in the right environment. Here is when the concept of niche becomes essential. For example, hematopoietic stem cells do not behave properly outside the bone marrow, which makes their culture difficult (Scadden, 2014).
- Relational: extrinsic property induced in a cell that would otherwise be a non-stem cell by its microenvironment. For example, in *Drosophila* and the mice, germinal progenitors could dedifferentiate in germinal stem cells after transplantation, irradiation or by aging (Barroca *et al*., 2009).
- Systemic: extrinsic property of a system (tissue), rather than an individual cell. For example, experiments performed in breast cancer cell lines showed that mature cells purified for a given phenotypic state based on cell-surface markers and then cultured, returned to equilibrium and generate a new population of cancer stem cells resembling the diverse phenotypic states before purification (Gupta *et al*., 2011). In the former example, the system is cancer *per se*, suggesting that stemness happens without a specific niche.

Thus, it is clear that stemness encloses distinct properties, depending on the tissue and context. The previous framework constitutes a philosophical approach to respond to the obvious but difficult question of what is a stem cell? This classification could be efficient in medicine to determine the treatment to follow in order to combat certain cancers. For example, in a case of a niche related stem cell, strategies that impact the niche may be efficient, such as immunotherapy. However, if a stem cell goes under systemic categorization, none of the current therapies would be efficient and relapse will eventually occur. Under this scenario, stemness will be hardwired in the organism even in the absence of the niche. Innovative therapies that focus on the regulation of the systemic properties (the hearth beating) of stemness, rather than the pure elimination of cancer stem cells will be of advantage.

1.3 Regeneration in the mammalian central nervous system

Here, I want to discuss the stemness properties of the adult mammalian central nervous system. First, I will present stem cells of the mammalian brain and what they are made for. Then, I will mention some factors that regulate either their proliferation and/or their differentiation. Additionally, I will talk about their endogenous potency under normal conditions and after injury. Finally, I will show the relationship between alterations in neurogenesis with the appearance of psychiatric disorders and the innovative therapies that utilize stem cells to investigate mechanisms of regeneration and to design therapeutic strategies for brain repair.

1.3.1 Discovery of adult NSCs and their function

While in mammals, organs like liver or lungs have adult stem cells that respond to injury to ensure tissue homeostasis; the brain was long considered an exception. The discovery of neural stem cells (NSCs) in the post-natal brain changed the idea that regeneration in the adult central nervous system (CNS) was impossible (Dantuma *et al*., 2010; Ma *et al*., 2009).

Studies in songbirds were the first to demonstrate adult neurogenesis, not only based by morphology, but also by their electrophysiological properties and integration into the song-control structure (Burd and Nottebohm, 1985; Paton and Nottebohm, 1984). Now, the presence of adult NSCs in mammals, including humans, is widely accepted but restricted to specifically brain regions (the olfactory bulb and hippocampus in the mouse) (**Figure 3**).

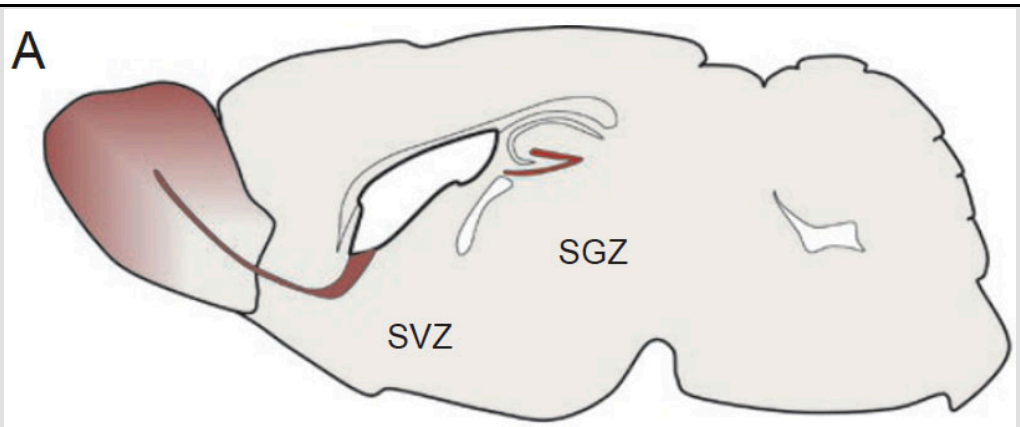


Figure 3. Adult NSCs in the SVZ and SGZ of the rodent brain. A schematic illustration of the adult mammalian brain in mice. Adult NSCs are primarily present in two germinal regions: the subventricular zone (SVZ) of the lateral ventricle wall and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Ma *et al*., 2009).

From rodent models, we know that newborn neurons of the olfactory bulb are required for its normal functioning and some olfactory behaviors, such as fine odor discrimination and odor-reward association (Grelat *et al*., 2018; Li *et al*., 2018; Lledo and Saghatelyan, 2005; Sakamoto *et al*., 2014). On the other hand, neurogenesis in the hippocampus plays important roles in learning, memory and pattern separation (Aimone *et al*., 2014; Deng *et al*., 2009; Ming and Song, 2011). In humans, neurogenesis in the olfactory bulb is still debated, since examination of the adult SVZ suggested that the putative NSCs remain quiescent (Lim and Alvarez-Buylla, 2016). On the contrary, the contribution of newborn neurons in the adult human hippocampus seems to be the same as that observed in rodents (Goncalves *et al*., 2016).

1.3.2. Regulation of NSCs

The regulation of NSCs *in vivo* is quite complex. There are intrinsic and extrinsic factors that regulate whether NSCs proliferate or differentiate. The list of

extrinsic factors that interact with NSC is quite extensive, since these cells receive multiple signals from the whole brain through their complex connections. The factors that regulate their behavior come from the immediate cellular neighbors (the immediate niche), the cerebrospinal fluid and from distant places through the blood vessels. The macrocosm of signalling molecules that regulate either positively or negatively neurogenesis includes neurotransmitters, growth factors, cytokines (Obernier and Alvarez-Buylla, 2019). How NSCs integrate all these factors remains unknown but it is likely that NSCs are heterogeneous (see below) and that subpopulations may respond differentially to such extrinsic factors (Obernier and Alvarez-Buylla, 2019).

1.3.3 Activity of mammalian NSCs under physiological conditions

While *in vitro* NSCs are multipotent cells with the ability of cell renewal and generation of neurons and glia; the multipotency of a NSC *in vivo* in the adult brain remains unknown (Obernier and Alvarez-Buylla, 2019).

Adult NSCs are largely quiescent *in vivo* (Morshead *et al*., 1994). In addition, rodents and human neurogenesis is known to decrease with aging (Spalding *et al*., 2013). By measuring the concentration of ^{14}C that is lastingly incorporated into the DNA of dividing cells, scientist measured neurogenesis in adult human brains. They observed a substantial human hippocampal neurogenesis with only a modest decline during aging compared with the aging in mice. Interestingly, exercise seems to affect positively neurogenesis in both rodents and humans; as seen in MRI studies showing that cerebral blood volume that correlates with neurogenesis, increased with physical activity (Small *et al*., 2004).

Studies *in vitro* showed the existence of two main populations of NSC, quiescent (qNSCs) and activated (aNSCs), which are thought to interconvert (Codega *et al*., 2014). Differences between the two populations include differential gene expression, preference to assembly neurospheres *in vitro* and changes in cell metabolism (Obernier and Alvarez-Buylla, 2019). While genes associated with signalling receptors, cell-cell adhesion and ion channels are enriched in qNSCs (upregulation of Notch, BMP and MAPK pathways), in

aNSCs there is a preference in genes involved in cell cycle control, protein synthesis and DNA repair. Interestingly, only qNSCs display chromatin structural patterns associated with gene repression (Cebrian-Silla *et al*., 2017), this configuration has been suggested to form heterochromatin compartments that are related to quiescence.

Further studies using single cell RNA sequencing showed distinct subpopulations of aNSC and qNSCs. These results also showed that the activation of protein synthesis genes in NSCs is required for exiting quiescence (Llorens-Bobadilla *et al*., 2015).

While most of the discoveries of adult neurogenesis is obtained from mice where activation, maintenance and differentiation of NSC have been shown; there is a big gap of information regarding the continual generation of neurons in the adult human brain. Obstacles including the protracted development, longer life-span and large size of human brains compared to mice; in addition of ethical restrictions, limit the progression of research. Data from different studies show discrepancy in the extent of neurogenesis in the adult human. While some of them suggest that adult neurogenesis occurs robustly even in elderly (Boldrini *et al*., 2018; Spalding *et al*., 2013), others reported a sharp decline (Dennis *et al*., 2016; Knoth *et al*., 2010).

1.3.4 Adult mammalian neurogenesis after injury

Several studies performed in murine models, that aimed to produce a traumatic brain injury, have shown to significantly increase cell proliferation in neurogenic niches (Sun, 2016). This response in proliferation, despite being common in all cases, is relatively transient, as a peak of proliferation is observed on the first week post-injury (Gao and Chen, 2013; Sun *et al*., 2005).

It was also shown that the response of neurogenesis after brain injury decrease with age, since youth animals displayed a more robust response (Sun *et al*., 2005). The difference in cell response related to aging has been also demonstrated in piglets (Costine *et al*., 2015). This correlates with the observation that under normal conditions, neurogenesis decreases with aging.

Unfortunately, in those models of brain injury, newborn neurons failed to significantly migrate to the site of injury (Costine *et al*., 2015; Ramaswamy *et*

al., 2005). Further studies need to focus in the guidance, survival and maturation of the newly formed NSC-derived neurons to the lesion site. Despite of its inefficacity, results showed that mature brain tries to repair injury trough the endogenous neurogenic response.

In humans, contribution of adult neurogenesis after brain injury is less studied, due to the difficulties to obtain brain samples. Moreover, results appear contradictory, while some report positive neural progenitor cells after brain trauma (Zheng *et al*., 2013), others did not find significant differences (Taylor *et al*., 2013). The discrepancy of the results may be associated with the source of samples and markers used, among other experimental variables.

1.3.5 Relation between altered adult neurogenesis and disease

Numerous studies link alterations of adult neurogenesis with neurological and psychiatric disorders. Impaired adult neurogenesis is associated with schizophrenia, major depression, addiction, and anxiety. For example, diminution of adult hippocampal neurogenesis have been proposed as a cause for depression, since antidepressants affects neurogenesis (Miller and Hen, 2015). Observations in rodents have supported the former observations.

Neurogenesis dysfunction is also thought to contribute to the emergence of epilepsy in adults (mesial temporal lobe epilepsy, mTLE). Although seizure activity increases adult neurogenesis, it also results in aberrant migration, morphology, and connectivity of new neurons (Parent *et al*., 1997).

Neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD), have been related with aberrant adult neurogenesis (Winner and Winkler, 2015).

1.3.6 Utility of NSCs in medicine

Following the confirmation that neurogenesis occurs in adult humans, scientists have explored ways to exploit these cells in health and disease. The use of stem cell technology in the nervous system could be applied to a broad range of disorders including trauma, stroke or neurodegeneration. The utility of stem cells is an appealing avenue of research, since it allows the modeling of

neurological diseases in a dish and additionally it provides a source of cells for clinical transplantation (Goncalves and Przyborski, 2018).

While embryonic stem cells (ESC) are pluripotent stem cells (PSC) obtained from the inner mass of the blastocyst that have been widely utilized to study *in vitro* the neural differentiation, development and regeneration (Clarke *et al*., 2017; Wichterle *et al*., 2002); they are rarely destined for clinical studies due to ethical reasons and to their tumorigenic properties (Andrews, 2002). To overcome these limitations, iPSC-derived neurons provide a potential alternative to obtain from patient skin biopsy, mature neurons that could be used for transplantation avoiding graft withdrawal (Sharma, 2016). Even more, iPSCs provide an opportunity for patient-specific drug screening (*in vitro* disease modeling) (Avior *et al*., 2016) (**Figure 4**). This system has been used to model disorders such as schizophrenia and bipolar disorder (Mertens *et al*., 2015; Yu *et al*., 2014).

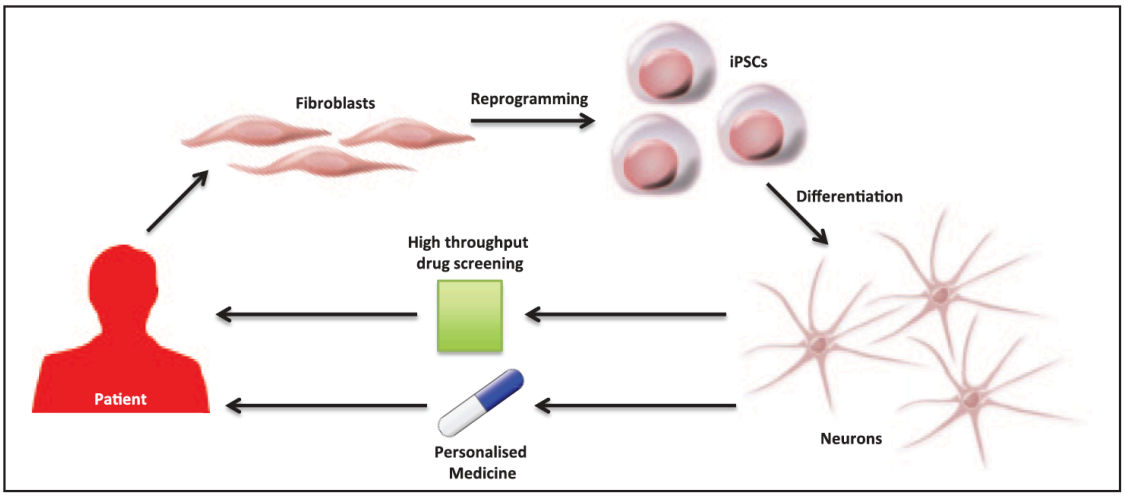


Figure 4. *In vitro* culture of induced pluripotent stem cells (iPSCs) and their use in drug development. Patient-derived somatic cells such as fibroblasts can be reprogrammed to form iPSCs that in turn can undergo robust differentiation to form mature neurons in culture. This process can be used to produce disease-specific neural subtypes such as neurons from patients with complex neurodegenerative disorders and *in vitro* models simulating aspects of the disease. These models can then be used for high-throughput screening (HTS) of test compounds that may combat conditions symptomatic of the disorder. iPSC technology also provides advantages in the field of personalised medicine as patient-specific *in vitro* models of neurological disease can be generated to test the efficacy of specific drug treatments prior to their administration (Goncalves and Przyborski, 2018).

Now, scientists can differentiate stem cells *in vitro* into mature neurons, this is achieved by the activation of signaling pathways involved in the development of the nervous system (Schwartz *et al*., 2008). Some of the most frequent

pathways used for neural differentiation include the retinoic acid pathway, the inhibition of the glycogen synthase kinase-3 and the induction of the fibroblast growth factor signaling (Goncalves and Przyborski, 2018). Efforts are being made to develop more efficient techniques of differentiation, like the design of molecules with improved robustness for neural induction (Clemens *et al*., 2013).
Nowadays, clinical trials based on stem cell transplantation are underway to treat neurodegenerative diseases such as PD and amyotrophic lateral sclerosis (Trounson and DeWitt, 2016). Nevertheless, more work in the field needs to be done to efficiently repair brain damage. It has been shown that replacing a unique cell type will not be enough for tissue restoration. The entire microenvironment and the phenotypic state of transplanted cells (i.e. ability to secrete growth factors) play an important role for a complete medical success for the treatment of neurodegenerative diseases (Goncalves and Przyborski, 2018).

1.3.7 Enhancing adult neurogenesis

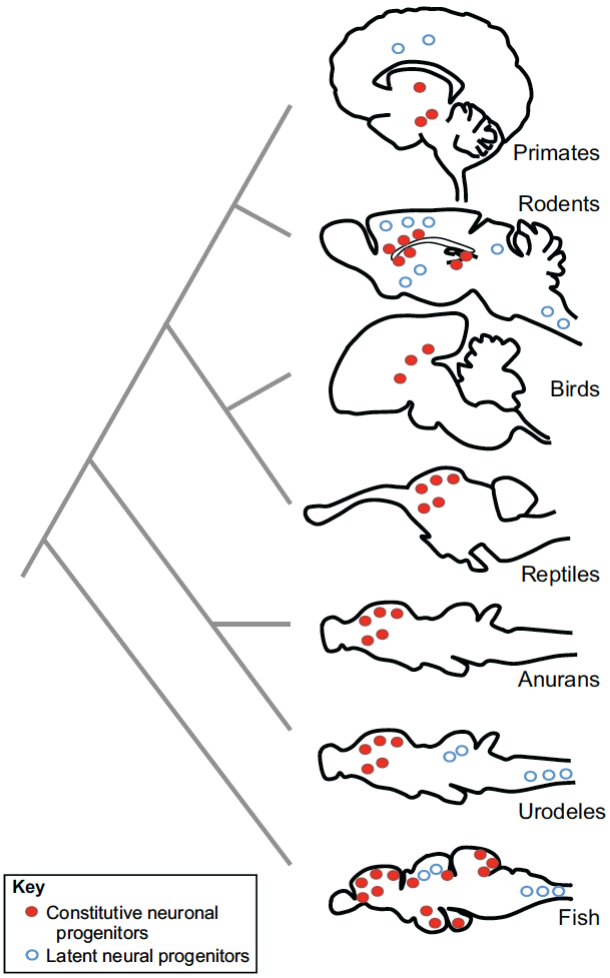
Different strategies to take advantage of endogenous NSCs have been considered. One possibility is their molecular stimulation *in situ* to promote their proliferation, migration and differentiation (Yu *et al*., 2013). In addition, NSCs could be cultured and then transplanted to the sites of lesions after being expanded and differentiated into the adequate cell fate through gene transduction (Gil-Perotin *et al*., 2013).
An innovative therapeutic approach for brain damage is through the activation of endogenous neurogenesis. Since it is observed that the innate response of mature neurogenesis is limited, new alternatives to augment this endogenous process via exogenous inputs is becoming an exciting area of research. An extensive list of factors has been proved to enhance neurogenesis (growth factors, including EGF and FGF2; transcription factors such as Gata3, Fezf2, Sox2; epigenetic regulators like Ezh2, Jmjd3, BAF). Particularly, growth factors have shown effectiveness in enhancing neurogenesis and improving functional recovery after brain trauma in adult animals (Sun, 2016). Alternatively, strategies such as hypothermia, environment enrichment or transcranial low-

laser treatment, among others, have been found efficient to improve neurogenesis after brain trauma.

Bringing new advances on the mechanisms that govern sustained stem cell proliferation and differentiation, should one day make it possible to stimulate efficiently and safely endogenous neurogenesis in humans for the treatment of brain diseases.

1.4 Regeneration in non-mammalian vertebrates

As mentioned before, regeneration of the adult brain in mammals is limited, however scientists have looked at other vertebrate species where post-natal regeneration is more robust. Advances towards the understanding of the mechanisms of human neurogenesis can benefit from comparative studies between different vertebrate models, such as rodents (mouse or rat), birds, reptiles, urodele amphibians (axolotl and salamander), anuran amphibians (*Xenopus*) or teleost fish (zebrafish or medaka). Each of these animals presents their own stemness properties. For example, while in birds and rodents the neurogenic niches are restricted into discrete zones, in amphibians they cover most of the forebrain ventricle and in fish the implication of other several brain subdivisions have been reported. Additionally, silent areas of neurogenic potential have been discovered across species (Alunni and Bally-Cuif, 2016) (**Figure 5**).



	Adult neurogenesis	Latent progenitors	Reparative neurogenesis
F	+	+	–
M	–	?	–
Sc	–	?	–
R	–	?	–
F	+	+	–
M	–	+	–
Sc	–	+	–
R	–	+	+/–
F	+	?	+
M	–	–	–
Sc	–	–	–
R	–	+	+
F	+	?	+
M	–	?	–
Sc	–	?	–
R	–	–	–
F	+	?	–
M	?	?	–
Sc	–	?	–
R	+	+	+
F	+	?	+
M	–	+	+
Sc	+	?	+
R	+	+	+
F	+	?	+
M	+	?	?
Sc	–	+	+
R	+	+	+

Figure 5. Phylogenetic tree of animal taxons used as models for neuronal regeneration. The location of adult neurogenic niches, which harbour constitutively active neuronal progenitors (red), and the presence of latent neural progenitors (blue) are indicated on schematic sagittal sections of the brain (left). Constitutive neurogenesis generates neurons in the adult brain under homeostatic conditions, whereas latent progenitors are activated in response to lesions to produce neurons and/or glial cells. The table summarizes the presence of (+), the demonstrated absence of (−), or the lack of experimental data on (?) constitutive neuronal progenitors, latent neural progenitors and reparative neurogenesis in the different central nervous system regions. F, forebrain; M, midbrain; Sc, spinal cord; R, retina (Alunni and Bally-Cuif, 2016).

The dorsal telencephalon, also known as the pallium, is the region of teleost fish brain that contains the homologous regions of the mammalian neurogenic niches. Different from mammals, teleost fish species have greater neural generation in the brain and retina thanks to active radial and astroglial cells in these regions throughout adult life (Alunni and Bally-Cuif, 2016).

Studies performed in the axolotl brain revealed the involvement of nerve-derived cues necessary for regeneration. Usually, regeneration in the brain of those animals is observed within 12-15 weeks after large ablation of the pallium,

but only if the olfactory nerve is intact (Maden *et al*., 2013). Despite of this discovery, the exact molecules involved remain to be elucidated.

In spite of the different regenerative capacities between non-mammalian and mammalian vertebrates, one common aspect of NSCs in all species is their quiescent state, a mechanism that helps to protect them from exhaustion. Researchers have focused their attention in finding the molecular mechanism that promotes exit from the quiescent state and that activate NSCs for neural repair. They identified Notch signaling pathway as a key for the maintenance of stemness of NSCs in both zebrafish and mice. While Notch3 helps to maintain radial glia quiescence (Alunni *et al*., 2013); Notch1 is necessary for the activation of the NSCs proliferation (Pierfelice *et al*., 2011).

1.4.1 Inflammation: different outcomes in the regeneration of vertebrates

In zebrafish, the first response after a mechanical insult in the adult pallium is the immune cell activation. The inflammatory response marks the initiation of a series of regeneration events. One of these is the activation of transcription factors such as Gata3. However, in mammals the inflammatory reaction following brain injury negatively regulates neurogenesis (Kizil *et al*., 2015; Kyritsis *et al*., 2014). After lesion, mammalian astrocytes become activated and express intermediate filament proteins to repair the wound by a glial scar, which ultimately impedes regeneration (Burda *et al*., 2016). A reason of such different outcome is that the zebrafish brain does not contain astrocytes. Thus, while in zebrafish the initial inflammatory response resolves relatively quickly with positive effects; in mammals, on the opposite, it is followed by a chronic phase with a negative impact on the regeneration process.

1.4.2 A model to study neural regeneration: the retina of non-mammalian vertebrates

The retina is an extension of the CNS. In vertebrates the retina is located at the back of the eye and is composed of layers of specialized neurons that are interconnected through synapses (**Figure 6**). Light that enters the eye is captured by photoreceptor cells in the outermost layer of the retina, which

initiates a cascade of neuronal signals that eventually reach the retinal ganglion cells (RGCs), the axons of which form the optic nerve (London *et al*., 2013).

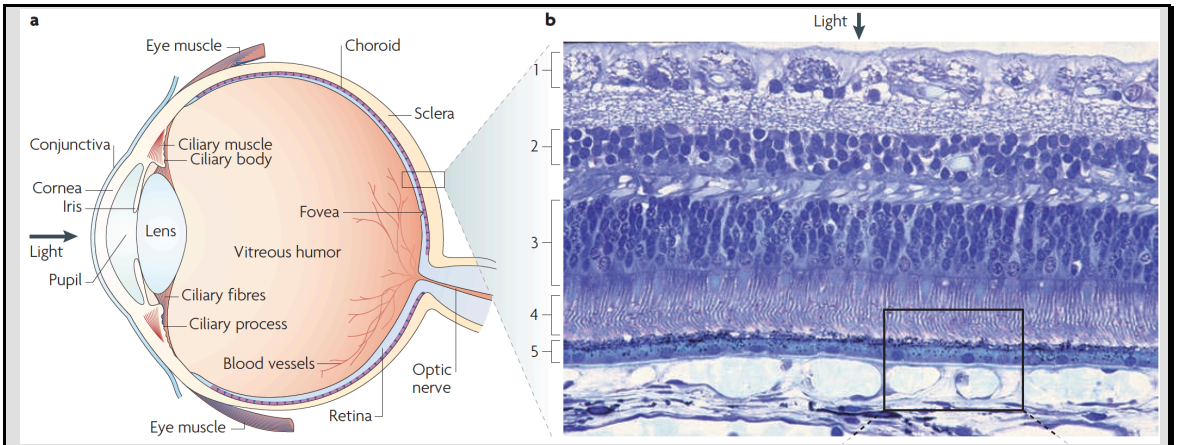


Figure 6. Structure of the human retina. A) Schematic representation of the human eye in which light passes through the pupil, lens and vitreous cavity before reaching the light-sensitive retina. B) Cross-section of the human retina, showing its laminated structure, which consists of: 1) the ganglion cell layer, 2) the inner nuclear layer which contain the bipolar, amacrine and horizontal cells, 3) the outer nuclear layer, which contains the cell bodies and nuclei of rod and cone photoreceptors, 4) the photoreceptor outer segments, and 5) the retinal pigment epithelium (RPE) (Wright *et al*., 2010).

The retina of non-mammalian species serves as a model to study the contribution of latent progenitors for regeneration. After injury in zebrafish retina, it is the Müller glia that acts as a source of new cells to repair damage following an event of reprogramming (Fausett and Goldman, 2006; Fimbel *et al*., 2007). Another example occurs with the retinal pigment epithelium (RPE) of newt that dedifferentiates following retinal ablation (Hasegawa, 1965; Stone, 1950).

1.4.3 The ciliary marginal zone, a powerful system to study neurogenesis

In addition to the afore-mentioned retinal cell types that can reprogram into stem-like cells upon injury, cells localized at the peripheral part of the retina in teleost fish and *Xenopus* are genuine stem cells that are active during the entire life of the animal (Ail and Perron, 2017). In contrast to mammals, the eyes of frogs and fishes grow throughout life, remaining in proportion to the size of the animal (Johns, 1977; Straznicky and Gaze, 1971). The region of the retina that harbors the neurogenic niche is known as the ciliary marginal zone (CMZ), also called *ora serrate* (**Figure 7**). Here, cells are disposed in a spatial gradient: the extreme edge is occupied by stem cells, followed by committed proliferating

progenitors and ending with post-mitotic progenitors (Centanin *et al*., 2011; Perron *et al*., 1998; Wetts *et al*., 1989).

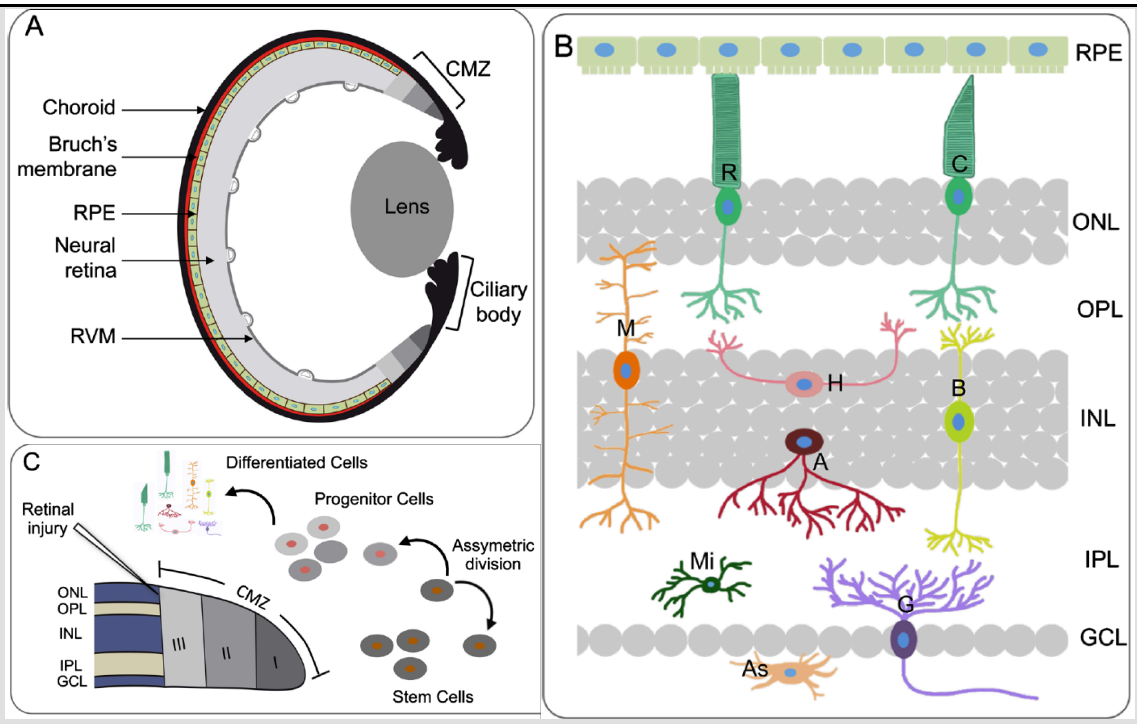


Figure 7. Structure of the non-mammalian vertebrate retina. A) Schematic representation of a cross section of the zebrafish or *Xenopus* eye showing the ciliary marginal zone (CMZ), retinal pigment epithelium (RPE), neural retina, choroid, Bruch's membrane, and the retinal vascular membrane (RVM). B) Cell types of the retina. The retina is composed of different cell types: the nuclei of the two types of photoreceptors, rods (R) and cones (C), form the outer nuclear layer (ONL), whereas the Müller cells (M), horizontal cells (H), bipolar cells (B), and amacrine cells (A) are present in the inner nuclear layer (INL), and the ganglion cells (G) in the ganglion cell layer (GCL). The axons of these neurons and glial cells form synaptic connections in the outer and inner plexiform layers (OPL and IPL). The astrocytes (As) are located near the blood vessels whereas the microglia (Mi) are mostly located in the plexiform layers but can be distributed through the different layers. C) Mode of regeneration and repair CMZ-mediated. In the constantly growing retinas of zebrafish and *Xenopus*, the spatial cellular gradient in the CMZ recapitulates embryonic retinogenesis with zone I, the most peripheral part of the CMZ, where stem cells reside, zone II encompassing retinal progenitor cells, and zone III consisting of late retinal progenitors including post-mitotic retinoblasts. The stem cells divide asymmetrically to self-renew and generate one progenitor cell, and this mode of asymmetric division is retained even in the case of retinal injury. RPE-mediated. Adapted from (Ail and Perron, 2017).

The deepest (also most peripheral) part of the CMZ, where the RPE folds over into the retina is the region that hosts the stem cells which give rise to other stem cells as well as neural retinal cells and RPE. The next most central cells in the CMZ, the progeny of the stem cells, are still mitotic; they do not give rise to further stem cells, but do generate clusters of cells containing neural cell types and Müller glial cells (Wetts *et al*., 1989). The cells of the CMZ present

neuroepithelial characteristics that differ from the radial glial cells found in mammalian brains; they resemble more to cells of the optic tectum margin in the adult zebrafish and medaka brains (Alunni *et al*., 2010; Ito *et al*., 2010) and the lateral edge of the zebrafish pallium (Dirian *et al*., 2014). In the later, it was discovered that these non-glial cells utilize other signaling pathways for their maintenance in particular, they do not depend on Notch for their maintenance and rather express the non-canonical *E (spl)* genes *her6* and *her9* (Dirian *et al*., 2014).

1.4.4 Characteristics of the CMZ for regeneration and repair

The presence of the CMZ seemed to have gradually disappeared during vertebrate evolution; its localization is shared in both teleost fish and amphibians and also exists in the post-hatched chick, but not in the adult chicken nor in mammals (Fischer and Reh, 2000; Kubota *et al*., 2002). Interestingly though, scientists reported the formation of CMZ-like cells *in vitro* in human ESC (hESCs)-derived optic cups (Kuwahara *et al*., 2015).

The genuine stemness properties of CMZ stem cells were demonstrated *in vivo* in medaka fish using transplantation experiments and lineage analysis of single cells over a long period of time (Centanin *et al*., 2011; Perron *et al*., 1998; Wetts *et al*., 1989). In *Xenopus*, CMZ cells are able to differentiate into all the different retinal cell types (Wetts *et al*., 1989). However in zebrafish, they generate all cells but rods as it is the Müller cells that give rise to rod photoreceptors (Lenkowski and Raymond, 2014; Raymond *et al*., 2006; Stenkamp, 2011).

Under normal conditions, neurogenesis from the CMZ contributes to continuous eye growth (**Figure 8**). In addition, it was showed by lineage analysis that stem cells from the CMZ utilized asymmetric cell division, after dividing one daughter cell remains quiescent in the niche while the other is pushed centrally to become a retinal progenitor and differentiate (Wan *et al*., 2016).

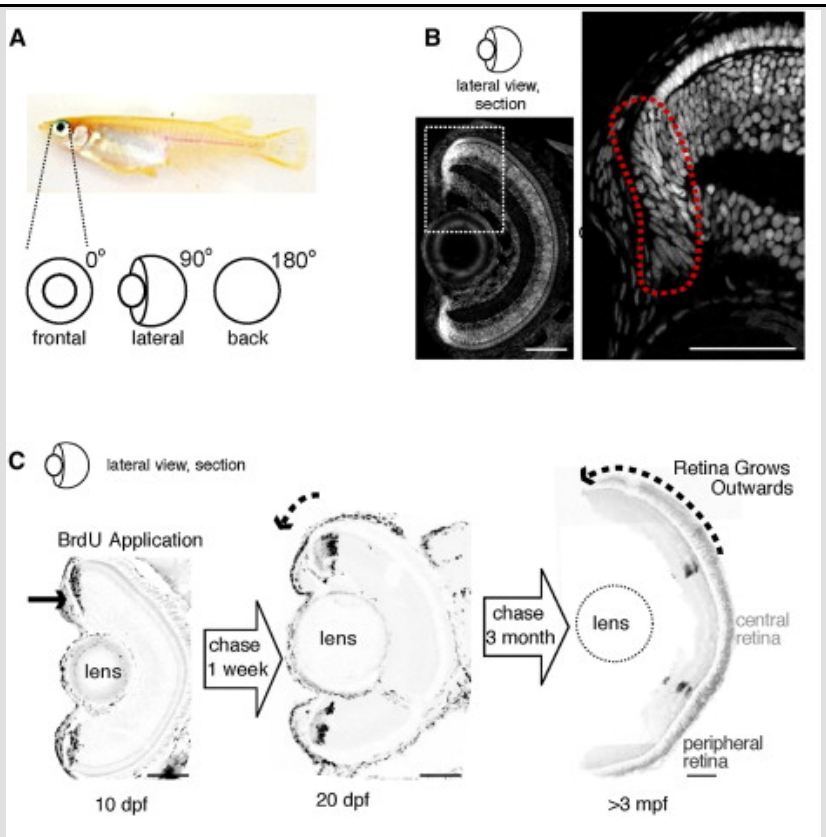


Figure 8. Spatio-temporal organization of the mature medaka retina. (A) Medaka retina is a highly 3D organ. (B) A transverse section shows the central layered retina and the ciliary marginal zone (CMZ) at the periphery. (C) Cells in the CMZ incorporate BrdU (left). When BrdU pulse is followed by a chase of 10 days, $BrdU^+$ cells are found in more central positions (center) and even more central after a chase of 3 months (right). Scale bars represent 50 µm. Adapted from (Centanin *et al*., 2011).

CMZ cells from zebrafish and *Xenopus* have been well characterized, and are an excellent model to study retinal development, since the different regions of the CMZ express particular combinations of transcription and post-transcription factors, signaling molecules and cell cycle genes (Agathocleous and Harris, 2009; Amato *et al*., 2005; Borday *et al*., 2012; Casarosa *et al*., 2005; Cerveny *et al*., 2012; El Yakoubi *et al*., 2012; Harris and Perron, 1998; Ohnuma *et al*., 2002; Perron *et al*., 1998; Raymond *et al*., 2006; Wehman *et al*., 2005).

Upon injury, the CMZ of fish contributes to some extent to retinal regeneration. It has been shown that the CMZ of goldfish preferably helps to regenerate the peripheral retina but not the central (Stenkamp *et al*., 2001), for the latter the regeneration is dependent on Müller glia cells. Moreover, between different *Xenopus* species the contribution of the CMZ to regeneration seems variable: while in the adult *X. tropicalis* the CMZ is capable to regenerate the retina after complete retinectomy (Miyake and Araki, 2014), *X. laevis* rather relies on its

RPE (Miyake and Araki, 2014). In *Rana pipiens*, the potency of the CMZ is restricted to only some cell types (Reh, 1987).
Overall, *Xenopus* CMZ cells offer a powerful model system to dissect *in vivo* signaling pathways underlying stemness features and regeneration to provide valuable information to stimulate the proliferation and neurogenic potential of dormant retinal stem cells in mammals. In this context, my laboratory main goal is to study and compare in both *Xenopus* and mouse, two models with very different regenerative capacities, the mechanisms underlying the maintenance, recruitment and activity of adult retinal stem cells under homeostasis and after injury.

1.5 The cell cycle of stem cells, an emerging stem cell-property

A notorious characteristic of stem cells is their unusual cell cycle structure that differs from somatic cells. As I will describe in the next paragraphs, this inherited characteristic is shared by multiple types of stem cells *in vitro* such as ESCs from mouse and human sources, iPSCs and NSCs, in addition to PSCs from early embryos of flies, fish and frogs.
Recent advances in research have unveiled some of the molecules that regulate the cell cycle structure of stem cells and make them unique from their counterpart-differentiated cells. However, an open question in the field is whether this peculiar stem cell cycle structure is just a mean to convey rapid maturation and development of ESCs during the first period of life or whether it sustains an active role in determining the general stemness state? If so, the manipulation of the cell cycle may represent an additional tool by which *in vitro* maintenance or differentiation of stem cells may be controlled in regenerative medicine.

1.5.1 The traditional cell-cycle regulation

The eukaryotic cell cycle refers to the sequential series of events that permit the reproduction of the cell. The basic cell cycle includes four phases: 1) G1-phase, a gap phase to allow cell growth; 2) the synthesis or S phase, in which the cell generates a copy of its genetic material; 3) G2-phase that serves to check the

integrity of genomic material, happening after S-phase; and 4) the M-phase that correspond to the cell division and distribution of all cellular components to the daughter cells(**Figure 9**). Additionally, when cells stop their proliferation depending on the microenvironment (for example, caused by antimitogenic signals), cells at G1 phase exit the cell cycle and enter into a quiescent, non-dividing state known as G0 (Malumbres and Barbacid, 2001).

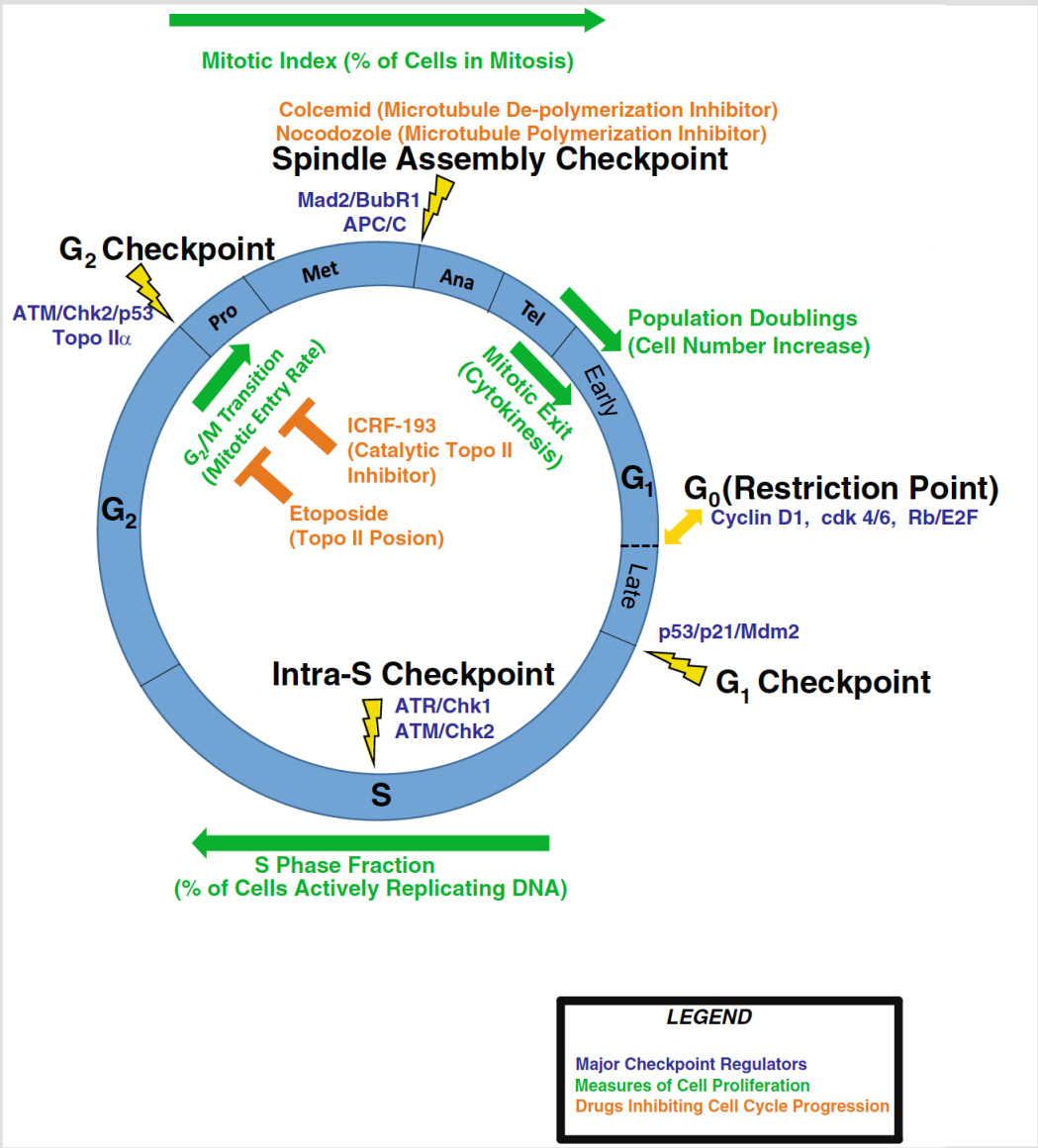


Figure 9. Diagram of cell cycle regulation. Phases of the cell cycle are shown inside the blue circle in the center of the figure (G_0, G_1, S, G_2, and mitosis which consists of several sub-phases: prophase (Pro), metaphase (Met), anaphase (Ana), and telophase (Tel)). The G_0 Restriction Point is designated with a *yellow dual headed arrow* to illustrate the reversible nature of cell cycle entry and quiescence. As cells progress through the cycle, exogenous perturbations can activate checkpoints that arrest cells during phase transitions (checkpoints are designated by *yellow lightning bolts*). Several measures of cellular proliferation are shown in green and span the cell cycle phases in which these markers are present. Drugs that inhibit cell cycle progression are shown in orange with their targets and mechanisms of action designated in subsequent

parentheses. Components of major regulatory pathways triggering each checkpoint are listed in *dark blue font* near the checkpoint in which they play a role. Precise control over the regulation of the cell cycle is a requirement for ensuring accurate DNA replication and cell division. Modified from (Bower *et al*., 2017).

Cell division and cell cycle progression are controlled by mechanisms that ensure the faithful transmission of genetic information from generation to generation. For cell cycle events to be maintained in the correct order, restrictive controls operate, which ensures that key events such as chromosome segregation does not proceed until DNA replication is achieved an vice versa (White and Dalton, 2005). These control mechanisms are named checkpoints, because they operate to check that prerequisites are properly satisfied before continuing to the next phase. Even more, they maintain genomic stability. For example, if errors in the DNA happened to occur then mechanism of senescence to arrest cell proliferation or apoptosis if damage is too severe, help to prevent transmission of mutations to daughter cells (Bower *et al*., 2017; Houtgraaf *et al*., 2006).

Throughout the entire length of the cell cycle the checkpoint response is active either between phases, for example G1/S and G2/M checkpoints or inside a phase, such as the intra-S- and the mitosis-associated spindle assembly checkpoints (SAC). Although distinct, the G1/S-, G2/M and intra-S- checkpoints respond to DNA damage and shared some proteins, however the intra-S phase is special in a way that it also recognizes problems in replication such as stalled forks progression (Houtgraaf *et al*., 2006). The SAC checkpoint functions in M-phase to ensure that all chromosomes exhibit bipolar attachment to the mitotic spindle (Taylor and McKeon, 1997).

The cell cycle is regulated at the molecular level by two classes of molecules, the phase-specific activity of cyclin-dependent kinases (CDKs) and their binding partners cyclins (Murray, 1993; Sherr and Roberts, 1999). In the classical model of cell-cycle regulation, the action of cyclin D (D1, D2, D3) and CDK4/6 drives progression through early G1 until a point known as the restriction point (R). Progression beyond R represents a point of no return that commits the cell to a new round of cell division (DNA synthesis, chromosome segregation and cytokinesis).

In late G1, when a cell commits to S phase progression based on the availability of growth factors and nutrients, cyclin D-CDK4/6 activity began to decrease not after initiating the phosphorylation of the tumor-suppressor retinoblastoma protein (Rb1). This inactivation by phosphorylation leads to the activation or derepression of E2F transcription factors, which then transactivate genes needed for the entry and progression into S phase, such as the DBF4-associated kinase, A-type and E-type cyclins. Then cyclin E (E1 and E2) together with CDK2 (but also CDK1 and CDK3) activity rises. Cyclin E-CDK2 further phosphorylates Rb1, whereas E2F stimulates its own transcription, which together create a positive-feedback-loop that promotes S phase entry (Budirahardja and Gonczy, 2009). During early S-phase, cyclin E is degraded and cyclin A2 complexes with CDK2 and CDK1 to drive progression through S-phase and into G2. From mid-G2 onwards, the activity of CDK2 decreases and cyclin A associates with CDK1 (formerly known as Cdc2 (cell division cycle 2)). Finally, at the entry in M-phase, cyclin B complexes with CDK1 to phosphorylate a number of targets involved in nuclear envelope breakdown, chromosome condensation, and segregation and cytokinesis. Degradation of cyclin B following cytokinesis signifies the start of the next G1 phase (Hindley and Philpott, 2013) (**Figure 10** & **14 top somatic cell cycle**).

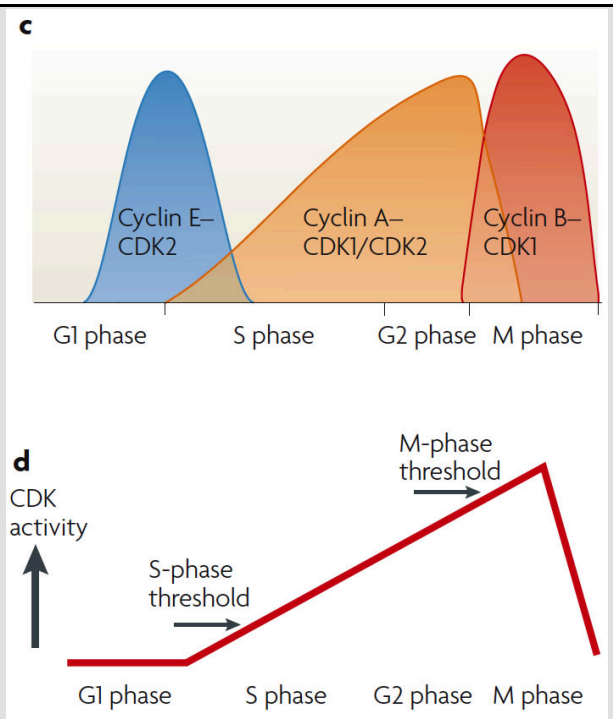


Figure 10. Cell-cycle control. C) According to the classical model of cell-cycle control, D-type cyclins and CDK4 or CDK6 regulate events in early G1 phase (not shown),

cyclin E-CDK2 triggers S phase, cyclin A-CDK2 and cyclin A-CDK1 regulate the completion of S-phase, and CDK1-cyclin B is responsible for mitosis. D) Based on the results of the cyclin and CDK-knockout studies, a minimal threshold model of cell-cycle control has emerged. Accordingly, either CDK1 or CDK2 bound to cyclin A is sufficient to control interphase, whereas cyclin B-CDK1 is essential to take cells into mitosis. The differences between interphase and mitotic CDKs are no necessarily due to substrate specificity, but are more likely to be a result of different localization and a higher activity threshold for mitosis than interphase (Hochegger *et al*., 2008).

In addition to the cyclin-CDK complexes that regulate positively the cell cycle, mammalian cells also have two classes of inhibitors; the INK proteins ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) that interact with CDK4/6 and block the association with cyclin D; and the KIP/CIP proteins ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) which form ternary complexes with cyclin-CDK2/CDK1 to inhibit their kinase activity (Morgan, 2007; Satyanarayana and Kaldis, 2009). Three inhibitors, homologues of the mammalian CIP/KIP family $p27^{Xic1}$, $p16^{Xic2}$ and $p17^{Xic3}$ were found in amphibian (Daniels *et al*., 2004).

1.5.2 Cell cycle of pluripotent cells: from early embryos to iPSCs

It has been shown that PSCs cultured *in vitro*, such as ESCs and iPSCs; as well as pluripotent cells from early embryos of different species including fly, frog, fish, rodent, and primate (humans) present a characteristic cell cycle structure different from somatic cells. They differ because their gap phases are not fully formed, or in some cases are completely absent, which as a consequence allows rapid cell division. Later in development, notably as differentiation commences, the cell cycle begins to gradually slow down and the cell cycle becomes longer due to the appearance of gap phases (Liu *et al*., 2019). An exciting avenue in the stem cell field is to understand how the cell cycle remodeling is associated with the decision of a stem cell to choose between remaining pluripotent or differentiate.

In early embryos of fruit flies, *Xenopus* and zebrafish, cell cycles are remarkably rapid consisting of alternate rounds of M- and S-phases (Boward *et al*., 2016). I will take the *X. laevis* embryo as an example. After fertilization occurs, the first cell cycle with duration of 90 min, involves the fusion of male and female nuclei and the termination of meiosis. Then, the following eleven cell divisions are fast, last between 20 to 30 min each, without gap phases, until the embryo reaches a

ball formed of 4,000 pluripotent cells, known as the mid-blastula embryo. It is at the end of the twelfth cycle that the two gap phases finally appear. Here, cell cycle duration takes 50 min and zygotic transcription initiates. Overall, this event is called the mid-blastula transition (MBT) (**Figure 11**). Post-MBT, the cell cycle is longer lasting 90 min. The fifteenth cycle marks the beginning of gastrulation and a period of mitotic quiescence (Heasman, 2006).

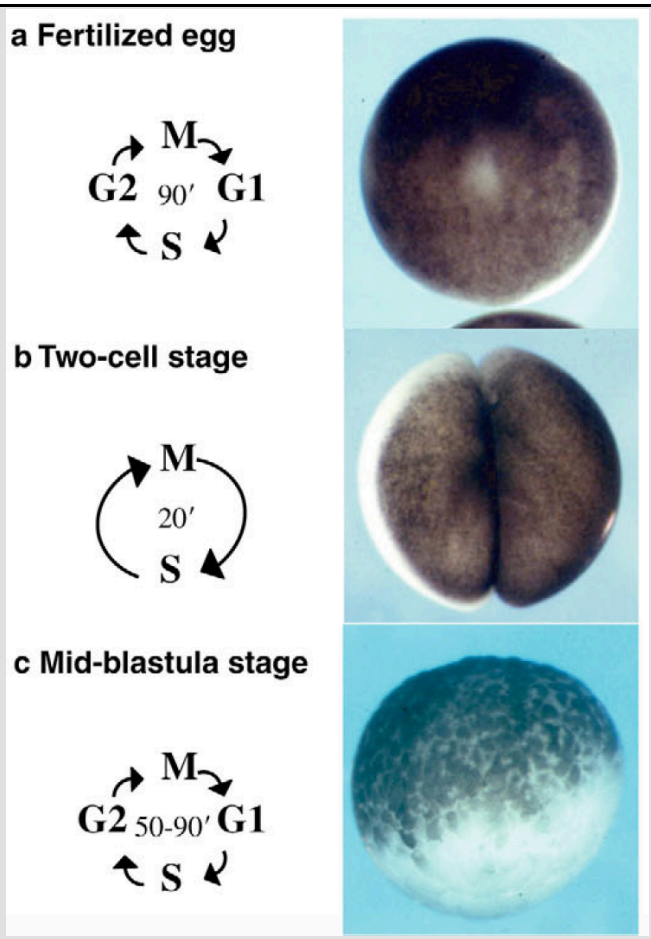


Figure 11. Characteristics of *Xenopus laevis* early development. The different cell cycles and the external appearance of (a) the fertilized egg, and (b) two-cell and (c) mid-blastula stages. a and b are views from the animal pole and c from the side. (a) Cycle 1 is approximately 90 minutes in length and has G1 and G2 phases. The next 11 divisions have no gap phases and occur every 20-30 minutes. (c) At the mid-blastula stage, the embryo consists of 4,000 cells, gap phases reappear, the cycle lengthens to 50 minutes and zygotic gene expression commences (Heasman, 2006).

The early embryonic cycles lacking gap phases are regulated by maternal components, which are used gradually until the onset of transcription from the zygote. It is thought that in response to the increased nuclear:cytoplasm ratio, the maternal components gradually decrease until a titration that results in the lengthening of interphase. In *Drosophila*, it is the reduction in Cyclin B and A

levels and the strong activation of checkpoint components ATR (Mei-41) and Chk1 (Grp), that together significantly increases interphase duration (Crest *et al*., 2007; Sibon *et al*., 1999).

The onset of zygotic transcription parallels the introduction a G2 phase. In zebrafish and *Xenopus* embryos it is Cdc25 that regulates the G2/M transition at the MBT (Dalle Nogare *et al*., 2009; Shimuta *et al*., 2002). In addition, Cyclin E is thought to control as well the transition to MBT in *Xenopus*, since maternal Cyclin E exhaustion correlates with the MBT (Howe and Newport, 1996) (**Figure 12**).

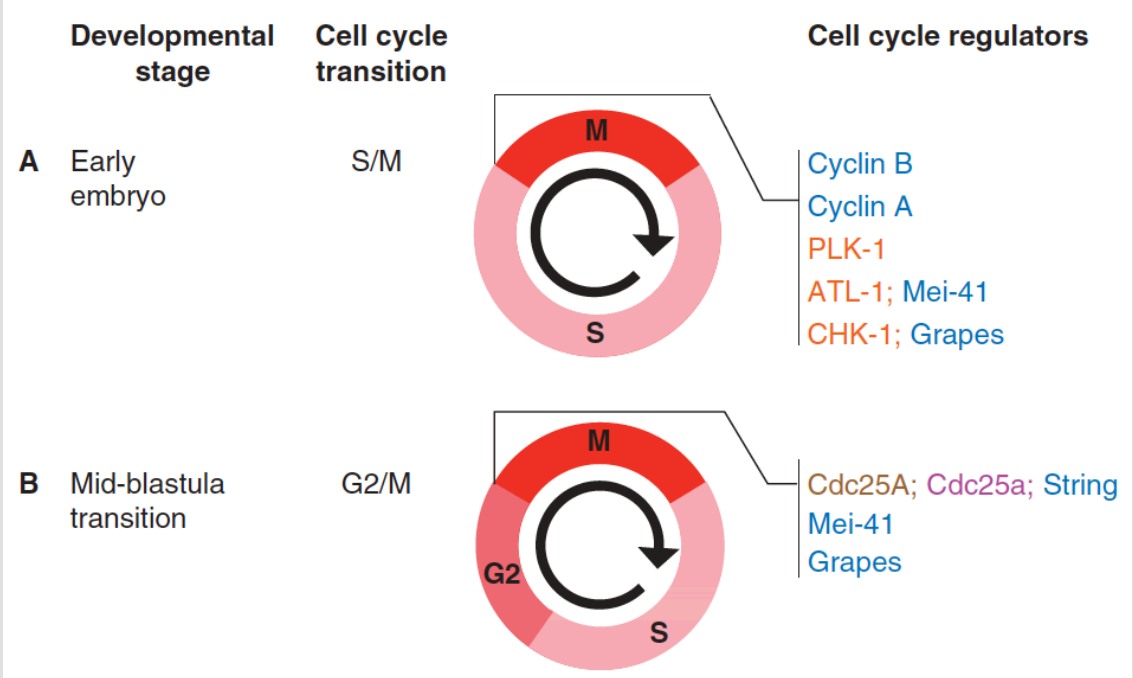


Figure 12. Modulation of the cell cycle during early development. Homologous cell cycle regulators are listed on the same line. Proteins from *Drosophila* are shown in blue, *Caenorhabditis elegans* in orange, *Xenopus* in brown, zebrafish in purple and mammals in green. (A) In the early embryo of many species, the cell cycle is driven by maternal components and alternates between S and M phases. In *Drosophila*, this embryonic cycle is regulated notably by Cyclin B and Cyclin A, as well as by Mei-41 (ATR) and Grapes (Chk1); in *C. elegans*, important regulators of the embryonic cycle include the Polo-like kinase PLK-1, as well as ATL-1 (ATR) and CHK-1 (Chk1). (B) At the mid-blastula transition (MBT), a broad switch from maternal to zygotic transcription is initiated and a G2 phase is introduced into the cell cycle in many species. The phosphatase Cdc25 is important for regulating MBT in *Drosophila*, *Xenopus* and zebrafish (Budirahardja and Gonczy, 2009).

Transition from maternal to zygotic transcription is present in metazoan animals including echinoderms, nematodes, insects, fish, amphibians and mammals (**Figure 13**), and even in plants. In all of them the transition occurs first after the

elimination of maternal mRNAs and secondly by the initiation of transcription by the zygotic genome. The major differences across species are the timing and scale of this process and the morphology of their embryos (Tadros and Lipshitz, 2009). Despite the fact that during mouse development zygotic transcription occurs early from the second cell division; a short G1 phase (1-2 h) has been reported at this stage that is accompanied by a long G2 phase (12-16 h) (Boward *et al*., 2016). Additionally, studies demonstrated that murine embryonic cells *in vivo* display short division times (~4.4-7.5 h) (Lawson *et al*., 1991; Liu *et al*., 2019; Snow and Bennett, 1978). Similarly to *Xenopus*, the murine cell cycle length increases because of an extension of gap phases occurring simultaneously with gastrulation (Dalton, 2015).

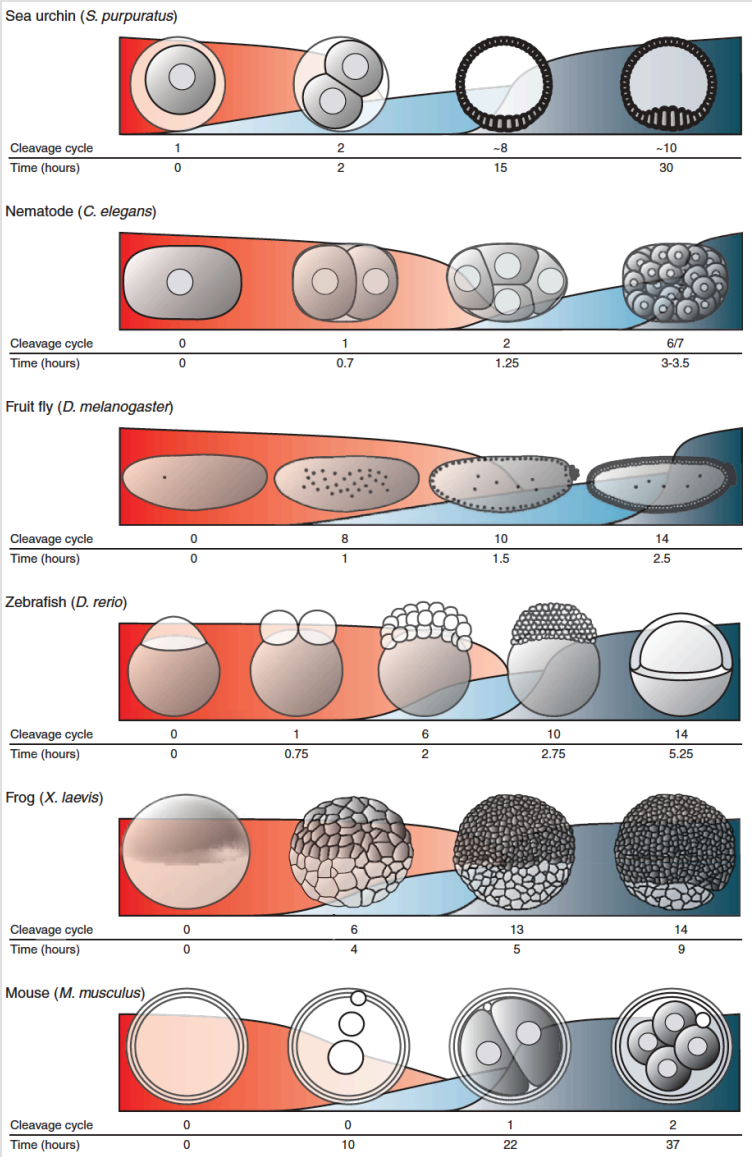


Figure 13. A comparative overview of the maternal-to-zygotic transition (MZT) in several model organisms. Key embryonic stages for each model organism are

depicted schematically above the corresponding cleavage cycle and time after fertilization. The red curves represent the degradation profiles of destabilized maternal transcripts in each species. The light and dark blue curves illustrate the minor and major waves, respectively, of zygotic genome activation. The last embryonic stage presented for each organism is the developmental point at which there is a major requirement for zygotic transcripts (Tadros and Lipshitz, 2009).

One explanation in the differences of development times between animal species is that in flies, fish and frogs, maternal pools of RNAs and proteins drive the rapid cell divisions before MBT. In the case of *X. laevis,* the list of maternal factors incudes: genome-wide transcriptional repressors (Xkaiso, Xtcf3), transcriptional activators (forkhead proteins), the T box protein VegT and cAMP response element-binding protein (CREB) (Heasman, 2006).

Interestingly, during the period of pre-MBT, cell signaling and transcriptional events are considered low. It was discovered that genes-repression until thirteenth cycle is associated with condensed, hypoacetylated and H3 methylated chromatin (Meehan *et al*., 2005). Moreover, before MBT transcriptional co-activators are inactive. It was suggested that transcription factors may be able to bind DNA but not efficiently enough to form active complexes, because of the repressive-state in architecture of the chromatin domains (Heasman, 2006).

Regardless the different animal models studied so far, a common thing in all of them is that gastrulation is accompanied by cell cycle structure remodeling with a marked diminution of proliferation rates. In other words, the rapid cell cycles observed in early embryos are correlated with pluripotency, while the beginning of commitment and cell specialization bring dramatic changes in the cell cycle length.

Strikingly, mESCs studies have revealed similarities in cell dynamics with that of fast-dividing embryos. Pluripotent cells in the rodent epiblast have a cell cycle structure lacking fully formed G1 and G2 phases, in which a high proportion of time (~60%) is devoted to S phase (Mac Auley *et al*., 1993; Stead *et al*., 2002). Additionally, it was observed that mESCs divide considerably rapidly (~12h) with short G1 phase (3h) (**Figure 14** & **Table 1**). At the molecular level, these cells express higher levels of Cdk1, Cdk2 and cyclins (E, A and B) compared to somatic cells. Contrary to somatic cells, the levels of Cdk2, cyclin-E- and cyclin-A-associated kinases are constitutively active throughout the entire cell cycle.

Only the cyclin B-Cdk1 remains oscillating. This peculiar expression of cyclins and Cdks maintain phosphorylated RB1, thus inactivated, and E2F activity is constitutively derepressed. There are different mechanisms that seem to participate in the upregulation of Cdk1 and Cdk2 in ESCs. One of these is the absence of KIP/CIP inhibitors (Liu *et al*., 2019).

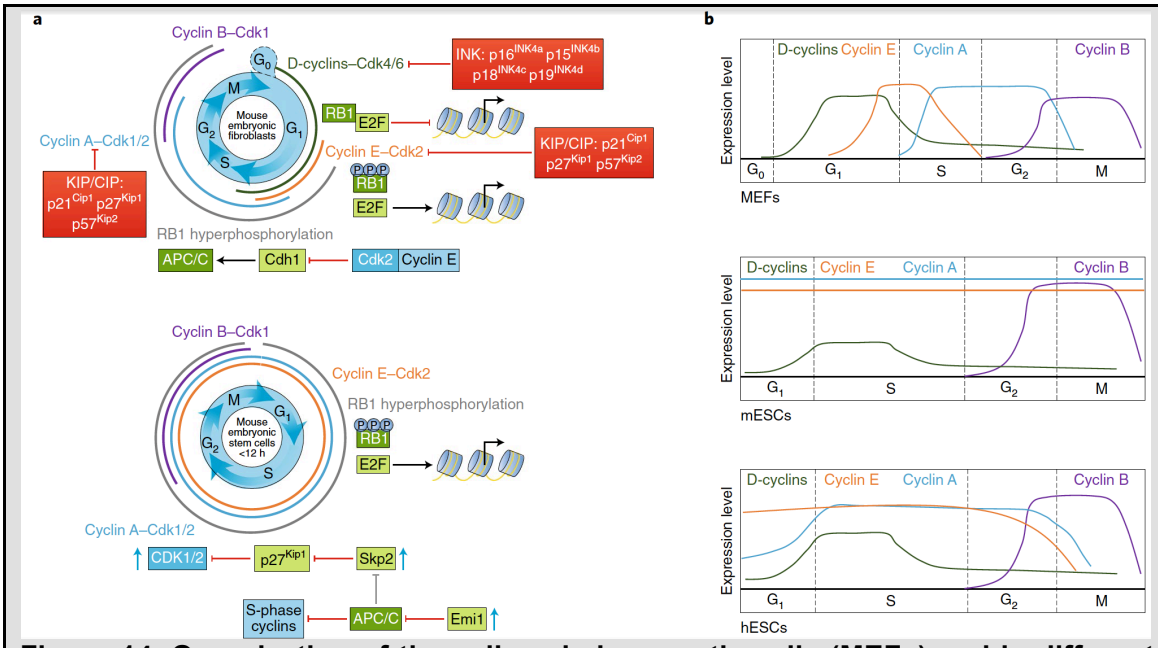


Figure 14. Organization of the cell cycle in somatic cells (MEFs) and in different types of ESCs. a) Differences in activity and expression of cell cycle components in MEFs and in murine ESCc (mESCs). In contrast to MEFs, mESCs lack expression of D cyclins and continuously express cyclin A and cyclin E. This allows them to maintain RB1 hyperphosphorylation throughout the cell cycle and results in a very short G1 phase. Cyclin-dependent kinase inhibitors are absent from mESCs. Upward blue arrows indicate increased expression. Gray cylinders represent histones. b) Oscillations of cyclin levels in MEFs, mESCs and hESCs. MEFs, mouse embryonic fibroblasts; mESCs, mouse embryonic stem cells; hESCs, human embryonic stem cells (Liu *et al*., 2019).

Table 1 | Comparison of cell cycle features in MEFs, mESCs and hESCs

	MEFs	mESCs	hESCs
Cell cycle length	24 h	4.4–7.5 h in vivo; up to 12 h in vitro	16 h
G1 phase length	11 h	3 h	3 h
CDK1 and CDK2 activity	Periodical	Very high and constant	Very high and periodical
D-cyclin expression	+++	+/−	+
RB1 phosphorylation status	Hypo- and hyperphosphorylated	Hyperphosphorylated	Hypo- and hyperphosphorylated
KIP/CIP inhibitor expression	Present	Absent	Present

MEFs, mouse embryonic fibroblasts; mESCs, mouse embryonic stem cells; hESCs, human embryonic stem cells.

Adapted from (Liu *et al*., 2019).

Furthermore, it was observed *in vitro* the existence of different subtypes of PSCs that represent the different types of cells observed during the peri-implantation development. Regarding this, mESCs were classified as naïve and

are maintained *in vitro* by the presence of leukemia inhibitory factor (LIF) and fetal calf serum (FCS) (Nichols and Smith, 2009). In addition, a second subtype of PSC was discovered, it is obtained by the treatment of naïve mESC with two small molecule inhibitors (2i) to block MEK/ERK and GSK3 signaling. These cells are called ground–state PSCs and are believed to proceed from an earlier developmental stage than naïve cells (Ying *et al*., 2008). A third type of PSC that resembles the primitive ectoderm in the embryonic epiblast was isolated from mouse. They are known as primed PSCs. Interestingly, it is possible to transform a naïve to a primed state in mESC through 2i withdrawal and FGF supplementation, which demonstrates that pluripotency has developmental plasticity (Boward *et al*., 2016).

The cell cycle structure between naïve to primed cells is slightly different, since the mechanisms of cell cycle control are not the same (Liu *et al*., 2019). Regarding hESC, these cells showed an organization of cell cycle resembling of a primed state (**Figure 15**). Shared aspects between naïve and primed cells from mouse and human are that they proliferate rapidly, they have a short G1 phase and they present high levels of CDK1 and CDK2 kinases. In addition, hESCs are similar to mESCs in that S-phase is highly populated (~50% of cells) and cyclin E expression does not display periodicity, but is constitutive (Filipczyk *et al*., 2007). However, only hESCs express KIP/CIP inhibitors, appreciable levels of cyclin D, present cell-cycle oscillations of CDK2 and contain hyper- and hypophosphorylated RB1 (Liu *et al*., 2019).

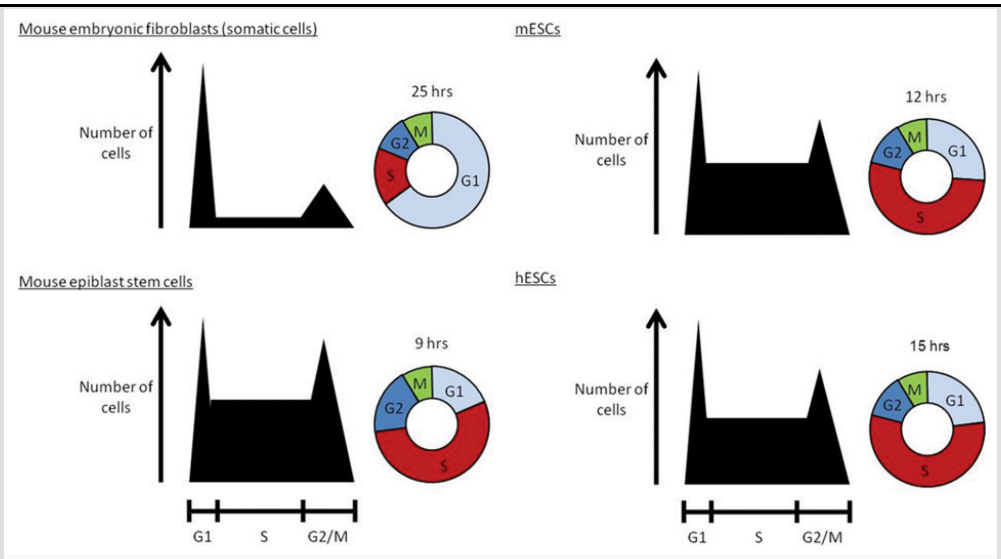


Figure 15. Schematic diagram comparing the cell cycle in somatic and pluripotent cells. For each panel, the first part is a graphical representation of the

number of cells in each phase of the cell cycle within a population, as assessed by propidium iodide staining and flow cytometric analysis. Peaks represent 2N and 4N DNA content. The second part of each panel is a summary for individual cell of the relative amounts of time spent in each cell cycle phase. In addition, the average total time taken to complete one cycle is represented for each cell type. It is clear that, proportionally, pluripotent cells have a shortened G1- and a longer S-phase for each cycle than somatic cells, although absolute S-phase length is comparable (Hindley and Philpott, 2013).

One of the first observations during the process of reprogramming was that the cell cycle suffers a strong acceleration (Cacchiarelli *et al*., 2015; Guo *et al*., 2014; Mikkelsen *et al*., 2008; Ruiz *et al*., 2011). Regarding this, a study reported that the ectopic expression of cyclins and CDKs increased the reprogramming efficiency, and conversely, their downregulation had the opposite effect (Utikal *et al*., 2009).

Overall, the organization of the cell cycle in iPSCs is quite similar to that of ESCs, consisting of a rapid division time and a short G1 phase (~2.5 h) (Ghule *et al*., 2011; Ruiz *et al*., 2011). Although reprograming clearly involves a cell cycle organization that resembles to a ESC state, the molecular mechanisms that drive these events in iPSC are largely unexplored (Liu *et al*., 2019).

In the Table 2 there is a summary of stem cell features shared by different PSC.

Table 2. Properties of stem cells regarding cell cycle.

Characteristic	Stem cell
Short or absent gap phases	Early embryos of fly, fish, frog, mESC, hESC and iPSC
Rapid cell division	Early embryos of fly, fish, frog, mESC, hESC and iPSC
Regulation of cell cycle by maternal components	Early embryos of fly, fish, frog
Absence of transcription	Early embryos of fly, fish, frog
Repressive state of chromatin domains	Early embryos of fly, fish, frog

1.5.3 Cell cycle machinery in NSCs

Remarkably, the cell cycle structure of embryonic neural stem/progenitor cells and adult NSCs is not the same. The first behave more like ESCs having rapid

cell divisions (~8h) (Salomoni and Calegari, 2010). On the other hand, adult NSCs present longer cell divisions (up to ~18h), which are related with the expression of $p57^{Kip2}$ (Furutachi *et al*., 2015). This increase in cell cycle length is due in majority to a four-fold increase of the G1 phase duration (Liu *et al*., 2019).

Moreover, it was observed that overexpression of cyclin D1, cyclin E1, or CDK4 to avoid G1 phase lengthening, has as a consequence the increase of self-renewal and the inhibition of neurogenic differentiation (Artegiani *et al*., 2011; Lange *et al*., 2009; Pilaz *et al*., 2009). In agreement, depletion of cyclin D1 and CDK4, treatment with inhibitors of CDK4 or Cdk3/Cdk4 double-knockout mice, stimulate neuronal differentiation (Calegari and Huttner, 2003; Lange *et al*., 2009; Lim and Kaldis, 2012; Roccio *et al*., 2013). Overall, these results showed that the activity of cyclin-CDK kinases block neurogenesis and stimulate self-renewal of NSCs. In addition, other cell cycle molecules have been associated with the regulation of neurogenesis such as $p27^{Kip1}$, $p57^{Kip2}$ and RB1 (Liu *et al*., 2019).

Mechanistic models have been proposed to explain whether NSCs decide to self-renew or to differentiate. Ali *et al* (Ali *et al*., 2011) postulated one of these models. They observed that in neural stem/progenitor cells, cyclin A- and B-dependent kinases phosphorylate the master regulator of neural differentiation, known as neurogenin 2 (Ngn2), resulting in its inhibition. Furthermore, they observed that when the length of G1 augments, CDK activity is diluted, allowing Ngn2 to active the transcription of neurogenic genes (**Figure 16**).

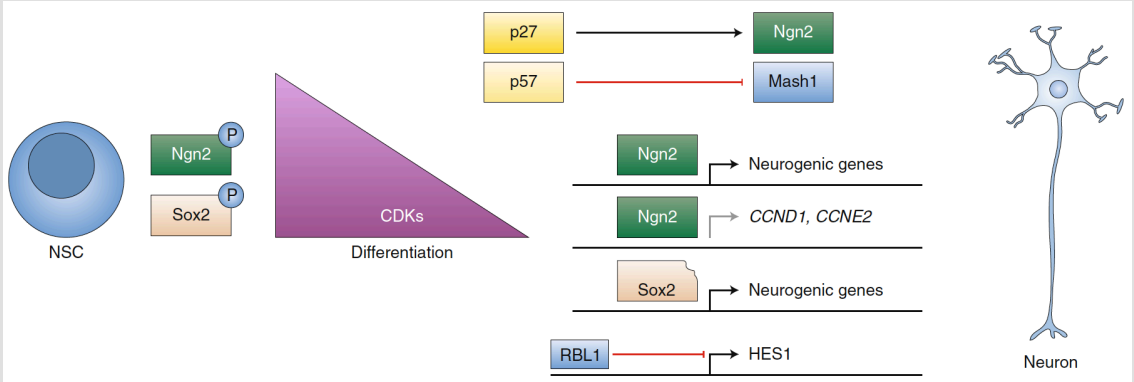


Figure 16. The cell cycle in neurogenesis. A decrease of CDK activity during neurogenic divisions enables the transactivation of proneural genes by a truncated form of Sox2 and Ngn2. Ngn2 in turn inhibits the expression of G1 cyclins. A cell cycle inhibitor $p27^{Kip1}$ stabilizes Ngn2, whereas $p57^{Kip2}$ interacts with proneural factor Mash1

and represses its transcriptional activity. RBL1 modulates the Notch pathway and affects the expression of its target genes. NSC, neural stem cell (Liu *et al*., 2019).

1.5.4 Cell cycle regulation in the CMZ of the retina

The tight co-ordination of cell cycle and retinal development is well demonstrated in adult/post-embryonic retinal neurogenesis of teleost fish and *Xenopus*. It was demonstrated that the CMZ region of the retina of these animals recapitulate the molecular processes occurring in the embryonic retina (Ohnuma *et al*., 2002; Perron *et al*., 1998).

More in detail, Ohnuma *et. al*, proposed that the CMZ can be broadly divided into five regions from peripheral to central retina. At the most peripheral edge of the CMZ, where RCSs reside, low mitotic activity is related to the transcriptional down-regulation of cyclins and CDKs (zone 1) (Ohnuma *et al*., 2002). Moving forward into the central CMZ (zones 2-3), proliferation is faster, which is triggered by the transcriptional up-regulation of cyclins-CDKs activity and allows the appearance of retinal progenitor cells. The next central region of the CMZ (zone 4) is characterized by a dramatically decrease of all major cell cycle activators (cdc2, cdk2, cyclin D1, cyclin A2, cyclin E1, and cdk4) and a strong expression of *atonal* proneural genes (*Xath5*, *XNeuroD*, *Xath3*, and *Xenopus neurogenin-1*) which produces a decrease in proliferation. Finally, progenitors exit the cell cycle and is associated with the upregulation of cell cycle inhibitors such as $p57^{Kip2}$ and $p27^{Xic1}$ (Dyer and Cepko, 2001; Ohnuma *et al*., 1999) in the most central region of the CMZ, where differentiation occurs (zone 5) (Bilitou and Ohnuma, 2010) (**Figure 17**).

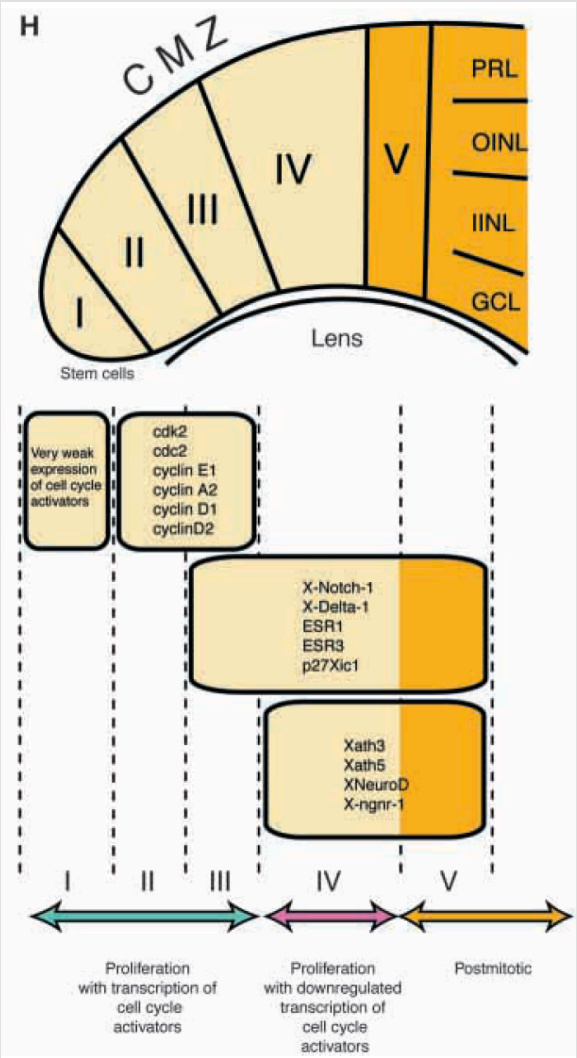


Figure 17. Cell-cycle progression in the CMZ. Schematic model of *Xenopus* CMZ representing the expression of cell cycle components and determination genes in the retina of stage 41 embryo. Adapted from (Ohnuma *et al*., 2002).

Using *in vivo* time-lapse imaging, the cell cycle division rates of the CMZ of post-embryonic zebrafish were confirmed. RSCs at the periphery divide slowly, then moving to the central retina the young retinal progenitor cells divide quickly, and finally, the old retinal progenitors leave the cell cycle and start to differentiate (Wan *et al*., 2016). This study agrees with the observations in the embryonic zebrafish retina where, early retinal progenitor cells divide symmetrically, while late progenitors slow down their cell cycle and differentiate (He *et al*., 2012). Moreover, it is observed that RSCs divide in an asymmetrical manner, while their daughter cells divide symmetrically (He *et al*., 2012).

Interestingly, it was observed that retinal cell-fate determinants collaborate with cell cycle genes to regulate proliferation of retinal progenitors and that cell cycle genes interfere with cell fate determination (Ohnuma *et al*., 2002). For example,

deletion of *Ath5*, the essential factor for RGC fate in zebrafish, caused alteration in the timing of cell-cycle exit (Kay *et al*., 2001). Moreover, it was demonstrated that overexpression of cyclin-CDKs inhibit cell-fate determination, while induction of cell cycle inhibitors had the opposite effect in *Xenopus* retinogenesis (Ohnuma *et al*., 1999).

It was shown that during *Xenopus* retinal development the cell cycle length of retinal progenitor cells increases over time (Decembrini *et al*., 2006). This observation suggests a correlation between cell cycle duration and cell fate. In addition, inhibitors of CDK are also important in the co-ordination between cell cycle and cell fate in the retina. It was observed that the expression of $p27^{Xic1}$ in the CMZ coincides with the timing of cell-cycle exit in *Xenopus* retina. Moreover, overexpression of $p27^{Xic1}$ activates cell cycle-exit, while its deletion has the opposite effect. At the molecular level, it was observed that $p27^{Xic1}$ activates neurogenesis by interacting with the fate determinant Neurogenin, which results in its stabilization (Vernon *et al*., 2003).

However, cell-cycle regulation is not an absolute factor deciding about cell fate determination. Back in 1991, Harris and Hartenstein showed that pharmacologically induced cell-cycle arrest in the frog retina does not prevent the arousal of all the different retinal cell types (Harris and Hartenstein, 1991). This suggests that an intrinsic mechanism may be involved in determining cell fate specification.

Taken all together, the CMZ represents an *in vivo* model to study the mechanisms behind self-renewal and differentiation. It was demonstrated that depending on the localization of the cell within the CMZ, cells exhibit different proliferation dynamics based on the differential expression of cell cycle activators/inhibitors (Ohnuma *et al*., 2002). Thus, the CMZ could serve as a model to study the unusual organization of the cell cycle of stem cells.

1.5.5 Molecular links between cell cycle and pluripotency or differentiation

In vivo data on the overall cell cycle structure of mammalian ESCs were obtained over the past 30 years, although molecular details have only been uncovered more recently with the development of techniques to culture PSCs *in vitro*. Several studies showed that cell cycle proteins play an active role in

ensuring pluripotency. For example, knockdown of CDK1, CDK2, cyclin E or B1, and treatments with CDK-inhibitors all resulted in the loss of pluripotent state and triggered differentiation. In agreement, ectopic overexpression of cyclins E or B1 promoted ESC self-renewal. At the molecular level, G1 cyclin-CDK kinases directly phosphorylate pluripotency factors such as Oct, Sox2, and Nanog, resulting in their stabilization (**Figure 18**). The inverse is also true, since pluripotency factors regulate cell cycle proteins. For instance, Nanog from hESCs binds to CDK6 and CDC25 genes to upregulate their expression (Liu *et al*., 2019).

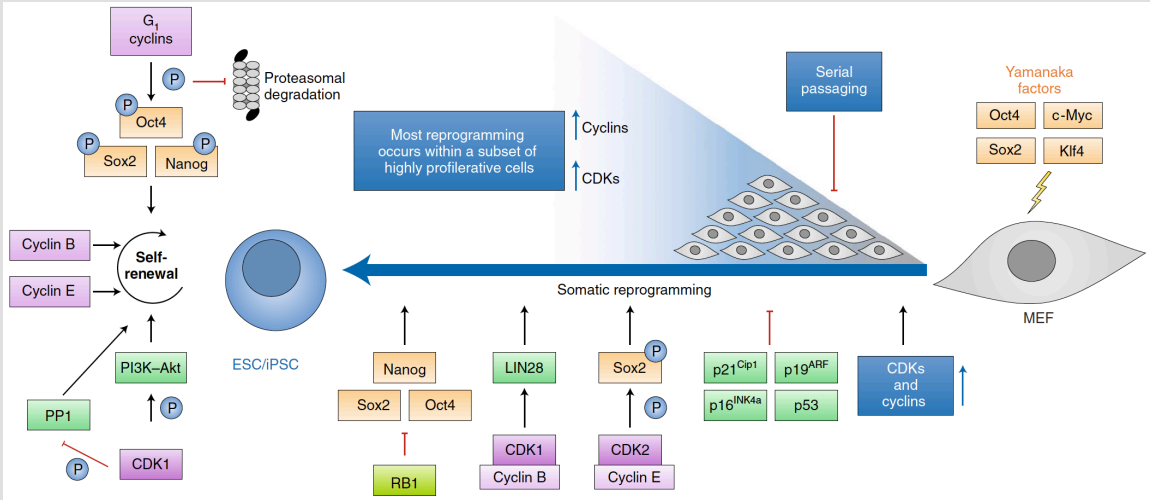


Figure 18. The cell cycle in somatic reprogramming and pluripotency maintenance. During somatic reprogramming by expression of Oct4, Sox2, Klf4 and c-Myc, somatic cells rapidly accelerate the cell cycle. Ectopic overexpression of cell cycle proteins or inactivation of cell cycle inhibitors increases the efficiency of reprogramming. Conversely, serial passaging leads to a decreased reprogramming rate. CDK2-dependent phosphorylation of Sox2 or cyclin B–CDK1-dependent upregulation of LIN28 was postulated to aid reprogramming. RB1 represses expression of core pluripotency factors. The cell cycle machinery is also important for the maintenance of ESC pluripotency. G1 cyclins stabilize core pluripotency factors through phosphorylation (P), thereby preventing their proteasomal degradation. High levels of cyclins and CDK1-dependent phosphorylation of PI3K–Akt pathway components likely contribute to the maintenance of pluripotency. CDK1 also inhibits Oct4 activity during the M phase, acting through PP1 and Aurkb. Upward blue arrows indicate increased expression. ESC, embryonic stem cell; iPSC, induced pluripotent stem cell (Liu *et al*., 2019).

It was observed that when ESCs start to differentiate, their cell cycle structure begins to change, particularly the duration of cell division increases, principally due to the extension of G1 phase. Interestingly, ESCs are also small in size when compared with somatic cells, a feature that is often attributed to a shortened period of growth in the truncated G1-phase (Singh and Dalton, 2009).

Upon differentiation, the cell cycle is restructured such that approximately 40% of an asynchronously dividing population of cells are found in G1 (Stead *et al*., 2002; White *et al*., 2005). This newly formed cell cycle is accompanied with upregulation of D cyclins, the decrease of activity of CDK1 and CDK2 by KIP/CIP inhibitors, and finally, CDK2-, cyclin E- and cyclin A-associated kinases becomes cell cycle phase-specific (Liu *et al*., 2019).

Many studies support that the differentiation of ESCs happens in G1 phase. Cells traversing G1 phase expressed higher developmentally regulated transcription factors. Additionally, it was proposed that a long G1 phase enables the accumulation of factors needed for differentiation (Lange and Calegari, 2010; Singh and Dalton, 2009).

Even if cells passing through G1 phase are prone to differentiate, pathways that operate in S and G2 phases may also play a role in differentiation. Gonzales *et al.* (Gonzales *et al*., 2015) found using a high-throughput RNAi screen that hESC passing throughout differentiation upregulate genes involved in DNA replication and G2 phase progression.

It is still unknown whether the reorganization of the cell cycle upon cell fate specialization represents the cause or the consequence of cell differentiation. In addition, the physiological role of very high activity of CDK1 and CDK2 in pluripotent cells remains a mystery. It was discovered that chromatin regulation such as histone modifications that change their accessibility to transcriptional factors, correlates with the onset of zygotic transcription in mice (Ura *et al*., 1997). In addition, drugs that alter chromatin structure are able to induce premature gene expression, suggesting the role of chromatin architecture with the regulation in the activation of gene expression during the maternal to zygotic transcription (Aoki *et al*., 1997). Recent advances in elucidating the 3D chromatin conformation could deliver important information about the activation/repression of certain genes in the control of pluripotency and differentiation.

2. DNA REPLICATION IN EUKARYOTES

As previously discussed, the proportion of S phase dedicated to the cell cycle of stem cells is longer when compared to non-stem proliferative cells. However, the biological significance of this is poorly understood. As a reminder, stem cells are regulated by different cues that include growth factors, transcription factors and more recently discovered, epigenetic regulators. Epigenetics can be understood, as the field of science in charge of the study of the biological mechanisms that regulate specific and heritable traits of genome function without the alteration of the DNA sequence (mutations). Evidence showed that stem-cell epigenetic state involves: 1) up-regulation of certain genes, 2) repression of others, and 3) transcription plasticity of genes for direct lineage specification. Thus, attention in research is focusing on the changes in the structure and function of the chromatin (complex of DNA plus associated proteins) that mediates the epigenetic maintenance of the genes on, off or poised states (Lim and Alvarez-Buylla, 2016). Interestingly, Hiratani, I and Gilbert, D. proposed that the way in which cells replicate their DNA might be considered as a distinct epigenetic signature since it is a feature that correspond to a particular cell differentiation state that dramatically changes during development (Hiratani and Gilbert, 2009). Thus, the coordination of DNA replication deserves to be studied in order to understand the mechanisms underling stemness.

I want to devote this second part of the introduction to the process of DNA replication, making special attention to the initiation part; since it is here that allegedly the length of S phase is decided. I will begin by a brief description of the two steps of DNA replication initiation: licensing and firing. After I will focus on the spatial-temporal program of DNA replication, which is a newly discovered cell-type specific feature. Later, I will present the different techniques and models to study this program, making special attention to the Xenopus egg extracts since it is one of the main models of my PhD project and then I will present what is known of this program in stem cells. Finally, I will mention what we know about its regulation, finishing by describing RIF1, an evolutionary conserved *trans*-acting factor of eukaryotic DNA replication.

2.1 DNA replication initiation

In eukaryotic cells, DNA replication is tightly controlled to ensure that an exact copy of the genetic material is inherited by two daughter cells. Contrary of bacteria, multicellular organisms have larger genomes, thus the mechanism of DNA replication has to be evolved. The duplication of the genome in eukaryotes is achieved by distribution of several regions in which DNA synthesis can initiate called replication origins (ORI) (**Figure 19**), which must fire no more than once per cell cycle to ensure a single error-free copy of the genome (Siddiqui *et al.*, 2013). In mammals, it was shown that between 30,000 - 50,000 origins are active at each cell cycle (Huberman and Riggs, 1966).

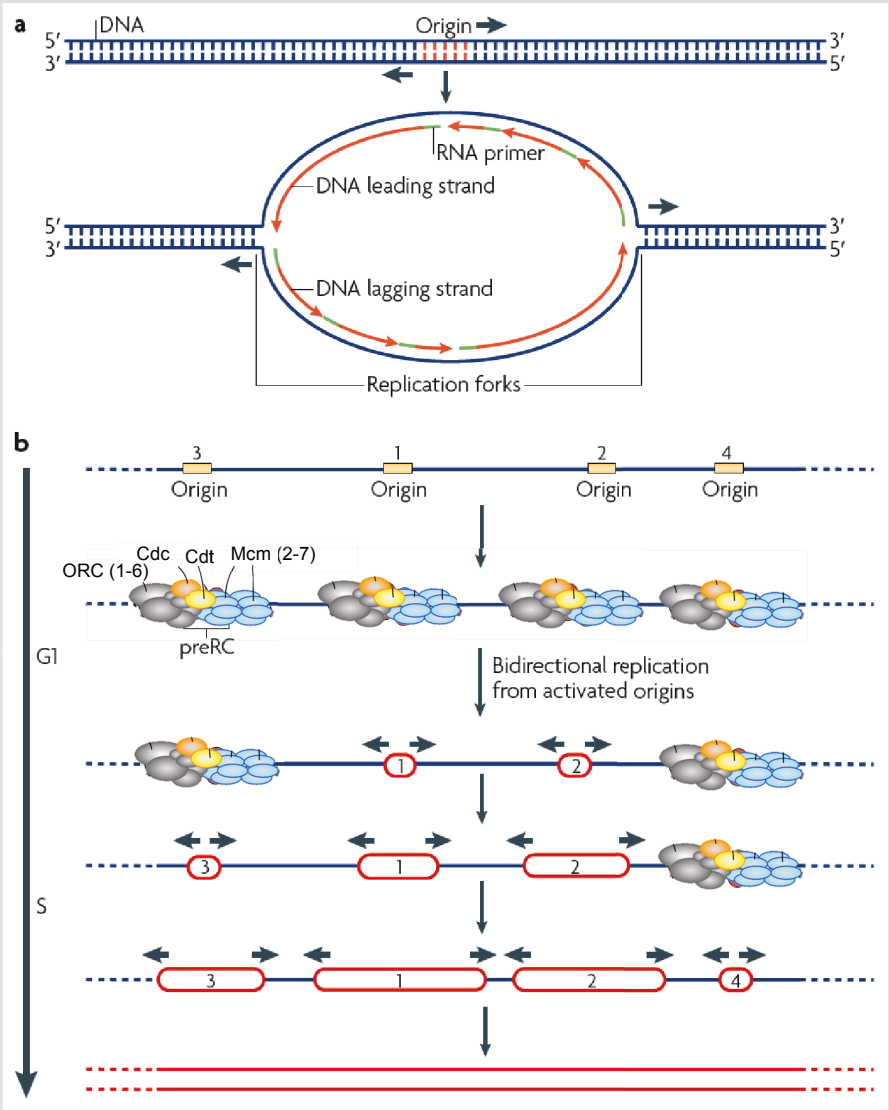


Figure 19. Replication origins. A) At each replication origin, DNA synthesis starts with short RNA primers that are synthesized by DNA polymerase-α. As DNA synthesis always occurs in the 5'-3' direction, one strand of the DNA (the leading strand) will be

synthesized continuously, whereas the other strand (the lagging strand) will be synthesized discontinuously by short RNA-primed DNA fragments. Two other DNA polymerases (δ and ε) are recruited for the elongation of lagging and leading strands, respectively. B) Activation of replication origins during S phase. Pre-replication complexes (pre-RCs) are assembled at replication origins during G1 phase. Activation of replication origins occurs throughout S phase, some during early (1 and 2), and some in mid (3) or late (4) S phase. Modified from (Mechali, 2010).

Cells achieve "once-per-cell-cycle replication initiation" by dividing the replication initiation process into two temporally separate phases: licensing and firing (Blow and Dutta, 2005; Siddiqui *et al*., 2013) (**Figure 20**). In mechanistic terms, licensing corresponds to the loading of inactive precursors of the Mcm2-7 helicase at replication origins by the pre-replicative complex (pre-RC) (Donovan *et al*., 1997; Evrin *et al*., 2009; Remus *et al*., 2009; Rowles *et al*., 1999; Seki and Diffley, 2000), while firing correspond to activation of the replicative helicase by association of additional accessory subunits (Aparicio *et al*., 2009; Costa *et al*., 2011; Gambus *et al*., 2006; Heller *et al*., 2011; Ilves *et al*., 2010; Moyer *et al*., 2006; Yeeles *et al*., 2017).

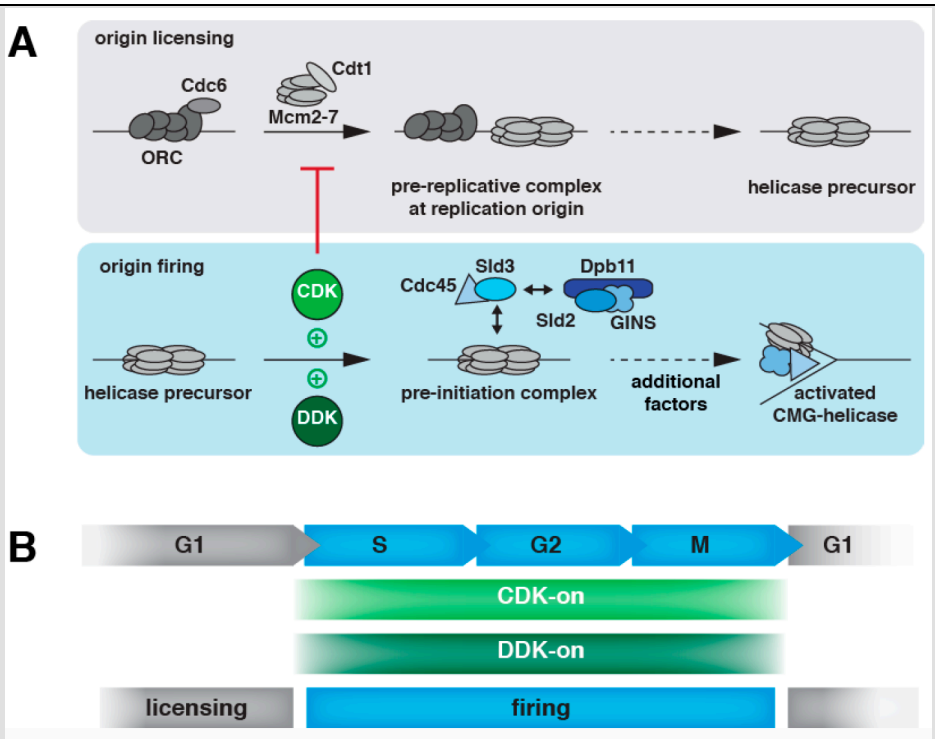


Figure 20. Two-step mechanism of DNA replication initiation. (A) Inactive helicase precursors are loaded during origin licensing (upper panel); CDK and DDK promote activation of these precursors to form active CMG helicases during origin firing (lower panel). In addition to the depicted factors, origin firing and helicase activation involve Sld7, DNA polymerase ε, and Mcm10, which are indicated as additional factors. (B) Changing activity of CDK and DDK couples licensing and firing strictly to distinct phases of the cell cycle (Reusswig and Pfander, 2019).

2.1.1 Origin licensing

Licensing generally occurs from late M phase to G1/S transition (Dahmann *et al*., 1995; Diffley *et al*., 1994; Seki and Diffley, 2000). Origin licensing involves sequential and interdependent anchoring of different proteins (Masai *et al*., 2010). First, the six-subunit origin-recognition complex (ORC) binds to all possible ORIs and recruits Cdc6 and Cdt1 proteins. Together these three licensing factors direct the loading of the helicase, the MCM complex, around dsDNA, resulting in the formation of the pre-RC. The MCM complex thus loaded is topologically linked to DNA and forms a double hexamer (Donovan *et al*., 1997; Evrin *et al*., 2009; Gambus *et al*., 2011; Remus *et al*., 2009; Rowles *et al*., 1999; Seki and Diffley, 2000) (**Figure 20 & 21**).

2.1.2 Origin firing

Firing occurs during S phase (Heller *et al*., 2011). The inactive pre-RC is converted into an active helicase that unwinds dsDNA, thus allowing DNA polymerases to access and copy the two template strands (Siddiqui *et al*., 2013). Firing involves the formation of the CMG complex, named after its components: Cdc45, the MCM proteins, and the GINS complex (Aparicio *et al*., 2009; Moyer *et al*., 2006).

Origin activation is achieved through a highly regulated series of phosphorylation events on the subunits of the MCM helicase complex, mainly by DBF4-dependent Kinase (DDK; also known as the CDC7-DBF4 complex) and by cyclin-dependent kinase (CDK) (Fragkos *et al*., 2015). This process also requires the activity of the Sld2, Sld3, Sld7, and Dpb11 proteins as well as the two kinases mentioned before, CDK and DDK (Siddiqui *et al*., 2013). These six firing factors are essential for initiating DNA synthesis from licensed origins. The active CMG helicase corresponds to the formation of the pre-initiation complex (pre-IC) (Heller *et al*., 2011; Ilves *et al*., 2010; Masumoto *et al*., 2002; Tanaka *et al*., 2007).

Once the DNA chains are unwound, replication in eukaryotes is initiated when DNA polymerase α (Pol α) is recruited, synthesizing a short RNA-DNA primer. This primer is recognized by replication factor C (RFC), which displaces the Pol

α and recruits PCNA, a processivity factor for DNA polymerase function (Maga and Hubscher, 2003). Finally, the DNA polymerase is coupled, either Pol ε for the leading strand or Pol δ for the lagging strand (Kunkel and Burgers, 2008) (**Figure 21**).

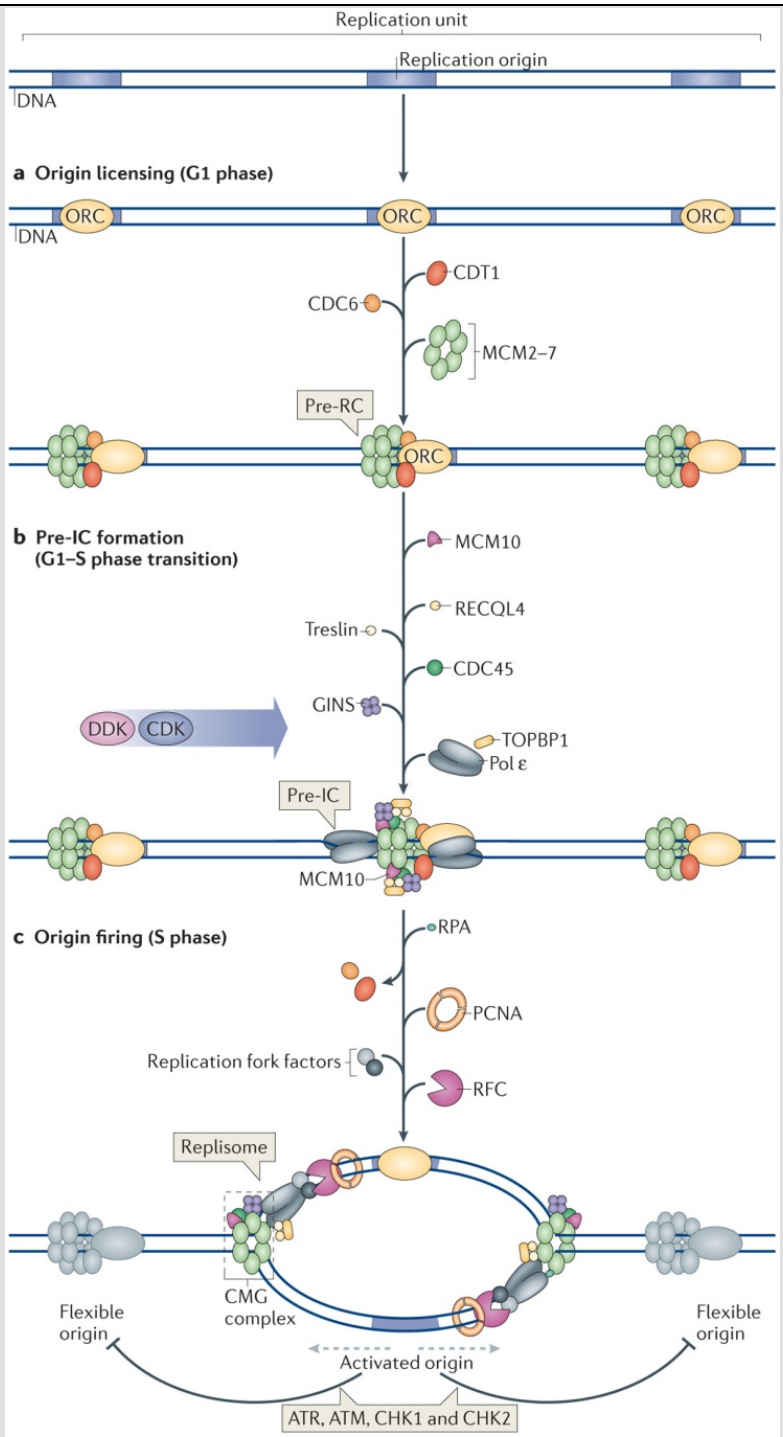


Figure 21. Formation and activation of DNA replication origins. The figure shows a replication unit with three potential replication origins. A) Licensing of replication origins is restricted to the G1 phase of the cell cycle and results from the sequential loading of pre-replication complex (pre-RC) proteins on all potential origins in the genome. First the origin recognition complex (ORC, comprising the six subunits ORC1-6), which has ATPase activity, is recruited to replication origins. This is followed by the binding of

CDC6 and CDC10-dependent transcript 1 (also known as CDT1). Loading of the mini-chromosome maintenance (MCM) helicase complex, which contains the six subunits MCM2-7, is the last step of the licensing reaction and can take only if ORC, CDC6 and CDT1 are already bound to origins. B, c) Origin activation involves the formation of a pre-initiation complex (pre-IC) and activation of the MCM helicase complex. Assembly of the pre-IC is triggered by DBF4-dependent kinase (DDK) and cyclin-dependent kinases (CDKs) at the G1-S phase transition and its activation into a functional replisome occurs in the S phase. DDK and CDKs phosphorylate several replication factors to promote their loading on origins. Moreover, DDK and CDKs directly phosphorylate several residues within the MCM2-7 complex, resulting in helicase activation and DNA unwinding. During helicase activation, activation the MCM2-7 double hexamer divides into two hexamers that function at the two replication forks emanating from the replication origin. Helicase activation induces the recruitment of other proteins that convert the pre-IC into two functional replication forks that move in opposite directions from the activated origin, with the replisome (a protein complex) at each replication fork. The functional helicase at the forks is the CMG complex. In a replicated unit, only one out of three origins on average is activated, whereas the other adjacent origins remain silent, although they have been licensed. Therefore, a replisome is only formed in the activated origin. In a given cell population, different origins can be used in individual cells; thus, a cell population contains a range of flexible origins. Inhibition of adjacent origins within a replication unit is controlled in part by the checkpoint kinases Ser/Thr protein kinase ATR and Ser protein kinase ATM that activate checkpoint kinase 1 (CHK1) and CHK2 (Fragkos *et al*., 2015).

2.2 Control of origin licensing

When DNA synthesis begins during S phase, the activated origins disassemble their pre-RCs, making impossible that the same origin starts initiation again. Inappropriate licensing (that is, licensing that takes place after the beginning of DNA synthesis), by over expression of CDT1, can lead to re-activation of origins that have already been used during S phase and subsequently to genome amplification, a proses known as re-replication or over-replication. Re-replicating cells show signs of DNA damage and genomic stress or instability (Neelsen *et al*., 2013; Vaziri *et al*., 2003), which are associated with cell cycle arrest, senescence and apoptosis (Melixetian *et al*., 2004; Vaziri *et al*., 2003; Zhu *et al*., 2004).

Multiple mechanisms have been discovered in different experimental organisms that prevent the pre-RC assembly once DNA replication has commenced. The response to inhibit re-replication varies between organisms, however in eukaryotes there is one major global regulator of origin licensing the APC, a multisubunit E3 ubiquitin ligase. The APC targets M- and S-CDK activity and

stabilizes CDKIs permitting a windows after mitosis and during G1 phase for origin licensing (Arias and Walter, 2007).

In early embryos of *X. laevis* characterized by cell cycles lacking gap phases, replication is dependent on Cdk2-Cyclin E, mitotic entry is driven by Cdk1-Cyclin B, and mitotic exit by APC^{Cdc20} (a version of APC that contains the activator protein Cdc20). Importantly, another mechanisms to prevent re-replication was discovered in *Xenopus* egg extracts which is the coiled-coil protein Geminin that acts by binding to CDT1 to inhibit its licensing activity (McGarry and Kirschner, 1998; Tada *et al*., 2001; Wohlschlegel *et al*., 2000) (**Figure 22**). Geminin has been identified in other metazoans, including mammals. Apart from geminin, early frog embryos use Cdt1 destruction and Cdk1 activity to prevent re-replication. Cdt1 ubiquitylation depends on the binding of PCNA to the chromatin. In *Xenopus* eggs extracts, Cdk1 activity prevents licensing by inhibiting the binding of ORC complex to DNA (Arias and Walter, 2007).

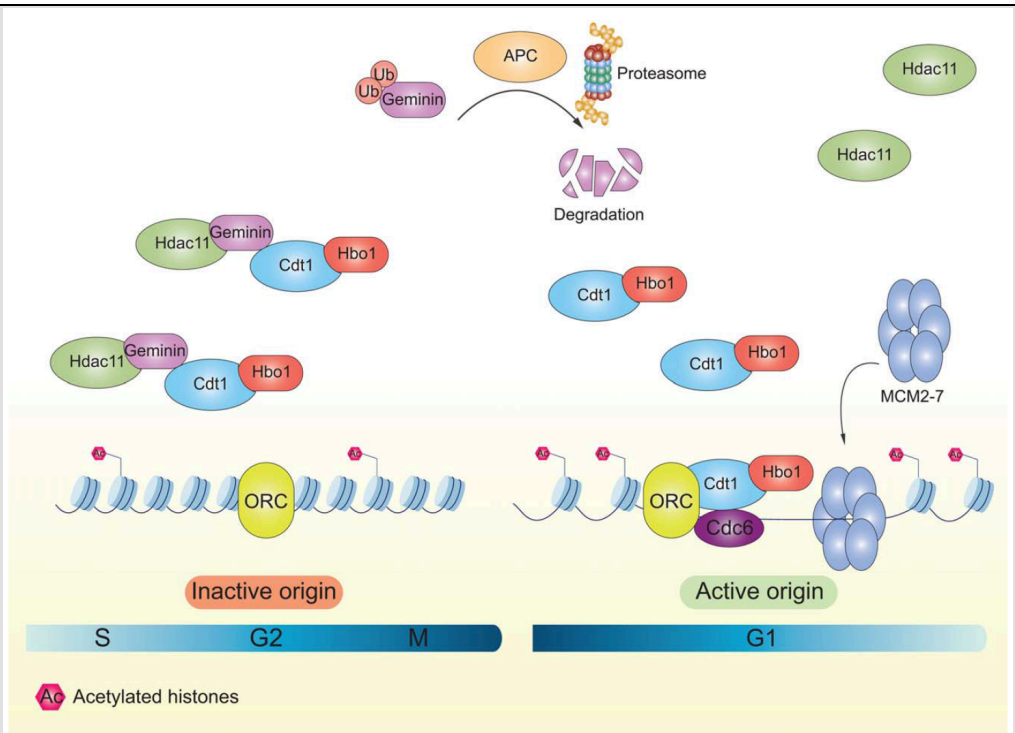


Figure 22. Regulatory role of Geminin on DNA replication. Interaction of Geminin with Hdac11 sequesters the Cdt1-Hbo1 complex and prevents it from being recruited onto origins, which are inactive during S-phase, G2 and M. During G1, degradation of Geminin permits the formation of Cdt1-Hbo1 complex which acetylates chromatin at active origins allowing the formation of the pre-RC. Abbreviations: APC, anaphase promoting complex; HDACs, histone deacetylases; ORC, origin recognition complex (Patmanidi *et al*., 2017).

The consequence of re-replication is seen as signs of replication stress such as chromosomes breaks visualized by γ-H2AX staining. The appearance of dsDNA breaks trigger DNA damage response, arresting cells in G2 phase. If the checkpoint is inefficient to repair damage, then it will lead the cell to apoptosis (Arias and Walter, 2007). Notably, deregulation of DNA replication is increasingly recognized as a critical factor during cancer development (Halazonetis *et al*., 2008; Kotsantis *et al*., 2018; Macheret and Halazonetis, 2015).

2.3 Types of replication origins

Importantly, only a subset of all licensed origins is activated (fire) in each cell cycle. The choice of origins to be activated varies from cell to cell, even in the same cell population, implying that origin usage is flexible in mammalian cells (Cayrou *et al*., 2011). The different usage of replication origins permits the classification into three categories: constitutive, flexible and dormant (**Figure 23**). The number of activated origins decreases over time in adult somatic cells only 20-30% of all potential replication origins are activated which contributes to lengthening of S-phase (Fragkos *et al*., 2015).

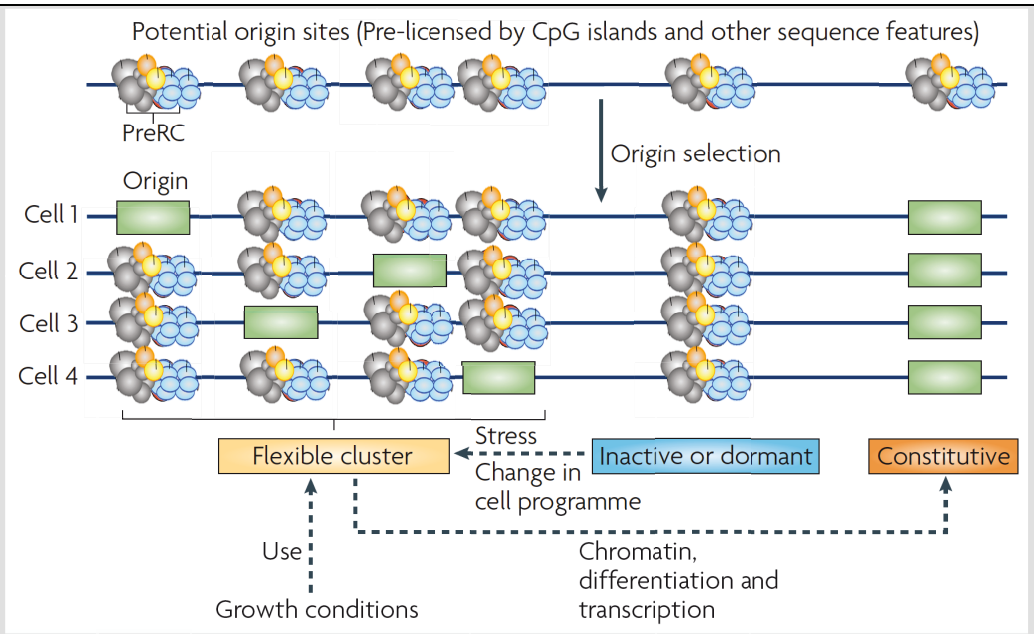


Figure 23. Different types of DNA replication origins. Potential DNA replication origins are set during mitosis-G1 phase by the assembly of pre-replication complex (pre-RC) proteins. The selection of the origins that will be activated at the next S phase occurs at G1 phase and may vary according to the cell fate or environmental

conditions. Four examples of DNA replication origin positions are shown in different cells in a growing cell population. A cluster of flexible origins contains origins that can be used differently in different cells. Their use could increase or decrease according to physiological or abnormal growth conditions. Inactive or dormant origins are rarely used or are not used at all. Constitutive origins are fixed origins that are always set at the same position by chromatin or transcriptional constraints. Replication stress can activate dormant origins or increase the use of flexible origins, resulting in an increased number of origins per replication cluster. Modified from (Mechali, 2010).

A major challenge is to understand how origins that are to be activated in the S phase are chosen from all the potential origins (Mechali, 2010). It is thought that the excess of licensed origins is crucial because enables the cell to respond to replication stress.

2.4 DNA replication timing program

In a mammalian cell division cycle lasting approximately 24 h, up to 10 h are allocated for DNA replication. The duplication of large genomes starts from multiple origins of replication, spaced every 100 Kb on average (Huberman and Riggs, 1968). Considering that new DNA is synthesized bidirectionally at a rate of 1-3 kb/min, the entire genome could be duplicated in less than one hour. However, this scenario would require all origins to fire simultaneously at the beginning of the S phase, and this is never observed. Instead DNA replication follows a replication timing (RT) program in which some chromosomal domains are replicated during early S phase and others are replicated late (Mendez, 2009) (**Figure 24**). Chromosomes are divided into domains with a specific RT (Mendez, 2009). Despite the RT program is erased during the S phase, it is restored again during the following G1 phase of the next cell cycle at the timing decision point (TDP) (Lu *et al*., 2010).

The TDP is when the chromatin domains take positions where they will stay for the remaining time of interphase and occurs during early G1 phase. These sites can be visualized cytogenetically (Dimitrova and Gilbert, 1999). Moreover, this time window happens before the origin decision point (ODP), which is the time when replication origins sites are selected (Wu and Gilbert, 2000). Interestingly, when replication is forced to initiate between the TDP and the ODP the DNA RT program occurs at the expected time, which indicates the relationship between

RT and genome architecture and independently of origin determination (Marchal *et al*., 2019).

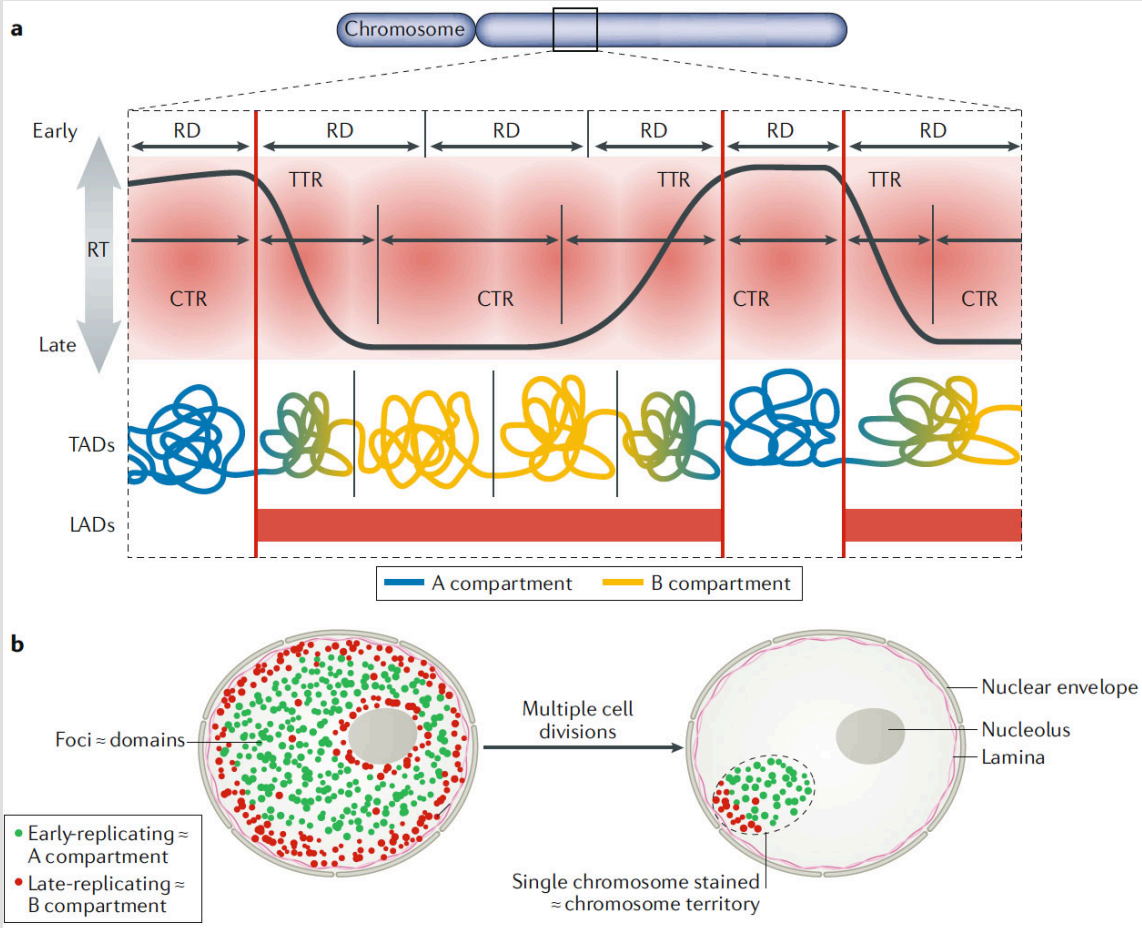


Figure 24. Current model of the relationship between replication timing (RT) and chromatin structure. a) Early and late constant timing regions (CTRs) are 1-5 Mb regions separated by timing transition regions (TTRs) as demarcated within the red shaded area. These CTRs consist of one to several replication domains (RDs), defined as chromatin segments that coordinately switch RT during cell fate changes (that is, between different cell types). RDs share the properties and approximate boundaries of a subset of topologically associated domains (TADs), aligning most closely with TADs that are at compartment boundaries. Early CTRs correspond to the A compartment, but late CTRs correspond to the B compartment and TTRs correspond to the transitions between compartments. Both TTRs and late CTRs correspond to lamina-associated domains (LADs). b) RT illuminates genome architecture: nuclei after an early S pulse label (green) followed by several hours of a chase period and then a late S pulse label (red). In this model, observable foci of DNA synthesis correspond to the replication domains in panel a and early/late-replicating chromatin corresponds to A/B compartments. After multiple passages, only one chromosome per cell remains labelled, marking the chromosome territory. However, the foci retain their label intensity and genetic continuity, demonstrating that the DNA that is synthesized during one cell cycle remains clustered together as structural unit of chromosomes for many generations (Marchal *et al*., 2019).

Replication origins have different levels of organization. The first level consists of the pre-RCs that are formed at all potential replication origins. The second is

the replication unit or replicon, each replicon may contain several potential pre-RCs, of which only one will be activated. When an origin is activated in a replication unit, all the other origins from the same replicon are repressed. This occur through a phenomenon termed negative origin interference (Brewer and Fangman, 1993).

The third level is the association of replicons in replication clusters, which are replication domains that form replication foci (**Figure 25**). In each replication cluster, origins fire synchronously (Coue *et al*., 1996) through a mechanism of positive interference (Marheineke and Hyrien, 2004). The replicon organization in clusters might involve chromatin looping to bring origins from different replicons into a single domain. Importantly, the 3D organization of chromosomes is related to RT domains in which DNA can fold so that sequences interact with other sequences within the same domain but not with sequences in adjacent domains. Early activation during the S phase occurs in the nuclear interior, which is more permissive to transcription, whereas late-replicating topologically associated domains (TADs) reside at the nuclear periphery or in other transcriptionally repressed compartments (Pope *et al*., 2014).

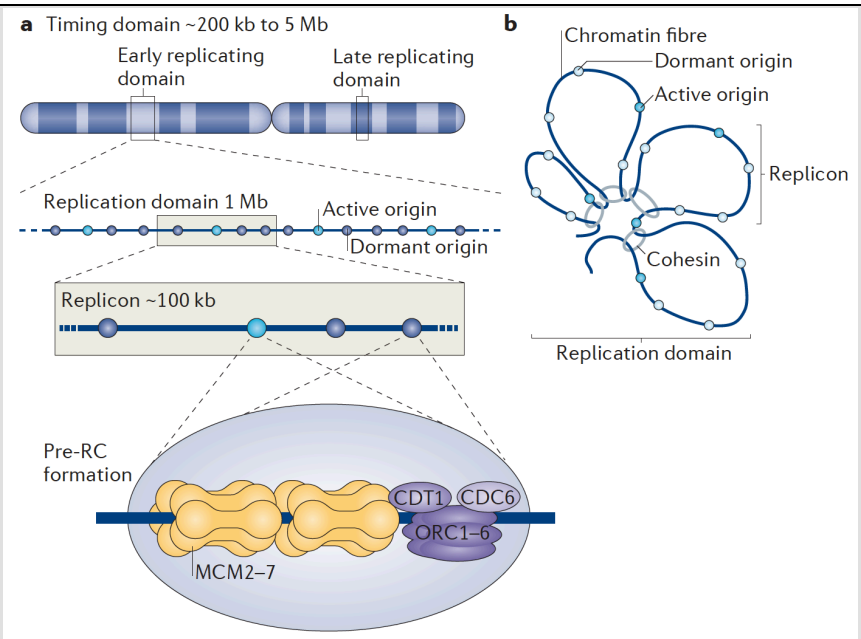


Figure 25. Replication initiation and genome organization. a. Timing domains correspond to large chromosomal regions that replicate at similar times, early or late in S phase. These domains are bordered by so-called transition zones. Each timing domain can include one or several replication domains, which in turn are composed of 5 to 10 adjacent replicons that fire simultaneously. A replicon corresponds to the stretch of DNA that is replicated bi-directionally from a single origin, with nearby

dormant origins being replicated passively. Pre-RCs (pre-replication complexes), the ORC (origin recognition complex), CDC6 (cell division control protein 6), CDT1 and MCM2–7 (minichromosome maintenance complex 2–7) double hexamers are assembled on both active and dormant origins, but only selected origins are activated in S phase. b. The loop model proposes that replication domains adopt a three-dimensional structure in which replicons are separated into loops by cohesin rings (Alabert and Groth, 2012).

However the temporarily coordination of DNA replication is not yet fully understood. It is not clear whether the coordination of origins activity is essential for the replication process and whether there is a causal relationship between DNA replication and gene transcription (Gilbert, 2002; McCune and Donaldson, 2003; Schubeler *et al*., 2002). For example, no universal signature or set of signatures that could predict all replication origins in metazoan genome has been identified (Fragkos *et al*., 2015). I will dedicate the section 2.4.5 to present and discuss the different elements that are known to regulate DNA RT either in *cis* or in *trans*. Our knowledge has shown that some of these factors are required for chromatin architecture or are directly involved in origin firing by allowing or inhibiting DNA synthesis.

2.4.1 Methods to study DNA replication dynamics

Current knowledge on DNA replication in metazoans is based on studies performed primarily using three model systems: *Xenopus* egg extracts, *Drosophila* embryos and cell lines (Siddiqui *et al*., 2013). Depending on the system there is a plethora of methods including biochemistry, molecular biology or microscopy techniques to study DNA replication simply because there are only two possible states, replicated or unreplicated DNA.

2.4.1.1 *Xenopus* egg extracts

The introduction of the *Xenopus* cell-free extracts have been of remarkably importance to the study of DNA replication, by allowing scientists the direct manipulation of proteins in an *in vitro* system whose replication performance is very robust. An advantage of this system is that they can be easily obtained, briefly eggs are collected and then lysed by centrifugation to release the

maternal stockpile material that characterize the fast cell division of the early embryos (Lohka and Masui, 1983) (**Figure 26**). Another advantage is that DNA replication can be studied without the perturbation of transcription and translation, since these events are practically absent in the cell following fertilisation (Blow and Laskey, 2016).

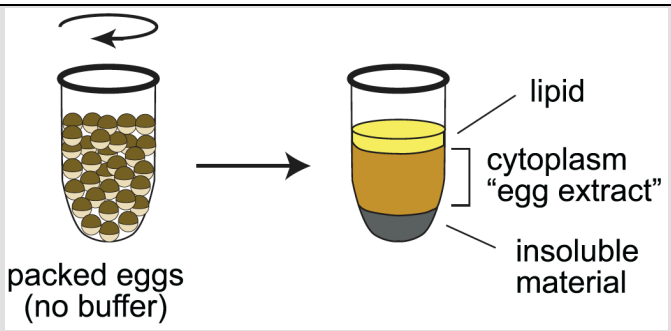


Figure 26. Making *Xenopus* egg extracts. Cartoon of the method of making *Xenopus* egg extracts ('low seed supernatants') according to the original protocol of Lohka and Masui. Dejellied eggs are packed into a centrifuge tube and any excess buffer is removed. Eggs are then lysed by relatively gentle centrifugation (~20,000 g). This separates the egg contents into a floating lipid plug, an insoluble pellet and in between, the cytoplasmic fraction (Blow and Laskey, 2016).

It is very well characterized that sperm nuclei from *Xenopus* incubated in *Xenopus* egg extracts is capable to decondense the sperm chromatin, then assemble the chromatin into nuclei to allow DNA synthesis, following semi-conservative replication. Additionally, when protein synthesis is blocked with the addition of cycloheximide, mitosis is not possible, thus only one round of DNA replication is achieved indicating that DNA replication is under normal cell cycle control (Blow and Laskey, 1986; Blow and Watson, 1987).

Interestingly, many fundamental aspects of DNA replication were discovered using the *Xenopus* egg extracts, for example it was thanks to them that we know that re-replication is prevented by making DNA replication initiation a two non-overlapping steps: licensing and firing (Blow and Laskey, 1988), as it was previously discussed here.

Now, it is possible to obtain cell-free extracts from other mammalian cells including human cells, which allows scientists to observe DNA replication mechanism in different systems (Blow and Laskey, 2016). Nevertheless, the *Xenopus* system has the advantage of a high concentration of proteins that can be identified using antibodies, and later characterize their function by their

immunodepletion. Additionally, mass spectrometry analysis is a powerful way to identify proteins and their interactions in those replicating extracts.

In addition, technology for using *Xenopus* egg extracts has been developed, now we can isolate the chromatin to observe protein recruitment dynamics on replicating chromatin and microscopy imagine is also available in the system. Notably, the DNA combing technique permits the visualization of origins at the level of single DNA fibers (**Figure 27**). This technique consist in pouring the DNA that has been incubated in *Xenopus* egg extract into a well, then a silanized glass coverslip is introduced inside the well and by a straight upward movement the DNA fibers will attach to the surface creating line tracks that will be later visualized by immunostaining (Marheineke *et al*., 2009). With the microscopic images of combed samples, researchers can measure different parameters of replication. Replication eyes are considered as the incorporation of biotin-dUTP that was added into the *Xenopus* egg extracts before initiating DNA replication. Other parameter is the replication extent, which is the sum of the eye lengths divided by the total DNA length, this value helps to assess the percentage of replication of the sample. Eye-to-eye distances (ETED) is the distance measured between the midpoints of two adjacent replication eyes and is a parameter of origin activation. Finally, fork speed can be inferred using the value of eye length (Platel *et al*., 2019). All of these parameters can be quantified in a context where a specific protein was depleted from the extracts to study its impact in replication.

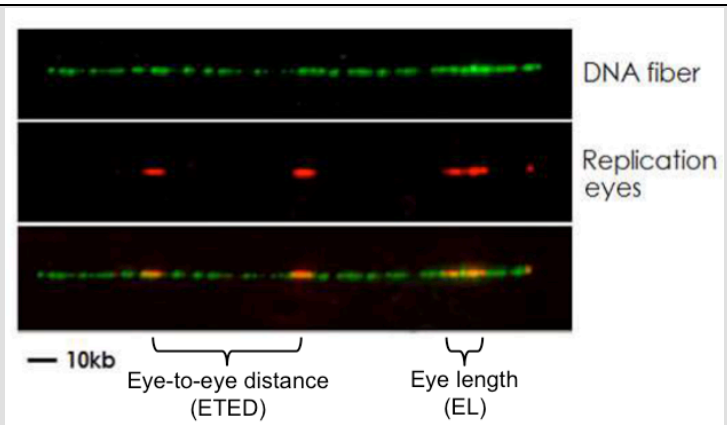


Figure 27. Epifluorescence image of a combed fragment of DNA. DNA from *Xenopus* sperm nuclei was incubated in *Xenopus* egg extract. Digoxigenin-dUTP single label. The red tracks are digoxigenin-labelled replication bubbles or eyes, the green stain between the tracks shows the whole DNA fiber counterstained with YOYO-1(Heller *et al*., 2011).

2.4.1.2 Techniques to study replication timing

In the earlier 60's it was the first time that scientists observed that different segments of mammalian chromosomes replicate at different time during S phase (Taylor, 1960). Nowadays, the subnuclear structures called replication foci or replication factories can be identified by the incorporation of labelled deoxynucleotides as discrete focal sites in the nucleus (Fragkos *et al*., 2015). Between 800 and 4,000 replication foci, each containing 4 to 6 origins on average, can be detected in a cell, depending on the microscopic resolution (Berezney *et al*., 2000; Cardoso *et al*., 2012; Cseresnyes *et al*., 2009). Replication foci appear as intranuclear punctuated structures (Nakamura *et al*., 1986), which form different patterns as the S phase progresses (**Figure 28**). Early in S phase, hundreds of small foci are distributed all over the nuclei. In mid S, foci are preferentially assembled around the nucleoli and nuclear periphery. Late S phase is characterized by clusters of foci that correspond to heterochromatic regions (Dimitrova and Gilbert, 1999; O'Keefe *et al*., 1992). Most foci remain active for 45-60 min before a different set of foci is activated (Jackson and Pombo, 1998). Each foci likely represents a replication domain with an average size of 1 Mb and contains a cluster of 5-10 origins that fire approximately at the same time (Ma *et al*., 1998; Nakamura *et al*., 1986).

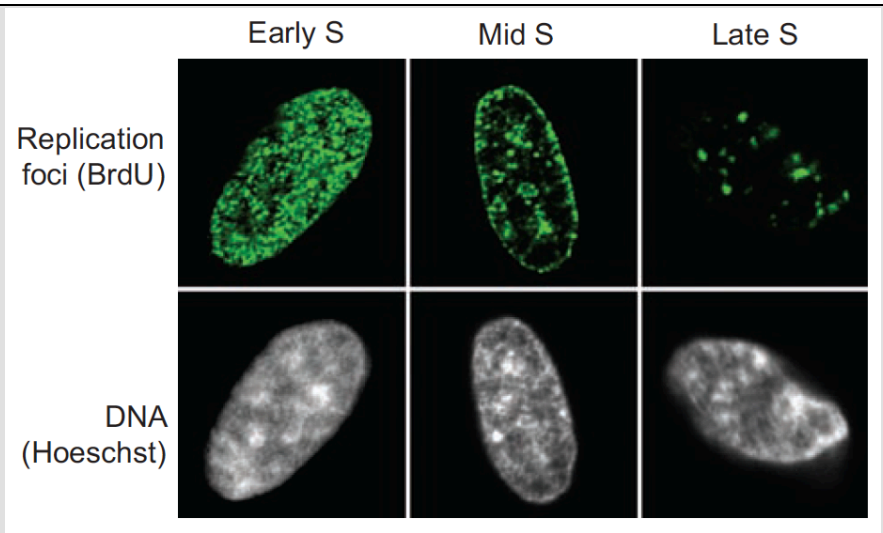


Figure 28. Patterns of replication foci in early, mid and late S phase. Replication foci were visualized by the immunodetection of BrDU after a 30 min pulse (Mendez, 2009).

Impressive advances in the state of the art has been done to study RT, several methods have been developed to map the replication domain at a genome-wide level in various mammalian cell types. For example, Véronique Azuara developed a method to separate a particular cell type according to its DNA RT, briefly she used cell populations BrdU-pulse labelled and FACS-sorted analysis to identify and separate early and late S phase cells (Azuara, 2006). Later, David Gilbert and colleagues used next-generation sequencing techniques to determine chromosomal position of each replication domain (**Figure 29**) (Ryba *et al*., 2011).. Moreover, the development of chromatin capture methods called Hi-C, permitted the high-resolution identification of the relationship between the 3D architecture of the genome and its RT (Hiratani *et al*., 2010; Hiratani *et al*., 2008; Pope *et al*., 2014; Ryba *et al*., 2010). This confirmed the existence of two different types of chromatin: the early replicating chromatin corresponding to the compartment A and the late replicating chromatin in the compartment B.

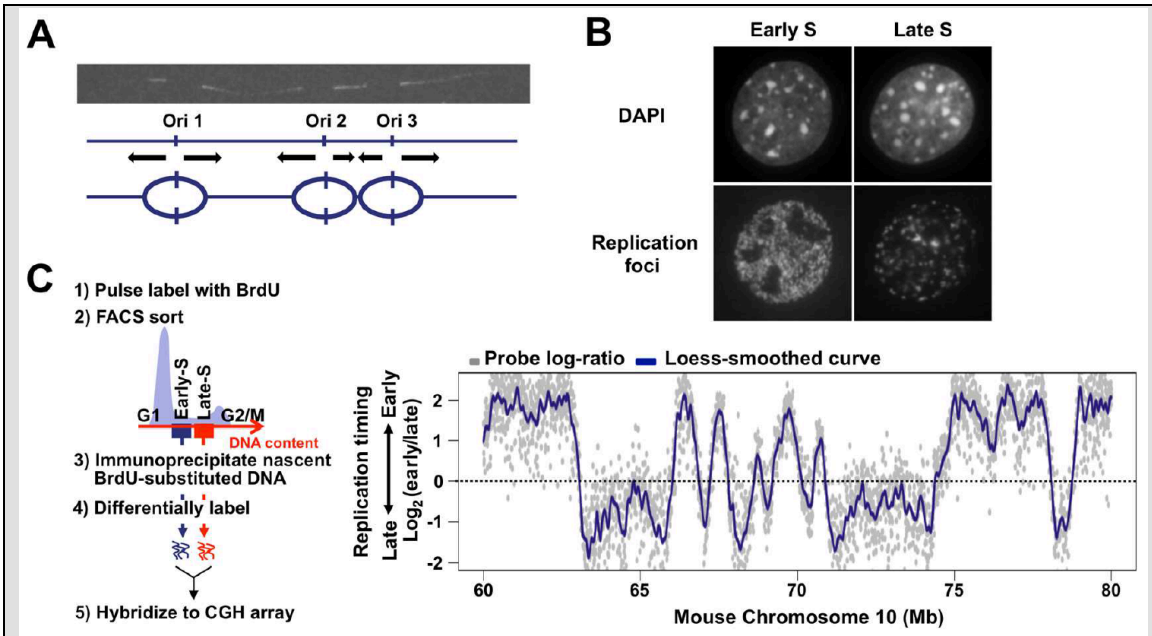


Figure 29. DNA replication in mammalian cells analysed by different methodologies. A) Multi-replicon structure of mammalian cells revealed by DNA fiber technique. The replicating cellular DNA was labelled with biotin-dUTP by the bead-loading method and detected with avidin-FITC on DNA fibers extended from the cell nucleus. Three origins (indicated by vertical arrows) were presumed to be activated simultaneously. To label replicating DNA, nucleoside analogues such as BrdU can also be used; B) Patterns of replication foci observed in early and late S phase of mammalian cells. Site of DNA synthesis in the nucleus were visualized by the incorporation of biotin-dUTP and subsequent detection with avidin-FIT (top). Cellular DNA was stained with DAPI (bottom); C) Flow chart of genome-wide replication domain analysis. Unsynchronized cells are pulse-labelled with BrdU. BrdU-substituted DNA

from early an late S phase fractions are collected, differentially labeled, and hybridized to a whole-genome CGH array. Alternatively, BrdU-substituted DNA from each fraction can be subjected to NGS (left). Exemplary replication domain organization from mouse embryonic stem cells for a 20 Mb region of chromosome 10. Log2 (early/late) raw values (the signal ration of early and late replicating DNA as shown in grey dots) for each CGH probe are plotted against the chromosomal position. Loess-smoothed plot is shown in blue (Takebayashi *et al*., 2017).

Recently, the RT of the entire human and mouse genomes has been mapped using different methods. A RT map of the human genome has been determined with 1 Mb resolution in a lymphoblastoid cell line with normal karyotype (Woodfine *et al*., 2005). Now, technology permits the study of RT at a single level, using single-cell DNA replication sequencing technology (Dileep and Gilbert, 2018; Takahashi *et al*., 2019) or the above mentioned Hi-C technique (Nagano *et al*., 2017), these powerful methodologies will have a positive outcome in understanding the biological importance of the RT, for example during the course of cell differentiation.

2.4.2 Replication timing in stem cells and differentiation

Stem cells provide a system to track down the relationship between RT with a specific developmental cell state. A substantial proportion of replication domains change from early to late and vice versa during cellular differentiation (Hiratani *et al*., 2008). These changes in RT are associated with nuclear reorganization (Hiratani *et al*., 2010). In addition, changes in origin usage seem to be linked to developmentally regulated modifications in transcriptional programs (Callan, 1974; Hyrien *et al*., 1995) (**Figure 30**).

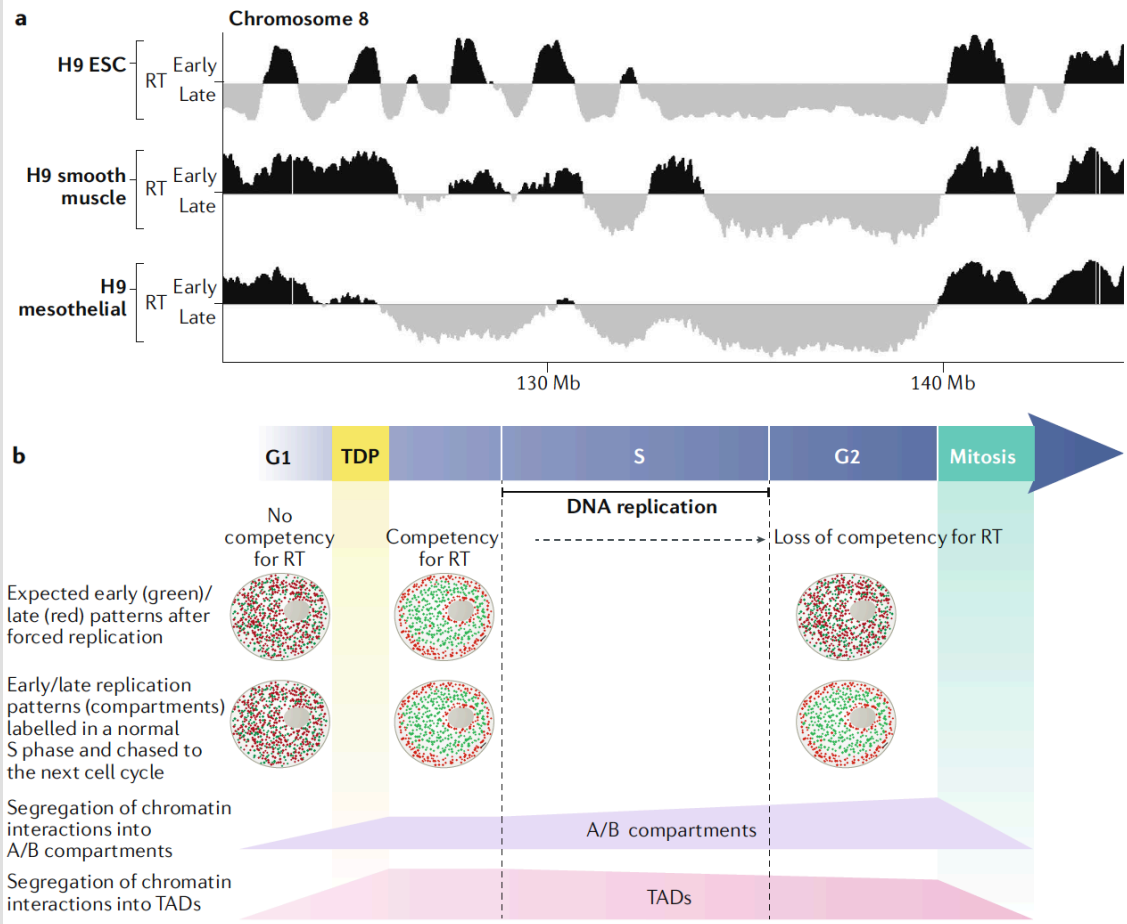


Figure 30. 3D chromatin structure and replication timing are dynamic during cell differentiation and during the cell cycle. A) RT is regulated during differentiation. Here, RT is shown for a region of chromosome 8, in hESC H9 (H) ESC) and two differentiated H9 cells. Some regions switch RT during differentiation (black to grey, or grey to black), whereas others remain constant. B) Both a defined RT program and interphase chromatin architecture are set up coincidently at the timing decision point (TDP) during the G1 phase. The information that defines RT is lost during the G2 phase. In nuclei that are artificially forced to replicate their DNA before the TDP or after the S phase, DNA replication does not follow any specific RT. The early- and late-replicating 3D compartments illuminated by replication labelling in the prior S phases are re-established at the TDP and persist through the remainder of interphase into the G2 phase, demonstrating that this spatial organization is not sufficient to dictate a RT program. 3D chromatin interactions- both the separation between large-scale spatial compartments and the distinction between topologically associated domains (TADs)- are dismantled during mitosis and re-formed at the TDP, coincident with the establishment of RT. Whereas compartments and TADs become slightly more or less distinct, respectively, during the course of the S phase, the major architectural changes in genome architecture occur during entry into and exit from mitosis (Marchal *et al.*, 2019).

The presence of a RT during early embryogenesis in numerous species before the onset of zygotic transcription argues the functional relevance of the RT program. For example, a distinctive RT could be observed in zebrafish and *Drosophila* embryos undergoing rapid cell divisions before zygotic genome

activation. More over, these RT changed after cell cycle remodelling and initiation of zygotic transcription (Hug *et al*., 2017; Hyrien *et al*., 1995; Kaaij *et al*., 2018; Sasaki *et al*., 1999; Seller *et al*., 2019; Siefert *et al*., 2017). The same was observed during one-cell mouse embryos in which changes in the spatio-temporal pattern of replication were associated with 3D chromatin architecture remodelling at the four-cell stage of embryonic development (Ferreira and Carmo-Fonseca, 1997; Ke *et al*., 2017).

Epigenetic modifications have been related with specific developmental cell stages. It was discovered using different blastocyst-stem cell lines that many lineage-specific genes replicate early in those cells, which is related with an accessible chromatin state. Further analysis of modified histones allowed their differential identification showing the importance of chromatin remodelling with cell specification and lineage identity (Santos *et al*., 2010).

The RT for at least some chromosomal regions is different depending on the tissue (Brown *et al*., 1987; Dhar *et al*., 1989; Gilbert, 2002; Hatton *et al*., 1988; Selig *et al*., 1992), and changes in replication timing can be detected during the course of differentiation (Hiratani *et al*., 2004; Perry *et al*., 2004). These differences appear to be related to differential gene expression. In the study of Hiratani *et al.* 2008 (Hiratani *et al*., 2008), they analyse the RT on the differentiation of ESCs to address whether RT is a static or dynamic property of chromosomes during the course of differentiation. They performed a genome-wide analysis of three mESC lines before and after differentiation to neural precursor cells (NPCs). They found that despite the disparate genetic and temporal histories of these three cell lines, their replication profiles were virtually identical. mESCs have more replication domains, smaller in size, than neural precursors. During differentiation, some of the smaller domains are fused into larger ones. This phenomenon, termed “replication domain consolidation” affects a significant (20%) fraction of the genome. During differentiation, GC-poor/gene-rich domains experience early-to-late transitions. Strikingly, these changes are reversible: the RT program of iPSCs, was similar to that of ESCs. They concluded that specific changes in RT take place during the course of neural differentiation, generating a novel replication profile that is characteristic of NPCs, suggesting that RT profiles are stable within particular cell lineages but change significantly in response to major cell fate decisions.

Nevertheless, RT has been associated with both chromatin architecture and transcriptional activity of chromosomal regions which all together become plastic along cell differentiation, the exact molecular link between them have not been proved (Hiratani and Takahashi, 2019). Remarkably, this has been very challenging since almost all gene alterations of the discovered factors related in replication do not have major impact in RT program (Marchal *et al*., 2019). A major challenge for the field is to unravel all the factors that regulate RT to finally understand its complex relationship with a particular cell state.

2.4.3 Factors that regulate the replication timing

The RT is probably imposed by the complex organization and folding of chromosomes in the nucleus, the regulation of transcriptional programs by epigenetic mechanisms and the direct activity of *cis*- and *trans*-acting elements (Marchal *et al*., 2019).

Early studies showed a correlation between transcriptionally active genes that replicate in early S phase, whereas transcriptionally inert genes replicate later (Goldman *et al*., 1984). These early replication regions were also enriched in replication origins and ORC (Cayrou *et al*., 2011; Dellino *et al*., 2013; MacAlpine *et al*., 2010). Conversely, late replication is observed in origin-poor regions that have low gene density and are enriched in repressive epigenetic marks that are heterochromatin hallmarks (**Figure 31**).

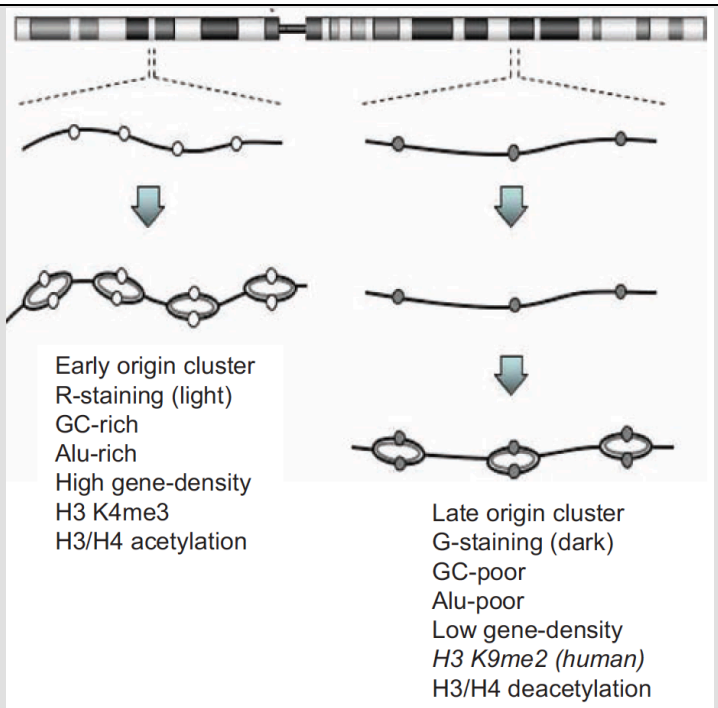


Figure 31. Genomic features that correlate with RT. Schematic of a human chromosome displaying Giemsa-staining banding. Reverse bands (R) correspond to early-replicating domains, and dark bands (G) correspond to late-replicating domains. Each domain consists of one or several clusters of origins arranged to fire simultaneously. Early origins are represented as white circles, late origins as grey circles (Mendez, 2009).

The open chromatin configuration of transcriptionally active promoter may favour the selection of replication origins that are located in the same area. Transcriptional regulators may positively (Knott *et al*., 2012) or negatively (Bellelli *et al*., 2014) affect the activation of specific origins. Moreover, there seems to be a general correlation between RT and GC content (Lucas and Feng, 2003).

The histone composition of the chromatin could also play a regulatory role in the replication process. In most organisms, the nucleosome is composed of the four core histones H2A, H2B, H3 and H4, plus the linker histone H1. The nature of this linker histone and its presence vary during developmental stages. It has been suggested that the association of certain H1 variants could trigger a more compact chromatin structure, resulting in late replication (Lucas and Feng, 2003).

The chromatin modification that correlates more strongly with early RT is histone acetylation. Tethering a histone deacetylase (HDAC) to the locus in an erythroid cell line was sufficient to change its RT from early to late (Goren *et al*.,

2008). The idea that RT has the capacity to influence chromatin structure, has been supported by the recent demonstration that DNA sequences packaged in nucleosomes characteristic of late replication (i.e. containing deacetylated histones) can be resembled with acetylated histones after shifting their RT to early S phase, and vice versa (Lande-Diner *et al*., 2009). Moreover, ectopic hyperacetylation of chromocentres leads to early replication of these regions (Casas-Delucchi *et al*., 2012), showing that histone acetylation has a positive role in the timing of early replication.

The nuclear envelope, chromatin domains and replication foci are the main nuclear structures that are involved in the regulation of origin activation. The formation of the nuclear envelope around chromatin at the end of cell division is a prerequisite for origin activation and initiation of DNA replication, but not for pre-RC assembly, which occur in the absence of nuclear membrane components (Coue *et al*., 1996; Newport and Spann, 1987; Sheehan *et al*., 1988). Laminins are intermediate filaments proteins that form a meshwork within the internal nuclear membrane and anchor chromatin, but they are also present in the nucleoplasm. Laminins are involved in DNA replication in *X laevis* egg extracts (Guelen *et al*., 2008). These findings suggest that nuclear membrane formation is an essential step in the selection of the origins that are to be activated.

Advances in genomics have enabled the correlation of DNA sequence with RT. These studies revealed the presence of regulators acting in *cis*, such as asynchronous replication and autosomal RNAs (ASARs) and early replication control elements (ERCEs). ASARs are long non-coding RNAs that drastically delay the replication of the chromosomes they are coating (Donley *et al*., 2015; Donley *et al*., 2013; Stoffregen *et al*., 2011), while ERCEs are DNA sequences identified to be required for early replication which happen to be sites of master transcription regulatory factor binding resembling transcriptional super enhancers (Rao *et al*., 2017; Schwarzer *et al*., 2017). Those findings provide an additional link between genome architecture and its function in RT.

Previously discussed, nuclear localization seems to correlate with the RT and may also be an important factor in the determination of the temporal program. Several lines of evidence indicate that proteins implicated in chromatin structure dynamics such as Sir3p (Stevenson and Gottschling, 1999), Ku70p (Cosgrove

et al., 2002), Rpd3p and Gcn5p (Vogelauer *et al*., 2002) and Rap1-interacting factor 1 (RIF1) (Foti *et al*., 2016) could influence RT by facilitating or impeding certain DNA-protein interactions. In addition, *trans* elements comprise factors directed involved in origin firing by allowing or inhibiting the activation of the replicative helicase (**Table 3**) (Marchal *et al*., 2019).

Table 3 | **Gene deletion effects on global replication timing and chromatin architecture in mammals**

Gene deletion	Effect on replication timing	Effect on 3D structure
Rif1 (KO and KD)	Genome-wide changes (MEF, mESC, HeLa)[164–166]	Localized changes (mESC)[166]
Cohesin (KD and KO of factors from the complex)	No change (HCT116)[77]	Loss of a part of TAD boundaries, no compartment change (HCT116, mouse hepatocytes)[73,80]
CTCF (KD and KO)	No change (KO: D.M.G., unpublished data, mESC)[75]	Loss of CTCF loops and local interactions, no compartment change (mESC, mNPC, HEK293T)[151,205]
Suz12 (KO)	No change (mESC)[66]	N/A
MeCP2 (KO)	No change (D.M.G., unpublished data)	N/A
G9a (KO)	Few local changes (mESC, mNPC)[112]	No change (mESC)[71]
H1a–H1d–H1e (triple KO)	No change (D.M.G., unpublished data)	Local changes (mESC)[206]
cMyc–nMyc (double KO)	No change (D.M.G., unpublished data)	N/A
BAF53a (KO)	No change (mESC)[207]	N/A
BAF250a (KO)	Local changes (mESC)[207]	N/A
Brg1 (KO)	Local changes (mESC)[207]	N/A

CTCF, CCCTC-binding factor; KD, knockdown; KO, knockout; MEF, mouse embryonic fibroblast; mESC, mouse embryonic stem cell; mNPC, mouse neural precursor cell (derived from ESC); N/A, data not available; TAD, topologically associated domain.

Adapted from (Marchal *et al*., 2019).

Most of our knowledge of trans-acting factors that regulate RT comes from studies in yeast. For example, in *Saccharomyces cerevisiae*, fork head protein homologue 1 (Fkh1) and Fkh2 are global regulators or early replication (Knott *et al*., 2012). Until now, RIF1 is the only *trans*-acting factor from yeast to humans that is capable to produce changes in RT similar as the ones observed during cell differentiation (Cornacchia *et al*., 2012; Dileep *et al*., 2015; Yamazaki *et al*., 2012).

2.4.4 RIF1 a global regulator of replication timing

This section is destined to the only genome-wide regulator of RT that is until now known, whose function is conserved among eukaryotes, RIF1. I will begin by a brief introduction of its discovery and function in yeast. Then, I will explain its role in mammalian cells and what has been shown in *Xenopus*. After that, I

will present how RIF1 achieve its role in RT. Finally, I will present how is RIF1 dynamics during the cell cycle and early embryogenesis.

RIF1 was originally discovered as a telomere-binding factor in budding yeast (Hardy *et al*., 1992), involved in telomere length regulation in both budding and fission yeast. There, it binds to subtelomeric regions and suppresses their replication. In *rif1*Δ fission yeast, twice as many origins are activated compared to checkpoint mutants. Interestingly, more than 100 early-firing origins are downregulated or their firing timings delayed in *rif1*Δ, suggesting that RIF1 negatively regulates late-firing origins and positively regulate early firing origins. These results indicate that RIF1 plays a global role in regulating the origin-firing program in fission yeast (Hayano *et al*., 2012).

On the contrary, metazoan RIF1 does not play a role in telomere maintenance but its major role is the establishment of RT domains (Cornacchia *et al*., 2012; Ryba *et al*., 2010). The most striking effect of RIF1 depletion in mammalian cells is the loss of the mid-S phase-specific pattern of replication foci. In RIF1-depleted cells, an early S phase-like pattern of foci prevails throughout S phase, and the late S phase pattern, characterized by replication foci at the heterochromatin segments, appears at the end of S phase. It was also observed that RT profiles also undergo dramatic changes when altering *Rif1*. Genome-wide analyses in *Rif1* knockout mouse MEF cells indicated that both early-to late and late-to-early changes in RT occurred in over 40% of the replication segments, resulting in fragmentation of RT domains (Cornacchia *et al*., 2012).

RIF1 is also present in *Xenopus* egg extracts, there it was found to be strongly recruited to replicating chromatin while its depletion increased DNA synthesis and origin firing. In addition, RIF1 depletion had a significant impact in the chromatin-binding behaviour of checkpoint proteins of the DNA damage response after induction of double strand breaks (Alver *et al*., 2017; Kumar *et al*., 2012).

RIF1 is highly expressed in undifferentiated ESCs. In differentiated cells, RIF1 levels are much lower, whereas replication domain sizes are larger (Hiratani *et al*., 2008). Further analysis of RIF1 sub-localization found it in nuclease-insoluble structures within nuclei generating chromatin loops. RIF1 labeling overlaps with mid-S replication foci, suggesting that RIF1 generates nuclear

structures that are specifically required for establishing mid-S replication domains (**Figure 32**). Chromatin loop sizes increase in *Rif1*-depleted cells, indicating that RIF1 is required for correct chromatin loop formation.

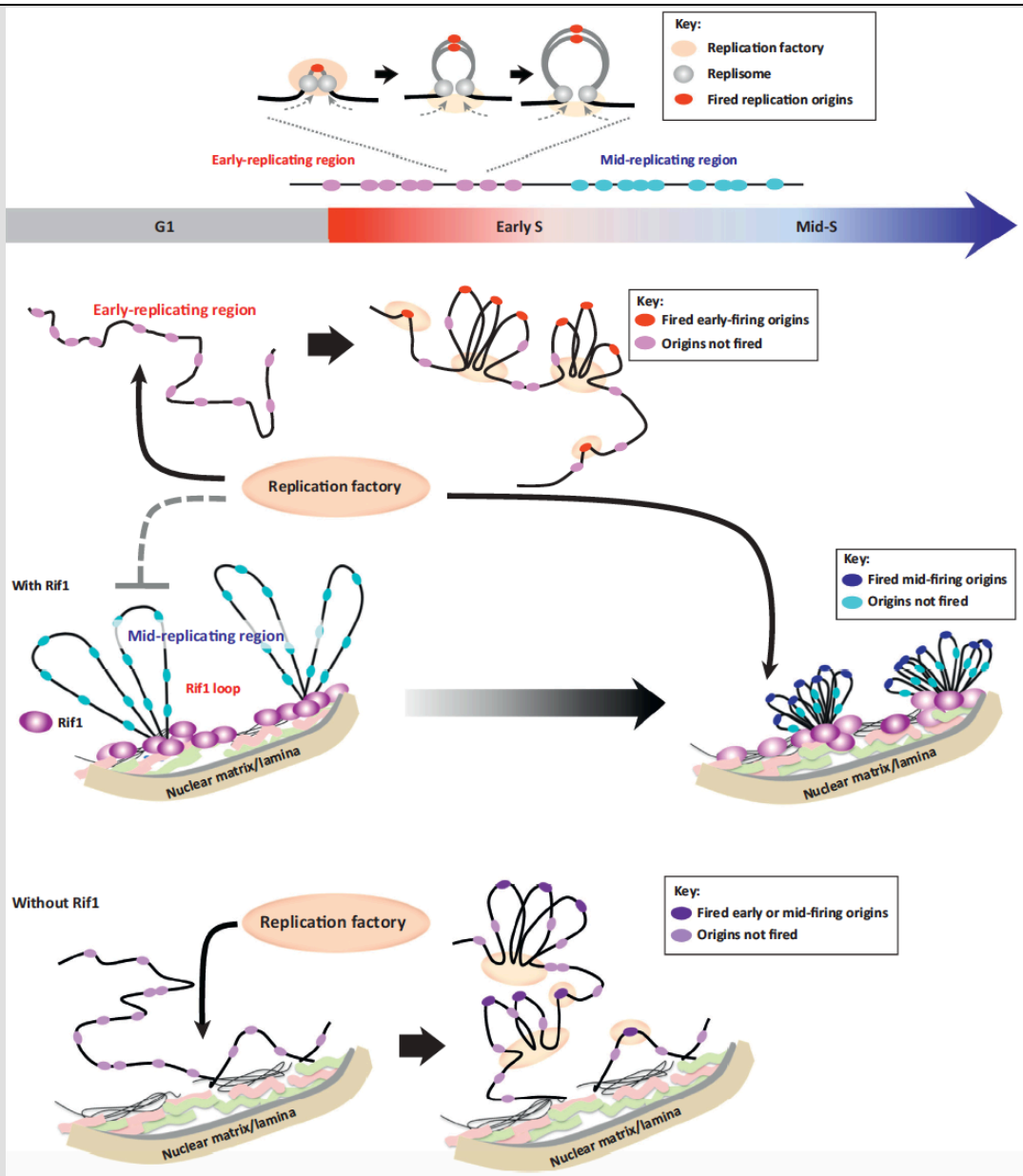


Figure 32. A model for regulation of replication timing domains in higher eukaryotes. Replication occurs at factories where two replisomes are held together and replicating DNA strands are passed through as bidirectional DNA synthesis proceeds, generating a loop consisting of the replicated daughter molecules (replication loops; shown in grey above the cell cycle bar). In the early-replicating domains (upper), chromosomes, whose conformations are not constrained during G1, can associate with replication factories where the clusters of early origins are simultaneously replicated. In the mid-replicating domains (middle), RIF1 generates specific chromatin loop structures (which we call "RIF1-loops" to distinguish them from replication loops) in G1, and origins present in the RIF1-loops are sequestered and kept inactive until mid-S phase. RIF1 associates with insoluble nuclear structures which could hold together multiple RIF1-loops. At mid-S phase, the origins in the RIF1-loop are activated through association with the axis of the RIF1-loop. Again, the selection of

origins to be activated could be dynamic and stochastic, and thus the sizes and numbers of replication loops generated from one RIF1-loop may vary from one cell to another and from one cell cycle to the next. How the origins in the RIF1-loop are kept from activation in early S phase and how they become activated after mid-S phase are unknown. We also do not know if any factors are responsible for replication loop formation during early S phase and if any factors sequester the late-replicating domains, which are not described here but show a distinct spatial distribution. In the absence of RIF1 (lower), the early S-phase domains re intact but the RIF1-loops are disrupted, releasing mid-S phase origins sequestration. Thus, the majority of the chromosomes (except the late-replicating heterochromatin segments) are replicated in the early S phase pattern throughout the S phase except for very late S phase. The replication loops in the main part of the figure are shown by single lines, even though they comprise two daughter molecules; both replicated and unreplicated DNA strands are shown in black. Below the cell cycle bar, darker-colored circles show fired origins whereas lighter-colored circles show origins not fired. Origins in early-replicating domains, in mid-replicating domains, and in disrupted replication domains (due to loss of RIF1) are shown in red, blue and purple, respectively (Yamazaki *et al*., 2013).

In addition, RIF1 was observed to interact with G-quadruplex (G4) structures that have been implicated in the establishment of the DNA RT. The presence of RIF1 with G4 generates specific chromatin domains, which replicate at mid/late S-phase (**Figure 33**) (Moriyama *et al*., 2017).

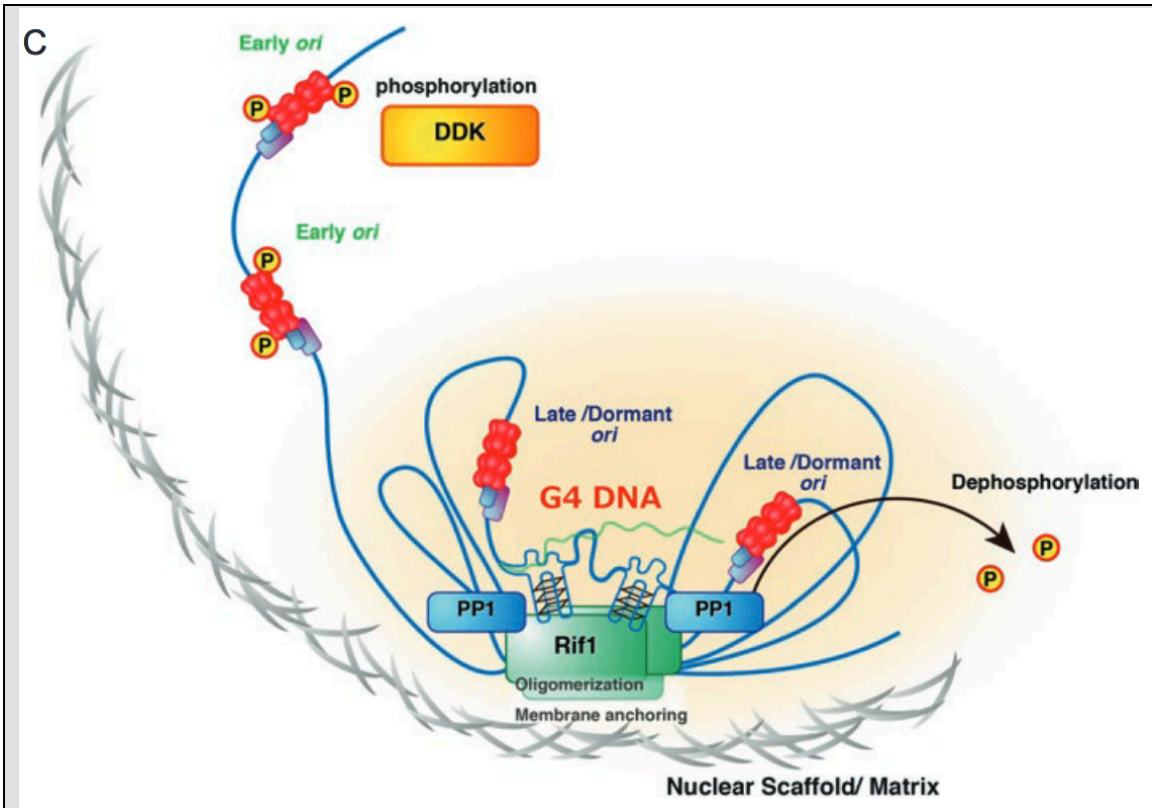


Figure 33. Interaction of RIF1 with G-Quadruplex. RIF1 binds to intergenic G4 structure to generate replication-suppressive schromatin domain near nuclear periphery by facilitating the chromatin loop formation through its G4 DNA-binding and oligomerization activities (Moriyama *et al*., 2017).

In mechanistic terms, RIF1 regulates origin firing through its interaction with PP1 phosphatase in yeast, *Drosophila*, mouse, human and *Xenopus* (Alver *et al*., 2017; Hiraga *et al*., 2014; Hiraga *et al*., 2017; Sreesankar *et al*., 2015; Sukackaite *et al*., 2017). PP1 has the ability to dephosphorylate the MCM complex at its Cdc7-dependent phosphorylation sites, suggesting that RIF1 delay DNA replication initiation of late replicating chromatin by antagonizing phosphorylation by Cdc7 (Hiraga *et al*., 2014) (**Figure 34**).

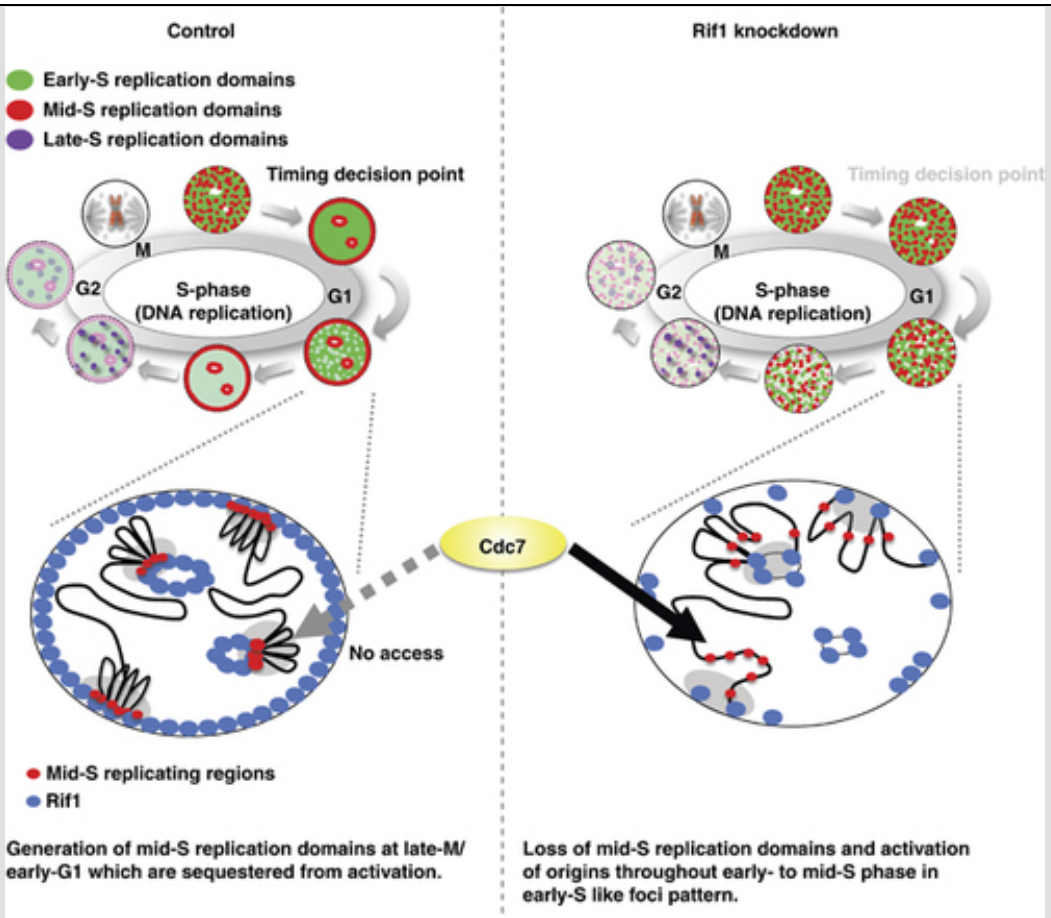


Figure 34. A model for RIF1-mediated determination of replication timing domains. (Left) Normally, RIF1 binds to nuclear-insoluble structures at late-M to early-G1, generating mid-S replication domains some of which are clustered at nuclear periphery as well as around nucleoli. This could be related to TDP known to occur at early-G1. The origins associated with the mid-S domains are sequestered from activation until mid-S-phase (shown with dotted grey arrow emanating from Cdc7). (Right) In RIF1-depleted cells, mid-S replication domains are not generated and the origins normally associated with mid-S domains are scattered throughout the nuclei. This permits access of Cdc7 (shown with solid arrow) and other replication factors to mid-S origins throughout early- to mid-S-phase, resulting in stimulation of initiation events (Cdc7-mediated phosphorylation of MCM and chromatin loading of Cdc45 and PCNA, etc.) at early-S-phase (Yamazaki *et al*., 2012).

Overall, RIF1 has at least three mechanisms to regulate RT, the first as a regulator of 3D genome organization (Foti *et al*., 2016), secondly by its role in counteracting origin activation with PP1 (Dave *et al*., 2014; Hiraga *et al*., 2014; Hiraga *et al*., 2017; Mattarocci *et al*., 2014) and finally it also has a less studied role in promoting origin licensing in human cells by protecting ORC1 degradation during G1 phase (Hiraga *et al*., 2017).

During mitosis, RIF1 dissociates from chromatin and re-associates with it at late M/early G1 in a manner that is resistant to nuclease treatment. Thus, RIF1 must generate mid-S replication domain structures by early G1. Interestingly, the timing decision point was reported to occur during early G1 concomitant with chromatin repositioning (Dimitrova and Gilbert, 1999; Li *et al*., 2001). Likewise, it is an intriguing possibility that RIF1 may play a role at the timing decision point in establishing RT domains.

The importance of RIF1 in early development has been underscored by its high expression level in undifferentiated ESCs and by the early embryonic lethality of the knockout mice (Buonomo *et al*., 2009; Loh *et al*., 2006). However, it was found in early *Drosophila* embryos that Cdk1 prevents RIF1 from slowing down DNA replication by driving its removal from the chromatin. Here, they propose that the normal down regulation of Cdk1 at the MBT, allows RIF1 localization at late sites of the chromatin thus initiating cell cycle slowing (Seller and O'Farrell, 2018). Thus, the chromatin architecture defined by RIF1 during early development continues to influence various biological events throughout the life of the organism.

Based on all the evidence, it seems that Rif1 could be the molecular link between domain-wide RT and the 3D genome architecture that has been extensively observed. Remarkably, it was found that RIF1 also contributes to the RT changes observed during developmental transitions. Since Rif1 is a big protein with over 250 kD, it contains several domains including the DNA and the PP1 interacting sites, however the function of its other domains holding possibly other interaction sites remains unsolved (Kobayashi *et al*., 2019).

Further elucidation of RIF1 mechanism of action as well as the discovery of its protein interactome will help to clarify the biological significance of RT regulation and its relationship with 3D genome architecture and development.

3. YAP, THE DOWNSTREAM EFFECTOR OF THE HIPPO PATHWAY

I would like to end this introduction by providing information about YAP, one of the major effectors of the Hippo pathway, on which I focussed my PhD project. This signalling pathway has been extensively studied because of its role in organ size control during development and regeneration. I will begin by a brief description of the Hippo pathway, pin-pointing the structural domains of YAP, as well as its regulation. Then, I will present the recent findings about its interaction with chromatin remodelling complexes and the role of YAP in stem cell biology. Finally, I will describe the recent findings of my laboratory showing the implication of YAP in the control of DNA RT of post-embryonic RSCs.

3.1 The Hippo pathway

The Hippo signaling pathway was first discovered in *D. melanogaster* in an attempt to find tissue growth regulators (Justice *et al*., 1995; Xu *et al*., 1995). Further studies in flies elucidated the distinct components of the pathway, which are remarkably evolutionarily conserved among metazoans. Since then, the study of the Hippo pathway has attracted scientists in search for the mechanisms that regulate organ development and regeneration.

The heart beating of the Hippo pathway is essentially a cascade of kinases, transcription coactivators and DNA-binding proteins (**Figure 35**). The phosphorylation cascade begins with the activity of Mammalian sterile 20-like 1/2 (MST1/2; Hpo is the ortholog in *Drosophila*) (Pan, 2010; Zhao *et al*., 2011), which then activates a second family of kinases the Large tumor suppressor homolog 1/2 (LATS1/2; Wts in *Drosophila*) (Pan, 2010; Zhao *et al*., 2011). Importantly, the kinase cascade of the Hippo pathway is conserved throughout the eukaryotic kingdom (Varelas, 2014). The principal targets of these kinases are the paralagous co-transcriptional regulators Yes-associated protein (YAP also known as YAP1) (Sudol, 1994) and transcriptional co-activator with PDZ-binding motif (TAZ) (Kanai *et al*., 2000) (Yki is the homolog in *Drosophila*).

YAP/TAZ regulation and function are shared in many aspects and thus here it will be mentioned collectively as YAP/TAZ unless specified otherwise.

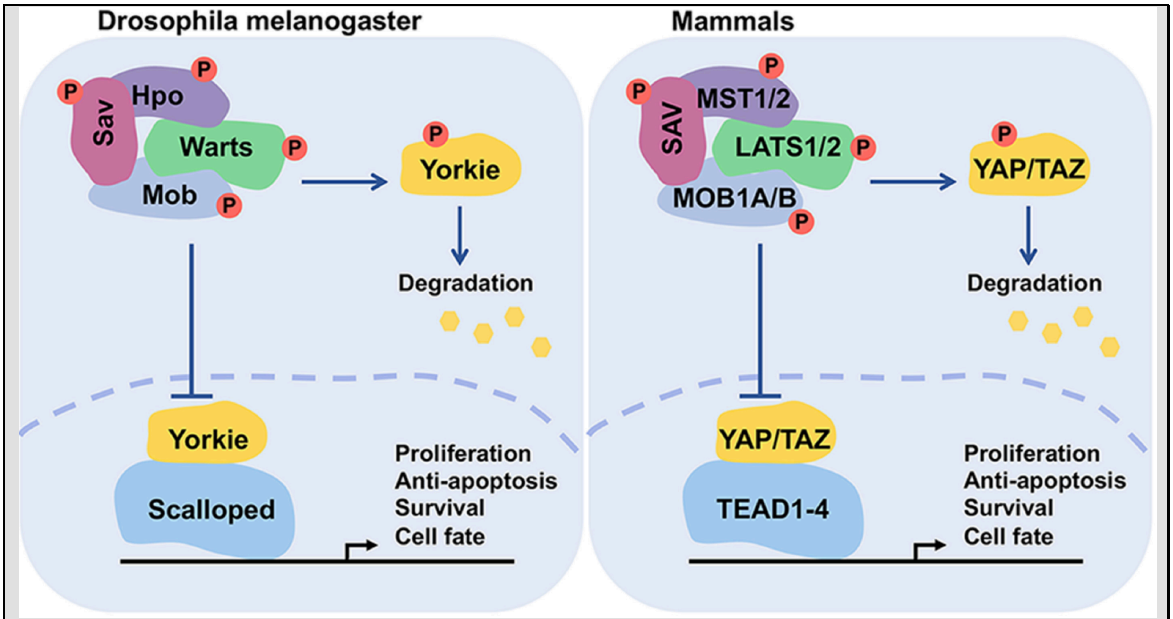


Figure 35. Functional conservation of the core components of Hippo pathway. The functional conserved factors of *Drosophila melanogaster* and mammals are matched by color. This network controls the transcriptional events for regulating cell proliferation, survival, and death (Chen *et al*., 2019).

When the Hippo pathway is on, the group of kinases in cooperation with the scaffold proteins Salvador (SAV1; Sav in *Drosophila*) and MOB kinase activator 1A and 1B (MOB1A and MOB1B; Mats in *Drosophila*), phosphorylate and thus impede YAP/TAZ localization in the nucleus by facilitating their binding with 14-3-3 proteins and promoting their proteosomal degradation (Dong *et al*., 2007; Lei *et al*., 2008; Liu *et al*., 2010; Zhao *et al*., 2010; Zhao *et al*., 2007), resulting in gene repression. On the contrary, when the Hippo kinases are inactive, non-phosphorylated YAP/TAZ enter the nucleus where they rely on DNA binding factors to execute their transcriptional functions, since they lack a DNA binding motif. For example, in the Hippo pathway, YAP/TAZ interact with the TEA domain-containing sequence-specific transcription factors (TEAD1 to TEAD4; Sd in *Drosophila*). Some of their target genes are *Ctgf, Cyr61, Ankrd1, Bic5, Axl* which are implicated in promoting proliferation and inhibiting apoptosis (Piccolo *et al*., 2014). Overall, Hippo signaling serves to prevent the transcriptional activity of the downstream effectors YAP/TAZ.

3.2 Structural features of YAP

YAP/TAZ share many structural domains. Interestingly, the gene duplication leading to YAP and TAZ happened in vertebrates, and their differential expression patterns are important in the development of various organs in *X. tropicallis* and zebrafish (Hong *et al*., 2005; Hu *et al*., 2013; Nejigane *et al*., 2011).

Peculiarly, YAP was originally identified as a protein interacting with the c-Yes tyrosine kinase (Sudol, 1994), before it was functionally related as an effector of the Hippo pathway. Subsequent studies have demonstrated that YAP is a transcriptional coactivator with a potent trans-activation domain in the C-terminal region (Yagi *et al*., 1999). Within this domain exists a conserved tyrosine residue (Y407 in human YAP) that can be phosphorylated and thus regulating its transcriptional role by a not very clear mechanism that depends on the cell type, cell environment and the responsible kinase (Jang *et al*., 2012). In addition to that, there are one or two WW domains in the central region of YAP, depending on alternative splicing. Finally, the N-terminal region of YAP is responsible for interaction with the transcriptional enhancer factor domain (TEAD) family (Vassilev *et al*., 2001) (**Figure 36**).

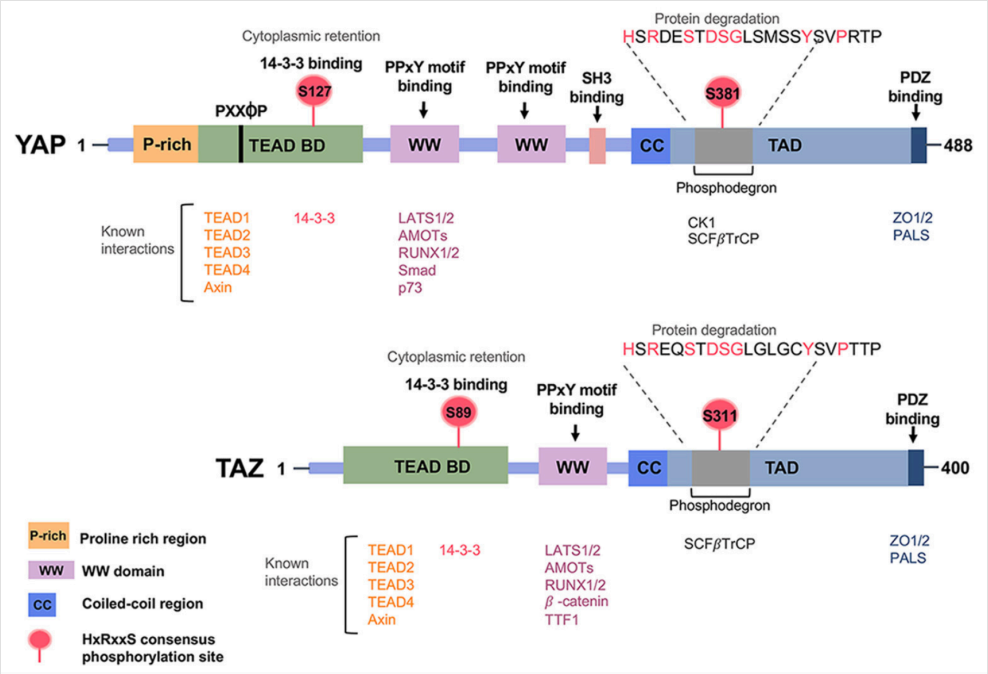


Figure 36. Regulatory domains of YAP/TAZ. Prominent regions include the WW domains, the coiled-coil (CC) domain, the SH3-binding domain, the TEAD transcription

factor-binding domain, the transcriptional activation domain (TAD) and the PDZ-binding motif (Chen *et al*., 2019).

In the C-terminal region, YAP contains a PDZ-binding domain motif, which interacts with other PDZ domains. The PDZ domains are commonly present in transmembrane or cytoskeleton associated proteins (Ye and Zhang, 2013) and it is suggested that this domain serves to direct YAP/TAZ localization (Oka and Sudol, 2009; Remue *et al*., 2010). In addition, inside the C-terminal region of YAP/TAZ there is a serine-rich phosphodegron motif, which phosphorylation targets YAP/TAZ ubiquitylation and proteasome-mediated degradation.

The N-terminal region of YAP, that mediates its binding with TEAD, consists of two short alpha helices with an extended loop containing a PxxΦP motif (Φ is a hydrophobic residue) (Chen *et al*., 2010; Li *et al*., 2010). It has been demonstrated that point mutations that disrupt the binding of YAP with TEAD decrease proliferation and tumorigenic phenotypes (Lamar *et al*., 2012; Zhang *et al*., 2009; Zhao *et al*., 2008).

Importantly, the most visible domain that confers signaling specificity is the WW domain that mediate interactions with many transcription factors. It recognizes a linear proline-rich motif found in a variety of proteins composed of two prolines (PP) followed by any aminoacid (x) and a tyrosine (Y), known as a PPxY or PY motif (Bork and Sudol, 1994; Sudol *et al*., 1995; Sudol and Harvey, 2010). This interaction with other transcription factors is important to regulate its activity for example by controlling its localization within the cell.

The key residue mediating YAP binding to 14-3-3 is Ser127 in human and Ser112 in mouse (Basu *et al*., 2003; Kanai *et al*., 2000). This LATS1/2-dependent phosphorylation is a signature of the Hippo pathway that regulates its nuclear localization. Regulation of YAP can occur at multiple levels, such as gene expression level (Wu *et al*., 2008), or protein level through both the ubiquitin-proteasome system and autophagy (Liang *et al*., 2014; Ma *et al*., 2019), and through post-translational modifications (**Table 4**) that affect the subcellular localization, protein-protein interaction partners and transcriptional activity of YAP (He *et al*., 2016).

Table 4. Post-translational modifications controlling TAZ/YAP activity

Modification	Regulatory enzyme	Functional consequence
YAP		
S61-p	LATS1/2	Cytoplasmic retention?
S109-p	LATS1/2	Cytoplasmic retention?
T119-p	CDK1	Cell cycle regulation
S127-p	LATS1/2	14-3-3 binding/cytoplasmic retention
S164-p	LATS1/2	Cytoplasmic retention?
S289-p	CDK1	Cell cycle regulation
S367-p	CDK1	Cell cycle regulation
S397-p	LATS1/2	Primer for S400/403-p
S400-p	CK1ε/δ	Degradation/β-TrCP recruitment
S403-p	CK1ε/δ	Degradation/β-TrCP recruitment
Y407-p	ABL/SRC/YES	Altered nuclear activity
K494-meth	SET-7	Cytoplasmic retention
TAZ		
S58-p	GSK3β	Degradation
S62-p	GSK3β	Degradation
S66-p	LATS1/2	Cytoplasmic retention?
S89-p	LATS1/2	14-3-3 binding/cytoplasmic retention
S117-p	LATS1/2	Cytoplasmic retention?
S311-p	LATS1/2	Primer for S314-p
S314-p	NEK1/CK1ε/δ	Degradation/β-TrCP recruitment
Y321-p	ABL	Altered nuclear activity

Adapted from (Varelas, 2014).

3.3 YAP integrate multiple inputs at cell and tissue level

In contrast to classic signal transduction pathways that are controlled by a dedicated ligand (for example Notch, Wnt, TGFb), activity of YAP is regulated by an ever-expanding network of factors and mechanisms. These include the Hippo pathway, cell-cell adhesions, cell polarity, extracellular forces exerted by the cell microenvironment (including the elasticity of the extracellular matrix, tissue stretching and shear forces), metabolic pathways and extracellular growth factors. Several of these inputs reflect the structure and organization of cells themselves, leading to the idea that YAP/TAZ integrate the “architectural” features of cells and tissues (Gaspar and Tapon, 2014; Halder *et al*., 2012; Irvine, 2012) (**Figure 37**).

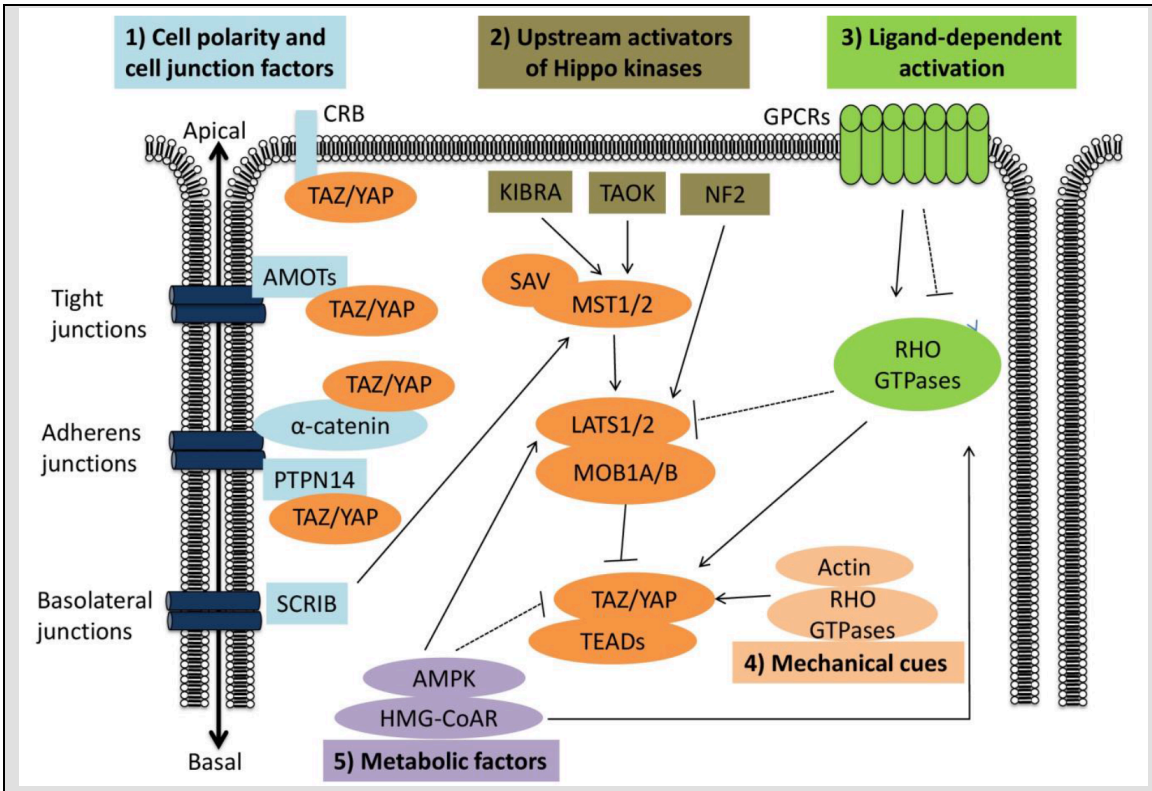


Figure 37. Regulation of the Hippo pathway. 1) Cell polarity and cell-cell junction factors that activate Hippo kinases or sequester YAP/TAZ, 2) Upstream positive regulators of the Hippo pathway, that activate MST1/2 or LATS1/2, 3) Soluble factors binding GPCRs: soluble factors and hormones can either activate or inhibit YAP/TAZ (via RHO GTPases), 4) mechanical cues, such as extracellular matrix stiffness and cell density, which activate YAP/TAZ independently on Hippo kinases, via RHO GTPases and the actin cytoskeleton, and 5) metabolic factors including the energy sensor AMPK that inhibits YAP/TAZ both directly and by activating LATS1/2, and HMG-CoA reductase, the central enzyme of the mevalonate pathway, that activates YAP/TAZ via prenylation of RHO GTPases (Maugeri-Sacca and De Maria, 2018).

3.4 YAP recruitment to the chromatin

YAP/TAZ are transcriptional co-activators that require other molecules to exert transcriptional control of their target genes. Interestingly, YAP/TAZ have been shown to associate with chromatin-remodeling complex proteins to alter chromatin structure and thus affect accessibility and activity of target genes. As mentioned before, YAP/TAZ canonically bind to TEAD family members (Vassilev *et al*., 2001), which is the best study interaction, however there are other DNA bound factors in which a direct interaction has been reported such as p73, Tbx5, SMADs, RUNX1/2 and PNOX1 (Cabochette *et al*., 2015; Grannas *et al*., 2015; Rosenbluh *et al*., 2012; Strano *et al*., 2001; Zaidi *et al*., 2004).

Recently, it has been suggested that YAP is able to modulate chromatin accessibility. Using chromatin conformation and transcript expression experiments, it was found that overexpression of YAP in cardiomyocytes made chromatin more accessible for TEAD binding motifs in the genome of those cells (Monroe *et al*., 2019). Moreover, Yki/YAP/TAZ recruitment to the chromatin has been demonstrated by the interaction with chromatin remodelers SWI/SNF complex, GAGA factor, Mediator complex, Ncoa6 and NuRD complexes which as a consequence impact on target gene transcription (Beyer *et al*., 2013; Jin *et al*., 2013; Kim *et al*., 2015; Monroe *et al*., 2019; Oh *et al*., 2013; Oh *et al*., 2014; Qing *et al*., 2014; Saladi *et al*., 2017; Skibinski *et al*., 2014; Song *et al*., 2017; Zhu *et al*., 2015). When Yki/YAP/TAZ interact with most of these chromatin-remodeling complexes, they up regulate target genes through remodeling DNA packing and organization (Bayarmagnai *et al*., 2012; Chang *et al*., 2018; Jin *et al*., 2013; Oh *et al*., 2013; Oh *et al*., 2014; Qing *et al*., 2014; Saladi *et al*., 2017; Skibinski *et al*., 2014; Song *et al*., 2017; Zhu *et al*., 2015). However, it has been shown that when binding to NuRD, YAP/TAZ/TEAD function negatively by repressing target genes (Beyer *et al*., 2013; Kim *et al*., 2015) (**Figure 38**).

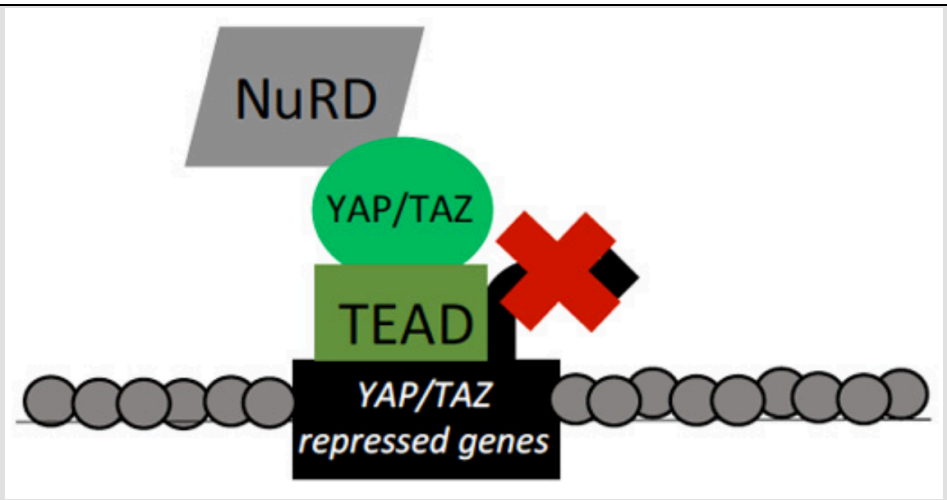


Figure 38. YAP/TAZ/TEAD interactions with the NuRD complex. YAP/TAZ-TEAD bind targets and recruit the NuRD complex to repress target gene expression. This repression is mediated through dual ATP-dependent chromatin remodeling and histone deacetylase (HDAC)-mediated thistone deacetylase functions of the NurD complex to ultimately reduce chromatin accessibility. YAP/TAZ targets repressed by NuRD recruitment included genes that drive apoptosis and promote senescence (Hillmer and Link, 2019).

Additionally, YAP/TAZ/TEAD has been associated with the activator protein 1 (AP-1) to function at distal enhancers (Zanconato *et al*., 2015). Genes regulated by this mean are involved in S phase and cell mitosis, making them relevant in tumorigenesis (Zanconato *et al*., 2015). It was discovered that AP-1 binding to YAP/TAZ helps to regulate the TGFβ/Smad3 signaling (Qin *et al*., 2018) and moreover AP-1 has been involved in the recruitment of BAF-SWI/SNF chromatin-remodeling complexes to alter chromatin accessibility to enhancers (Vierbuchen *et al*., 2017).

Overall, the response of Yki/YAP/TAZ is suggested to be context-specific and depends on the interaction with protein complexes. It is expected that further studies between Yki/YAP/TAZ and the machinery that manages chromatin structure will bring more insights of the role of the Hippo pathway in the control of transcription. For example, it was discovered in the mouse brain development that inactivation of LATS1/2 (the upstream inhibitors of YAP/TAZ) produced a major increase in transcription activity known as hypertranscription that included genes related to proliferation. This observation was associated with YAP/TAZ activation, since additional deletion of these genes restored the brain development. Interestingly, following a peak in cell proliferation with an accelerated cell cycle, the neural progenitors failed to differentiate and eventually died because of replication stress, DNA damage and p53 activation (Lavado *et al*., 2018).

3.5 Hippo pathway in stem cell regulation and early development

The best-known role of the Hippo pathway is to orchestrate organ development and control tissue homeostasis through modulation of cell proliferation, apoptosis, migration, and differentiation (Fu *et al*., 2017; Wang *et al*., 2017). Hippo pathway also regulates stem cell self-renewal and expansion and tissue regeneration (Camargo *et al*., 2007; Mo *et al*., 2014; Ramos and Camargo, 2012). In the next, section I will focus in the role of Hippo pathway in stem cell biology.

3.5.1 Pre-implantation embryonic development

During early animal development, YAP/TAZ localization is crucial for the first cell fate events that include the renewal of stem cells and the control of their dedifferentiation (Varelas, 2014). It was discovered that the nuclear/cytoplasmic distribution of YAP/TAZ defines the first cell fate decision in the mouse embryo which involves cells to become either trophectoderm (TE) or inner cell mass (ICM). In the blastocyst YAP/TAZ accumulates in the nuclei of outer TE and in the cytoplasm of ICM (Nishioka *et al*., 2009) (**Figure 39**). Nuclear YAP/TAZ binds with TEAD inducing the expression of *Cdx2* (Home *et al*., 2012). Accordingly, deletion of *Tead4* results in the loss of *Cdx2* expression and blocks the establishment of the TE (Nishioka *et al*., 2008; Yagi *et al*., 2007). Moreover, deletion of both *Yap* and *Tead* results in the embryos dying at the morula stage caused by cell fate specification defects in TE or ICM (Nishioka *et al*., 2009). Interestingly, deletion of only *Yap* or *Taz* does not impact pre-implantation defects, indicating redundancy activity at this embryonic stage (Hossain *et al*., 2007; Morin-Kensicki *et al*., 2006).

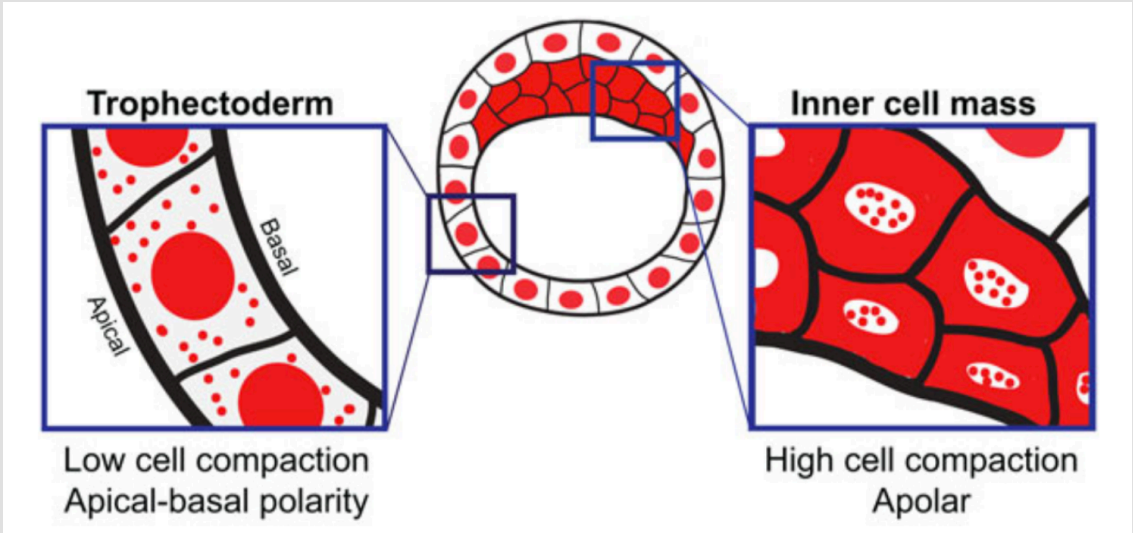


Figure 39. Dynamic changes in YAP/TAZ localization direct pre-implanttion development. As the mouse embryo develops from the morula toblastocyst stage (illustrated), the inner and outer cells acquire differences in apical-basal polarity that alter the localization of TAZ/YAP (red). TAZ/YAP is nuclear localized in the less compacted, but polarized, outer cells that give rise to the trophectoderm. By contrast, compactation of the apolar cells within the inner cell mass promotes cytoplasmic YAP/TAZ localization (Varelas, 2014).

Deletion of Lats/Lats2 increase nuclear localization of YAP/TAZ and amplified expression of *Cdx2*, which results in defects of ICM specification (Nishioka *et al*., 2009). Additionally, knockout of *Mob1a* and *Mob1b* results in developmental defects and embryos dye at embryonic day 6.5, prior to gastrulation (Nishio *et al*., 2012). Although the exact mechanism is still unknown, YAP/TAZ localization in the pre-implantation embryo is linked to cell polarity changes.
In addition, the Hippo pathway interacts with other signaling pathways such as TGFβ/SMAD and Wnt/β-catenin signaling to control cell fate in the developing embryo (Varelas and Wrana, 2012). Interestingly, localization of YAP/TAZ correlates with SMAD2/3 localization in pre-implanted embryos (Varelas *et al*., 2008; Varelas *et al*., 2010b). In response of Wnt, YAP/TAZ interact and affect the function of Dishevelled (DVL) and β-catenin proteins (Heallen *et al*., 2011; Imajo *et al*., 2012; Varelas *et al*., 2010a).

3.5.2 Hippo pathway in ESCs

Evidences demonstrate that nuclear YAP/TAZ activity is required for the maintenance of ESCs pluripotency. For example, YAP/TAZ form complexes with SMAD2/3 (Varelas *et al*., 2008; Varelas *et al*., 2010b), furthermore this complex binds to TEAD and the core stem regulator OCT4, mediating the pluripotent state (Beyer *et al*., 2013). This complex associates with other molecules that make up the nucleosome remodeling and deacetylation (NuRD) complex to regulate the expression of pluripotency genes and limit genes for mesoderm specification (**Figure 40**).

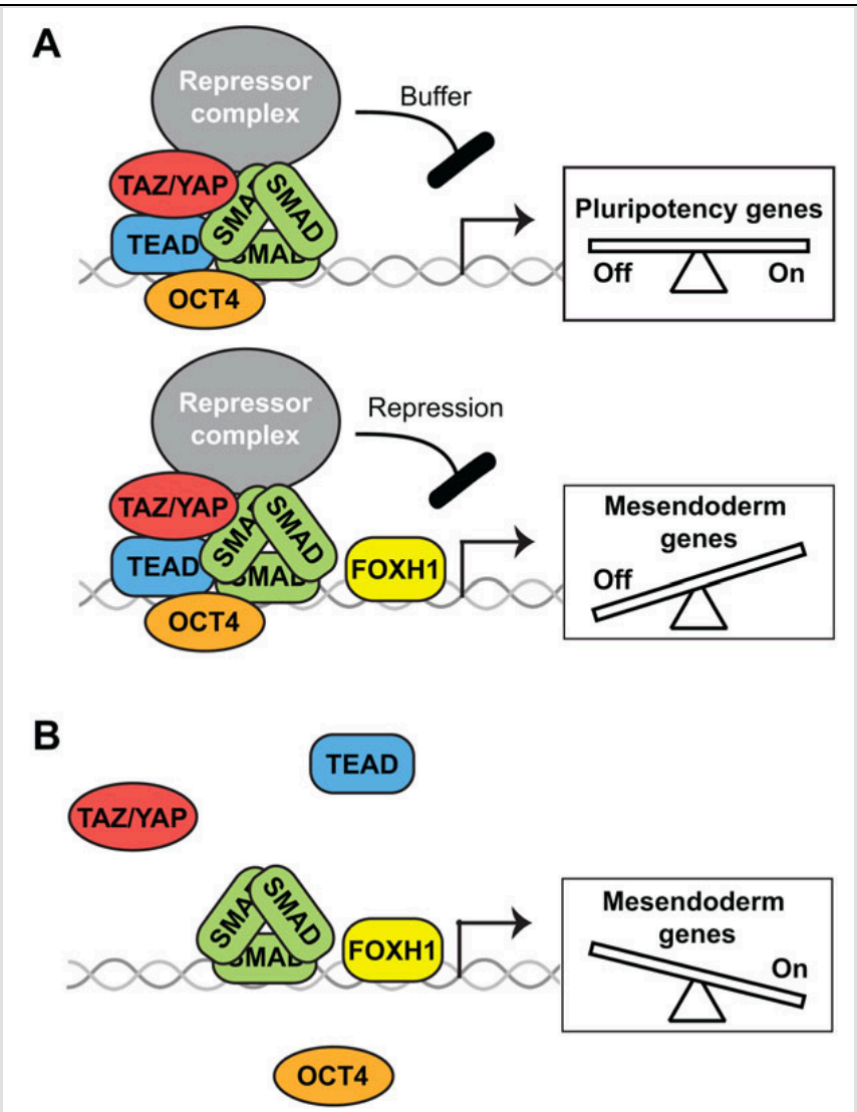


Figure 40. Roles for YAP/TAZ in human embryonic stem cell (ESC) specification. YAP/TAZ, TEADs, TGFβ-induced SMAD2/3-SMAD4 complexes, and OCT4 assemble on the promoters of genes important for controlling embryonic pluripotency and mesendoderm specification in human ESCs. (A) TAZ and YAP recruit the NuRD repressor complex (gray) to buffer and maintain an optimal expression level of pluripotency genes (top), while suppressing the expression of mesendoderm genes (bottom). (B) Upon mesendoderm specification, the TAZ/YAP-TEAD-OCT4 complex dissociates, allowing the TGFβ-induced SMAD2/3-SMAD4 complexes to activate the FOXH1 transcription factor, consequently driving mesendoderm gene expression (Varelas, 2014).

Notably, YAP was shown to be essential in the reprogramming of fibroblast into iPSCs (Takahashi and Yamanaka, 2006). Moreover, ectopic expression of YAP in the nucleus of mESC promotes their self-renewal and increases the efficiency of reprogramming (Lian *et al*., 2010). Additionally, in human iPSCs, LATS2 knockdown was shown to increase reprograming efficiency (Qin *et al*., 2012).

3.6 Hippo pathway in the central nervous system

In the CNS, YAP was found to affect the homeostasis of NSCs, NPCs and glial cells during development. For example in *Drosophila*, Yki overexpression promotes the expansion of neuroepithelial cells and blocks their differentiation (Reddy *et al*., 2010). *In vivo*, YAP/TAZ activation increase the self renewal of embryonic NSCs, since injection of YAP virus vector into the mouse brain of E13.5 embryos promoted NSCs proliferation via its binding with TEAD (Han *et al*., 2015). However, it was recently found that YAP is not essential for the normal self-renewal of NSCs since YAP knockout experiments did not significantly affect the proliferation of NSCs *in vivo* and *in vitro* (Huang and Xiong, 2016).

Regarding the differentiation of NSCs, it was shown that YAP limits neurogenesis through its interaction the ECM microenvironment blocking the nuclear localization of β-catenin thus inhibiting NSCs differentiation (Rammensee *et al*., 2017). It was discovered in the hindbrain of zebrafish that YAP/TAZ function as mediators of the mechanical forces that take place during brain development. When YAP/TAZ activity begins to decrease, NPCs proliferation also decreases and neural differentiation occurs (Voltes *et al*., 2019).

In addition it was found that YAP plays an important role in the homeostasis of NPCs, which are the precursor of NSCs. Interestingly, the proliferation of neuroepithelial cells is strongly influenced by YAP activity. It was found in the developing chick neural tube and *X. laevis* embryos that YAP expression correlates with $SOX2^+$ neuroepithelial progenitors and that YAP knockdown results in a decrease number of neuroepithelial cells. Accordingly, YAP gain-of-function by the injection of *yap* RNA into *Xenopus* embryos expanded neural progenitor cells (Cao *et al*., 2008; Gee *et al*., 2011). Additionally, YAP overexpression in the chick stimulated neural crest migration *in vivo* (Kumar *et al*., 2019). Moreover, YAP loss of function by shRNAs or a YAP dominant-negative construct leaded to cell death in the chick neural tube (Cao *et al*., 2008).

The mechanism of action dictating the control of YAP/TAZ in neural progenitor expansion is not well understood, but evidence suggest that it involves the activation of genes related to cell cycle, such as cyclin D1, and inhibition of pro-differentiation factors, such as NeuroM (Cao *et al*., 2008). Moreover, YAP attenuation by shRNA was found to inhibit the cell cycle exit by an accumulation of FoxD3 expression, reduced proliferation, and enhanced apoptosis in the chick dorsal neural tube (Kumar *et al*., 2019). As a result, YAP has an impact on both the activation and repression of transcriptional events that dictate neuroepithelial progenitor fate.

PAX3 is a paired box transcription factor which expression is localized in cells that give rise to the neural plate and thus is important for neural crest induction. Interestingly, YAP and TEAD are factors that directly regulate the expression of *Pax3* (Gee *et al*., 2011; Milewski *et al*., 2004). Moreover, YAP overexpression also expands *Pax3* expression in *X. laevis* embryos (Gee *et al*., 2011), while expression of a dominant-negative TEAD2 mutant decreases *Pax3* expression and impairs neural crest development (Milewski *et al*., 2004).

Interestingly, the knockout of NF2, a protein important for mammalian brain hippocampus and neocortical layer development, was found to increase total YAP and YAP localization in the nucleus. Moreover, YAP overexpression produced a phenotype similar to NF2 inactivation, resulting in hippocampus malformation, which can be restored when both proteins are knocked out together (Lavado *et al*., 2013). Mechanistically NF2, inhibit YAP function in NPCs to promote their differentiation. When this inhibition is lost, YAP function is up regulated, leading to excessive proliferation and dysplasia of the corpus callosum (Bao *et al*., 2017).

Evidence shows that YAP is also important in the homeostasis of cortical astrocytes. YAP is localized in NSCs and astrocytes and its deletion resulted in reactive astrogliosis (Rojek *et al*., 2019). YAP knockout was found to decrease the proliferation and differentiation of cortical astrocytes (Ouyang *et al*., 2020). It was proposed that YAP promote astrocytic proliferation and differentiation trough the stabilization of BMP2-SAMD1 signaling (**Figure 41**) (Huang *et al*., 2016).

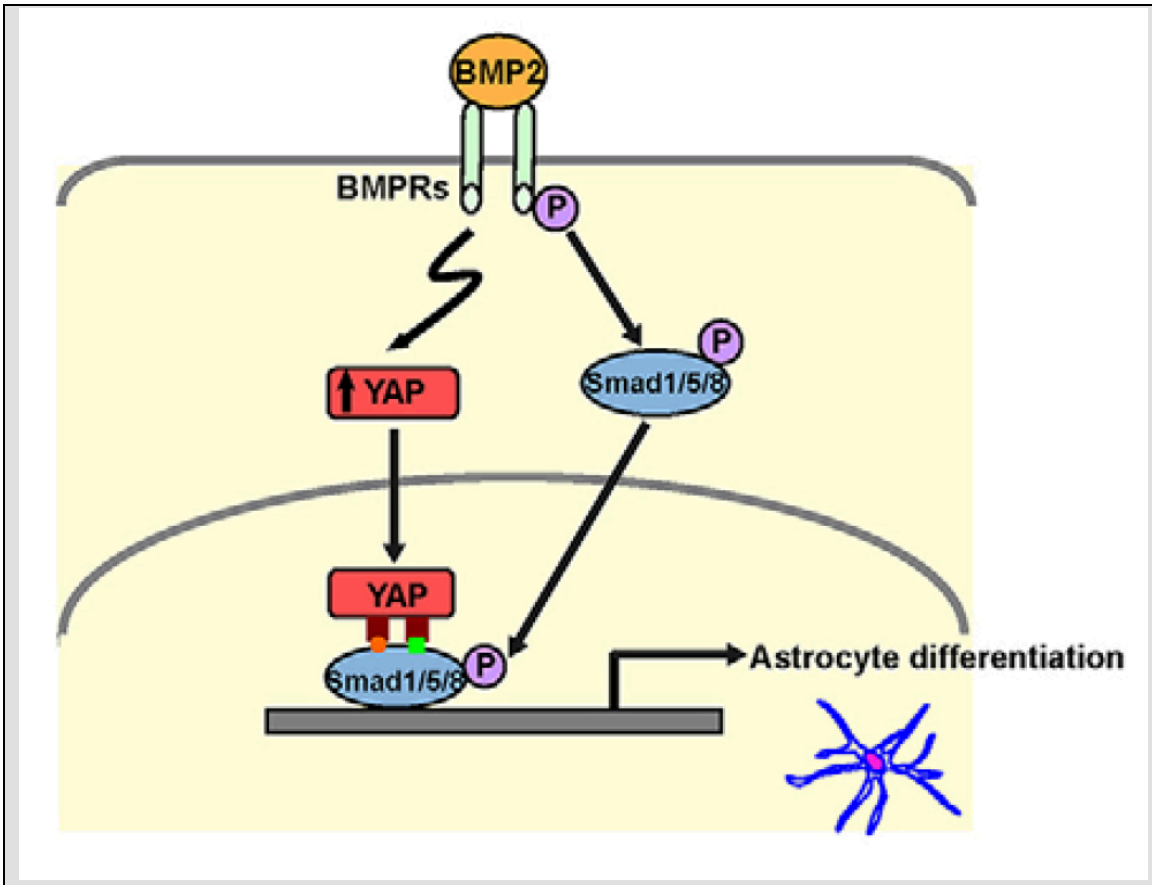


Figure 41. YAP stabilizes the BMP2-SMAD1 signaling. Model of YAP functions in neocortical astrocytic differentiation. BMP2 treatment promotes YAP nuclear translocation, and the nuclear/active YAP interacts with and stabilizes SMAD1 and is required for BMP2-induced pSMAD1/5/8 signaling and astrocytic differentiation (Huang *et al*., 2016).

YAP was found to be important for the morphology and maturation of oligodendrocytes, the glial cells that myelinate neural axons in the CNS. It was found that the mechanical stress in cell cultures of oligodendrocytes that were YAP knock-down had alterations in their axon morphology. Moreover, YAP overexpression in mice prevents oligodendrocytes to extend their axons and have an appropriate cell morphology (Shimizu *et al*., 2017).

Interestingly, it was found that the Hippo pathway was related with the transcriptional dysregulation that occurs during Huntington's disease (HD). Nuclear localization of YAP was decreased in HD post-mortem cortex and in NSCs derived from HD patients. Additionally, YAP was found to interact with huntingtin and Hippo pathway genes were altered in HD. Overall, they found the implication of YAP in the pathogenesis of HD through the activation of the Hippo

pathway kinases that reduce YAP's nuclear activity thereby causing neuronal death in HD (Mueller *et al*., 2018).

Recently, YAP was proposed to be responsible of the neocortex expansion during mammalian evolution, since YAP is highly expressed in ferret and human basal progenitors that are related with higher proliferation rates, but low in mouse basal progenitors, which do not have the same proliferation capacity. The genetically activation of YAP in mouse basal progenitors leaded to its proliferation and production of upper-layer neurons. Finally, YAP dysregulation in ferret and human developing cortex resulted in a decrease in cycling basal progenitors (Kostic *et al*., 2019).

3.6.1 Implication of YAP in the retina

In zebrafish embryos, knockdown of YAP decreases neurogenesis affecting brain, eyes and neural crest development (Jiang *et al*., 2009). Differentiation of mouse retinal progenitor cells is controlled by YAP activity. It was found that YAP overexpression enhances proliferation and decreases differentiation of postnatal mouse retinal progenitors, in part by repressing the activity of pro-neural transcription factors (Zhang *et al*., 2012).

The Hippo pathway also mediates neural fate decisions in the developing eye of *D. melanogaster*. In this context, Hippo pathway and retinoblastoma pathway were found to direct differentiation of photoreceptors. More precisely, mutations in wts (LATS1/2 orthlog) or hpo (MST1/2 ortholog), together with retinoblastoma mutations resulted in dedifferentiation of photoreceptors into a progenitor-like state (Nicolay *et al*., 2010). However, these effects are not a consequence of altered cell cycle that are typically associated with Retinoblastoma mutations, suggesting that mutations of both Hippo pathway and Retinoblastoma promote to stem cell-like state.

Interestingly, during optic vesicle development, the differentiation of optic vesicle progenitors into RPE is compromised in *yap*$^{-/-}$ zebrafish embryos (Miesfeld *et al*., 2015). YAP/TAZ were identified as key elements for RPE genesis by its interaction with TEAD and its nuclear localization. Noteworthy, mutation in the YAP-binding domain of TEAD1 causes Sveinsson's chorioretinal

atrophy, a genetic eye disease characterized by chrorioretinal degeneration (Fossdal *et al*., 2004; Li *et al*., 2010)(31,32).

Studies from my laboratory showed that the Hippo pathway is related to photoreceptor degeneration. They found for the first time that YAP and TEAD1 are expressed in mouse Müller cells. Interestingly, they expression was increased in a photoreceptor degenerative context using a mouse model of retinitis pigmentosa in which two well-characterized target genes, *Ctgf* and *Cyr61* were upregulated (Hamon *et al*., 2017).

Recently, my laboratory found in *Xenopus* that YAP is required for Müller cell-cycle re-entry and they showed in mouse that YAP is sufficient to activate Müller cell proliferation (**Figure 42**). Using conditional *Yap* deletion in Müller cells they found in moue retinas that these cells stay quiescently after damage related to reactive gliosis. Moreover, in *Xenopus* they showed that YAP is responsible for Müller cell proliferation in response to injury since blocking YAP function greatly impairs their proliferative response. Finally, they demonstrate that YAP's effect relies on EGFR signalling which is necessary for Müller cell proliferation (Hamon *et al*., 2019).

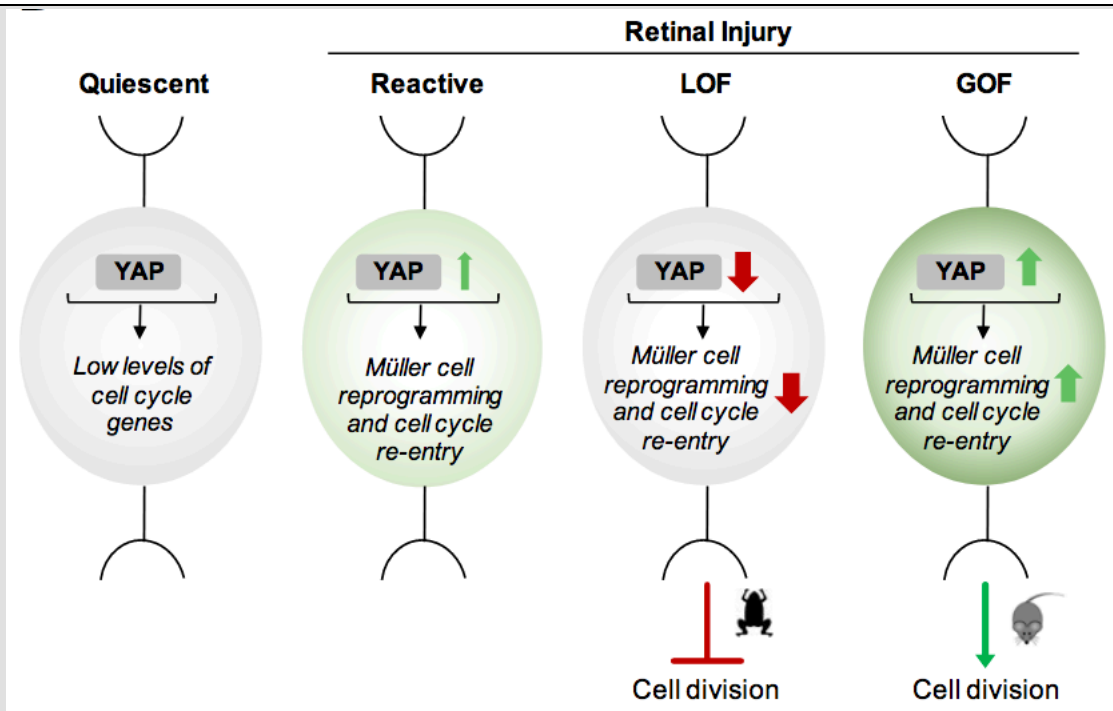


Figure 42. Model of YAP in the regulation of Müller glia cell cycle. In a quiescent Müller cell (grey), YAP expression maintains a basal level of cell cycle genes. Upon retinal degeneration, YAP level rises in reactive Müller cells (light green), which triggers the upregulation of reprogramming and cell cycle genes. YAP loss of function (LOF) impairs Müller cell reprogramming and cell cycle re-entry. In Xenopus, which is

endowed with regenerative properties, this prevents Müller cell proliferation. In mouse, YAP gain of function (GOF) is sufficient to enhance gene expression levels (dark green) and to trigger Müller cell proliferation (Hamon *et al*., 2019).

3.6.2 YAP in *Xenopus* retinal stem cells

Since YAP has an important role in the stemness properties of several tissues, our laboratory decided to study YAP function in post embryonic NSCs. They use as a model the retina of *X. laevis* since it is a well-characterized model to study stem cell biology. They found that YAP was specifically expressed in the CMZ of the retina and in Müller glial cells (Cabochette *et al*., 2015) (**Figure 43**).

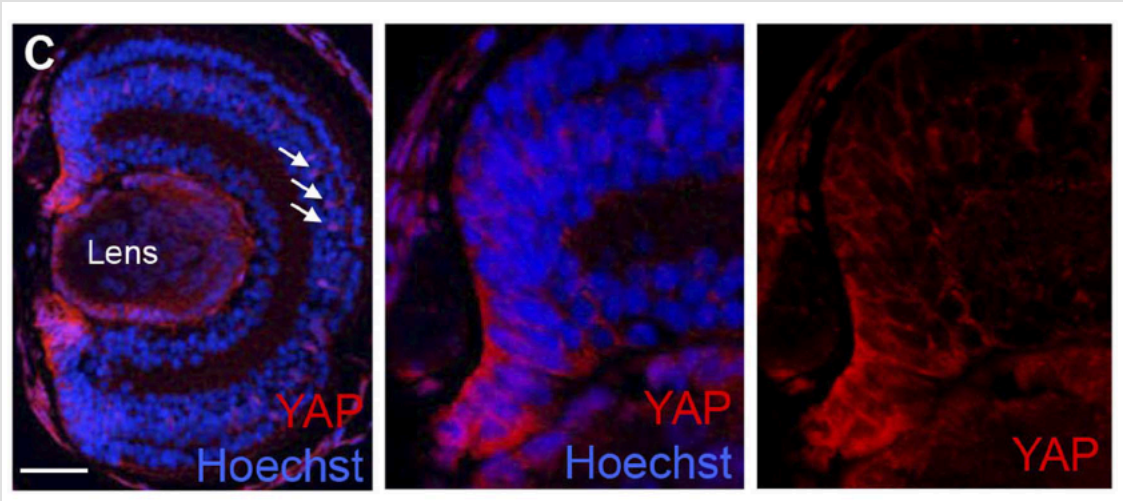


Figure 43. Localization of YAP in the retina of Xenopus laevis. C) Immunostaining with anti-YAP antibody on stage 42 retinal sections. YAP labeling is detected in the CMZ as well as in Muller glial cells (arrows) (Cabochette *et al*., 2015).

Then, they wanted to investigate whether YAP was essential for the growth of the post-embryonic retina. They knockdown *Yap* by photo-cleavable morpholino injections (allowing for inducible or reversible gene knockdown), which indeed suggested that YAP is required for the homeostatic control of post-embryonic retinal growth (Cabochette *et al*., 2015). Interestingly, *Yap* knockdown does not affect the number of stem cells but rather affects the proportion of time these cells spend in S-phase. Neither the total number, nor size-area within the CMZ was affected upon knockdown. *Yap* loss of function led to an accelerated S phase and an abnormal progression of DNA replication foci, a phenotype likely mediated by upregulation of *c-Myc* which has been associated to accelerate S-phase by increasing firing and origin density (Robinson *et al*., 2009; Srinivasan

et al., 2013). *Yap* knock-down increased the expression of p53 and p21 related to cellular stresses including DNA damage (Cabochette *et al*., 2015) (**Figure 44**). Together these findings suggest that YAP is required in adult retinal stem cells to regulate the temporal firing of replication origins and quality control of replicated DNA.

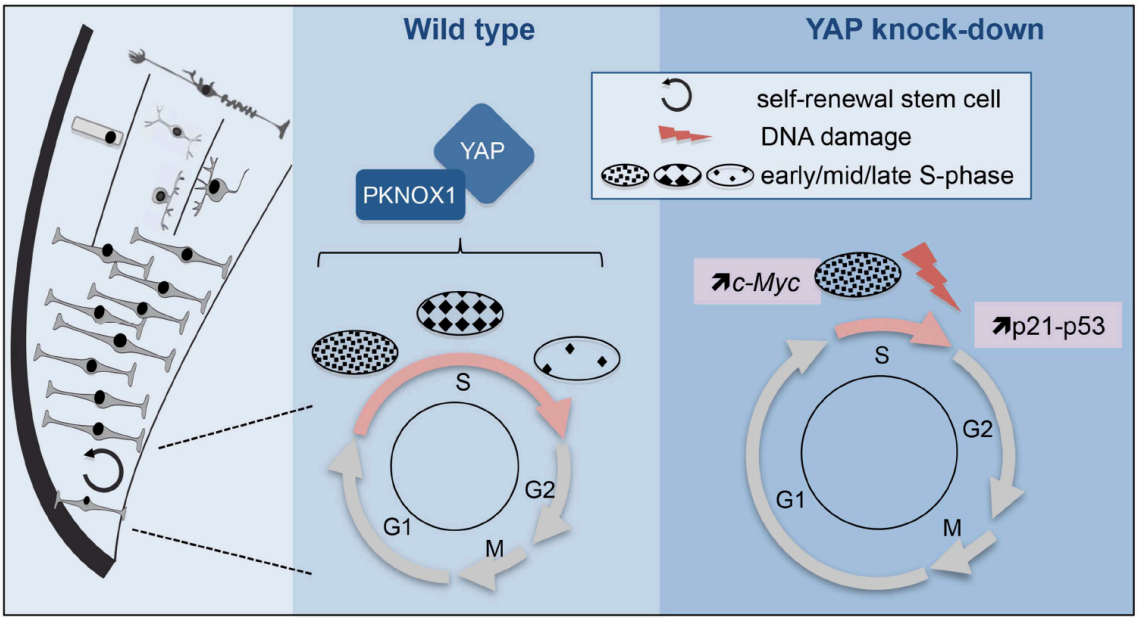


Figure 44. Model illustrating YAP function in retinal stem cells. YAP is expressed in the retinal stem cells (left panel). The middle panel shows the cell cycle of wild type retinal stem cells and the putative role of YAP/PKNOX1 complex in the control of S-phase temporal progression (represented by the distinct patterns of DNA replication foci). YAP knockdown (right panel) leads to a dramatic reduction of S-phase length likely due to c-Myc-dependent premature firing of late replication origins. This result in increased occurrence of DNA damage enhanced p21 and p53 expression and eventually cell death (Cabochette *et al*., 2015).

As it has been described, YAP is a pleiotropic protein with different functions regarding stem cells biology. The recent data obtained by my laboratory raised the question to investigate what is the molecular mechanism behind YAP function in the control of DNA replication. With this objective in mind, my PhD project will try to characterize YAP function using *Xenopus* egg extracts (*in vitro*) and early embryos of *X. laevis* (*in vivo*) to assess its action on DNA replication dynamics and find potential partners that mediate its function. Our work will assess whether YAP functions as an additional *trans*-acting factor that regulates the spatial-temporal program of replication in stem cells.

OBJECTIVES

Recently, the study of the mechanisms that govern stemness have been on the spotlight for the research community. Innovative therapeutic approaches can be envisaged that rely on the transplantation of iPSC or the reactivation of endogenous quiescent stem cells for the treatment of diseases that imply cell-loss. A property of stem cells that has attracted some attention is their peculiar RT, a signature that is shared among this cell state. Moreover, it is a stable and heritable property that dramatically changes upon differentiation and reprogramming, thus it has been suggested to be an epigenetic mark. However, little is known about its molecular regulation. With this in mind, discovering the molecules implicated in the regulation of DNA RT program, would provide insights about its biological relevance.

My host laboratory is interested in studying the molecular cues that regulate stem cell homeostasis to eventually develop stem cell based-treatments for retinopathies. Because the eyes of the frog *Xenopus* have a constant population of post-embryonic RSCs, they offer a powerful system to search for molecules that may be relevant for the function of adult stem cells. It was before my arrival to the laboratory, that they discovered the implication of the Hippo pathway in the homeostasis of RSCs. Precisely, they found that YAP, the downstream effector of the Hippo signaling pathway, was expressed specifically in the CMZ region and more interestingly they showed a novel function of YAP at regulating the time at which those cells replicate their DNA. In this context, the main goal of my project was to study whether YAP is directly involved in DNA replication dynamics and to further elucidate the molecular mechanisms behind this effect.

Objective 1. To characterize the function of YAP during DNA replication using the *Xenopus* egg extract system.

To know whether YAP is directly involved in DNA replication, the first aim was to take advantage of *Xenopus* egg extracts. This cell-free *in vitro* system is particularly well suited to the study of the mechanisms and dynamics of DNA replication.

Objective 2. To identify YAP binding partners during DNA replication.

To understand how YAP is recruited into the chromatin, our second objective was to identify its protein interactants, because YAP structure does not contain a DNA binding motif. We opted for a large-scale screen based on mass spectrometry followed by validation by co-immunoprecipitation assays.

Objective 3. To investigate the expression and role of YAP binding partners in retinal stem cells in *X. laevis,* as a manner to compare previously YAP findings.

Following the discovery of YAP partners that could mediate its role during DNA replication, we aimed at characterizing their function in the retina of *X. laevis* in a similar way as my host laboratory did before with YAP. Briefly, this includes studying the expression by *in situ* hybridization, performing knockdown experiments using a morpholino approach and analyzing replication foci and cell death.

Objective 4. To assess the function of YAP and its partners *in vivo* during DNA replication using early *X. laevis* embryos.

In *Drosophila*, zebrafish and *Xenopus* major changes in DNA RT happen before and post zygotic transcription activation. Moreover, the cell cycle of these organisms at early stages is characterized by fast cell divisions lacking gap phases. With this in mind, they offer a simplified *in vivo* system to study DNA replication dynamics. Our fourth objective was to study the effect of YAP and its partners in DNA dynamics taking advantage of early *X. laevis* embryos. The goal was to measure the rate of embryonic cell divisions after their protein depletion. To this aim, we decided to set up a recently discovered technique called Trim-away (Clift *et al*., 2018) to efficiently deplete maternal expressed proteins using their antibodies.

Overall, these objectives were expected to demonstrate whether YAP *per se* affects DNA replication dynamics *in vitro* and *in vivo* and provide insights about its protein interactions in this context.

MATERIAL & METHODS

In this section I would like to explain briefly some of the techniques that I used in my project that are not familiar in the field of developmental biology. The rest of the techniques and reagents can be found in the material and methods section of the article manuscript.

Replication in *Xenopus* egg extracts and immunodepletions

Egg extracts were obtained from unfertilized eggs and sperm nuclei from testis of *X. laevis* as described in (Blow and Laskey, 1986). Briefly, fresh eggs were collected from female frogs injected the day before with chorionic gonadotropin (500 U) to induce egg laying. Then eggs were first dejellied and activated since those eggs are arrested at metaphase of meiosis II. Extracts from activated eggs are able to enter the first mitotic interphase. Activation is done by the addition calcium ionophore (0.25 µg/mL). After activation, eggs are rinsed and by centrifugation eggs are crushed to release their cytoplasm (low speed supernatant) (**Figure 45**). To obtain protein depleted (YAP-depleted, ΔYAP and Mock depleted, ΔMock) extracts, protein A-sepharose beads were incubated with either anti-YAP rabbit antibody or purified anti-IgG rabbit (GE Healthcare) overnight/4°C. These beads were then incubated in the egg extracts for 30 min/4°C with rotation and separated by filtration using compact reaction columns (Thermo Fischer). Sperm nuclei (2000 nuclei/µl) were incubated in untreated, ΔMock or ΔYAP extracts in the presence of cycloheximide (250 µg/ml) and energy mix (7.5 mM creatine phosphate, 1 mM ATP, 0.1 mM EGTA, pH 7.7, 1 mM $MgCl_2$). Depending on the assay 20 µg biotin-dUTP (Roche Appled Science) or a-^{32}P-dCTP (3000 Ci/mmol) were added for DNA combing or radioactive nucleotide incorporation, respectively. Replication was allowed to continue, then samples were withdrawn at indicated time points and replication was stopped for posterior analysis. *In vitro* fertilization of *Xenopus* eggs with sperm was performed according to standard techniques (Sive *et al*., 2007), and developmental stages of embryos were determined according to Nieuwkoop and Faber (Gordon *et al*., 1994).

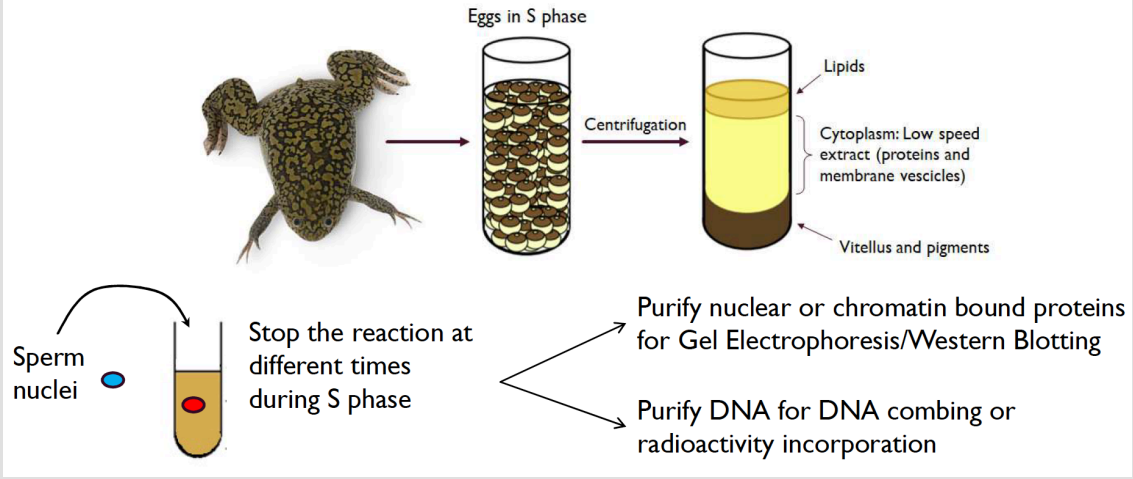


Figure 45. Preparation of *Xenopus* egg extract. Unfertilized fresh laid eggs are collected in a tube, then activated and by centrifugation (100,000 x *g*) three major layers are obtained: top layer contains lipids, bottom layer contains pigments and vitellus, and middle layer contains the cytoplasmic fraction with membranes, also known as low speed supernatant (LSS). This last one, when mixed with DNA from different sources initiates the DNA replication process.

DNA combing and detection by fluorescent antibodies

Sperm nuclei were incubated with the addition of 20 µg biotin-dUTP (Roche Appled Science). Replication was allowed until determined time points and samples were obtained and stopped by dilution. DNA fibers embedded in low melting agarose cubes were subjected to purification and DNA combing as described in (Marheineke *et al*., 2009) (**Figure 46**). Briefly, the melted block with the sample is poured into the reservoir of the combing apparatus, then a silanized coverslip is immersed into the solution and being removed vertically by a straight upward movement. DNA will be attached into both sides of the coverslip forming parallel lines. Biotinilated DNA replication is detected by immunofluorescence using AlexaFluor594 conjugated streptavidin followed by anti-avidin biotinylated antibodies. This was repeated twice, then followed by anti-DNA antibody, AlexaFluor488 rabbit anti-mouse, and goat anti-rabbit antibodies for enhancement (Gaggioli *et al*., 2013).

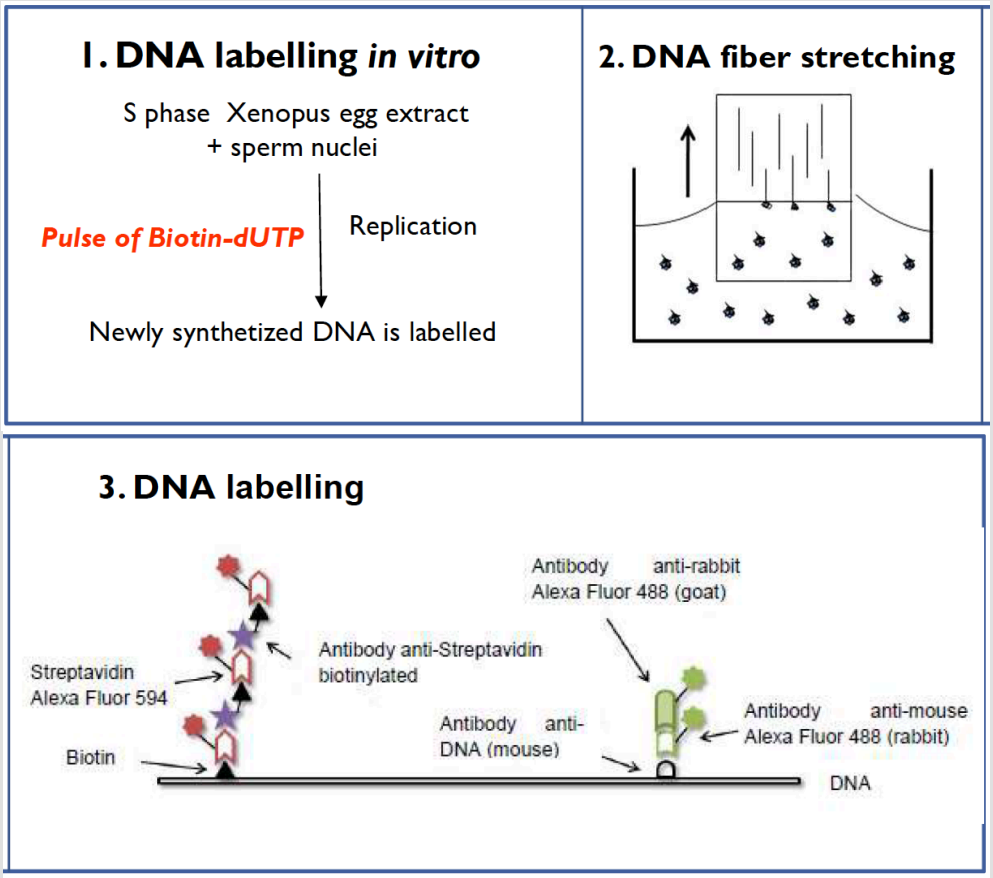


Figure 46. Visualization of DNA replication by DNA combing. Addition of biotin-dUTP to replicating egg extracts allows the labeling of newly synthetized DNA fibers. Then DNA is stick to a silanized coverslip forming straight fibers, which will be detected by immunofluorescence.

Molecular combing measurement and data analysis

DNA combing images were acquired and measured as in (Marheineke *et al.*, 2009). For each combing experiment a total of 24-35 Mb DNA was measured. The fields of view were chosen at random, unless mentioned otherwise. Measurements on each molecule were made using Image Gauge version 4.2 (Fujifilm) and compiled using macros in Microsoft Excel (2010). Replication eyes were defined as the incorporation tracks of biotin–dUTP. Replication eyes were considered to be the products of two replication forks, incorporation tracks at the extremities of DNA fibers were considered to be the products of one replication fork (**Figure 47**). Tracts of biotin-labeled DNA needed to be at least 1 kb to be considered significant and scored as eyes. When label was discontinuous, the tract of unlabeled DNA needed to be at least 1 kb to be considered a real gap. The replication extent was determined as the sum of eye

lengths divided by the total DNA length. Replication eye density was calculated as the total DNA divided by the total number of replication eyes. The midpoints of replication eyes were defined as the origins of replication. Eye-to-eye distances (ETED), also known as inter-origin distances, were measured between the midpoints of adjacent replication eyes. The means of fiber lengths were comparable inside each individual experiment in order to avoid biases in eye to eye distances. Incorporation tracks at the extremities of DNA fibers were not regarded as replication eyes, but were included in the determination of the replication extent, calculated as the sum of all eye lengths (EL) divided by total DNA. Box plots of ETED (with n ranging from 71-286) and EL (n=143-409) were made using GraphPad version 6.0 (La Jolla, CA, USA). Statistical analysis of repeated experiments have been included as means including ranks where possible. When experiments were repeated with a different egg extract replication extent differs at identical time scales because different egg extracts replicate nuclei with different replication kinetics. It is therefore difficult to include statistics of independent kinetics experiments.

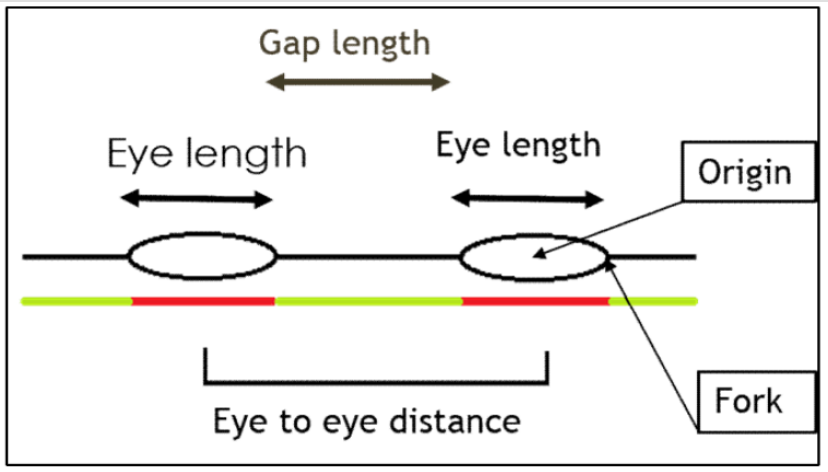


Figure 47. Schematic representation of the features analyzed by DNA combing. Cartoon representing different values that can be obtained by the DNA combing technique to study DNA dynamics. The green line represent a fiber of DNA and the red segments of the line are sites of DNA synthesis also known as replication eyes.

Protein depletion in early *Xenopus* embryos

Protein depletion of YAP and Rif1 proteins was obtained using a combined approach of microinjection of morpholinos and the recently developed Trim-Away technique (Clift *et al*., 2018). Briefly, the Trim-Away technique is based on

the activity of TRIM21, an E3 ubiquitin ligase with affinity to the Fc-region of an antibody and subsequent autoubiquitination of TRIM21 (**Figure 48**). In this study, anti-Rif1, anti-YAP or purified rabbit IgG were used to target endogenous proteins for degradation. Then movies of the embryos were taken with a time-lapse camera (Zeiss) and analyzed with Image Gauge version 4.2 (Fujifilm) to quantify the number of cells in the embryo.

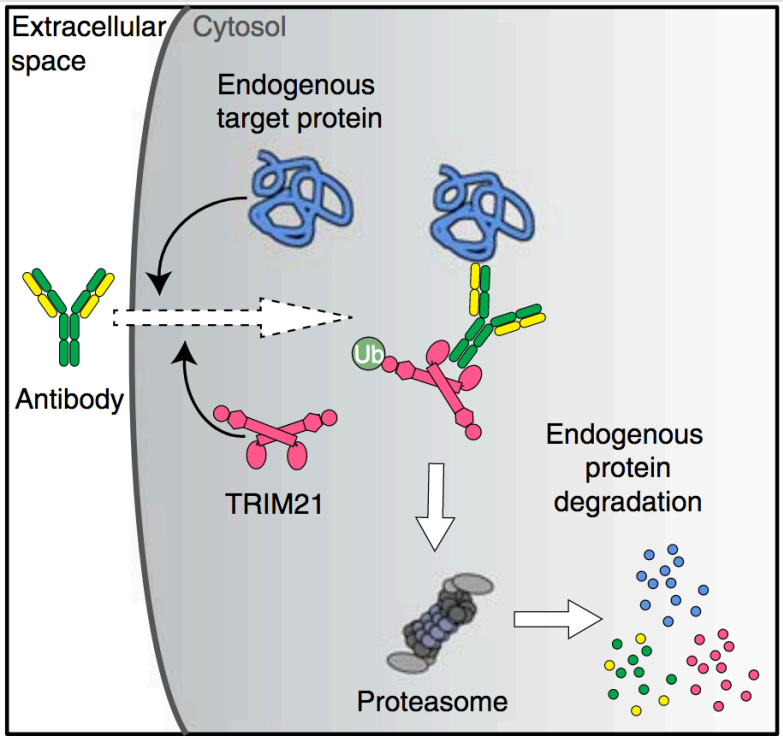


Figure 48. Schematic of the principle of Trim-Away. Exogenously antibody targeted to the protein to deplete is delivered to the interior to the cell. Then it binds to the endogenous protein and TRIM21 recognizes the Fc region of the antibody and mediates its degradation by the proteasome (Clift *et al*., 2018).

RESULTS

The ambition of my project was to determine whether YAP could directly be involved in the eukaryotic DNA replication process. This work has its roots in the previously findings of my host laboratory about YAP implication in the control of RT of retinal stem cells of *Xenopus*. Because YAP is a factor strongly associated to stem cells dynamics, that has been implicated in the regulation of transcription factors related with cell cycle and that has been shown to interact with chromatin remodelers, we found it very pertinent to further study its implication in the DNA replication process. To better characterize this novel YAP function, we utilized the *Xenopus* eggs extracts system, since it allows YAP depletion and the dissection of the molecular mechanism of YAP in DNA replication in absence of any transcription. Furthermore, we were interested to study YAP binding partners that could mediate the effect of YAP, since YAP needs to interact with other molecules to be recruited into the chromatin because it does not contain any DNA binding motif. For *in vivo* studies, we used *X. laevis* embryos since their retinas offer a unique system to study neural stem cells activity in their niche. In addition, the pre-MBT embryos of *Xenopus* offer a simplified *in vivo* system to study cell division rates of cell cycles composed only by M- and S-phases and where zygotic transcription is not active. My study shows that the Hippo pathway effector YAP is implicated in DNA replication and we propose a mechanism of interaction with RIF1 to control the speed of DNA synthesis and thereby of cell division rate in *Xenopus*.

Associated publication (to be submitted):

Rodrigo Meléndez García, Olivier Haccard, Hemalatha Narassimprakash, Muriel Perron, Kathrin Marheineke and Odile Bronchain. YAP interacts with RIF1 and sustains proper DNA replication dynamics and temporal program in *Xenopus.*

A. Models used in this study

In my study we used the *Xenopus* egg extracts to molecularly characterize *in vitro* the role of YAP during DNA replication as well as *Xenopus* early embryos for *in vivo* approaches. The advantage of the *Xenopus* egg extracts is that it allows the efficient immunodepletion of YAP and then the study of its function by a vast amount of pharmacological tools and techniques that offer this system, such as the DNA combing technique to directly visualize newly synthetized DNA fibers and the quantification of origin activation. Additionally, this system permitted us to find YAP interactants during DNA replication by the realization of YAP-IP and then mass spectrometry analysis. In the case of *in vivo* studies, we also took advantage of *Xenopus* retina. This system was helpful to compare the phenotype of genetic ablation of *Rif1*, the novel partner of YAP that we discovered, with the results previously obtained with *Yap* knockdown. Finally, we also decided to study the impact of YAP or RIF1 depletion in early division rates of pre-MBT embryos by setting up a recently developed technique to deplete maternally expressed proteins, the Trim-away technique.

B. Main results

- We found that YAP is recruited to the chromatin as early as DNA synthesis initiates and notably its presence in the chromatin is dependent on the assembly of pre-RC proteins.
- We showed that the immunodepletion of YAP in *Xenopus* egg extracts leads to accelerated DNA synthesis and increased activation of origins, suggesting that YAP function is to slow-down DNA replication.
- We identified RIF1 as a novel partner of YAP during DNA replication.
- *Rif1* knockdown in *Xenopus* embryos leads to a phenotype very similar as the one observed with *Yap* down regulation, in particular in DNA RT regulation of retinal stem cells.

- Finally, early protein depletion of YAP or RIF1 accelerated cell division rate of pre-MBT *Xenopus* embryos, presumably by shortening S-phase length due to accelerated DNA synthesis.

Taken together our results showed a direct implication of YAP in DNA replication dynamics and we identified RIF1 as a novel partner of YAP. We propose that together they act as breaks during the process of DNA replication.

ARTICLE

Rodrigo Meléndez García, Olivier Haccard, Hemalatha Narassimprakash, Muriel Perron, Kathrin Marheineke and Odile Bronchain.

YAP interacts with RIF1 and sustains proper DNA replication dynamics and temporal program in *Xenopus.*

YAP interacts with RIF1 and sustains proper DNA replication dynamics and temporal program in *Xenopus.*

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Key words:

Hippo/YAP pathway, RIF1, DNA replication, retinal stem cells, *Xenopus*

Highlights:

- YAP is recruited to chromatin during DNA replication in a manner dependent on the pre-replicative complex assembly.
- YAP controls DNA replication dynamics by limiting origin firing and slowing down DNA synthesis.
- RIF1 is revealed as a novel YAP binding-factor in replicating *Xenopus* egg extracts.
- RIF1, as previously shown for YAP, controls retinal stem cell DNA replication timing *in vivo*.
- Both YAP and RIF1 act as breaks during the process of early embryonic cell divisions in *Xenopus*.

Abstract

In eukaryotic cells, the initiation of DNA replication occurs asynchronously throughout S phase, from discrete sites on chromatin yielding early- and late-replicating territories on the genome. This process, known as the replication-timing program (RT), appears highly stable within a cell type and provides a robust epigenetic signature of cellular differentiation state. The RT can drastically changes during cell fate transitions and is deregulated in many disease states, including cancer. A major issue is thus to understand the mechanisms that orchestrate where and when a given segment of DNA is replicated.

We previously identified YAP (Yes-associated protein), a downstream effector of the Hippo signalling pathway, as a *bona fide* regulator of the RT in adult retinal stem cells. Here, we show that YAP is directly required for the control of DNA replication dynamics in *Xenopus* egg extracts. We find that YAP chromatin recruitment follows the process of DNA replication and is dependent on pre-replicative complex assembly. YAP negatively impacts the rate of DNA replication by limiting both the number of activated origins and rate of DNA synthesis. Besides, we unravel RIF1, a critical determinant of the RT, as a novel YAP-binding factor in replicating egg extracts. *In vivo*, using a Trim-Away approach, we find that both *Yap* and *Rif1* knock-downs lead to accelerated cell divisions during early cleavage stages in *Xenopus* embryos. Finally, we demonstrate that as YAP, RIF1 is required *in vivo* for the control of the RT in adult retinal stem cells.

Altogether, our findings unveil YAP implication in the regulation of replication dynamics and show RIF1 as a novel partner. We propose that YAP and RIF1 function as breaks during the process of replication to control the overall rate of DNA synthesis.

Introduction

Prior to cell division, DNA must be entirely and accurately duplicated to be transmitted to the daughter cells [1]. This is of utmost importance in stem cells which continuously self-renew and produce new cells needed for organ growth or maintenance. In most metazoan cells, replication initiates at several thousands of fairly specific sites called replication origins in a highly-orchestrated manner in time and space although no strict consensus DNA sequences have been identified so far [2,3]. In late mitosis and G1 phase, origins are first "licensed" for replication by loading onto chromatin the six ORC (origin recognition complex) subunits, then Cdc6 (cell-division-cycle 6) and Cdt1, and finally the MCM (mini-chromosome maintenance) 2-7 helicase complex, thus forming the pre-replicative complex (pre-RC, for review see Bell, S. P. & Dutta, A. [4]). Pre-RC is subsequently activated during S phase by cyclin- and Dbf4/Drf1-dependent kinases (CDKs and DDKs) which leads to the recruitment of many other factors, DNA unwinding and start of DNA synthesis at origins. In eukaryotes, segments of chromosomes replicate in a timely organized manner throughout S-phase. It is now widely accepted that the genome is partitioned in two regions of coordinated activation that can be visualized by pulse labelling experiments using nucleotide analogues [5]. During the first half of S-phase, the early-replicating chromatin, mainly transcriptionally active and localized to central regions of the nucleus, duplicates, while late replicating chromatin spatially located at the periphery of the nucleus awaits until the second half [6]. Recent advances in high-resolution chromatin capture methods confirmed chromatin allocation into these two domains [7,8]. This spatiotemporal pattern of DNA replication, also called DNA replication timing program (RT), has been found to be stable, somatically heritable, cell-type specific, and associated to cellular phenotype. Altogether these features makes the RT compatible with the definition of an epigenetic mark [9], and provides a specific signature associated to the cell state. This signature is indeed dramatically modified upon cell state changes [8,10–15] and deregulation of the RT is associated with many diseases, including cancer [16–20]. Despite major advances in technology and wealth of protocols to study DNA replication, the elucidation of the regulatory machinery

involved in the control of replication timing has been challenging and consequently the biological relevance of the RT remains elusive.

Very few gene knockouts have been shown to trigger alterations in the RT [5,21]. Until now, Rap1-interacting factor 1 (RIF1) is one of the very few trans-acting factors whose loss of function has been found to result in major RT modifications [22,23]. Besides, we unravelled a novel role for YAP, the downstream effector of the Hippo signalling pathway, in the control of RT [24]. We indeed found that YAP is specifically expressed in neural stem cells in the *Xenopus* retina and that its knockdown in these cells leads to altered RT associated with a dramatic S-phase shortening. However, whether YAP is directly involved in RT regulation remains to be investigated. Here, we took advantage of *Xenopus* egg extracts, a cell-free system that recapitulates the key nuclear transitions of the eukaryotic cell cycle *in vitro*, to further assess YAP function in DNA replication. This system is uniquely suited to the study of the mechanisms and dynamics of DNA replication [25,26]. We found that YAP is recruited onto chromatin during replication in a manner that is dependent on the pre-RC formation and that it regulates DNA replication dynamics by limiting both the activation of replication origins and the overall rate of DNA replication. We also identified RIF1 as a binding partner. Interestingly, as previously shown for *Yap* [24], we found that *Rif1* is expressed in retinal stem and early progenitor cells and involved in their RT signature. We also found similar phenotypes for *in vivo Yap* and *Rif1* knock-down during early cleavage stage *Xenopus* embryos, e.g. an acceleration of the speed of cell divisions, likely resulting from S-phase shortening. Altogether, our findings unveil YAP implication in the regulation of replication dynamics and identify RIF1 as a novel partner. We propose that YAP, like RIF1, acts as a brake during replication, to control the rate of DNA synthesis.

Materials and Methods

Ethics statement

All animal experiments have been carried out in accordance with the European Community Council Directive of 22 September 2010 (2010/63/EEC). All animal care and experimentation were conducted in accordance with institutional guidelines, under the institutional license C 91-471-102. The study protocol was approved by the institutional animal care committee CEEA #59 and received an authorization by the Direction Départementale de la Protection des Populations under the reference APAFIS#998-2015062510022908v2 for *Xenopus* experiments.

Embryo, tadpole and eye collection

Xenopus laevis embryos were obtained by conventional methods of hormone-induced egg laying and *in vitro* fertilization [27], staged according to Nieuwkoop and Faber's table of development [28], and raised at 18-20°C. Before whole eye dissection, tadpoles were anesthetized in 0.4% MS222 (Sigma-Aldrich). Dissected eye area was measured using AxioVision REL 7.8 software (Zeiss).

Antibodies

A detailed list of the antibodies used in this study for immunohistochemistry (IHC) and western blot (WB) is depicted in Supplementary Table 1. HLTV-hTRIM21 was a gift from Leo James (Addgene plasmid # 104973; http://n2t.net/addgene:104973; RRID: Addgene_104973). Recombinant His-geminin, GST-p21 and His-TRIM21 were prepared as described, respectively [29–31]. C-terminal *Xenopus Rif1* cloned in pET30a vector (a gift from B. Dunphy and A. Kumagai [32], was expressed in *Escherichia coli* C41 cells, purified by Nickel-Sepharose chromatography (Amersham Bioscience), and used as an antigen to raise antibodies in rabbits at a commercial facility (Covalab, Villeurbanne, France). A cDNA encoding recombinant His-tagged *Xenopus* YAP was cloned in pFastBac1vector, expressed in the baculovirus Bac-to-Bac expression system (Invitrogen), purified by Nickel-Sepharose chromatography as described by the supplier (Amersham Bioscience) and then dialyzed over

night against 25 mM Hepes pH 7.8, 250 mM NaCl, 5 mM imidazole, 5% glycerol, 7.5 mM MgCl2, 1 mM DTT, 1 mM EDTA. Purified His-YAP was then used as an antigen to raise antibodies in rabbits at a commercial facility (Covalab, Villeurbanne, France).

Morpholinos and TRIM21 microinjections

For *in vivo* depletion experiments, 2 pmol of *Yap*-MO and 1 pmol of *Rif1*-MO together with a fluorescent tracer (dextran fluorescein lysine, Thermo Fisher Scientific) were microinjected into fertilized oocytes. The TRIM21 experiments were conducted in a similar way using a mixture of recombinant hTRIM21, anti-RIF1 or anti-YAP antibody and *Rif1*- or *Yap*- or control-MO. Morpholinos used in this study can be found in Supplementary Table 2.

Replication of sperm nuclei in *Xenopus* egg extracts

Replication competent extracts from unfertilized *Xenopus* eggs and sperm nuclei from testis of male frogs were prepared as described [25]. Egg extracts were used fresh unless stated otherwise. Sperm nuclei (2000 nuclei/µl or 7000 nuclei/µl) were incubated in untreated, mock or YAP depleted extracts in the presence of cycloheximide (250 µg/ml, Sigma), energy mix (7.5 mM creatine phosphate, 1 mM ATP, 0.1 mM EGTA, pH 7.7, 1 mM $MgCl_2$).

Immunoprecipitations and Immunodepletions

Rabbit anti-*Xenopus* YAP serum, rabbit anti-YAP antibody (ab62752, Abcam), pre-immune serum or rabbit IgG (Sigma) were incubated overnight at 4°C with protein A Sepharose beads (GE Healthcare). Anti-YAP or rabbit IgG coupled beads were washed with EB buffer (50mM Hepes, pH 7.5, 50mM KCl, 5mM $MgCl_2$) and incubated 1 hour at 4°C in egg extracts (volume ratio 1:3).

Neutral and alkaline agarose gel electrophoresis

Sperm nuclei were incubated in fresh extracts complemented with indicated reagents and one-fiftieth volume of [a-^{32}P] dCTP (3000 Ci/mmol). DNA was recovered after DNAzol® treatment (Invitrogen protocol) followed by ethanol precipitation, separated on 1.1% alkaline agarose gels, and analysed as described [33]. From one extract to another, the replication extent (percent of

replication) differs at a specific time point, because each egg extract replicates nuclei with its own replication kinetics. In order to compare different independent experiments, performed using different egg extracts, the data points of each control sample were independently fitted to a logistic curve and scaled by the inferred maximum incorporation value to 0-100 %. To include statistics, the scaled data points were grouped into 4 bins (0-25% = early; 26-50% = mid; 51-75% = late; 76-100% = very late S phase); mean and standard deviation were calculated for each bin and the t-test was used to assess statistically significant differences between the data in each bin.

Western blot

For analysis of chromatin-bound proteins, we used a protocol slightly modified from [34]. Briefly, reactions were diluted into a 13-fold volume of ELB buffer (10 mM Hepes pH 7.5, 50 mM KCl, 2.5 mM $MgCl_2$) containing 1 mM DTT, 0.2% Triton X100, protease inhibitors and phosphatase inhibitors; chromatin was recovered through a 500 mM sucrose cushion in ELB buffer, at 6780g, 50 sec, 4°C. Interphase was washed twice with 200 µl ELB, 250 mM sucrose and resuspended in SDS sample buffer. Western blots were conducted using standard procedures on *Xenopus* embryo/tadpole protein extracts. Proteins were loaded, separated by 7.5%, 12% or 4-15% SDS-polyacrylamide gels (Bio-Rad) and transferred into nitrocellulose membranes. Western blots were then conducted using standard procedures. Immunodetection was performed using appropriate horseradish peroxidase-labelled antibodies (1/10000, company), followed by chemiluminescence using Super Signal West Pico or Femto Chemiluminescence Kit (Pierce). Quantification was done using Fiji software (National Institutes of Health [35]. For immuno-depleted or immuno-precipitated samples, horseradish peroxidase-labelled protein A (1/20000, Invitrogen 101023) was used for immunodetection to minimize denatured IgG chains recognition.

Molecular combing and detection by fluorescent antibodies

DNA was extracted and combed as described [36]. Biotin was detected with AlexaFluor594 conjugated streptavidin followed by anti-avidin biotinylated antibodies. This was repeated twice, then followed by mouse anti-human

ssDNA antibody, AlexaFluor488 rabbit anti-mouse, and AlexaFluor488 goat anti-rabbit for enhancement [37]. For dilutions and antibodies references refer to Supplementary Table 1. Images of the combed DNA molecules were acquired and measured as described [36]. For each combing experiment a total of 24-35 Mb DNA was measured. The fields of view were chosen at random, unless mentioned otherwise. Measurements on each molecule were made using Image Gauge version 4.2 (Fujifilm) and compiled using macros in Microsoft Excel. Replication eyes were defined as the incorporation tracks of biotin–dUTP. Replication eyes were considered to be the products of two replication forks, incorporation tracks at the extremities of DNA fibers were considered to be the products of one replication fork. Tracts of biotin-labelled DNA needed to be at least 1 kb to be considered significant and scored as eyes. When label was discontinuous, the tract of unlabelled DNA needed to be at least 1 kb to be considered a real gap. The replication extent was determined as the sum of eye lengths divided by the total DNA length. Fork density was calculated as the total DNA divided by the total number of forks. The midpoints of replication eyes were defined as the origins of replication. Eye-to-eye distances (ETED), also known as inter-origin distances, were measured between the midpoints of adjacent replication eyes. The means of fiber lengths were comparable inside each individual experiment in order to avoid biases in eye to eye distances. Incorporation tracks at the extremities of DNA fibers were not regarded as replication eyes, but were included in the determination of the replication extent, calculated as the sum of all eye lengths (EL) divided by total DNA. Box plots of ETED (with n ranging from 71-286) were made using GraphPad version 6.0 (La Jolla, CA, USA). Statistical analyses of repeated experiments have been included as means including ranks where possible. When experiments were repeated with a different egg extract replication extent differs at identical time scales because different egg extracts replicate nuclei with different replication kinetics. It is therefore difficult to include statistics of independent kinetics experiments.

Immunostaining, EdU labelling, and TUNEL assay

For immunostaining, tadpoles were anesthetized in 0.4% MS222 (Sigma-Aldrich) and fixed in 1X PBS, 4% parafolmaldehyde 1h at room temperature,

and were then dehydrated, embedded in paraffin and sectioned (12 µm) with a Microm HM 340E microtome (Thermo Scientific). Immunostaining on retinal sections was performed using standard procedures. For proliferative cell labelling, tadpoles were injected intra-abdominally, 1-hour prior fixation, with 50-100 nl of 1 mM 5-ethynyl-20-deoxyuridine (EdU, Invitrogen) at stage 41. EdU incorporation was detected on paraffin sections using the Click-iT EdU Imaging Kit according to manufacturer's recommendations (Invitrogen). Detection of apoptotic cells was carried out with the DeadEnd fluorometric TUNEL system (Promega, Fitchburg, WI, United States) according to the manufacturer's instructions. Cell nuclei were stained with Hoechst (1 µg/ml, Sigma-Aldrich).
Fluorescent images were taken with the AxioImagerM2 with Apotome (Zeiss) coupled to digital AxiocamMRc camera (Zeiss) and processed with the Axio Vision REL 7.8 (Zeiss) and Adobe Photoshop CS4 (Adobe) software. For quantifications of labelled cells by manual cell counting in the CMZ, 4 sections per retina and a minimum of 12 retinas were analysed. All experiments were performed at least in duplicate. All results are reported as mean ± SEM. Statistical analysis was performed for parametric data using Student's *t*-test and for non-parametric data Mann-Whitney test. *p*-value is shown in each graph, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$, n.s. not significant.

Co-IP

Immunoprecipitation assays on HEK293T protein extracts were performed with the Dynabeads Protein A Immunoprecipitation Kit (Invitrogen) according to the manufacturer's protocol. Antibodies used for immunoprecipitation are listed in Supplementary Table 1. For antibody-bead incubation (anti-FLAG or anti-HA) 5 µg of antibody was used.

Mass spectrometry

Rabbit anti-YAP antibody (ab62752, Abcam) or rabbit IgG were coupled to Protein A Sepharose beads (GE Healthcare) as described above and incubated with *Xenopus* egg extracts for 30 min at 4 °C. Beads were isolated by centrifugation and washed three times with EB buffer. For the elution of the immunoprecipitated proteins, 2X laemmli buffer was incubated into the beads for 10 min at room temperature and collected by centrifugation. Approximately

20 ng of immunoprecipitated YAP protein fraction was loaded in a 7.5% polyacrylamide gel and send it to the mass spectrometry facility (Protéomique Paris Saclay-CICaPS). Protein samples were reconstituted in solvent A (water/ACN [98: 2 v/v] with 0.1% formic acid) and separated using a C18-PepMap column (Thermo Fisher Scientific) with a solvent gradient of 2–100% Buffer B (0.1% formic acid and 98% acetonitrile) in Buffer A at a flow rate of 0.3 µl/min. The peptides were electrosprayed using a nanoelectrospray ionization source at an ion spray voltage of 2300 eV and analyzed by a NanoLC-ESI-Triple TOF 5600 system (AB Sciex). Protein identification was based on a threshold protein score of > 1.0. For quantitation, at least two unique peptides with 95% confidence and a P-value < 0.05 were required.

Results

YAP is recruited to chromatin in a pre-RC-dependent manner in *Xenopus* egg extracts

In order to characterize the role of YAP during S phase, we took advantage of the *Xenopus* egg extract system, widely used to study DNA replication *in vitro* [25,26]. By quantitative western blot, we found that YAP protein is present in oocytes at a concentration of 11 ng/µl (169 nM, Figure S1A-C). We next incubated permeabilized sperm nuclei in S phase egg extract, and collected purified chromatin fractions starting from pre-RC assembly until after the start of DNA replication. Western blot analysis revealed that YAP recruitment onto chromatin coincides with the recruitment of PCNA, marking the start of DNA synthesis (Figure 1A). YAP further accumulates on chromatin as S phase progresses. Our results show that during normal DNA replication, YAP is recruited to chromatin after the recruitment of the pre-RC proteins (MCM2, MCM7, ORC2) in the *Xenopus in vitro* system. We next asked whether the recruitment of YAP could be dependent on pre-RC assembly on chromatin. Loading of the MCM complex can be prevented by adding recombinant geminin, an inhibitor of Cdt1, necessary for MCM loading [38,39]. After addition of 100 nM recombinant geminin, we confirmed that MCM loading and DNA replication were inhibited and in addition, YAP chromatin recruitment was severely delayed (Figure 1A). We conclude that YAP is recruited to chromatin at the start of DNA replication and its recruitment is dependent on functional pre-RC assembly in the *Xenopus* egg extract system.

YAP depletion triggers acceleration of sperm nuclei DNA synthesis in egg extracts

To further assess the potential direct role of YAP in the DNA replication process, we monitored nascent strand DNA synthesis after incubating sperm nuclei in YAP-depleted egg extracts in the presence of ^{32}P-dCTP (Figure 1B, C). Replication reactions were stopped at indicated times during S phase and then quantified (Figure 1D). We found that YAP depletion increased DNA synthesis during the early stages of DNA replication (30-60 min: low molecular

weight nascent strands), but to a lesser extent at later stages (75-150 min: high molecular weight strands). We calculated the ratio between YAP and mock-depleted synthesis at four different intervals of percentages of incorporation reflecting early (0-25 % replication extent), mid (26-50 %), late (51-75 %) and very late (76-100%) S phase. We found that YAP depletion increased DNA synthesis 1.8 fold during early S phase, 1.7 fold during mid S phase and 1.6 fold during late but only 1.2 fold during very late S phase. We wondered whether the observed increase in DNA replication after YAP depletion could be simply due to a quicker entry into S phase, maybe as a consequence of a more rapid chromatin assembly, rather than an effect on DNA replication itself. We however ruled out this hypothesis by analysing nascent strands during very early S phase, which did not reveal any precocious start of DNA synthesis after YAP depletion (Figure S2). We therefore conclude that YAP depletion leads to accelerated DNA synthesis, mainly during early stages of S phase.

YAP depletion increases replication origin firing

The higher rate of DNA synthesis observed in absence of YAP could result from either an increase in origin firing, fork speed, or both. In order to directly monitor origin activation in single DNA molecules, we performed a DNA combing experiment in control and YAP depleted extracts, and determined the density and spacing of replication origins (Figure 2A). We found that after YAP depletion, the replication content increased during early to mid S phase by 2.15-fold (Figure 2B), consistent with the nascent strand analysis shown in Figure 1C. YAP depletion also increased the density of active replication forks (1.57-fold), which shows that the absence of YAP leads to an increase of activated replication origins. This was consistent with a significant decrease (1.5 fold) in eye-to-eye distances. Replication eye lengths were also significantly increased (1.24 fold). Together, we conclude that YAP depletion leads to an increase in both replication origin activation and fork speed.

YAP depletion accelerates the rate of cell division in early developing embryos

To assess whether YAP function in DNA replication also holds true *in vivo*, we took advantage of the early embryonic divisions of *Xenopus* that provide a

simplified system of cell cycle analysis. Indeed, during early development, prior the mid-blastula transition (MBT), cells divide very rapidly, rather synchronously for a series of 12 divisions and present a cell cycle structure without gap phases. As a result, variations of the number of cells at a given time during this developmental period would reflect alteration of the time spend in the 2 remaining phases (S or M). We thus decided to deplete embryos from YAP and assess the outcomes on the rate of embryonic cell division. Since YAP protein is expressed maternally, we employed the recently developed Trim-away technique for cells and mouse oocytes [31,40] to trigger direct YAP degradation *in vivo* and combined it with injections of translation blocking morpholinos to further prevent *de novo* protein synthesis (Figure 3A). By western blot, we confirmed that this strategy triggers efficient YAP protein depletion in embryos. We found that cells appeared smaller and more numerous in YAP depleted embryos than in controls at stage NF 7 (Figure 3B, C). We conclude that YAP depletion leads to an increase of the speed of cell divisions in pre-MBT *Xenopus* embryos. In order to assess whether the depletion of a known factor implicated in the control of the RT program could lead to similar phenotypes, we undertook the same strategy to deplete RIF1 in *Xenopus* embryos using both the Trim-away technique and *Rif1* Morpholinos (*Rif1*-MO). RIF1 depletion was previously found to increase the rate of DNA replication in *Xenopus* egg extracts [32,41]. Here we found that its depletion in embryos leads to an increased number of cells at stage NF 7, indicative of a faster rate of cell division, similarly to what we observed upon YAP depletion (Figure 3B, C). Considering the well-known function of RIF1 in DNA replication and the occurrence of only S- and M-phases in pre-MBT embryos, this strongly suggests that the increased rate of cell division in absence of RIF results from DNA replication acceleration and shortening of S-phase length. We therefore propose that YAP could be similarly involved in controlling the rate of DNA replication in pre-MBT embryos.

YAP interacts with RIF1

In order to identify YAP partners in the context of DNA replication, we conducted an exploratory search for interacting proteins by co-immunoprecipitation coupled to mass spectroscopy (co-IP-MS) in control or YAP-depleted S-phase egg extracts (Supplementary Table 3). Among the

proteins enriched more than 3 fold in YAP-co-IP versus control co-IP conditions, we identified some factors known to be involved in DNA replication, including RIF1. We verified YAP/RIF1 interaction in egg extracts by reciprocal co-immunoprecipitated assays (Figure 4A). We next also confirmed this interaction following expression of tagged proteins in HEK293 cells (Figure 4B). Altogether, our data reveal RIF1 as a potential interactant for YAP in the context of DNA replication.

Rif1 **is expressed in retinal stem cells and its knockdown affects their temporal program of DNA replication**

Since RIF1 has been recently shown to function in a tissue-specific manner [42], we investigated its expression and function in the post-embryonic *Xenopus* retina and compared the results with our previous findings regarding YAP retinal expression/function [24]. Immunostaining experiment revealed prominent RIF1 expression in the peripheral region of the ciliary marginal zone (CMZ) of the retina containing stem and early progenitor cells, and where YAP is also specifically expressed (Figure 4C, D). We next undertook a knockdown approach using *Rif1*-MO (Figure 5E). Morphant tadpoles exhibited significantly reduced eye size compared to controls (Figure 5F,G), similarly to *Yap* morphants. We next determined the level of proliferation within the CMZ in morphant tadpoles (Figure 5). Unlike the observed decreased EdU cell number in *Yap* morphant CMZ [24], we here did not find any significant difference in the number of EdU+ cells in *Rif1*-MO-injected tadpoles compared to a control situation (Figure 5B, C). Interestingly however, as observed in *Yap* morphants [24], we found a drastic change in the distribution of EdU-labelled replication foci in retinal stem and early progenitor cells, where *Rif1* is normally expressed (Figure 5B, D). The spatial distribution of these foci evolves in a stereotype fashion during S phase: from numerous small ones located throughout the nucleus in early-S phase, to few large punctuated ones in mid/late-S phase [43–46]. Our analysis revealed decreased proportion of cells exhibiting a mid-late versus early S-phase patterns in *Rif1* morphants. These data highlight that *Rif1* knockdown alters the temporal program of DNA replication in retinal stem/early progenitor cells.

Discussion

We recently revealed a novel role for YAP in governing DNA RT in *Xenopus* retinal stem cells [24]. Whether and how YAP could directly regulate DNA replication was however unknown. Here, we used the *Xenopus'* synchronous *in vitro* replication system and early *Xenopus* embryos, where RNA transcription is absent, to study the role of YAP in S phase, independently from its role in transcription. Our study shows that YAP negatively regulates DNA replication. First, we found that YAP is recruited to chromatin at the start of DNA synthesis, in a pre-RC-dependent manner. Second, our *in vitro* and *in vivo* data reveal a non-transcriptional role for YAP in the initiation of DNA replication and in the regulation of replication fork speed. Third, we identified RIF1 as a novel YAP partner, a major regulator of RT program.

YAP negatively controls initiation of DNA replication in a transcriptional-independent manner

We found that YAP is recruited to replication competent chromatin at the start of S phase and accumulates over S phase. We showed that its binding does not only correlate with active replication forks, but it also depends on a functional pre-RC assembly. We do not know how YAP is recruited to chromatin in the first place since our proteomic analysis did not reveal a direct interaction with any members of the MCM complex (Supplementary Table 3), indicating that YAP might be recruited by proteins involved in steps downstream of pre-RC assembly. Since inhibition of replication does not prevent completely but rather delays YAP recruitment, additional mechanisms of recruitment may also be at work after prolonged incubation times.

Next, we showed increased DNA synthesis and replication origin activation in YAP depleted egg extracts compared to controls. Our study thus reveals a so far unknown direct role for YAP in the initiation of DNA replication in *Xenopus* egg extract system. It will be important to address whether YAP role in DNA replication similarly occurs in mammalian cells.

Adding back recombinant *Xenopus* YAP protein did not rescue the YAP depletion induced increase in DNA synthesis (not shown). This could be

explained by the fact that we co-immunodepleted one or more factors important for the replication process. Another explanation could be one or more missing post-translational modifications of the recombinant YAP produced in baculovirus-infected insect cells, as YAP localization and function can be modified by many different types of PTMs (phosphorylation, ubiquitinylation, sumoylation, methylation, acetylation) [47].

Our results demonstrated an enhanced origin usage, especially early in S phase in the absence of YAP. Unscheduled origin usage can give rise to genomic instability. We previously showed that YAP knock-down leads to DNA damage [24]. Therefore, it would be interesting to see whether YAP depletion in the *Xenopus in vitro* system induces DNA damage. Similar to YAP, it was reported that the transcription factor and protooncogene c-Myc has a non-transcriptional role in initiation of DNA replication [48,49]. Interestingly, overexpression of c-Myc, in culture cells and in the *Xenopus in vitro* system, leads to a similar phenotype than the one obtained following YAP loss of function, e.g. enhanced activation of early replication origins. Whether c-Myc mediates increased replication origin activation in YAP depleted extract remains to be analysed.

YAP-RIF1 interplay in replication timing regulation

DNA combing analysis after YAP depletion shows that the overall fork density is increased to a higher extent than local origin distances are decreased. This suggests that YAP controls origin firing more at the level of replication clusters than on the level of single origins, therefore regulating the temporal control of origin activation. Consistent with this proposed role in replication timing, we identified RIF1 as a novel YAP-interacting partner. Among all the gene knockouts and knockdowns assessed so far, that of *Rif1* caused major alterations in RT in higher eukaryotes [22,23,50,51] and in *Drosophila* [52]. RIF1 has been shown to recruit protein phosphatase 1 (PP1) which can modulate binding of pre-initiation complex components to DNA via the dephosphorylation of the Cdc7-target sites in the MCM complex [53–57]. In the *Xenopus in vitro* system, it was shown that the loss of the interaction between RIF1 and PP1 increases the rate of replication in RIF1 depleted-extracts [41], similarly to what we observed after YAP depletion. It is thus tempting to speculate that RIF1 and YAP, both

hub proteins (having a large number of interacting partners) with multifunctional roles, act in concert to regulate the temporal DNA replication program. Furthermore, we provide evidence that RIF1 and YAP functions are required to sustain proper speed of the early cleavage divisions in developing embryos before the onset of transcription. These rapid divisions result from an unusual cell cycle structure that alternates between S and M phases [58]. The duration of mitosis has been previously described as short, constant and uncoupled to variation that may occur in other phases of the cell cycle [59]. We thus favour the hypothesis that RIF1 and YAP slow down S phase in early embryonic cleavage cycles, which is consistent with the *in vitro* replication data for both proteins. Of note, our data demonstrates that the Trim-away technique can be efficiently used to deplete maternal proteins in *Xenopus* early embryos, similar to what has been shown in human culture cell lines and mouse oocytes [31].

Short pulse labelling experiments allow the visualization of so-called "replication foci" in cells. In RIF1-depleted Hela cells, the overall replication foci was found to be extensively rearranged with cells displaying predominantly early-S-phase-like patterns [22]. Here, we provide *in vivo* evidence for RIF1 requirement in the control of the RT in *Xenopus* retinal stem cells. *Rif1* morphants RT defects in the retina are similar to those observed in *Yap* morphants [24]. However, the changes in late/early foci ratio is not accompanied by a decrease in EdU cell number in the CMZ, as found in *Yap* morphant retinas, suggesting a lesser impact on S phase length. Nevertheless, their similar expression pattern in retinal stem and early progenitor cells is consistent with *in vivo* interaction. Whether they work in concert remains to be demonstrated but it is tempting to speculate that they could belong to the same regulatory network in retina cells. Along this line, the literature reports common interactants for YAP and RIF1 such as PP1 (see above and [60]).

Combined observations point to a role for RIF1 in higher order chromatin architecture and its relationship with RT [50]. RIF1 indeed localizes in late-replicating sites of chromatin and acts as a remodeler of the 3D genome organization and as such defines and restricts the interactions between replication-timing domains [50]. It would therefore be interesting in the future to assess whether YAP function in DNA replication could also be linked to a role as an organizer of nuclear architecture. In this context, it is interesting to note

that interactions between YAP and chromatin-remodeling complexes have been established. YAP has indeed been found to associate with chromatin remodelers of the SWI/SNF complex, GAGA factor, Mediator complex, Ncoa6, and NuRD complexes [61].

DNA replication timing regulation and signalling pathways

Not much is known about signalling pathways regulating the RT or RIF1 activity. Growing evidence show that the RT represents a stable epigenetic feature and that specific RT signatures are associated to given cellular states [9]. It is also becoming clear that deregulation of the RT is associated with many diseases, including cancer [16–20]. As an epigenetic mark, the RT is considered stable. However, drastic modifications are observed upon cell fate changes for instance, during differentiation or upon reprogramming [8,10–15]. Recent studies further suggest that a disruption of the RT acts upstream of the establishment of the global epigenetic landscape and subsequent genome compartmentalization [62]. Since both intrinsic and extrinsic cues regulate cell lineage decisions, mechanistic links between signalling pathways and the regulation of the RT are expected to be found. So far, the prominent signalling pathway identified upstream of the RT is the ATM/53BP1 signalling that relays information onto RIF1 activity in response to DNA double strand breaks [63]. Other connections between the RT and upstream signal transduction machineries remain elusive. Here, we are providing evidence that the Hippo pathway downstream effector YAP can convey information to the RT and we are proposing a model in which YAP-RIF1 interaction could act as an integrating hub (Fig. 8). Interestingly, LATS1, another component of the Hippo pathway, has been involved in the ATR-mediated response to replication stress [64]. Several Hippo pathway components may thus regulate, independently or in concert, the RT. Altogether, these data indicate that strategies aiming at targeting the Hippo pathway activity may provide upstream means to modulate the RT *in vivo*. Further research in this direction could provide major perspectives in the fields of cellular reprogramming, regeneration or cancer.

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Figure legends

Figure 1. YAP is recruited onto the chromatin and implicated in the process of DNA replication. (A) Sperm nuclei were incubated in *Xenopus* egg extracts in the presence or absence of geminin (+ Gem, 100nM). Chromatin was isolated at indicated time points for immunoblotting. A graphical representation of the relative amount of YAP intensity values relative to Histone 3 (H3) is shown below the blot. Values were normalised to the control condition (CTL) at 90 min (attributed value of 1, red dashed line). Statistical analysis was performed for parametric data using Student's *t*-test. *p*-value is shown in the graph, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$, n.s. not significant. Data is reported as mean ± SEM. **(B)** Schematic representation of the immunodepletion procedure used to reveal YAP implication in the process of DNA replication illustrated in (C). Low speed supernatant (LSS) extracts were incubated with protein A-coupled beads coated with either an anti-YAP rabbit antibody (DYAP) or a random rabbit IgG at an equivalent antibody concentration as a control (DMock). Beads were removed by centrifugation and aliquots of the extracts obtained were processed for immunoblotting with either anti-YAP or anti-tubulin (loading control) antibodies. The remaining extracts were then supplemented with sperm nuclei and incubated with [α-^{32}P]dCTP for different times in order to label nascent DNA during replication. **(C)** Nascent DNA strands synthesized were analysed by alkaline gel electrophoresis after the indicated times. The level of radioactivity incorporation was quantified for each lane and the ratio of the values in DYAP over DMock conditions was calculated for each time point. The ratio at 60 minutes is indicated. **(D)** Violin plot showing DYAP/Dmock ratios from 8 independent experiments including the one depicted in C. The time scale was fractionated in 4 periods to roughly distinguish early, mid and late phases of the replication process. Red dots indicate the mean and red error bars the SEM.

Figure 1

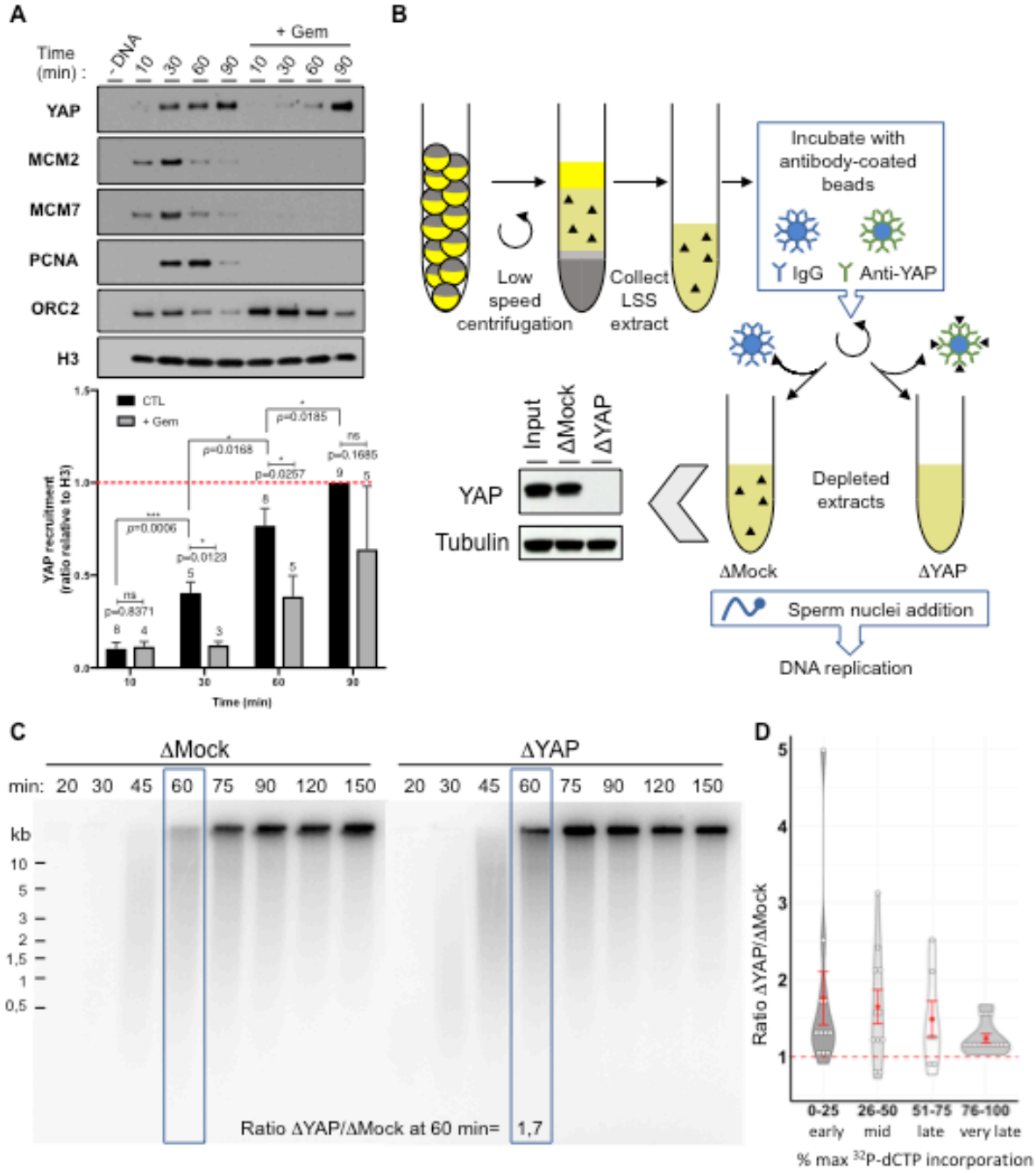

A
Time (min) :
- DNA
10
30
60
90
+ Gem
10
30
60
90
YAP
MCM2
MCM7
PCNA
ORC2
H3
CTL
+ Gem
YAP recruitment (ratio relative to H3)
Time (min)
B
Low speed centrifugation
Collect LSS extract
Incubate with antibody-coated beads
IgG
Anti-YAP
Input
ΔMock
ΔYAP
YAP
Tubulin
Depleted extracts
ΔMock
ΔYAP
Sperm nuclei addition
DNA replication
C
ΔMock
ΔYAP
min: 20 30 45 60 75 90 120 150
20 30 45 60 75 90 120 150
kb
10
5
3
2
1,5
1
0,5
Ratio ΔYAP/ΔMock at 60 min= 1,7
D
Ratio ΔYAP/ΔMock
0-25 early
26-50 mid
51-75 late
76-100 very late
% max 32P-dCTP incorporation

Figure 2. Egg extracts lacking YAP exhibit more replication origins and enhanced DNA replication velocity. Sperm nuclei were incubated in egg extracts in the presence of Biotin-dUTP and DNA combing was performed. **(A)** Three representative combed DNA fibers replicated in either the DMock- or DYAP-depleted extracts (green: whole DNA labelling; red: biotin labelled replication eyes). **(B-E)** The mean replication extent (B), mean fork density (C, number of forks/100kb), the eye-to-eye distance distributions (D, ETED, scatter dot plots with median) and the eye length distributions (E, EL, scatter dot plots with median) were measured for each condition. Statistics: Mann-Whitney test.

Figure 2

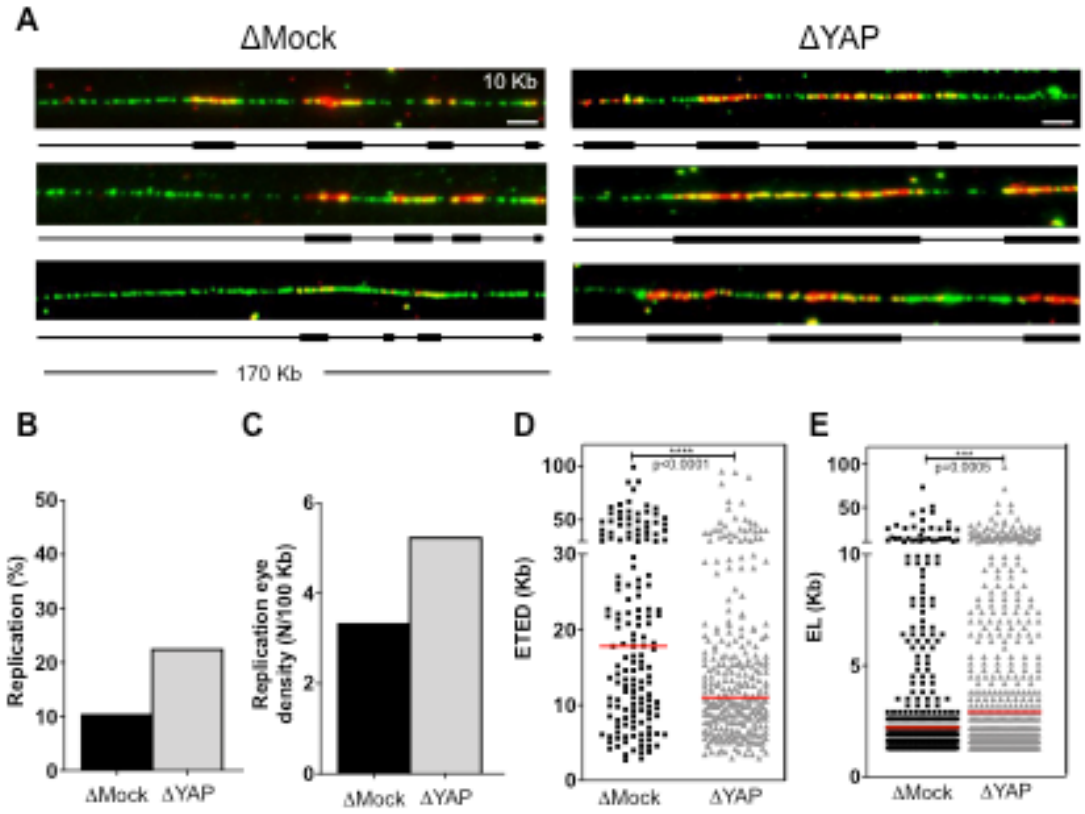

A
ΔMock
ΔYAP
10 Kb
170 Kb
B
Replication (%)
ΔMock
ΔYAP
C
Replication eye density (N/100 Kb)
ΔMock
ΔYAP
D
ETED (Kb)
ΔMock
ΔYAP
E
EL (Kb)
ΔMock
ΔYAP

Figure 3. YAP or RIF1 depletion increases cell cycle kinetics of early *Xenopus* embryos. (A) Diagram of the experimental procedure used in (B) and western blot showing the efficiency of RIF1 or YAP depletion at stage 7. *X. laevis* embryos were microinjected at one-cell stage with (i) control MO + anti-IgG rabbit + TRIM21 (TRIM-control); (ii) *Rilf1*-MO + anti-RIF1 antibody + TRIM21 (TRIM-RIF1) or (iii) YAP-MO + anti-YAP antibody + TRIM21 (TRIM-YAP). **(B)** Images from stage 7 embryos injected as in (A). The number of cells per embryo in a defined area was quantified as shown on the top right panel. Data are represented as violin plots for two independent experiments. Mann-Whitney test, **** $p \leq 0.0001$, ns: non significant.

Figure 3

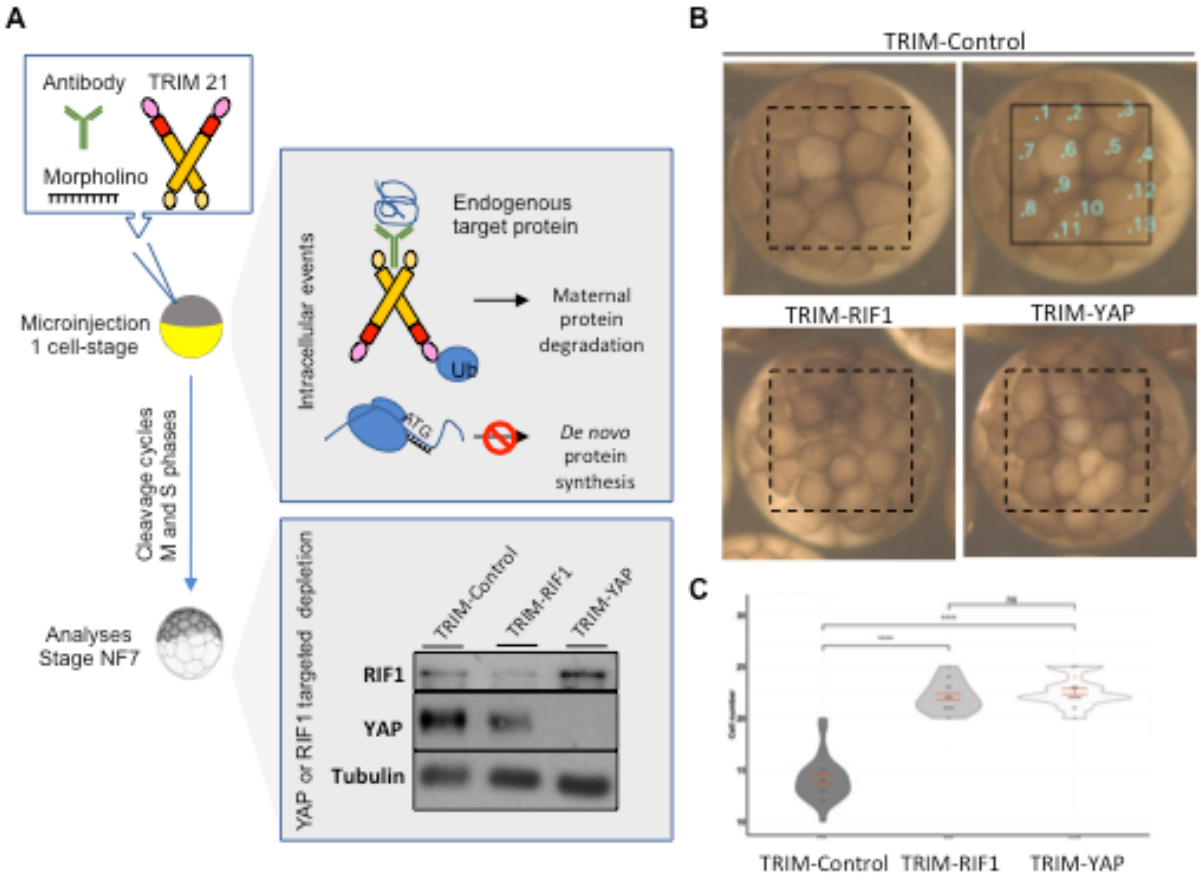

A
Antibody
TRIM 21
Morpholino
Microinjection
1 cell-stage
Cleavage cycles
M and S phases
Analyses
Stage NF7
Intracellular events
Endogenous
target protein
Ub
Maternal
protein
degradation
ATG
De novo
protein
synthesis
YAP or RIF1 targeted depletion
TRIM-Control
TRIM-RIF1
TRIM-YAP
RIF1
YAP
Tubulin
B
TRIM-Control
TRIM-RIF1
TRIM-YAP
C
TRIM-Control
TRIM-RIF1
TRIM-YAP

Figure 4. **RIF1 interacts with YAP, is expressed in retinal stem cells and its knock-down leads to small eye phenotype (A, B)** Co-immunoprecipitation assays from egg extracts (A) or HEK293T cells transfected with the indicated tagged constructs (B). **(C)** Schematic transversal section of a *Xenopus* tadpole retina (RPE: retinal pigmented epithelium; NR: Neural retina; ON: optic nerve). Within the central marginal zone (CMZ; right panel), retinal stem cells (RSC) reside in the most peripheral margin while early (P1) and late (P2) progenitors are located more centrally. **(D)** Retinal sections from stage 41 *Xenopus* tadpoles, immunostained for YAP and RIF1 (red). Nuclei are counterstained with Hoechst (blue). **(E)** Diagram showing the experimental procedure used in (F). One cell-stage embryos are microinjected with Control MO or *Rif1*-MO and analysed at stage 41. The western blot shows the efficiency of the MO to deplete RIF in embryos. **(F)** Tadpoles microinjected with MO as shown in (E). **(G)** Dissected eyes from tadpoles microinjected with MO as shown in (E). The quantification indicates the mean area of the eyes.

Figure 4

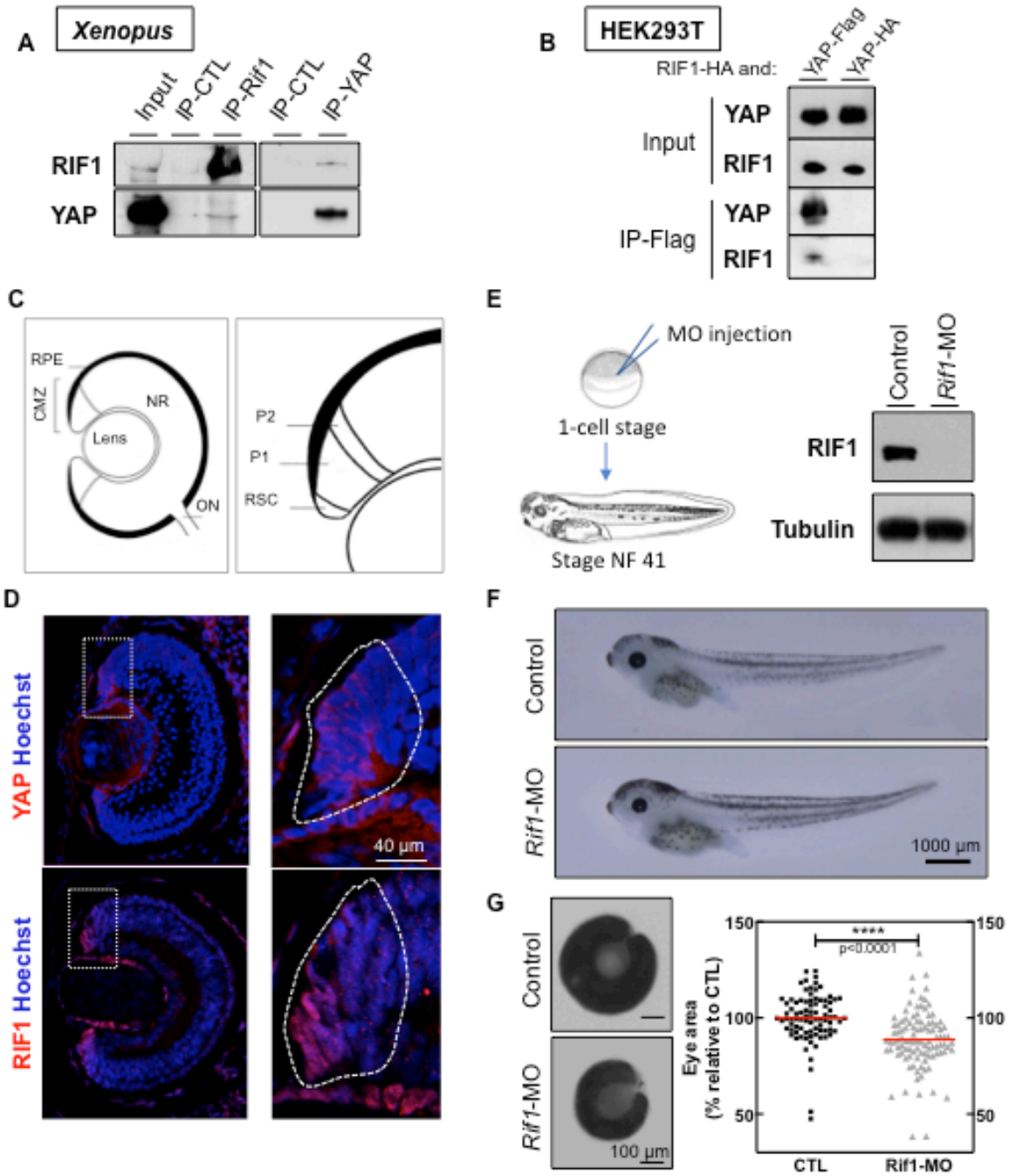

A
Xenopus
Input
IP-CTL
IP-Rif1
IP-CTL
IP-YAP
RIF1
YAP
B
HEK293T
RIF1-HA and:
YAP-Flag
YAP-HA
Input
YAP
RIF1
IP-Flag
YAP
RIF1
C
RPE
CMZ
NR
Lens
ON
P2
P1
RSC
D
YAP Hoechst
RIF1 Hoechst
40 µm
E
MO injection
1-cell stage
Stage NF 41
Control
Rif1-MO
RIF1
Tubulin
F
Control
Rif1-MO
1000 µm
G
Control
Rif1-MO
100 µm
Eye area
(% relative to CTL)
150
100
50
****
p<0.0001
CTL
Rif1-MO

Figure 5. *Rif1* loss of function affects DNA replication timing in retinal stem/early progenitor cells. (A) Diagram showing the experimental procedure used in (B). One cell-stage embryos are microinjected with Control MO or *Rif1*-MO and injected with EdU one-hour prior fixation at stage 41. **(B)** Retinal sections from tadpoles microinjected with MO and EdU as shown in (A). Early (red arrowheads) and late (white arrowheads) profiles were distinguished. (B) Quantification of cells inside the whole CMZ (delineated by doted lines) compartment. (C) Quantification of EdU-positive cells in the same manner as in B. (D, E) Quantification of the ratio mid-late/early pattern is shown. Data are represented as means ± SEM. Statistics: Mann-Whitney test.

Figure 5

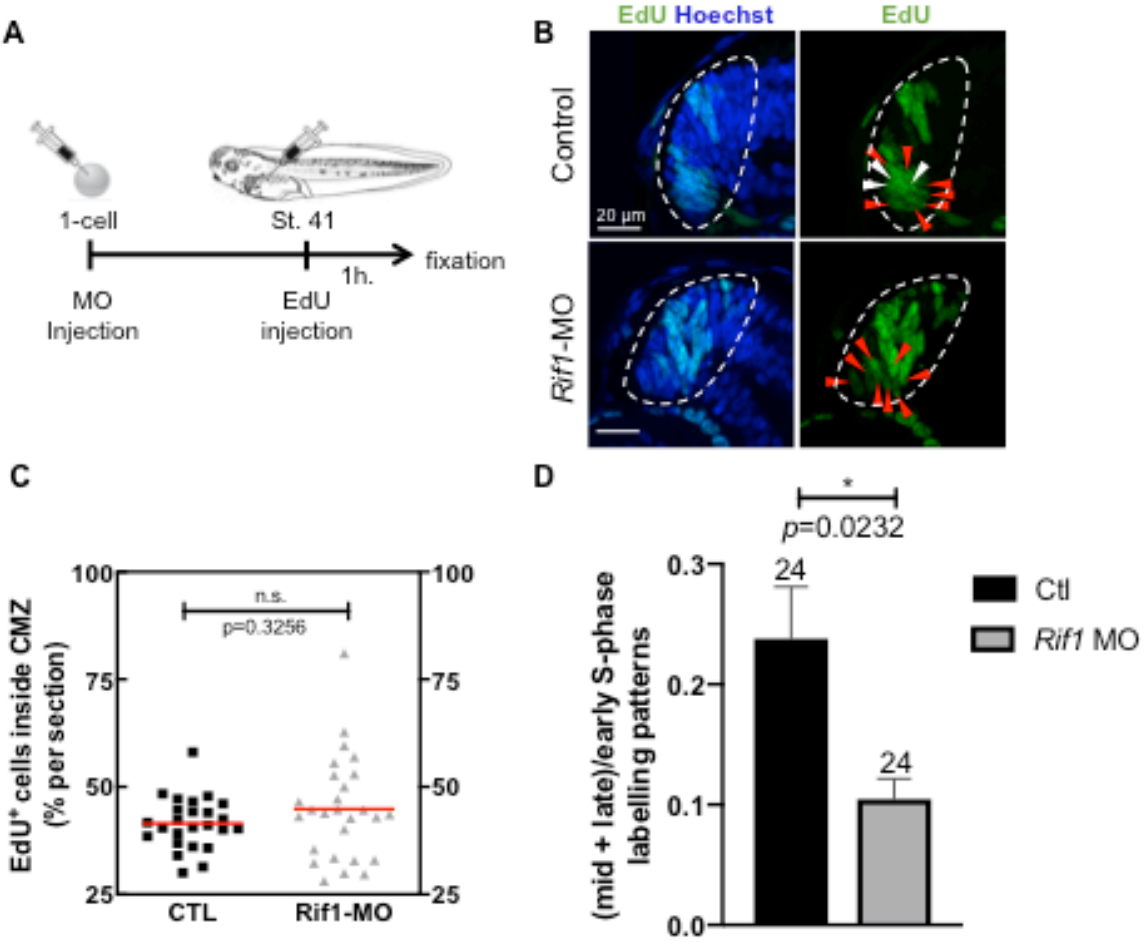
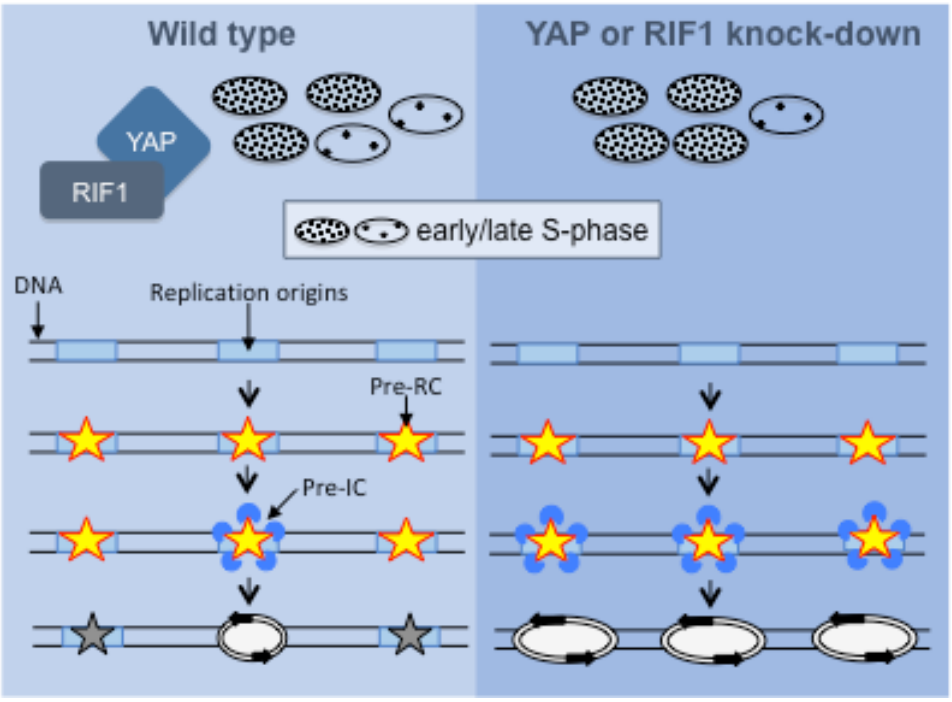

A
1-cell
St. 41
fixation
1h.
MO
Injection
EdU
injection
B
EdU Hoechst
EdU
Control
Rif1-MO
20 µm
C
EdU+ cells inside CMZ
(% per section)
n.s.
p=0.3256
100
75
50
25
CTL
Rif1-MO
D
*
p=0.0232
(mid + late)/early S-phase
labelling patterns
0.3
0.2
0.1
0.0
24
24
Ctl
Rif1 MO

Figure 6. Model illustrating YAP/RIF1 interaction in the control of retinal stem/early progenitor cell RT program. In wild type cells (left panel), YAP/RIF1 interaction limits the formation of pre-initiation complexes and thereby the total number of activated replication origins. When either YAP or RIF1 is knocked-down (right panel), both the number of activated origins and the rate of replication are increased. This is associated with an increased proportion of cells with early replication pattern.

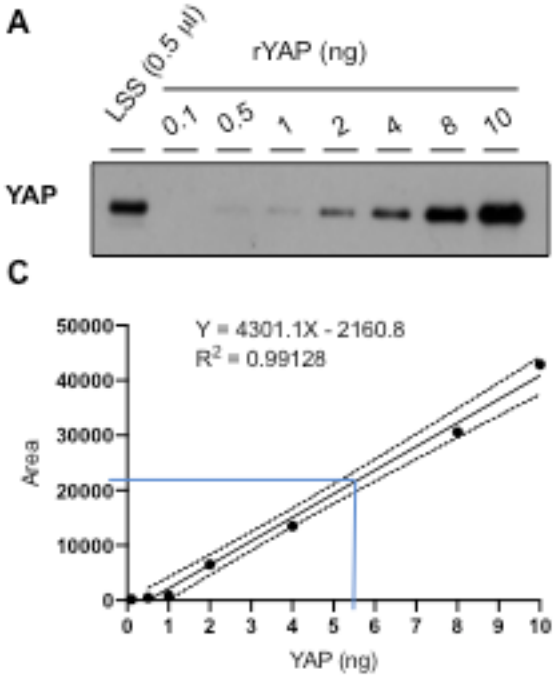
Figure 6


Wild type
YAP or RIF1 knock-down
YAP
RIF1
early/late S-phase
DNA
Replication origins
Pre-RC
Pre-IC

Supplementary data

Figure S1. YAP protein expression in *Xenopus* egg extracts. A. Western blot showing different amounts of recombinant YAP used to estimate endogenous YAP expression in egg extracts (LSS). B-C) Signals derived from the protein bands obtained in A were used to make a standard curve to calculate YAP concentration in the LSS extract.

Figure S1

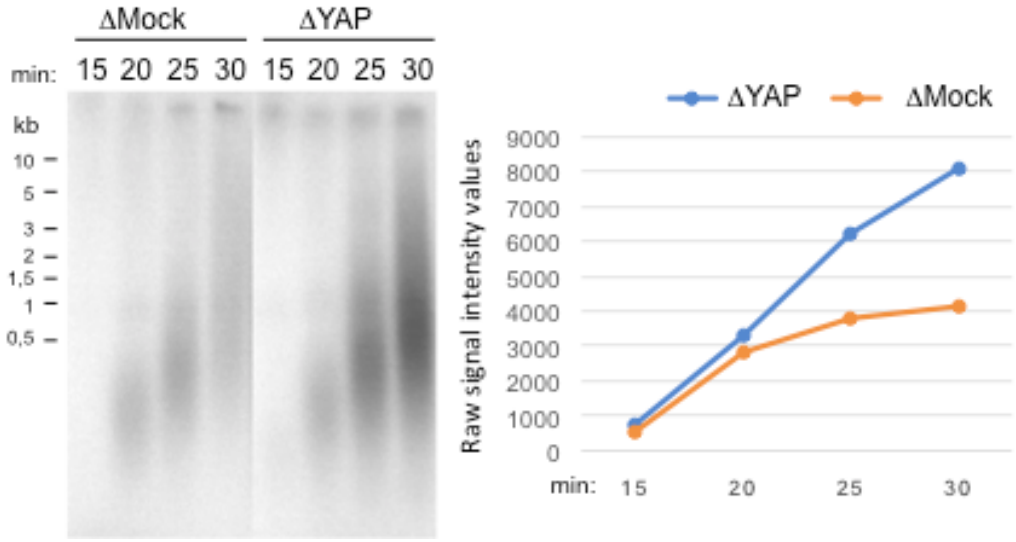


B

X = ng	Y = area
0.1	182.607
0.5	498.021
1	862.335
2	6508.388
4	13461.016
8	30592.543
10	42877.291
LSS (0,5 µl)	21795.836

Figure S2. YAP depletion does not affect entry into S phase. Nascent DNA strands synthesized were analysed during early S phase by alkaline gel electrophoresis after the indicated times. The level of radioactivity incorporation was quantified for each lane and plotted as raw intensity values. Similar signals are initially observed at the earliest time points before getting higher in YAP depleted (DYAP) compare to control depleted extracts (DMock).

Figure S2


ΔMock
ΔYAP
min: 15 20 25 30 15 20 25 30
kb
10
5
3
2
1,5
1
0,5
ΔYAP
ΔMock
Raw signal intensity values
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
min: 15 20 25 30

Supplementary Table 1: List of antibodies. IHC: immunohistochemistry, WB: western blot.

	Antigene	Host	Supplier	Reference	Dilution (IHC)	Dilution (WB)
Primary antibodies	Anti-human MCM2	Mouse	Bethyl Laboratories	A300-191A		1:2000
	Anti-*Xenopus* MCM7	Rabbit	Gift from R. A. Laskey			1:1000
	Anti-*Xenopus* ORC2	Rabbit	Gift from R. A. Laskey			1:1000
	Anti-α Tubulin	Mouse	Sigma	T5168		1:10000
	Anti-recombinant rat PCNA	Mouse	Thermo Fisher Scientific	MA5-11358		1:50
	Anti-recombinant human YAP	Mouse	Abcam	Ab56701		1:1000
	Anti-human YAP	Rabbit	Abcam	Ab62752		1:1000
	Anti-human H3	Mouse	Abcam	Ab1791		1:1000
	Anti-human HA	Mouse	Sigma	H9658		1:1000
	Anti-Flag	Rabbit	Cell signaling	F7425		1:1000
	Anti-human H2AX	Mouse	Millipore	05-636	1:50	
	Anti-human ssDNA monoclonal antibody	Mouse	Millipore	MAB3034	1:50	
Home-made antibodies	Anti-*Xenopus* YAP	Rabbit	Covalab		1:100	1:2000
	Anti-*Xenopus* RIF1	Rabbit	Covalab		1:100	1:2000
Secondary antibodies	Alexa 448 anti-mouse or rabbit	Goat	Thermo Fisher Scientific	A11001	1:1000	
	Alexa 594 anti-mouse or rabbit	Goat	Thermo Fisher Scientific	A11005	1:1000	
	Anti-streptavidin biotinylated	Mouse	Abcyss	BA-0500	1:50	
	AlexaFluor594 streptavidin	Mouse	Invitrogen	S11227	1:50	

	AlexaFluor488 rabbit anti-mouse	Rabbit	Invitrogen	A11059	1:50	
	AlexaFluor488 goat anti-rabbit	Goat	Invitrogen	A11008	1:50	
	HRP anti-mouse IgG	Goat	Sigma-Aldrich	A4416		1:10000
	HRP anti-rabbit IgG	Donkey	GE Health	NA934		1:10000

Supplementary Table 2: List of Morpholinos.

Yap-MO	5' TAGGAGACTGTGPGTCACTTCACC 3'
Rif1-MO	5' AATCCACAGAACAGACGACAGCCAT 3'
Control (GeneTools Standard Control)	5' CCTCTTACCTCAGTTACAATTTATA 3'
Rif1 5MM MO	5' AATCCAGACAAGAGACCACACCCAT 3'

Supplementary Table 3: List of proteins enriched in YAP-IP sample. The list provided here is restricted to proteins showing enrichment equal or over 3 fold compared to control-IP.

Accession Number	Molecular Weight	Fold Change	Ctrl IP	YAP IP
KW11_Step3_007429_rpc1__sp|O14802|RPC1_HUMAN__POLR3A__DNA-directed	156 kDa	INF		5
KW11_Step3_008946_hnrpm__sp|P52272|HNRPM_HUMAN__HNRNPM__Heterogeneous	78 kDa	INF	0	2
KW11_Step3_004563_rent2__sp|Q9HAU5|RENT2_HUMAN__UPF2__Regulator	147 kDa	INF		3
KW11_Step3_006138_aass__sp|Q9UDR5|AASS_HUMAN__AASS__Alpha-aminoadipic	103 kDa	INF		4
KW11_Step3_009027_ddx23__sp|Q9BUQ8|DDX23_HUMAN__DDX23__Probable ATP-dependent RNA helicase DDX23	?	INF	0	2
KW11_Step3_004305_prc2c__sp|Q9Y520|PRC2C_HUMAN__PRRC2C__Protein	374 kDa	INF		2
KW11_Step3_003251_kcc2g__sp|Q13555|KCC2G_HUMAN__CAMK2G__Calcium/calmodulin-dependent (+1)	63 kDa	INF		2
KW11_Step3_001179_l14aa__sp|Q8ND56|LS14A_HUMAN__LSM14A__Protein	51 kDa	INF		3
KW11_Step3_005249_saps1__sp|Q9UPN7|PP6R1_HUMAN__PPP6R1__Serine/threonine-protein	98 kDa	INF		2
KW11_Step3_001830_rl31__sp|P62899|RL31_HUMAN__RPL31__60S	22 kDa	INF		2
KW11_Step3_009010_sfpq__tr|Q9BSV4|Q9BSV4_HUMAN__SFPQ__SFPQ	74 kDa	INF		2
KW11_Step3_006419_srsf7__sp|Q16629|SRSF7_HUMAN__SRSF7__Serine/arginine-rich	27 kDa	INF		2
KW11_Step3_004549_yap1__tr|F5H202|F5H202_HUMAN__YAP1__Yorkie	50 kDa	INF		2
KW11_Step3_009038_pyr1__sp|P27708|PYR1_HUMAN__CAD__CAD	249 kDa	10	3	29
KW11_Step3_001014_hnrpc__tr|B4DY08|B4DY08_HUMAN__HNRNPC__Heterogeneous	32 kDa	9	1	8
KW11_Step3_004457_rl6__sp|Q02878|RL6_HUMAN__RPL6__60S	34 kDa	8	1	7
KW11_Step3_007675_rl13a__sp|P40429|RL13A_HUMAN__RPL13A__60S	36 kDa	4,5	2	5
KW11_Step3_004582_ilf3b__sp|Q12906|ILF3_HUMAN__ILF3__Interleukin	98 kDa	4	1	4
KW11_Step3_010697_ddx20__sp|Q9UHI6|DDX20_HUMAN__DDX20__Probable	87 kDa	4	1	4
KW11_Step3_006247_rfc3__sp|P40938|RFC3_HUMAN__RFC3__Replication factor C subunit 3	?	4	1	4
KW11_Step3_008706_djc13__sp|O75165|DJC13_HUMAN__DNAJC13__DnaJ	255 kDa	4	1	4
KW11_Step3_009543_cul9__sp|Q8IWT3|CUL9_HUMAN__CUL9__Cullin-9	208 kDa	3,2	4	12
KW11_Step3_009358_rif1__sp|Q5UIP0|RIF1_HUMAN__RIF1__Telomere-associated	221 kDa	3	1	3
KW11_Step3_010633_gemi4__sp|P57678|GEMI4_HUMAN__GEMIN4__Gem-associated	115 kDa	3	1	3
KW11_Step3_007790_rfc2__sp|P35250|RFC2_HUMAN__RFC2__Replication	39 kDa	3	1	3
KW11_Step3_005242_pimt__tr|H7BY58|H7BY58_HUMAN__PCMT1__Protein-L-isoaspartate	32 kDa	3	1	3
KW11_Step3_003318_ilf2__sp|Q12905|ILF2_HUMAN__ILF2__Interleukin	43 kDa	3	1	3
KW11_Step3_000638_edc4__sp|Q6P2E9|EDC4_HUMAN__EDC4__Enhancer	154 kDa	3	1	3
KW11_Step3_000681_elv1a__sp|Q15717|ELAV1_HUMAN__ELAVL1__ELAV-like	37 kDa	3	1	3

DISCUSSION & PERSPECTIVES

YAP, commonly identified as the downstream effector of the Hippo pathway, functions as a co-transcriptional factor for gene expression important in organ growth (Dong *et al*., 2007) and regeneration (Moya and Halder, 2019). Recently, my host laboratory found that it may also regulate the RT program of RSCs (Cabochette *et al*., 2015). Here, we found YAP direct implication in DNA replication dynamics using *Xenopus* egg extracts, a well-characterized eukaryotic cell-free replication system. Furthermore, since this system lacks transcriptional capacity, our data shows a novel role of YAP that is independent of its commonly transcriptional function. Interestingly, we showed that YAP normal function is to slow-down DNA replication dynamics by limiting origin firing and overall speed of replication. Moreover, we discovered RIF1, the major *trans*-acting factor in mammalian DNA RT (Yamazaki *et al*., 2013), as a novel partner for YAP.

YAP is recruited to replicating chromatin in a similar fashion as RIF1

Xenopus egg extracts represent the golden standard to study eukaryotic replication, since they mimic with fidelity the fast replicating cell cycle of early embryos (Hutchison *et al*., 1989). One of its characteristics is the abundant presence of maternal factors necessary for DNA replication (Jones and Smith, 2008). Here, we showed that YAP is one of those factors, which gets recruited to chromatin as early as DNA synthesis begins, that parallels the recruitment of PCNA and gradually accumulates. Often, most of the proteins that have a role at the DNA replication cascade have concerted functions, that is being called at certain steps of the process and as replication progress they are eventually removed. For example, there are molecules which become necessary at licensing (i.e. pre-RC proteins) and others that appear later at firing (i.e. DDK and CDK) (Fragkos *et al*., 2015). In our study, we found that this was not the case for YAP, since its time of recruitment happens after the time of pre-RC formation and continues to increase with the following events of replication. This behavior strongly suggests that like RIF1, YAP holds important roles during several steps of the DNA replication cascade.

Assemble of RIF1 has been seen to start at the beginning of anaphase and during early G1, notably at the timing decision point (TDP) (Yamazaki *et al.*, 2013). Perhaps, the recruitment of YAP after RIF1 serves as an accessory protein conceding protein stabilization, since we observed that co-expression of both proteins in HEK293 cells greatly increase the expression of the two proteins (**Figure 49**).

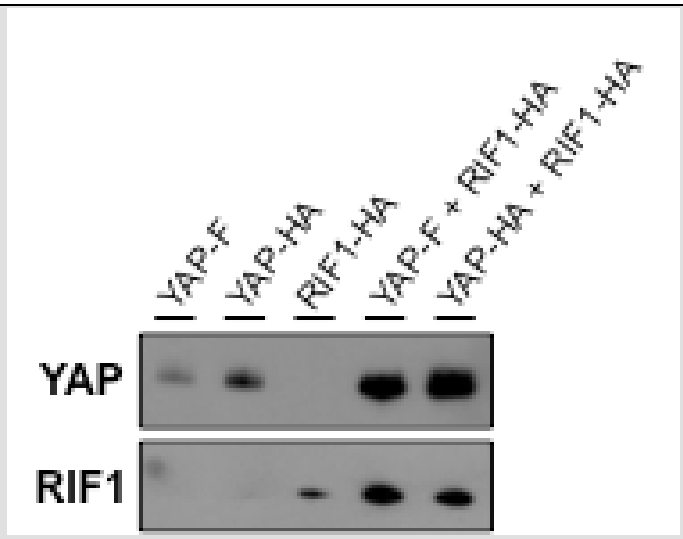


Figure 49. Co-expression of YAP and RIF1 increases their stability. Western blot showing the expression levels of YAP and RIF1 when either YAP or RIF1 are expressed alone or together in HEK293 cells.

This important difference in expression levels when both proteins are co-expressed made the use of single transfections inappropriate as negative IP controls. For this reason, we opted to use a YAP construct flagged with another epitope (HA-YAP) than the one used for IP (FLAG-YAP) to evaluate nonspecific binding. Of note, we were able to detect RIF1 overexpression following transfection using the primary RIF1 antibody but unable to efficiently do so by revealing the added HA-tag for unknown reasons. This constituted a technical drawback which prevented us from doing reciprocal co-IP.

Focusing on the timing of recruitment of YAP vs RIF1 onto chromatin, we observed that both of them are tethered very early in replicating extracts (**Figure 50**). However, we noticed that RIF1 is recruited before YAP, since RIF1 can be observed as early as 5 min after the addition of the sperm DNA into replicating extracts. Moreover, we observed that YAP depletion did not have any impact on RIF1 recruitment, meaning that its recruitment is YAP-independent. Importantly, since YAP does not contain a DNA binding domain

(Pocaterra *et al*., 2020), we speculate that during DNA replication YAP needs another binding partner that could be RIF1. Unfortunately, we were unable to deplete RIF1 from *Xenopus* egg extracts to a sufficient level so as to abrogate its recruitment onto chromatin as we could do for YAP. RIF1 is indeed highly abundant in those extracts (Kumar *et al*., 2012) and even two consecutive rounds of immunodepletion were unsuccessful. The use of different types of cellular extracts in which RIF1 could be depleted by other means such as siRNA as previously tested in HeLa cells extracts (Alver *et al*., 2017) could help answering the question whether or not RIF1 is required for YAP chromatin recruitment during DNA replication.

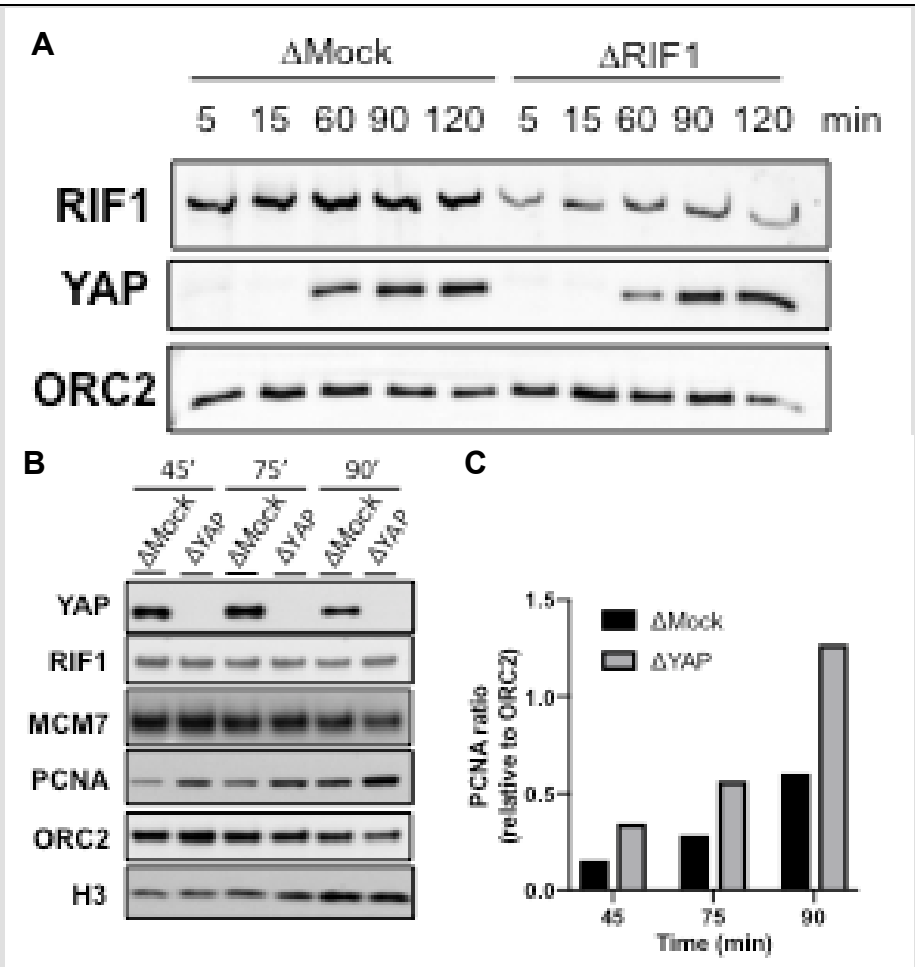


Figure 50. Protein dynamic recruitment in ΔRIF1 and ΔYAP chromatin. A. Western blot showing protein recruitment into chromatin in ΔMock and ΔRIF1 extracts. RIF1 could not efficiently depleted after two rounds of RIF1 antibody-beads into extracts. B. Western blot showing protein recruitment into chromatin in ΔMock and ΔYAP extracts C. Quantification of PCNA signal ratio relative to ORC2.

DNA replication has the advantage to be a process separated in steps that are mutually exclusive, licensing is restricted to late M- and G1-phase while firing

occurs in S-phase (Tanaka and Araki, 2013), this aforementioned segregation of licensing and firing is recapitulated in *Xenopus* egg extracts, thus facilitating researchers to study individual steps thanks to pharmacological manipulations. We observed that YAP recruitment was impaired after both origin licensing and firing inhibition using geminin and $p21^{Cip1}$, respectively (**Figure 1A of the article and Figure 51**). As opposed to geminin treatment, MCM2 recruitment into the chromatin was not inhibited by the addition of $p21^{Cip1}$, as expected. Efficiency of $p21^{Cip1}$ is observed by PCNA inhibition. Here, we observed that even if we do not affect pre-RC complex assembly, YAP recruitment is somehow inhibited after impairment of DNA synthesis, this could imply that YAP has different effects according to the step of DNA initiation. Thus, not only pre-RC is needed for YAP recruitment but also impacting the stability of the chromatin has a negative effect in YAP recruitment. In addition, even after blocking pre-RC assembly by geminin, we only delayed YAP recruitment but it was not completely blocked as we could find it at later time of replication. This supports the idea that YAP has multiple roles during DNA replication, similarly to RIF1.

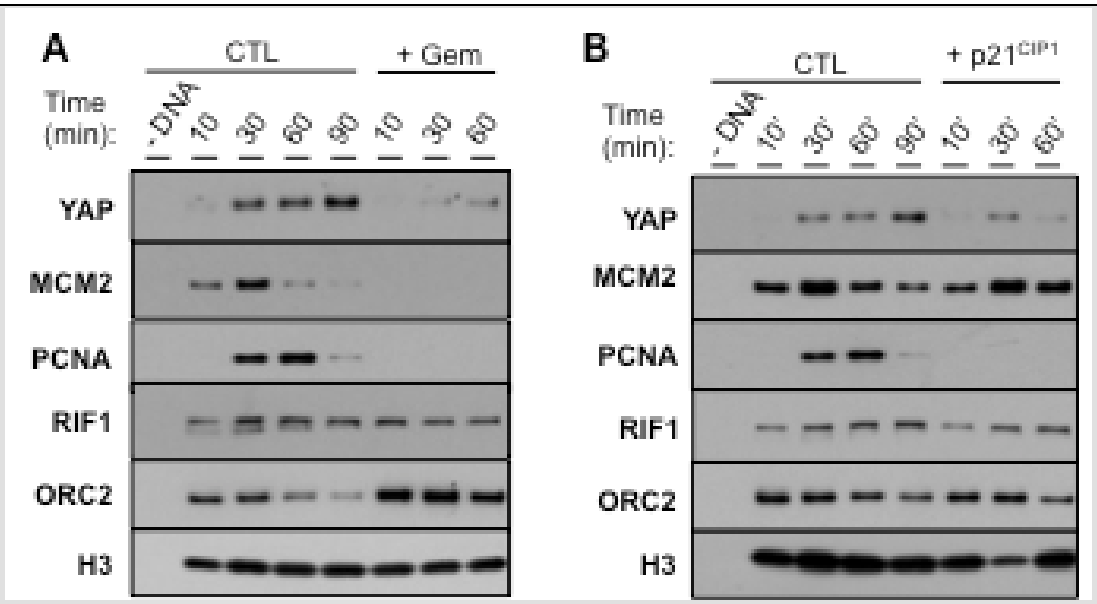


Figure 51. Effect of geminin and $p21^{CIP1}$ addition on YAP recruitment in *Xenopus* egg extracts. (A) Demembranated sperm nuclei (2000 nuclei/µl) were incubated in *Xenopus* egg extracts in the presence (+ Gem) or absence (CTL) of geminin (100nM) to block origin licensing. Chromatin was isolated at indicated time points for immunoblotting. (B) Chromatin was isolated in the presence (+ $p21^{CIP1}$) or absence (CTL) of $p21^{CIP1}$ (5 ng/µl) to block CDK activity and origin firing. Isolated chromatin was subjected to immunoblotting.

Before, it was demonstrated that RIF1 is dispensable for pre-RC formation (Yamazaki *et al*., 2013), our results found the same thing for YAP since YAP depletion does not impairs ORC2 or MCM7 recruitment (**Figure 50B**). On the opposite, we observed that YAP recruitment was greatly decreased after geminin treatment an event that was not appreciable for RIF1 by western blot (**Figure 51**). Interestingly, RIF1 in *Drosophila* embryos could not be detected at late replicating foci after geminin treatment, but did not blocked the initial binding occurring after mitosis exit nor the nuclear accumulation of RIF1 (Seller and O'Farrell, 2018). In other words, only the localized binding of RIF1 into late S foci was impacted. This reveals a clear difference between YAP and RIF1.

YAP parallels the function of RIF1 in *Xenopus* egg extracts

We were interested in studying whether YAP affects origin firing, in view that RIF1 depletion in *Xenopus* egg extracts increased DNA initiation (Alver *et al*., 2017). Alver *et al*. showed that hyperphosphorylation of MCM4 and increased chromatin binding of Cdc45 and PCNA were a consequence of depleting RIF1 in *Xenopus* egg extracts (Alver *et al*., 2017). They concluded that RIF1 changed the structure of chromatin in order to be more accessible for the phosphorylation of pre-RC components by Cdc7. Interestingly, we showed that YAP depletion also recruits more PCNA to the chromatin compared to the control (**Figure 50**). To exploit this result further, we could assess whether MCMs phosphorylation is also increased in YAP depleted extracts.
Additionally, we observed that YAP depletion in *Xenopus* egg extracts produced an increase of DNA synthesis. When confronting our YAP results with RIF1 literature, we found that immunodepleted RIF1 extracts had a rate of replication increased compared to Mock depleted extracts (Alver *et al*., 2017). In addition, RIF1 depletion in HeLa cells by siRNA produced a slight but reproducible increase in DNA synthesis assessed by BrdU and ^{3}H thymidine incorporation (Yamazaki *et al*., 2013). By observing the alkaline gel of YAP depleted samples, it was not the time of origin activation that was changed, since DNA synthesis started at the same time but rather the rate of DNA replication that was increased. This hypothesis was later confirmed by DNA combing assay.

To determine whether the effect observed after YAP immunodepletion was specifically generated by YAP removal, we tried to perform rescue experiments adding back recombinant YAP protein in the extracts and then evaluating DNA synthesis (**Figure 52**). However, we could not restore the dynamics of DNA replication upon the addition of recombinant YAP. Perhaps this is due to the loss of other factors when we immunodeplete YAP or to the lack of proper YAP post-translational modifications that impair its activity. For example, we observed among the proteins enriched in the YAP-IP sample the presence of replication factor C subunits 2 and 3 (RFC2 and RFC3), so it could be that YAP interacts with a complex of several proteins that when we immunoprecipitated YAP we also remove some of them. Additionally, it has been shown that phosphorylation, deacetylation and glycosylation modulate the activity and stability of YAP by controlling its subcellular localization (Yan *et al*., 2020). These types of modifications could thus be crucial for the correct function of YAP during replication dynamics.

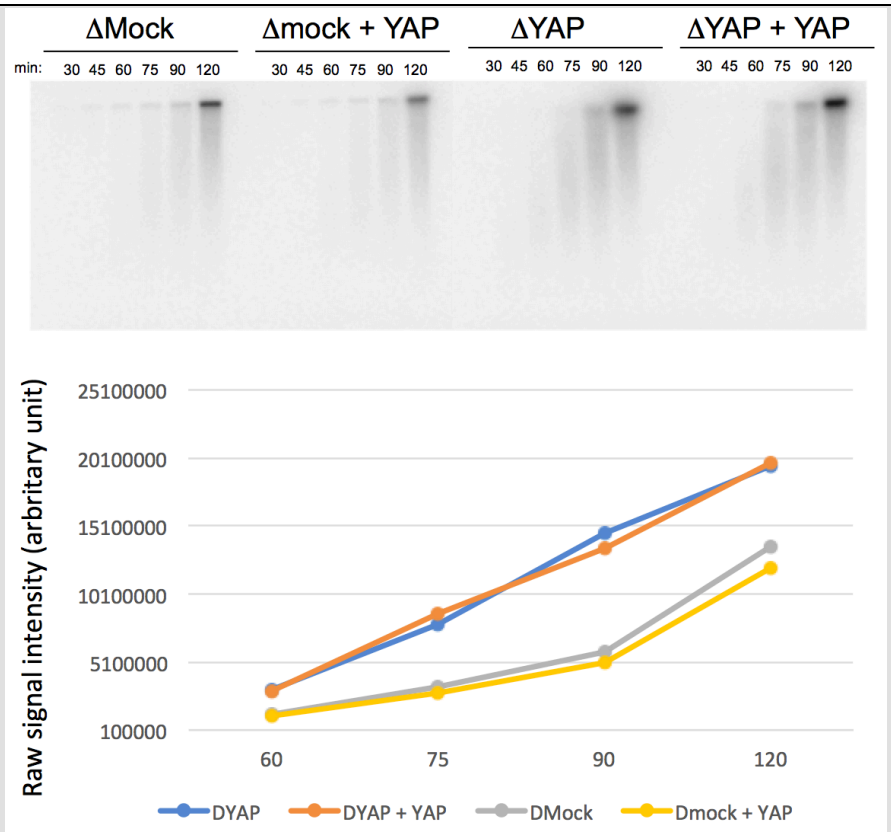


Figure 52. Addition of recombinant YAP into depleted YAP extract does not rescue the effect on DNA replication. Alkaline gel showing DNA synthesis in ΔMock, ΔYAP and with the addition of recombinant YAP. Graphic showing the intensity of the signal observed in the alkaline gel.

In the manuscript presented in the result section, we showed the results of one combing experiment (**Figure 2 of the article**) in which all the data were consistent and converged to the conclusion that YAP depletion leads to an acceleration of DNA replication as well as an increase in the number of replication origins. However, we performed this experiment a second time and we obtained slightly different outcomes regarding the ETED (eye to eye distance) (**Figure 53**). The ETED is the distance between two adjacent origins of replication, a shorter value represents more activated origins while longer distances reflect less and more spaced activated origins. In the first one (**Experiment A in Figure 53 and Figure 2D of the article**), we observed a reduction in the overall ETED that suggests more origin activation, which is in accordance with our results of increased replication extent and fork density. However, when looking at the second experiment (**Experiment B in Figure 53**), we surprisingly observed the opposite, that is an increase in ETED. We have one possible explanation for this: the replication extent and the EL (eye length) are bigger in experiment B compared to experiment A (Figure 52A & 52D), suggesting that eye fusions could have occurred at late phases of replication in experiment B, altering the interpretation of the ETED measure. This result could be studied more deeply with the addition of two dinucleotides at different times of replication to label newly synthetized DNA. However, this kind of experiments is quite difficult to accomplish in *Xenopus* extracts due to the high speed of replication in this system. In our hands, full replication was observed nearly at 90 min. If we compare this time with other systems, such as the extracts from HeLa cells, their replication takes place in intervals of 8h, making it possible to discriminate eye fusions and the apparition of replication stress marks such as fork stalling or unidirectional fork progression with the use of double labeling markers. In addition, the use of cells could help to unveil the sites of YAP binding inside the nucleus, for instance if they mimic the sites of RIF1 in the heterochromatin.

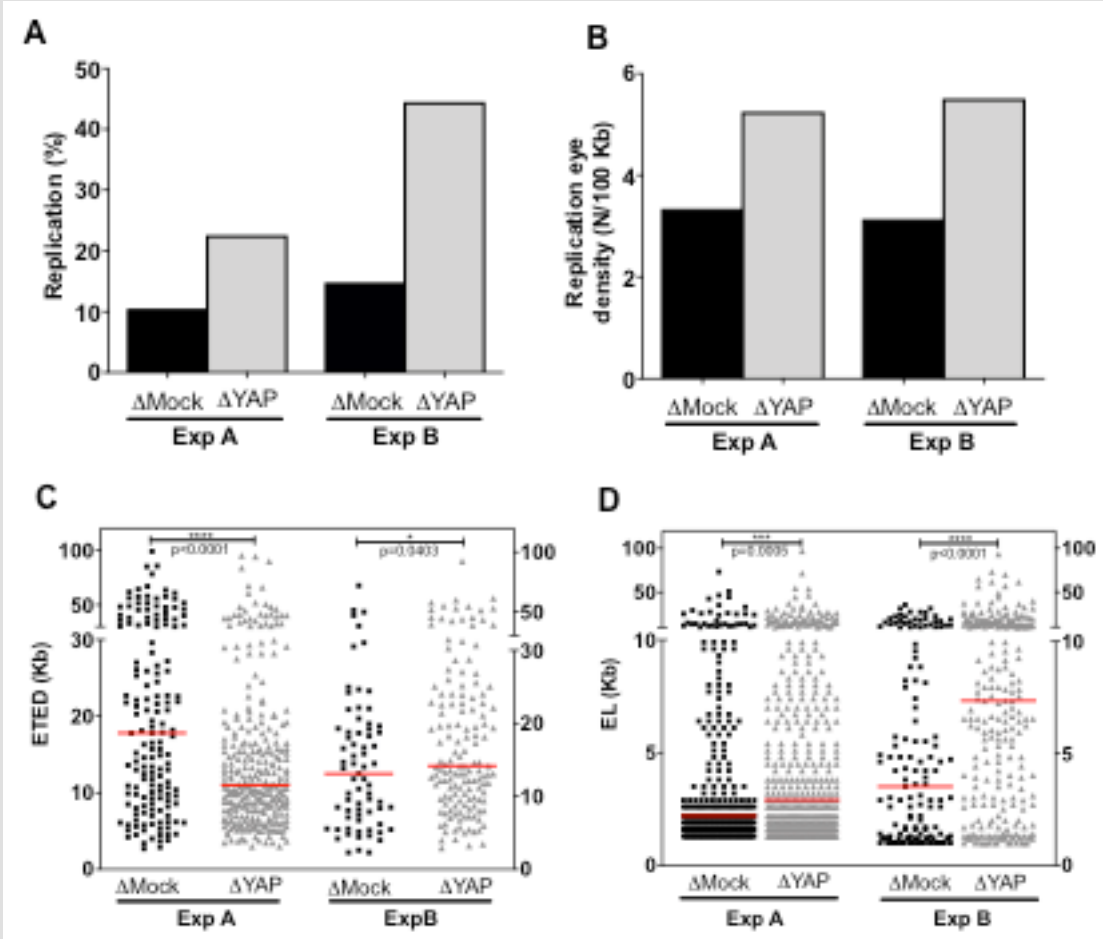


Figure 53. DNA replication dynamics increases after YAP depletion. Sperm nuclei (2000 nuclei/µl) were replicated in egg extracts in the presence of Biotin-dUTP. (A) Percentage of replication calculated for two independent combing experiments, (B) Mean replication eye density (N/100kb) of two independent experiments, (C) Eye-to-eye distance distributions (ETED) (scatter dot plots with median) (D) Eye length distributions (EL) (scatter dot plots with median). Data were evaluated for significance using an unpaired, two-tailed t test followed by a Mann-Whitney test.

Interestingly, in contrast to our results showing that YAP depletion increases replication fork speed, DNA fiber analysis performed in cells transfected with siRif1 could not confirm alteration in fork speed (Alver *et al*., 2017). Inter origin distances were not affected in mESC *Rif1*$^{-/-}$ and no signs of unidirectional or collapsed forks were found. As a conclusion, they stated that the increase in replication was due to the loss of slow replicating origins and an increase in the activation of replication domains. As a perspective, it will be interesting to study whether dormant origins are activated after YAP depletion. For example, we could use caffeine that allows dormant origin firing thereby increasing overall active origins (Woodward *et al*., 2006) and then see the effect after YAP depletion.

RIF1, similar to YAP, controls RT of *Xenopus* retinal stem cells

Similarly to YAP, we observed that RIF1 localization is restricted to RSCs and early progenitor cells of the CMZ region of *Xenopus* post-embryonic embryos. Interestingly, we showed that *Rif1* depleted cells in *Xenopus* exhibited altered replication foci cell patterns having predominantly early S-phase foci. Notably, this event was also observed after *Yap* depletion in the same model (Cabochette *et al*., 2015) reinforcing the idea of an *in vivo* cooperation. RIF1 outstands in the control of RT through remodeling the chromatin and changing the 3D chromosomal architecture (Yamazaki *et al*., 2013). With a higher resolution microscopy, RIF1 was observed to localize in nucleolar and nuclear periphery regions of heterochromatin (Foti *et al*., 2016). It will be interesting to test whether YAP is involved in nuclear architecture and look for its ultrastructure localization. This could be studied using DNA halo assays that indicate alterations in the size of chromatin loops (Gerdes *et al*., 1994; Yamazaki *et al*., 2012) and electron microscopy, respectively.

Unlike YAP that clearly affects S phase progression of RSCs in *Xenopus*, RIF1 effect on S phase progression seems more ambiguous and differs between different studies. For example, FACS analysis performed in HeLa cells transfected with siRif1 showed no alterations on S phase length compared to control cells (Yamazaki *et al*., 2013). On the opposite, it was observed that RIF1 is important for the lengthening of S phase that occurs in post-MBT embryos of *Drosophila*, since *Rif1* mutant embryos displayed shortened S phase (Seller and O'Farrell, 2018). We cannot rule out that the effect observed by YAP in RCS is a mixture of effects, on one hand the control of DNA replication *per se* and on the other hand the effect on transcription due to its role as the effector of the Hippo pathway. Additionally, YAP has been shown to bind chromatin remodelers to modify transcription. In this context, it is difficult to discriminate YAP specific function in origin firing regulation since evidence describe YAP as a hub where several inputs connect such as cell density, ECM components and mechanical forces, just to mention a few. By contrast, RIF1 major function is associated principally to the regulation of RT, where it marks the timing of mid-late replication foci in mESC, an event that we also observed in *Xenopus* RSCs.

It was observed by my laboratory that CMZ retinal stem cells posses longer S-phase compared to late progenitors (Cabochette *et al*., 2015). Similarly, it was observed that mammalian cortical NSCs exhibit longer S-phase than their committed progenitors (Arai *et al*., 2011; Turrero Garcia *et al*., 2016). Thus, our findings showing that both YAP and RIF1 are specifically expressed in RSCs and in early progenitors is consistent with their role in slowing-down S-phase progression. We propose that YAP and RIF1 serve to decelerate DNA replication dynamics in order to preserve stem cells genome quality.

The biological importance of RT is still not well understood, however it is clear that inappropriate DNA replication may trigger the DNA damage response and the S-phase checkpoint activation, in order to give enough time to the cell to repair errors in duplication. Before, my team observed that YAP deficient cells in the retina of *Xenopus* present genome instability observed by γ-H2AX labeling as a marker for dsDNA breaks and TUNEL assay for apoptosis. Regarding RIF1, studies have implicated it in the activation of cell-cycle checkpoints in yeast and *Xenopus* (Kumar *et al*., 2012). According to our results *Rif1* depletion increased cell death and genomic instability in *Xenopus* retina, however the positive cells appear to be in some cases outside the zone where RIF1 is expressed in the CMZ (**Figure 54**).

To tackle down this issue that could be derived from off target effects of the morpholino, we tried to perform rescue experiments by co-injecting a non-targeted morpholino mRNA of *Rif1* to verify the specificity of the effect. However, we could not succeed in the production of a full-length product of RNA likely due to its large size sequence of 7 kbp. As an alternative, we could try a co-injection of an anti-p53 morpholino, since it was observed that some of the off target effects that produce neural toxicity are due to the activation of p53 after morpholinos injections in zebrafish (Bedell *et al*., 2011).

Even if we cannot confirm that the activation of cell death and genomic instability is indeed due to *Rif1* knockdown, other studies associate *Rif1* expression with genomic instability, for example RIF1 co-localized with γ-H2AX labeling in mESCs after inducing DNA damage (Buonomo *et al*., 2009; Silverman *et al*., 2004). In addition, it would be interesting to test whether the DNA damage response in *Xenopus* egg extracts is altered in a YAP depleted context.

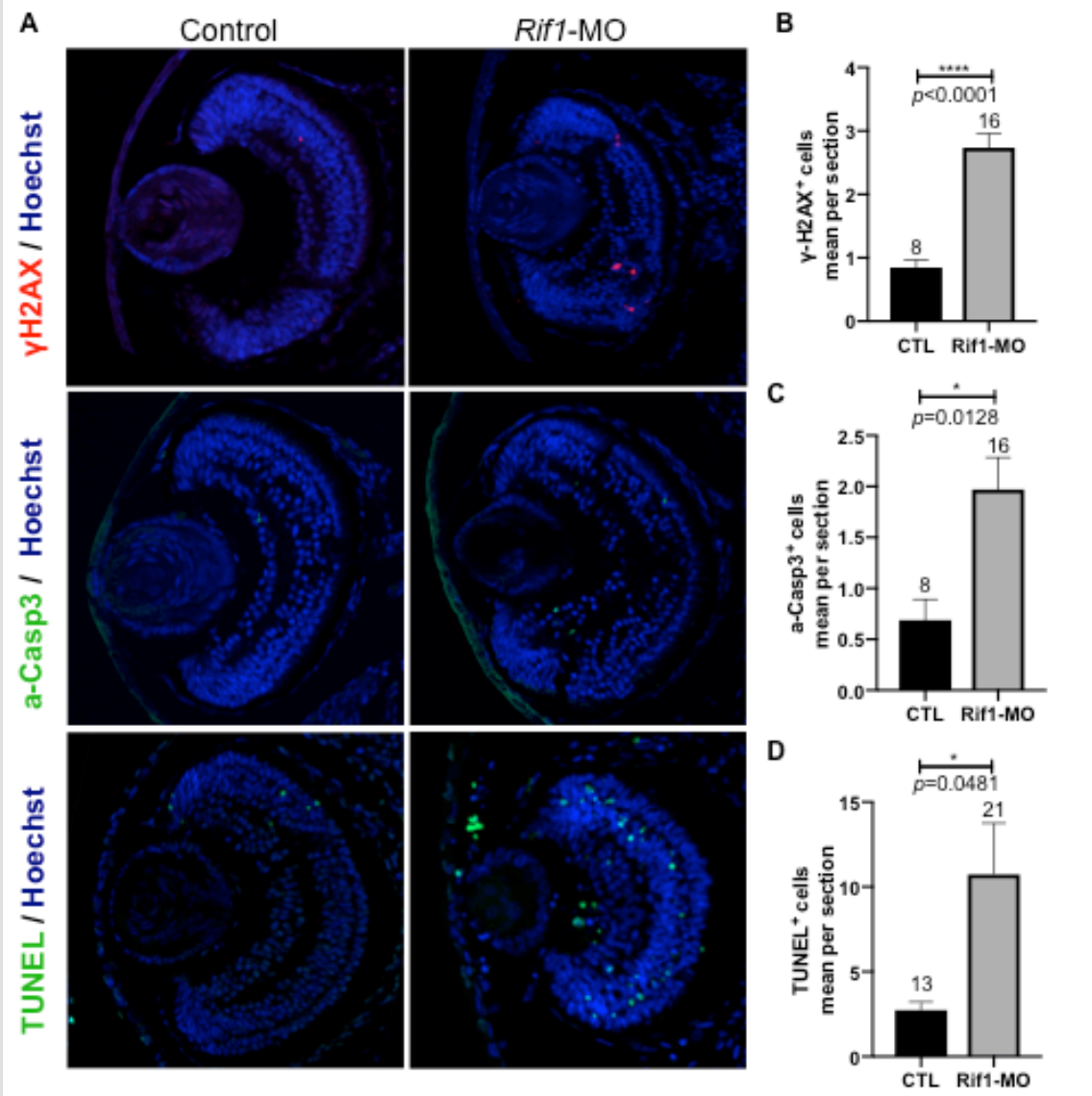


Figure 54. *Rif1* knockdown decreases eye size and leads to DNA damage in the retina. (A) TUNEL assay and immunolabelling analyses using either an anti-activated caspase3 (a-Casp3) or an anti-γ-H2AX (γ-H2AX) antibodies performed on retinal sections from stage 41 tadpoles injected as in Fig. 5F. (B-D) Quantification of TUNEL (B), a-Casp3 (C) and γ-H2AX (D) positive cells. Data are represented as means ± SEM. Data were evaluated for significance using an unpaired, two-tailed t test followed by a Mann-Whitney test. Scale bare: 40 μM.

YAP and RIF1 control cell division timing in *Xenopus* early embryos

In the whole embryo of *Xenopus*, *Yap* and *Rif1* have similar patterns of expression. *In situ* hybridization performed in *X. tropicalis* for *Yap* showed that at neurula stage it localizes at neural tissue such as neural crest, neural plate and neural groove (Nejigane *et al*., 2011), localizations that resemble our results with RIF1 expression (**Figure 55**). Later in development, *Yap* localizes at

the nervous system including head, eye and spinal cord, all of them sharing *Rif1* expression.

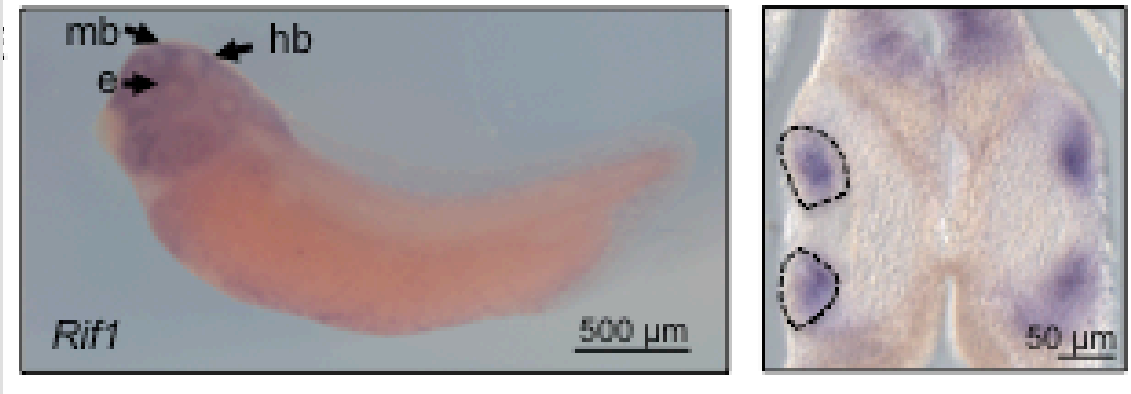


Figure 55. *Rif1* expression in *Xenopus laevis*. *Rif1 in situ hibridization*, (left) whole embryo of *X. laevis* stage 35/36, mid-brain (mb), hind-brain (hb), e (eye); (right) cross section of the head, showing both retinas, encircled areas show one CMZ.

Formerly, it was proposed that transcription and RT were likely correlated. This speculation was evident after seeing that early replicating sites of the chromosomes correspond to sites of active gene expression, also known as euchromatin. On the other hand, regions of late replication tend to be silent zones of expression, the heterochromatin (Marchal *et al*., 2019). However, recent studies suggest that those events can be separated from each other. For example, it was discovered that RT occurs before transcription as seen in the early embryos of fast dividing organisms, such as zebrafish before MBT period (Kaaij *et al*., 2018; Siefert *et al*., 2017). In mouse embryos, the presence of spatiotemporal patterns of replication is observed before transcription starts at one cell-stage embryos (Ferreira and Carmo-Fonseca, 1997). In our case, we found that RIF1 and YAP are proteins whose regulation is developmentally regulated. Interestingly, both proteins are maternally expressed and present variation throughout early *Xenopus* embryogenesis (**Figure 56**). While RIF1 seem to decrease at later stages of development, concentrating on neurogenic niches, YAP on the contrary tends to increase. In this regard, during the first cell divisions of *Xenopus*, the DNA:cytoplasm ratio increases until MBT when zygotic transcription begins and cell cycle lengthens. Interestingly, it was shown that S phase slows down shortly after MBT due to a genome wide decrease of replication origins (Platel *et al*., 2019). Thus, it would be interesting to investigate whether the differential expressions of YAP and RIF1 could regulate

these embryonic RT features during early embryogenesis before and after zygotic transcription.

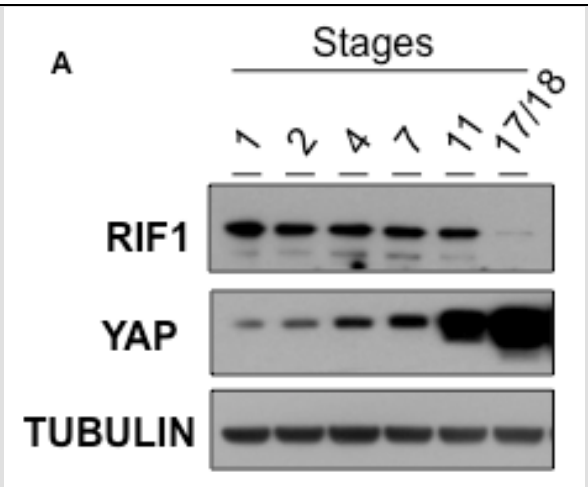


Figure 56. RIF1 and YAP expression in early *Xenopus* embryos. (A) Western blot showing the developmental expression of RIF1 and YAP during early *X. laevis* embryogenesis. Tubulin serves as a loading control.

Here, we observed that both YAP and RIF1 alter the cell division timing upon depletion in early *Xenopus* embryos (**Figure 57**). Interestingly, it was observed that RIF1 helps to prolong S phase duration in *Drosophila* post-MBT embryos (Seller and O'Farrell, 2018). Our results in early pre-MBT *Xenopus* embryos are consistent with a shortening of S phase in absence of YAP or RIF1. As a perspective, it will be interesting to observe whether the timing of the MBT is changed after YAP and RIF1 depletion.

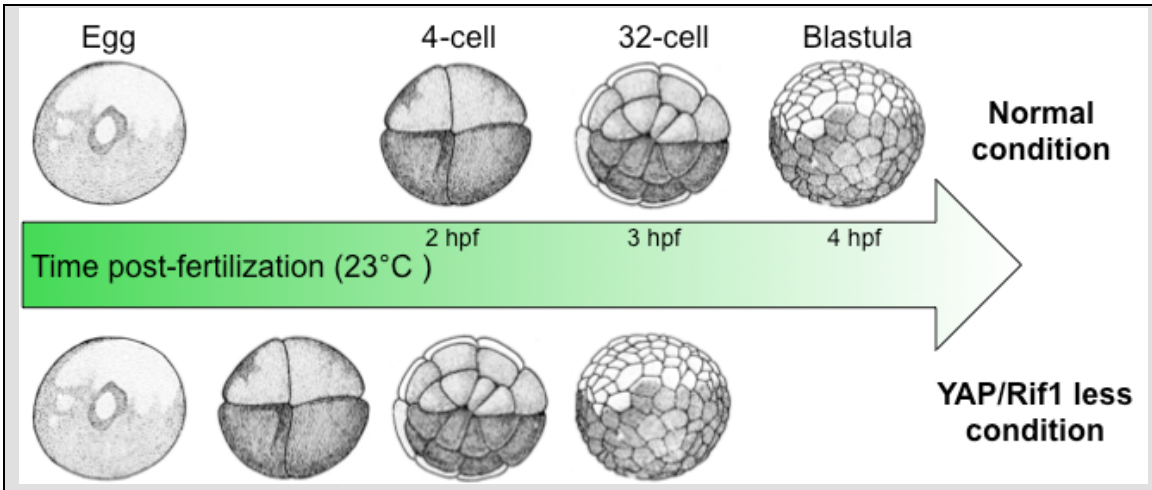


Figure 57. Cartoon showing the effect on acceleration of cellular division after YAP/RIF1 depletion of *X. laevis* early embryos. Under normal conditions (top of the arrow), embryonic development takes place leading to blastula stage 4 hours post fertilization (hpf) at 23°C. When YAP, RIF1 or both proteins are depleted early in development, overall cell division is accelerated.

Besides the effects of RIF1 in DNA replication, it was observed that it also localizes to the midbody of dividing cells where it recruits PP1 to counteract Aurora B kinase activity and allow abscission timing (Bhowmick *et al*., 2019). Similarly, YAP was localized in the midbody and spindle of HeLa cells (Bui *et al*., 2016). In this study, it was found that YAP interacts with a scaffold protein to regulate the cytokinesis independently of transcription. We could imagine that the accumulation of disturbed DNA RT joined with perturbed abscission timing in this early developmental period by the lack of two maternally expressed proteins have important consequences in the internal cellular clock. According to the literature, improper cell division generates uneven segregation of genetic material to daughter cells and aneuploidy, features observed in cancer cells (Bui *et al*., 2016). Further studies involving live cell imaging will give us more insights about this interaction.

In human embryogenesis, it was observed that reactivation of gene expression from the embryo starts between 4- to 8-cell stage (Braude *et al*., 1988). Thus the 8-cell stage represents a moment in which the embryo starts to guide by itself its development and it is here that cell cycle and chromosome machinery need to work perfectly for normal human development. Using improved methods of sequencing and whole human microarray analyses, scientist characterized the gene expression appearing in 8-cell human embryos to understand the molecular pathways that control human development. Interestingly, genes involved in circadian rhythm, cell cycle division and DNA replication were highly up regulated (Kiessling *et al*., 2009). In the future it will be important to assess whether our results showing that YAP and RIF1 control cell division rates in early *Xenopus* embryos also hold true during mammalian embryogenesis. Moreover, it has been proposed that the rate of embryo cleavages could serve as a tool to determine the embryo viability as a manner to improve the outcomes of assisted reproduction. In fact, women producing early-cleaving embryos had higher pregnancy and implantation rates than those who did not (Lundin *et al*., 2001; Sakkas *et al*., 1998; Salumets *et al*., 2003). After a large-scale time-lapse analysis of human blastocyst, it was observed that embryos that cleave earlier have better chances to continue developing than embryos that develop more slowly (Cruz *et al*., 2012). This produces the notion that the speed at which embryos develop is linked to their quality and

success to form a baby. According to this information, YAP and RIF1 might be of relevance for the viability of embryos and as such their study will be important to know more about the relationship between the correct parameters of healthy embryos used for *in vitro* fertilization.

Importance of RT in neurosciences

The relevance in the regulation of DNA replication has been demonstrated for normal organism development (Champeris Tsaniras *et al*., 2014; Hua and Orr-Weaver, 2017). Here, we observed that RIF1 and YAP depletion in early *Xenopus* embryos leads to alteration in cell division, body malformations and a phenotype of dwarfism. Interestingly, recent data associates changes in DNA replication with normal development of the mammalian brain (Kalogeropoulou *et al*., 2019). Additionally, alterations in their regulation may be associated with microcephaly and mental diseases.

Changes in the duration of the cell cycle have been seen during murine neurogenesis, when NSCs begin to differentiate. The relatively short cell cycle of ~12 h elongates to have a duration of ~17 h as a consequence of an increase of the G1 phase length during NSC differentiation (Calegari *et al*., 2005; Noctor *et al*., 2002; Takahashi *et al*., 1995). It has been proposed that this change in cell length is due to changes in origin licensing which happens to be at G1 phase. Studies of hESCs differentiation into neural progenitor cells showed a decreased expression of licensing factors (Matson *et al*., 2017), while non differentiated hESCs maintain high levels of CDT1 and CDC6 to ensure abundant origin licensing (Fujii-Yamamoto *et al*., 2005). An open question is whether NSCs deploy a distinct mechanism to regulate licensing. Analysis at different developmental stages of neural progenitor cells is needed to address the developmental regulation of licensing. In our study, we found that RIF1, a major regulator of RT had an impact in the correct development of *Xenopus* embryos, highlighting the importance of DNA replication timing program and development.

It was observed that deregulation of DNA replication leads to a decrease in proliferation and associates with developmental retardation and brain malformations such as microcephaly (Khetarpal *et al*., 2016; Mazouzi *et al*.,

2014). Notably, microcephaly has been detected in patients with Meier-Gorlin Syndrome (MGS) (Bicknell *et al*., 2011b; Burrage *et al*., 2015) which in addition present mutations in the pre-RC and geminin genes (Bicknell *et al*., 2011a; Burrage *et al*., 2015; de Munnik *et al*., 2012). It is thought that improper early licensing of NSCs impairs their rapid proliferation having as a consequence a low pool of cells and leading to incomplete development, as observed by dwarfism and microcephaly in patients with this syndrome. However, the exact mechanism causing the decreased proliferation of NSCs in MGS is not known. It is intriguing, whether RIF1 expression could be affected in this context, since we observed that RIF1 is expressed in the proliferating populations of neural cells in the early *Xenopus* embryos (**Figure 55A**).

Additionally, microcephaly can be caused by virus infection, such as Zika virus (ZIKV). Interestingly, ZIKV targets principally proliferating neural progenitor cells (Garcez 2016, Tang 2016). It was observed that those cells after infection decrease their proliferation due to altered cell cycle kinetics (Li *et al*., 2016; Wu *et al*., 2016) and present downregulation of genes involved in DNA replication progression (Zhang *et al*., 2016). Perhaps the preference of this virus to early proliferating NSCs is associated with the particular cell cycle of those cells. To further investigate the differential regulation of DNA replication and the mechanism behind it, innovative animal models related to impaired-licensing syndromes are needed. In this context, we showed using *Xenopus* that RIF1 and YAP are molecules important for the regulation of DNA replication timing program *in vivo* during development.

Moreover, regulation of DNA replication has been implicated with neuroplasticity and the occurrence of neurodegenerative diseases. For example, genes associated with AD and amyotrophic lateral sclerosis have been found to be located in regions where DNA RT switch from early to late S phase (Watanabe *et al*., 2002; Watanabe *et al*., 2014). Additionally, some of these genes are located in these transition zones in neural precursor cells but not in ESCs, suggesting that gene localization is important for the development of neurological diseases (Watanabe *et al*., 2014). Many neural genes implicated in learning and memory have been found in regions of human chromosomes between early to late replication, corresponding as well with zones that are susceptible to epigenetic modifications. Watanabe and Maekawa proposed a

model where RT influences the expression of neural genes located in early/late zones (Watanabe and Maekawa, 2016) (**Figure 58**). They hypothesized that alterations in the number of origin firings in the mid S phase are related to transitions into early S phase when there is an increase, or to late S phase when a decrease happens. It will be interesting to test whether RIF1 and YAP are associated with changes in the expression of neural genes due to their role as regulators of RT and their neural localization in *Xenopus* embryos.

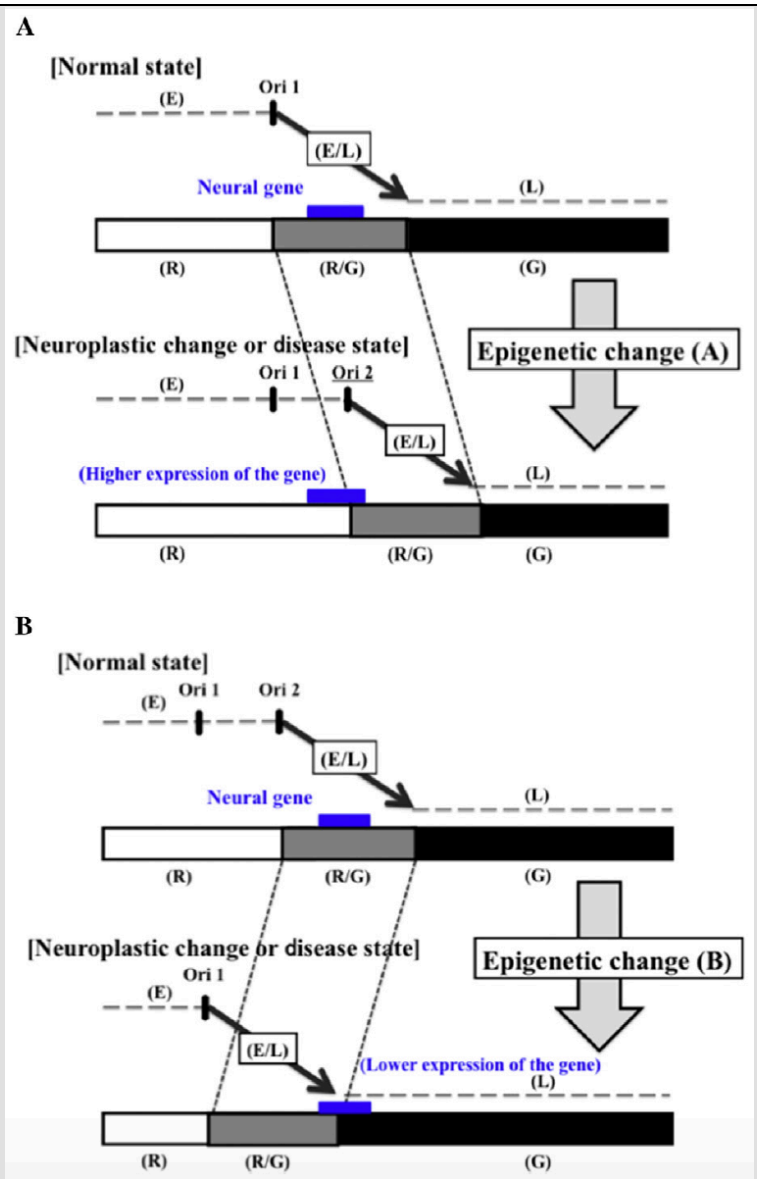


Figure 58. Proposed mechanism for how changes in RT of a neural disease gene located in a transition zone affect its expression. RT might switch from mid S phase to early or late S phase due to in crease (A) or decrease (B) of active early replication origins at the edge of the early replication zone. Additionally, the chromatin environment of the neural disease gene might change from an R/G chromosome band boundary to an R or a G band. Stalling of the replication fork in the vicinity of neural disease genes might induce chromosomal amplification (Triplet repeat expansions) or chromosome rearrangements that affect gene function, possibly through influencing the

rate of expression. The position of the neural disease gene (large gene) is indicated by the blue rectangle. E, early replicating zone; L, late replicating zone; E/L, early/late-switch region; R, R band; G, G band; R/G, R/G band boundary; Ori, replication origin (Watanabe and Maekawa, 2016).

Future of RT: the 4D nucleome project

A very promising project that aims to understand the 3D organization of the genome and its dynamics across time, called the 4D nucleome project is now taking place between nations (Dekker *et al*., 2017). Ultimately, it will develop, validate and standardize techniques; integrate the data to provide models of a comprehensive view of the 4D nucleome; and finally, investigate the implication of structural features of the genome in transcription, DNA replication and pathologies, among others (**Figure 59**). This major effort will implicate multidisciplinary involvement between distinct fields of science and it promises to clarify the relation between 3D architecture, transcription and RT and find its biological relevance.

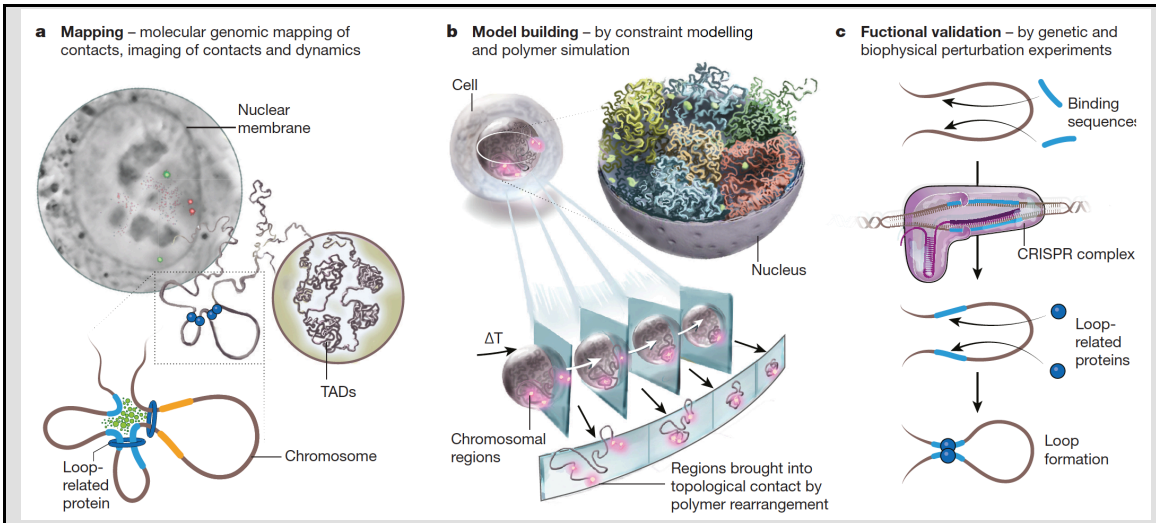


Figure 59. The 4D Nucleome project. The project encompasses three components. a) Experimental mapping approaches are used to measure a range of aspects of the spatial organization of the genome, including chromatin loops, domains, nuclear bodies and so on. b) Computational and modeling approaches are used to interpret experimental observations and build (dynamic) spatial models of the nucleus. c) Perturbation experiments, for example, using CRISPR-Cas-9-mediated genome engineering, are used for functional validation. In these studies chromatin structures are altered, for example, by removing chromatin loops, creating novel loops at defined positions or tethering regulatory components in selected regions to test their architectural function. These perturbation studies can be complemented with functional implications of chromatin folding (Dekker *et al*., 2017).

This ambitious project united with the development of powerful techniques to study RT, such as mapping the chromosomal domains and the acquisition of RT profiles at the single-cell level of different animal species with characterized developmental stages such as *Xenopus,* would permit the use of RT dynamics as property of stem cells that has been underscored but potentially could be manipulated to obtain stem cells for regenerative medicine.

CONCLUSION

In this study, we conclude that YAP is a direct and active regulator of DNA replication dynamics, having a mechanism that works independently from its well-known role in gene transcription. We confirmed that its implication is not merely limited to RSCs of the frog but it also exist in the wide-known model of eukaryotic DNA replication, the *Xenopus* egg extracts, as well as in the early development of fast-dividing *Xenopus* embryos, in both cases where transcription is absent.

Despite our knowledge about the major proteins required for eukaryotic DNA replication, the molecules that regulate the timing of this process are largely unknown. Here, we found that YAP slow-down DNA replication through a mechanism that limits origin firing and overall DNA synthesis.

Moreover, we unveiled RIF1 as a novel interactant of YAP, which is likely to mediate the effects observed in the regulation of DNA RT. We were the first to look at the expression of *Rif1* during early embryogenesis. RIF1 is most strongly expressed in the nervous system after gastrulation and confined to stem/early progenitor cells in the post-embryonic retina, similar to YAP. Our knockdown experiments strengthen the idea of a YAP/RIF1 *in vivo* interaction.

Overall, our studies link YAP and RIF1 cooperation at setting up the timing of DNA replication, an event that correlates with stemness. Further research aimed at elucidating how these molecules interact during the RT program will bring essential information to decipher the importance of DNA replication in stem cell biology.

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ÉCOLE DOCTORALE
Signalisations et réseaux intégratifs en biologie (BIOSIGNE)
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Titre : YAP comme régulateur du programme de réplication de l'ADN.....

Mots clés : réplication de l'ADN, cellules souches, voie de signalisation Hippo/YAP.

Résumé : Une cellule souche est capable de s'auto-renouveler et de générer des cellules différenciées après division cellulaire. La duplication complète de son génome doit être exempte d'erreurs afin d'éviter la propagation aux cellules filles de mutations délétères. Chez les eucaryotes, il a été montré que des segments d'ADN sur les chromosomes se répliquent de manière coordonnée et à des moments définis pendant la phase de synthèse, un processus appelé programme spatio-temporel de réplication de l'ADN (RT). Des changements majeurs dans le RT sont corrélés avec les changements de détermination des cellules souches et associés à l'organisation et à l'expressivité du génome. Malgré ce rôle central, les mécanismes qui sous-tendent le contrôle du RT restent méconnus. Mon laboratoire a mis en évidence que YAP, l'effecteur en aval de la voie de signalisation Hippo impliquée dans la croissance cellulaire, régule la vitesse et la chorégraphie de la réplication de l'ADN des cellules souches rétiniennes chez l'amphibien xénope. Ces données révèlent YAP comme un nouvel acteur moléculaire dans le contrôle du RT.
Pour tester l'implication directe de YAP dans la dynamique de réplication de l'ADN, nous avons tiré profit du système *in vitro* d'extraits d'œufs de xénope dans lequel toutes les étapes du processus sont reproduites de manière synchrone. Nous montrons que YAP est recruté à la chromatine pendant la réplication et que ce processus se produit seulement après la phase d'initiation des origines de réplication. Des extraits déplétés de la protéine YAP présentent une accélération de la vitesse de réplication et une augmentation du nombre de sites d'activation de la synthèse de l'ADN. Par ailleurs, nous avons identifié RIF1 (Rap1-Interacting Factor 1 ou Replication Timing Regulatory Factor 1), un des rares régulateurs connus du RT, comme un nouveau partenaire de YAP. Comme pour YAP, la perte de fonction de RIF1 dans les embryons de xénope conduit à un phénotype de petit œil et à la dérégulation du RT dans les cellules souches rétiniennes.
Dans l'ensemble, nos résultats montrent l'implication de YAP dans le contrôle de la dynamique de réplication de l'ADN et révèlent RIF1 comme un nouveau partenaire dans ce processus. Ce travail ouvre de nouvelles perspectives d'étude quant à l'importance biologique de cette interaction YAP-RIF1 dans le contrôle du RT et sa pertinence comme cible pour influencer le devenir des cellules souches.

Title : YAP as a regulator of DNA replication timing.....

Keywords : DNA replication, stem cells, Hippo/YAP signaling pathway.

Abstract : Stemness could be defined as a state in which a cell is able to self-renew and/or to differentiate after cell division. Before this happens, exhaustive duplication of the genome free of errors must occur in order to avoid deleterious mutations, a hallmark of cancer. Thus, DNA replication is particularly important to stem cells because of their continuous division capacities. Regarding DNA replication in eukaryotes, it was discovered that segments of chromosomes close in space, replicate in a coordinated manner during S phase, a process called replication timing. Moreover, major changes in replication timing correlate with cell differentiation, 3D chromatin architecture and transcription. However, the molecules that govern its regulation are poorly understood.
Previously, my laboratory found that YAP, the downstream effector of the Hippo pathway, regulates S phase progression of retinal stem cells in *Xenopus laevis*. To test YAP function in the direct control of replication timing, we took advantage of the powerful *in vitro* DNA replication system of *X. laevis* egg extracts. Briefly, we discovered that YAP is recruited to replicating chromatin dependently of origin licensing. In addition, YAP depleted extracts showed increased DNA synthesis and origin activation; revealing that YAP normal function is to slow-down replication by limiting origin firing. Interestingly, we found RIF1, a major regulator of replication timing, as a novel partner of YAP. *In vivo*, RIF1 expression overlaps that of YAP within the stem cell compartment of the *Xenopus* retina. Knockdown of *Rif1* leaded to a small-eye phenotype and alterations in replication foci of retinal stem cells, resembling the effect observed in *yap* deficient cells. Finally, early-embryonic depletion of both molecules resulted in a strikingly acceleration of cell division.
Altogether, our findings unveil YAP implication in the regulation of replication dynamics and show RIF1 as a novel partner. Further investigation to analyze this interaction would help us to understand the biological relevance in the control of replication timing and whether it could be used as a target in regenerative medicine.