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DOCTORAL THESIS

Mechanisms that control the growth of cells in the presence of replication stress

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“Soy una abierta ventana que escucha,
por donde ver tenebrosa la vida.
Pero hay un rayo de sol en la lucha
que siempre deja la sombra vencida.”

Miguel Hernández

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En especial a mi abuelo Ángel

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pero también sé que estarías orgulloso.

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ABBREVIATIONS

40HT	4-hidroxi-tamoxifen
aa	Aminoacid
AT	Ataxia-Telangiectasia (related)
ATM	Ataxia-Telagiectasia Mutated
ATR	ATM and Rad3 Related
ATRIP	ATR Interacting Protein
CDK	Cyclin Dependent Kinase
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CFS	Common Fragile Site
CNV	Copy Number Variation
CSR	Class Switching Recombination
DAPI	4', 6-Diamine-2phenylindol, DNA intercalating agent
DSB	Double Strand Break
dsDNA	double stranded Deoxyribonucleic acid
ERFS	Early Replication Fragile Sites
IF	Immunofluorescence
IRIF	Ionizing radiation induced foci
DDR	DNA Damage Response
DNA	Deoxyribonucleic acid
DSB	Double strand break
γH2AX	Phosphorylated form of histone H2AX (S139)
G0, G1, G2	cell cycle phases; G0Gap1 and Gap2
HR	Homologous Recombination
HTM	High throughput Microscopy
HU	Hydroxyurea
IF	Immunofluorescence
IR	Ioinizing radiation
KO or -/-	Knock-Out
MEF	Mouse Embryonic Fibroblasts
MMR	Mismatch Repair
NHEJ	Non-Homologous End Joining
PCR	Polymerase Change Reaction
RNA	Ribonucleic acid
ROS	Reactive Oxigen Species
RPA	Replication Protein A
RS	Replication Stress
shRNA	Short hairpin Ribonucleic acid
ssDNA	Single Stranded Deoxyribonucleic acid
SV40	Simian vacuolating virus 40 or Simian virus 40
V (D) J	Somatic recombination during lymphocyte maturation

T121	First 121 aminoacids of T-Large antigen, part of SV40 virus
TopBP1	Topoisomerase II Binding Protein 1
UV	Ultraviolet radiation
WB	Western Blot
WT or +/+	Wild Type
+/-	Heterozygous

RESUMEN EN CASTELLANO

El ADN está expuesto a muchos tipos de daño durante la vida de un organismo. La mayor parte de las lesiones son detectadas y reparadas sin llegar a comprometer la viabilidad celular. Sin embargo, ciertos tipos de daño pueden poner en peligro la integridad del genoma. Durante la replicación, por ejemplo, se pueden dar gran variedad de lesiones que darán lugar a lo que conocemos como estrés replicativo. Si la cantidad de daño excede unos determinados límites, se activarán programas de apoptosis o senescencia celular que pueden comprometer la capacidad regenerativa de los tejidos y derivar en patologías asociadas al envejecimiento.

Así pues, mantener la integridad del genoma es fundamental para cada una de nuestras células. Sin embargo, a diferencia de otras proteínas y macromoléculas, no existe un recambio constante de ADN, lo que hace fundamentales la precisa detección, señalización y reparación de cualquier tipo de daño. Toda esta respuesta se denomina Respuesta al Daño en el ADN y está regulada por las quinasas ATM y ATR. Estas proteínas comienzan una cascada de fosforilaciones que logra establecer diferentes puntos de control en el ciclo celular. Estos puntos de control ralentizarán el ciclo celular, lo que dejará tiempo para reparar el daño en el ADN.

El estrés replicativo es un tipo especial de daño que ha sido asociado al envejecimiento y al cáncer; y que es controlado por la quinasa ATR. En ratones, la reducción de los niveles de ATR en un modelo del síndrome de Seckel se traduce en un incremento de los niveles de estrés replicativo y envejecimiento acelerado. Del mismo modo, los fibroblastos embrionarios derivados de este modelo murino acumulan estrés replicativo y entran en senescencia de forma prematura. En trabajos anteriores de este laboratorio se demostró que la eliminación de p53 no logra rescatar esta senescencia. Sin embargo, en esta tesis doctoral probamos cómo la deleción genética del locus INK4a/ARF logra rescatar por completo no sólo la senescencia prematura de los fibroblastos de ATR-Seckel, sino también la inducida por otras condiciones que generan estrés replicativo, como dosis bajas de hidroxiurea o inhibidores de ATR. Además, demostramos que una exposición

constante a estrés replicativo induce la expresión de los productos del locus INK4a/ARF; lo que revela que este locus se comporta como un punto de control del estrés replicativo.

En resumen, mediante el empleo de diferentes técnicas de biología molecular (Western Blot, Southern Blot, inmunofluorescencias…), biología celular (infecciones, estudios de la proliferación y ciclo celular…) y modelos animales, nuestros datos revelan un nuevo papel para el locus INK4a/ARF en la limitación de la expansión de células que sufren una exposición repetida al estrés replicativo. De este modo, logramos establecer un nuevo enlace entre el conocido supresor tumoral INK4a/ARF y el mantenimiento de la integridad genómica.

ABSTRACT

During our lifetime, DNA encounters many kinds of damage, both from endogenous and exogenous origin. Most lesions are detected and repaired without compromising cell viability. However, there are special types of damage that can endanger genome integrity. During replication, for instance, a wide range of lesions can occur. The different types of damage that appear during DNA replication give rise to Replication Stress (RS). If the amount of damage is over a certain threshold, cells can activate apoptotic and/or senescence programs, which can compromise the regenerative ability of tissues and lead to ageing related pathologies. This way, the integrity of the genome is a crucial event in the life of every cell. Nevertheless, unlike proteins and other molecules, DNA is not replaced. Thus, proper detection of DNA damage, precise signaling and potent repair machineries are needed. The response that cells establish upon DNA damage is what we call the DNA Damage Response (DDR). Protein kinases such us ATM and ATR are the key activators of this transduction pathway. They start a phosphorylation cascade in order to establish the different cell cycle checkpoints, which will slow down the cell cycle, leaving time for DNA repair.

Replication stress is a particular source of DNA damage that has been linked to cancer and ageing, and which is suppressed by the ATR kinase. In mice, reduced ATR levels in a model of the ATR-Seckel Syndrome lead to RS and accelerated ageing. Similarly, ATR-Seckel embryonic fibroblasts (MEF) accumulate RS and undergo cellular senescence. We previously showed that senescence of ATR-Seckel MEF cannot be rescued by p53-deletion. In this thesis work we show that the genetic ablation of the INK4a/ARF locus fully rescues senescence on ATR mutant MEF, but also that induced by other conditions that generate RS, such us low doses of HU or ATR inhibitors. In addition, we show that a persistent exposure to RS leads to increased levels of INK4a/ARF products, revealing that INK4a/ARF behaves as a bona fide RS- checkpoint. Our data revealed an unknown role for INK4a/ARF in limiting the expansion of cells suffering from persistent replication stress, linking this well-know tumor suppressor to the maintenance of genomic integrity.

Mechanisms that control the growth of cells
in the presence of replication stress

Introduction

INTRODUCTION

A. DNA DAMAGE

Our genetic material is exposed to continuous aggressions. One of the main biological functions of a cell is to maintain genome integrity. DNA lesions have been related to different aspects of human physiology and pathology. The most obvious one, due to its capacity to mutagenise, is cellular transformation and cancer development (Hanahan and Weinberg, 2000). Another one is the loss of regenerative potential in different tissues, by the reduction of their proliferative rates, what leads to ageing of the whole organism (Garinis et al., 2008). DNA damage has also been related to a wide range of illnesses such as neurodegenerative diseases, immunodeficiencies, or sterility (Jackson and Bartek, 2009). But DNA damage is also the driving force of evolution. DNA lesions are fundamental to boost the small variations in our genetic material that allow the wide adaptative range required for evolution (Stamatoyannopoulos et al., 2009).

There are a great variety of DNA lesions: from lack of complementarity of a pair of nucleotides, to the breakage of one or both of the DNA strands, lesions from exogenous or endogenous sources… And we need to take into account that chromosomes, unlike other cellular components, cannot be replaced. Therefore, a complex protein network that detects, signals and repairs any damage in the DNA is essential for the cell.

1. Sources of DNA Damage

1.1.Exogenous damage

Most exogenous sources of DNA damage come in the form of radiations that are able to penetrate membranes and tissues, reaching the cell nucleus and causing different lesions. We can classify radiations by their mechanism of action in two types: ionizing and non ionizing radiation.

Ionizing radiations, like X rays, gamma (γ) radiation and electromagnetism, can generate single strand or double strand breaks in the DNA helix (Lindahl and Barnes, 2000). They can also generate reactive oxygen species (ROS) upon their

impact in water, which will give rise to clustered DNA breaks along the radiation path (Hoeijmakers, 2001).

Non ionizing radiation is less potent, and therefore less damaging to DNA. However, we are exposed to it every day, since one type is ultraviolet (UV) radiation. Even though the ozone layer blocks most of the UV rays, part of the radiation is able to go through it and impact our skin, giving rise to highly stable and mutagenic covalent bonds that form pirimidine's dimmers (Jiang et al., 2009).

Besides the influence of radiations, there are several chemicals that we can be exposed to accidentally, or in purpose; such as in the case of many of the chemotherapies used in cancer treatment.

Still nowadays, cancer is still treated with surgery, radiotherapy and classical chemotherapy. Both, classical chemotherapy and radiation are based on the generation of DNA damage and the consequent activation of apoptosis or senescence pathways in order to kill cancer cells (Toledo et al., 2011a). Through their use, both cancer and healthy cells will incorporate some damage in their DNA, which in some occasions contribute to relapses or secondary tumors. However, cancer cells usually have high proliferation rates, what makes them particularly sensitive to DNA damage inducing agents. In contrast, healthy cells do not grow – most cells in a tissue are in G0 or G1- or grow at a slower rate, what keeps them somewhat protected from DNA damage.

1.2. Endogenous damage

A great portion of the DNA damage that we are exposed to and that is relevant to mutagenesis, carcinogenesis and aging is of endogenous origin. More specifically there are three main types of endogenous damage: reactive oxygen species (ROS), eroded telomeres and replication stress. There are also spontaneous changes in DNA bases, approximately 10^7 a day in a standard human body (Lindahl and Barnes, 2000).

But DNA is not only broken accidentally. DNA damage repair, as well as other physiological processes – like meiosis or limphocyte maturation - selectively produces DSBs. During meiotic recombination, chromosomes are broken in a

control fashion in order to interchange sequences with their homolog. This process increases the variability in gamete's genome (Zickler and Kleckner, 1998). Moreover, during class switching and V(D)J recombination there are different somatic recombination events that also require DNA breakage and reapair (Dudley et al., 2005). Accordingly, mutations in proteins implicated in DNA repair, in many cases lead to immune and fertility problems (Jackson and Bartek, 2009).

1.2.1. Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. The most dangerous ROS are the superoxide ion, hydrogen peroxide and the hydroxyl radical (De Bont and van Larebeke, 2004). In aerobic organisms the energy needed to for biological functions is produced in the mitochondria via the electron transport chain (Han et al., 2001). In addition to energy, ROS are also produced in this process. ROS can damage RNA, proteins and DNA - giving rise to single strand breaks (DNA nicks) and double strand breaks (DSBs) - what in theory, contributes to the physiology of ageing.

1.2.1. Eroded telomeres

Eroded telomeres are another important source of endogenous DNA damage. Telomeres are highly conserved structures, which function is to protect and stabilize the end of the chromosomes. Telomere erosion makes the cell unable to distinguish between a DSB and a normal telomere end. This causes irregular fusions and recombinations, that lead to chromosomal aberrations (Ludérus et al., 1996). Telomere erosion is due to the inability of DNA polymerases to completely replicate the 5' DNA end. Therefore, telomeres suffer a shortening that varies between 50- 200 base pairs in every cell division (Martens et al., 2000). This telomere shortening behaves like a biological clock that regulates the proliferative potential of every cell. Once a certain critique telomere length is reached, cells usually enter senescence. If they continue to proliferate, they will be predisposed to telomere fusions and genomic instability that could eventually lead to genetic disorders (Günes and Rudolph, 2013).

1.2.3. Replication stress

Replication stress (RS) is a not very well defined concept that refers to a diversity of alterations in the normal progression of the replication fork, caused by lesions encountered in the DNA, dNTPs deficiency or other problems at the replication fork. When RS is prolonged or, in the absence of ATR, the replication fork collapse and DSB are generated. Importantly, there is no such a thing as RS-free replication. Thus, RS repair pathways are essential, even in the absence of exogenous DNA damage.

Somehow it is believed that DNA synthesis magnifies the common effects of DNA damage (Ruzankina et al., 2008). For instance, a mutation in one single base that does not pair properly with its complementary one, will only give rise to a transitory ssDNA stretch during G1. However, the same mutation during S phase can lead to replication fork stall, which could eventually generate multiple DSBs.

Part of the errors that can occur during replication may be due the incorporation of the wrong nucleotide or a chemically modified one. Proof reading and Mismatch Repair (MMR) look after these mistakes and correct them during the replication process (Fu et al., 2012).

Also, in order to duplicate DNA content, replication forks need to unwind the double helix. Thus, ssDNA is constantly being exposed during this process. Any obstacle that blocks the advance of the replication machinery during this unwinding step, will give rise to long stretches of ssDNA; putting in risk DNA integrity (Paulsen and Cimprich, 2007).

In addition to this, loss of function of some components of the replication machinery can cause tension in the replication fork, which may lead to a DNA break. Topoisomerases are enzymes that are able to wind and/or unwind DNA. Some of them are responsible for releasing the tension caused by the characteristic supercoilling found in the front part of the replication fork (Koster et al., 2010). Their way of action implies cutting and pasting DNA, what makes them a potential source of DNA breaks. If the ligase activity of topoisomerases is blocked, they will not be able to repair the breaks that they have already made (D'Arpa et al., 1990).

Importantly, activation of oncogenes has recently been postulated as a source of replication stress. The first evidences revealed an activation of the DDR in precancerous lesions and cancer (Bartkova et al., 2005; Gorgoulis et al., 2005). Later on, and consistent with the previous observation, oncogene activation was proven to generate replication stress and senescence (Bartkova et al., 2006; Gorrini et al., 2007). In principle, the activation of oncogenes increases replication rates. These high replication rates can compromise replication control mechanisms, causing a greater ssDNA exposition and even DSBs. The underlying mechanism behind RS accumulation due to oncogene activation is not fully understood. Recent works propose that the activation of oncogenes induces RS through increased firing of replication origins, what leads to an insufficient nucleotide pool unable to support normal replication and genome stability (Beck et al., 2012; Bester et al., 2011; Poli et al., 2012). Consistently, addition of nucleosides counteracts the effects of oncogene activation on fork speed and DSB formation (Beck et al., 2012).

Altogether, these events would contribute to the characteristic genomic instability observed in most human cancers. This constant basal damage caused by oncogene activation is able to activate the DDR through the ATR and ATM kinases.

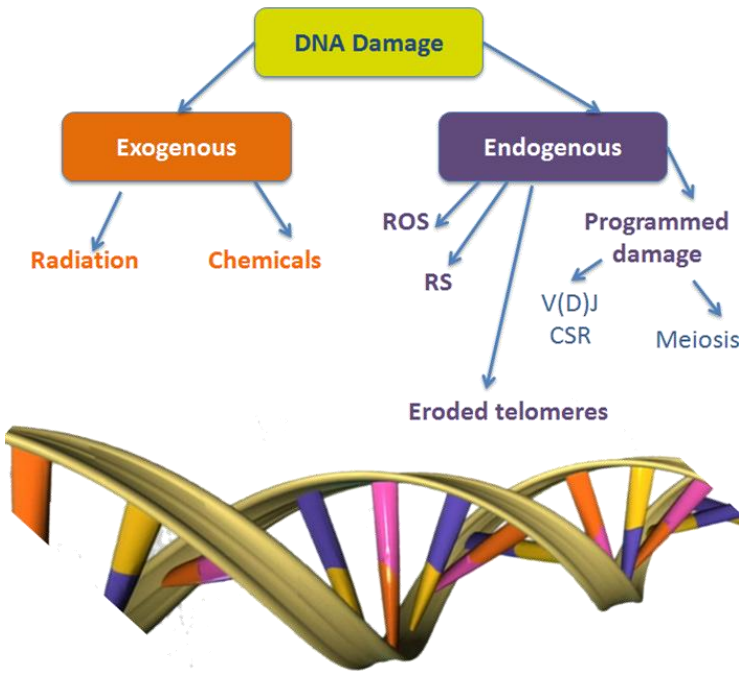

Figure 1. DNA damage sources. There are different sources of DNA damage that can alter the structure and function of DNA. DNA damage sources can be classified according to their origin in exogenous and endogenous sources. Different types of DNA damage can arise from these sources: from simple nicks or nucleotides alterations to single strand or double strand breaks, being these last ones the more severe ones.

2. The DNA Damage Response (DDR)

When a cell detects damage in its DNA, it activates a response that leads to DNA damage repair in order to keep genome integrity (Harper and Elledge, 2007). DNA damage Response refers to the group of processes that allow DNA repair, cell cycle control and, depending on the cell type or the amount of DNA damage, the induction of apoptosis or senescence. The type of response depends on the type of damage. Most of the lesions are repaired without affecting cell cycle control. However, in certain occasions the amount damage is high and/or the type of lesion severe and a cell cycle stop signal is activated. This stop signal is known as checkpoint and it delays the entry into the next phase of the cell cycle. There are cases in which the damage exceeds the repair capacity of the cell. In this situation, and depending on the cell type, healthy cells activate either a cell death program – apoptosis - or an irreversible stop program – senescence - . The most severe types of DNA damage that a cell can suffer are ssDNA and DSBs accumulation. ssDNA stretches are a highly recombinogenic structures and DSBs can cause chromosomal translocations and/or fusions. These events will lead to an improper segregation during mitosis, with the possible loss of genetic material or complete mitotic failure and catastrophic dead. In this scenario, most cells will die. However, low amounts of DNA damage can promote cellular transformation and the onset of cancer, by inducing the expression of oncogenes or the loss of tumor suppressors.

The DDR is a hierarchical process that activates several groups of proteins in a specific order (Figure 2). These proteins take care of the different tasks needed to repair DNA damage (Shiloh, 2003). First of all, lesions are detected by sensing proteins that stay near chromatin. Next, break ends are processed to gain a recognizable structure that can serve as a substrate for the different repair mechanisms. After this, the signal is transmitted through transducers. At this stage, the signal is diversified by the action of a variety of modulators and adaptators that spread it throughout the whole nucleus until it reaches effectors. Effectors will finally carry out the necessary actions to repair the damage. Finally, the entire signaling cascade must be turn off and return to its basal state, mainly thanks to the action of phosphatases (Bakkenist and Kastan, 2004).

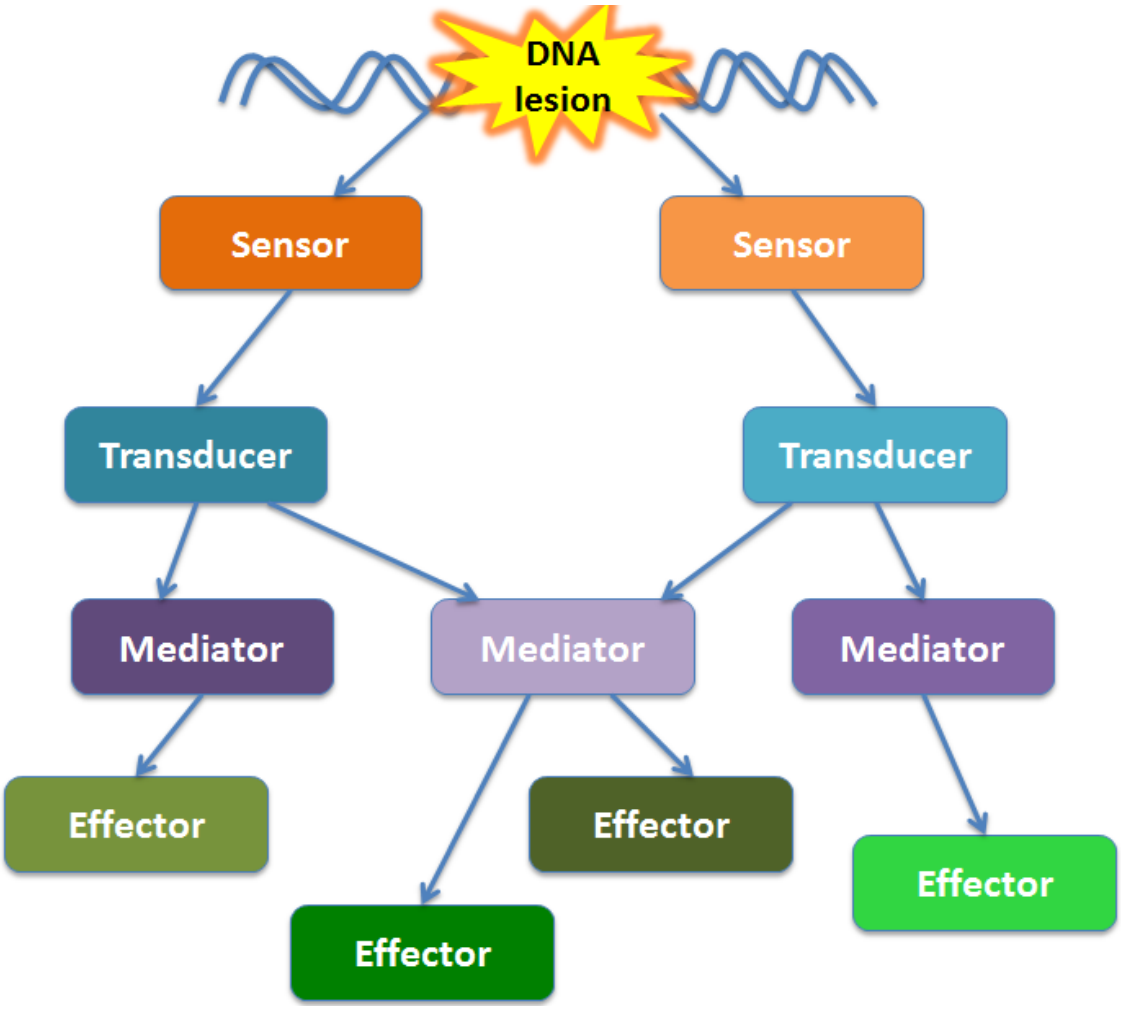

Figure 2. DDR hierarchy. Different types of damage are initially recognized by sensors, that recruit transducers to trigger the signal. This signal is amplified by mediators. Mediators guide the signal until it reaches effectors, in charge of executing the appropriate response.

3. Detecting the lesion and initiating the signal: DDR sensors and transducers

As mentioned before, DNA damage signaling starts with the recognition of the lesion, accomplished by sensors. These proteins are in constant contact with chromatin. Sensors are activated upon different types of damage and have an essential role in the activation of the DDR (Zou et al., 2002).

The most significative transducers of the DDR are three kinases: ATM (Ataxia Telangiectasia Mutated), ATR (ATM and Rad3 related), and DNA-PKcs (DNA protein kinase catalytic subunit).

3.1. DNA damage Kinases

ATM, ATR and DNA-PKcs transmit the DNA damage signal, mainly, by the most common post translational modification: phosphorylation. ATM, ATR and DNA-PKcs, together with mTOR, SMG1 and TRRAP, are part of the phosphatidylinositol-3-kinase like (PIKK) family (Shiloh, 2003). ATM, ATR and DNA-PKcs are high molecular weight proteins in which the kinase domain is highly conserved. In contrast to PI3K, which phosphorylate lipids, PIKKs exclusively phosphorylate proteins in their serine and treonine residues, preferentially when they are followed by a glutamic acid -(S/T)Q-. Their kinase domain represents only about 5-10% of the protein, which leaves the majority of it to regulatory functions. All these three proteins have a similar structure: a long aminoterminal region with several HEAT (Huntingtin, Elongation factor 3, a subunit of protein phosphatase 2A, and TOR1) repeats, a caboxiterminal region composed by FACTC (FACT C-terminal), and a kinase (catalytic domain PI3K), PRD (PiKK Regulatory Domain) and FAT (FRAP, ATM and TRRAP) domains.

Despite the structural similarities between ATM, ATR and DNA-PKcs, they all have different functions. On the one hand, ATM and DNA-PKcs are activated in response to DSBs in every phase of the cell cycle (Gottlieb and Jackson, 1993; Pandita et al., 2000). On the other hand, ATR is responsible for signaling ssDNA and its activity is restricted to S and G2 phases (Zou and Elledge, 2003). Finally, DNA-PKcs activity is restricted to the site of the lesion and it mainly promotes DSB repair (Collis et al., 2005), while ATM and ATR have an essential role in the global signaling of damage.

3.1.1DNA PKcs

DNA-PKcs is attracted to the break site by the damage sensor Ku70/Ku80 heterodimer, a DSB sensor that binds to DNA ends and stabilizes them (Smith and Jackson, 1999). The complex Ku70/ku80/DNA-PKcs forms a catalytically active kinase DNA-PK (Gottlieb and Jackson, 1993), that promotes DSB repair through Non Homologous End Joining (NEHJ) (Mahaney et al., 2009). NHEJ consists of the approaching and later ligation of two DNA ends with the need of very little or no homology. Although DNA-PK kinase activity is restricted to the damage site it can phosphorylate certain substrates in common with ATM and ATR. This fact

increases the signaling capacity of ATM and ATR and attracts other mediators to the lesion (Stiff et al., 2004). Also, a recent work shows that DNA-PK can replace some of the functions of ATM when the later is absent, such as H2AX or p53 phosphorylation (Callén et al., 2009).

3.1.2. ATM

The ATM mediated response is initiated by the complex Mre11- Rad50-Nbs1 (MRN) (Lee and Paull, 2005; Petrini and Stracker, 2003). This complex recruits ATM and collaborates for its activation (Uziel et al., 2003). However, the exact mechanism by which ATM is activated is still a matter of debate (Lavin, 2008). One group proposed that, in basal conditions, ATM is an inactive homodimer which is dissociated and activated in response to DSB, due to its autophosphorylation (Bakkenist and Kastan, 2003). Of note, it has later been shown that ATM autophosphorylation is not necessary for its activation *in vivo* (Daniel et al., 2008).

Although DNA-PK is the main kinase promoting DSB repair, it has been proposed that ATM also contributes to this process, especially when the break lies in heterochromatin. The repair of heterochromatic DSBs is slower and more difficult and ATM has been reported to be able to phosphorylate certain substrates in order to increase chromatin accessibility (Goodarzi et al., 2008). However, ATM is essential for repair during meiosis, and meiotic DSBs are thought to be generated within accessible chromatin domains. Also, AT patients, develop progressive degeneration of cerebellar Purkinje neurons, in which chromatin is highly euchromatic. Thus, the importance of ATM-mediated facilitation of DSB repair in heterochromatin is still a matter of debate (Fernandez-Capetillo and Nussenzweig, 2008).

3.1.3. ATR

In contrast to ATM, ATR is an essential protein at the cellular and organism level (Brown and Baltimore, 2000). ATR is activated in response to abnormally long ssDNA regions, which can appear at S or G2 phase. ssDNA in S phase can be a consequence of the uncoupling between the replication machinery and the movement of the replicative helicases (Byun et al., 2005). However, ssDNA can also appear as a byproduct of DBS resection during S and G2 phases (Raynard et

al., 2008). Replication forks can be blocked in response to DNA alterations. In this scenario, ATR activity maintains the stability of the replication complexes so that they can re-start DNA synthesis when the problem is solved (Paulsen and Cimprich, 2007). ATR exists only as part of a heterodymeric complex with ATRIP (ATR interacting protein). ATRIP is necessary for ATR signaling activity and it is responsible for the correct loading of ATR to ssDNA sites (Cortez et al., 2001). The ATR-ATRIP complex needs to be activated by interaction with TopBP1 (Topoisomerase II binding protein). TopBP1 functions as an allosteric inductor of ATR's kinase domain (Kumagai et al., 2006). This allosteric inductor only interacts with the ATR-ATRIP complex when the three of them are recruited to ssDNA, what ultimately triggers ATR kinase activity.

This complex control mechanism is possible because different sensors are in charge of attracting ATR-ATRIP and TopBP1 to ssDNA. On one hand, Replication Protein A (RPA) coats ssDNA stabilizing it and attracting ATR-ATRIP (Walter and Newport, 2000) and Rad17. Independently, Rad 17 interacts with the Replication Factor C and loads 9-1-1 (Rad9, Hus1, Rad1) (Bermudez et al., 2003) to the double stranded DNA adjacent to the RPA coated ssDNA (Zou et al., 2002). Finally the 9-1-1 complex would recruit TopBP1, thus bringing it into close proximity to ATR. In addition to this, recent data have shown that TopBP1 is also recruited to ssDNA/dsDNA junctions through interactions with Nbs1 (Duursma et al., 2013).

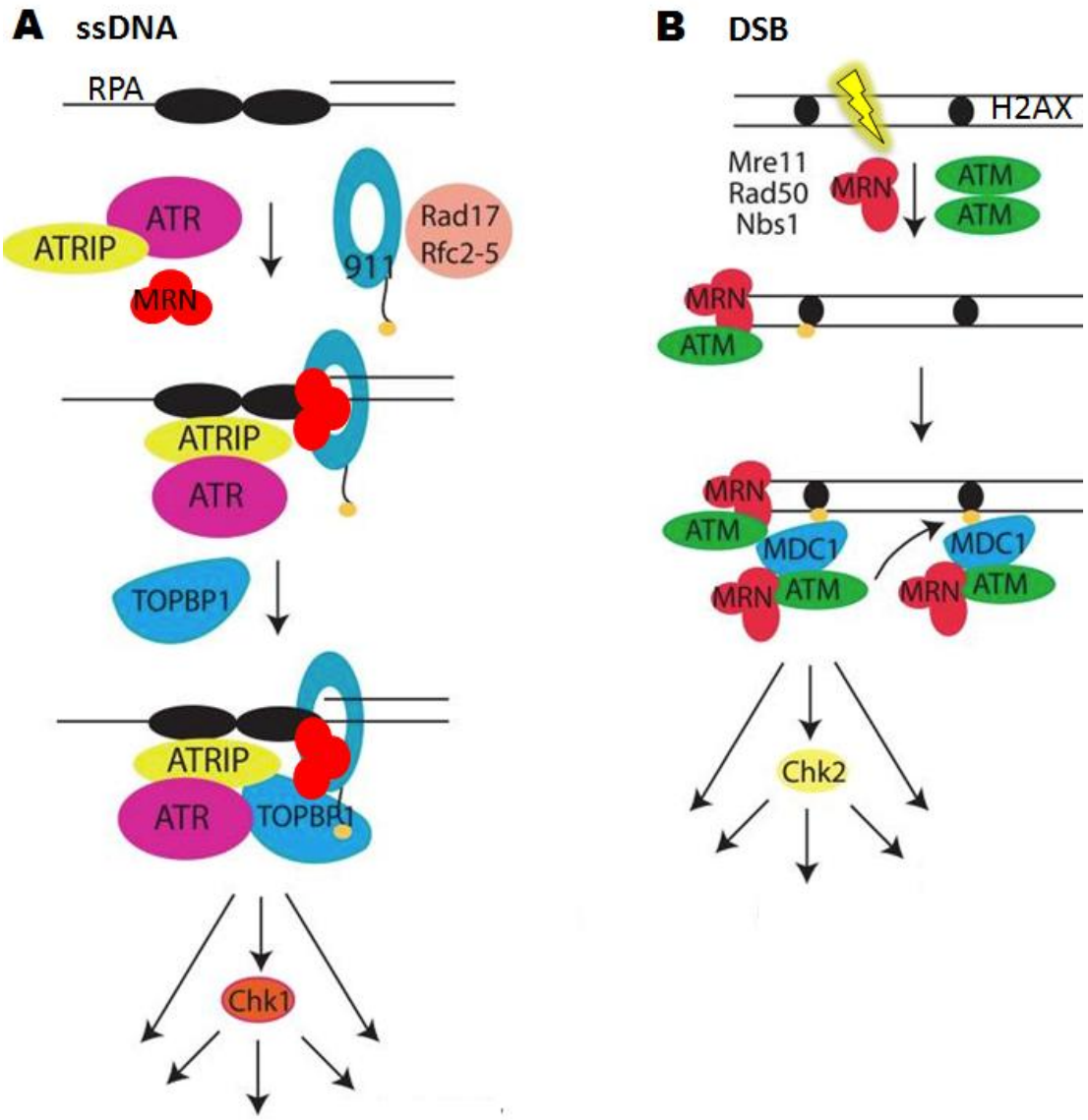

Figure 3. ATM and ATR activation mechanism A) ATR and TopBP1 are recruited to ssDNA by RPA and –possibly- the MRN complex. TopBP1 activates ATR, which triggers the corresponding phosphorylation cascade. B) ATM goes to DSB attracted by the MRN complex. H2AX phosphorylation recruits MDC1 and amplifies ATM signal. Modified from (Cimprich and Cortez, 2008)

3.2. Human diseases related to the DDR Kinases

3.2.1. SCID

The absence of DNA-PKcs activity in mouse results in severe combined immunodeficiency (SCID), a profound defect in the mouse immune system that is accompanied by ionizing radiation hypersensibility (Smith and Jackson, 1999), ((Biedermann et al., 1991). In humans SCID can appear due to defects in different proteins that lead to a severe immunodeficiency (Buckley, 2004).

3.2.2. Ataxia - Telangiectasia

Lack of ATM activity was identified as the cause of Ataxia-Telangiectasia (A-T) (Savitsky et al., 1995), a recesive autosomal disease. A-T patients present every sign of a deficiency in DSB repair: immunodeficiency, genomic instability, predisposition to cancer of lymphoid origin and ionizing radiation hypersensitivity (Becker-Catania and Gatti, 2001; Shiloh and Kastan, 2001). In addition, ATM mutations are frequent in cancer (Vorechovský et al., 1996).

3.2.3. Seckel Syndrome

Seckel syndrome (SS) is a congenital autosomic rare disease. It is characterized by a delay in intrauterine development, mental retardation, microcephaly, proportional dwarfism and a characteristic craniofacial malformation that includes a receding forehead and micrognatia (O'Driscoll et al., 2003), which confers patients a peculiar face. This last sign was what allowed Rudolf Wirchow, who described the illness for the first time in 1892, to call it "Bird Headed Dwarfism". Later on, in 1960, Helmut Seckel characterized the whole syndrome in depth. It is estimated that less than 1/10.000 births suffer this syndrome, affecting women and men and without any ethnic or geographic predominance.

Apart from the previously mentioned signs, patients show a progeroid appearance and dwarfism, frequent hallmarks of genomic instability syndromes. The syndrome has a complicated etiology; mutations in different loci contribute to the same symptoms. It was not until 2003 when the first genetic defect was associated to the Seckel syndrome: a mutation in ATR (O'Driscoll et al., 2003).

In studies in several families with SS, some residual ATR activity was detected. This was enough to allow patient viability, although with severe clinical consequences (O'Driscoll et al., 2003). The study of cellular lines obtained from patients' skin identified a point mutation (2101A-G) that affects ATR splicing. The mutation described, localized at the beginning of exon 9, generates an aberrant non-functional protein. However, the patients still express about 5% of normal ATR protein levels, what allows their survival. Based on this mutation, a mouse model of the Seckel Syndrome was developed in our lab (Murga et al., 2009). ATR-

Seckel mice are dwarf and die prematurely due to a pleotropic progeroid disease. In addition, these mice present the same craniofacial abnormalities describe on the patients. Thus, ATR-Seckel mice provide an excellent model for the study of ATR function in mammals.

4. Amplifying the signal: mediators

In order to spread the alarm signal and orchestrate the global cellular response to DNA damage, ATR and ATM cooperate very tightly with other proteins. At this level, mediators play an essential role: they are proteins with very diverse functions that modulate ATR and ATM activity. Mediators regulate the spatiotemporal assembly of protein complexes in the chromatin regions next to the lesion (Kastan and Bartek, 2004). Many of the mediator proteins share the capacity to be loaded into DNA and be concentrated on DSBs or ssDNA in protein aggregates that can be visualized by immunoflorescence as foci.

In response to IR, many of these proteins form ionizing radiation induced foci (IRIF). In fact, many of the components of the DDR colocalize at foci that present an accumulation of phosphorylated H2AX (γH2AX). H2AX is phosphorylated by ATM, ATR and DNA-PKcs through megabases of DNA (Rogakou et al., 1998). After the phosphorylation of histone H2AX in its serine 139, MDC1 is recruited through direct phosphobinding. Altogether, these events recruit additional proteins from the ATM pathway, amplifying the signal. According to the current model, although most of the mediators are recruited to the lesion in a H2AX independent manner, their accumulation at foci depends on the phosphorylation of H2AX by ATM (Celeste et al., 2003).

Phosphorylation is not the only modification that recruits factors to foci. For instance, BRCA1 and 53BP1recruitment depends on histone ubiquitination by RNF8 or RNF 168 (van Attikum and Gasser, 2009). Additionally, when ssDNA is exposed, RPA is accumulated coating the single strand in a γH2AX independent fashion.

Of note, protein accumulation at foci is a posterior and functionally distinct process to protein recruitment to damaged regions. In the absence of γH2AX or MDC1, fundamental factors of the DDR, like 53BP1or BRCA1 cannot longer form

foci. However, this does not have severe consequences because they maintain their ability to go to DSBs (Celeste et al., 2003; Celeste et al., 2002; Stewart et al., 2003)). One possible explanation for this is that foci just facilitate an efficient signaling of the damage (Fernandez-Capetillo et al., 2002) which is critical when only a few breaks are present. That is, the concentration of mediators in a particular area avoids that they uncouple from chromatin before the damage has been repaired (Kruhlak et al., 2006a; Kruhlak et al., 2006b).

5. Executing the response: effectors

The execution of the signal generated by ATM and ATR in response to DNA damage is carried out by two effector kinases: CHK1 and CHK2. ATM specifically phosphorylates CHK2 in its treonine 68 (Matsuoka et al., 1998) and ATR phosphorylates CHK1 in its serines 317 and 345 (Zhao and Piwnica-Worms, 2001). Altogether, the four kinases constitute the main guides to stop the cell cycle in response to DNA damage. Although they are under the control of ATM and ATR, CHK1 and CHK2 kinases are essential for the correct activation of the different checkpoints. In fact, whereas CHK1 is phophorylated at the break site, it then spreads to the whole nucleoplasm (Lukas and Bartek, 2004). Given that this thesis work is mainly focused in the ATR/CHK1 pathway, CHK1 function is explained in greater detail in the following paragraphs.

ATR was described as the mammalian equivalent to the main yeast checkpoint regulator MEC1p (Cimprich et al., 1996). ATR has several substrates, among which CHK1 plays a central role (Matsuoka et al., 2007). CHK1 is responsible for spreading the DDR signal throughout the nucleus by regulating CDKs activity via CDC25A (Smits et al., 2006). Moreover ATR and CHK1 absences have a similar phenotype: embryonic lethality due to massive RS accumulation (Brown and Baltimore, 2000; Liu et al., 2000). Thus, the ATR/CHK1 axis is essential even at the cellular level.

CHK1 is the main DDR effector of the G2/M checkpoint. However, it also has many other functions. For instance, CHK1 an essential activator of homologous repair (Syljuåsen et al., 2005) and it is required for the proper control of the mitotic spindle checkpoint (Zachos and Gillespie, 2007), among others.

As stated above, CHK1 activation is ATR dependent. Nevertheless, a protein called Claspin, that facilitates ATR binding to CHK1, is also required for CHK1 activation (Kumagai et al., 2006). Claspin levels are tightly regulated through the cell cycle, which restricts CHK1 activity to S/G2 phases of the cell cycle (Mailand et al., 2006). In addition to Claspin, several complexes take part in the CHK1 activation process: ATR-ATRIP, Rad17/RFC and the 9-1-1 complex need to be recruited to the damaged site in order to induce the activation of CHK1 by ATR (Zou et al., 2002).

CHK1 is required for the establishment of every cell cycle checkpoint in vertebrates (Bartek et al., 2007). In addition, there are also evidences of its role in the control of replication progression (Syljuåsen et al., 2005). This makes CHK1 essential during embryogenesis, when most of the cells are undergoing active replication (Sørensen et al., 2003). In fact, CHK1 complete deletion renders embryonic lethal mice (3.5 embryonic days).

In addition to knockouts, a mouse strain carrying one extra allele of CHK1 ($CHK1^{Tg}$) was recently generated in our laboratory. The insertion of a third CHK1 copy was able to partially rescue the phenotypes of ATR-Seckel mice. In contrast to this, the extra copy did not affect the phenotypes linked to a deficiency in DSBs repair (López-Contreras et al., 2012). Moreover, $CHK1^{Tg}$ MEF were found to be resistant to various sources of RS, including oncogenes, which facilitated *in vitro* transformation with RAS/E1A oncogene.

6. Consequences of the DDR

The main function of ATM and ATR is to coordinate the response to DNA damage in cell cycle progression. They avoid that cells keep proliferating with damaged DNA with the resulting transmition of mutations to their daughter cells. Thus, cell cycle transitory arrest allows the repair of DNA damage. Depending on the tissue, and if damage is excessive, the DDR can activate a permanent stop of the cell cycle – senescence- or cell death –apoptosis-. Moreover, recent works have also described that the DDR can activate differentiation (Inomata et al., 2009).

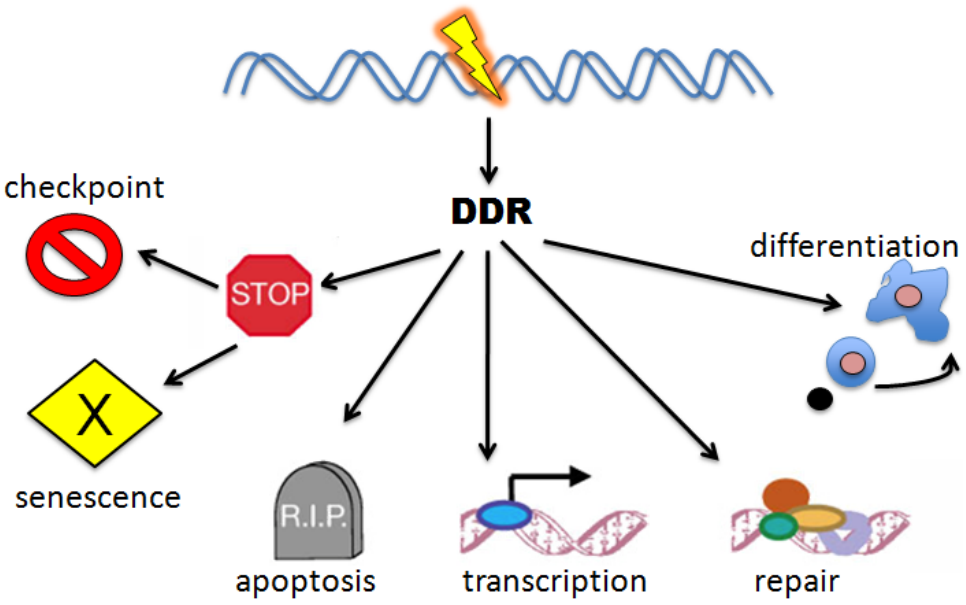

Figure 4. Effects of the activation of the DDR. Once the lesion is detected, the DDR activation results in multiple effects: cell cycle arrest in a transitory way through checkpoints or in an irreversible manner by the onset of senescence, effects in transcription, cellular differentiation and in the activation of repair mechanisms. Depending on the type and amount of damage it can lead to apoptosis. Modified from (Hanahan and Weinberg, 2000).

6.1. Repair

The cell has two main pathways to repair chromosome breaks: Non Homologous End Joining (NHEJ) and Homologous Recombination (HR) (Hoeijmakers, 2001). NHEJ is essential for DSB repair during G1. However, it tends to introduce mistakes in the DNA sequence. This is because NHEJ binds the two ends of the break, at the expense of causing possible local microdeletions. HR is not error prone, but it can only take place during S and G2, when there exists a homologous chromatid for recombination.

During NHEJ, break ends are detected by the Ku70/80 heterodimer, which then recruits DNA-PKcs. After this, the break is repaired by a complex formed by DNA ligase IV and XRCC4 (Hefferin and Tomkinson, 2005). Despite its low fidelity, this pathway is able to act in a very urgent way and it can be used in any part of the cell cycle.

However, when the cell is in S or G2 it preferentially uses the HR, which is a high fidelity repair mechanism. The expression of the different components of this pathway is restricted to S and G2 phases because HR activity during G1 could

result in deleterious consequences, like loss of heterozygosity (LOH) or chromosomal translocations (Richardson and Jasin, 2000). The first step of the HR pathway involves seeking homologous regions in the sister chromatid. To this aim, ssDNA must be generated by DSB resection. Long ssDNA with 3' ends are generated thanks to the action of different helicases and exonucleases (Hiom, 2000). After resection, ssDNA is coated with RPA, that will later be substituted by another protein with invasive properties: Rad51 (Benson et al., 1994) in a process that is facilitated by Rad52. At this point, ssDNA coated with Rad51 invades double stranded DNA, displacing the two complementary strands. Next, the replication machinery fills the lacking sequence using ssDNA ends as primers. This way, a structure called Holyday junction is formed (Bzymek et al., 2010). This structure will later give rise to two separated sister chromatids by a mechanism that is under debate. There are two possible mechanisms in which this can happen: On the one hand, Mus81, Gen1 and SLX4 endonucleases can make breaks to resolve the recombination intermediates (Constantinou et al., 2002; Svendsen and Harper, 2010; Wu and Hickson, 2003). On the other hand, BLM (Bloom Syndrome) helicase together with TopIII topoisomerase are able to dissolve these intermediates generating only DNA nicks in one of the strands (Wu and Hickson, 2003). Not all the components of the pathway or their mechanisms of action are known. However, what is certain is that these intermediates are highly recombinogenic structures that suppose a threat for the cells. If cells proceed to mitosis before repairing these structures, these links between sister chromatids may lead to chromosome breaks and asymmetric segregation.

Defects in repair pathways cause serious diseases. Programmed physiological breaks, essential to lymphocytes' V(D)J recombination and class switching recombination (CSR), are repaired via NHEJ. Therefore, the absence of NHEJ factors gives rise to a severe immunodeficiency. Moreover, some of the factors implicated in HR are of extreme importance in human tumor suppression. For instance, BRCA1 and BRCA2, which are frequently mutated in familiar breast and ovarian tumors, are fundamental to recruit Rad51 and their absence seriously compromises HR (Welcsh et al., 2000).

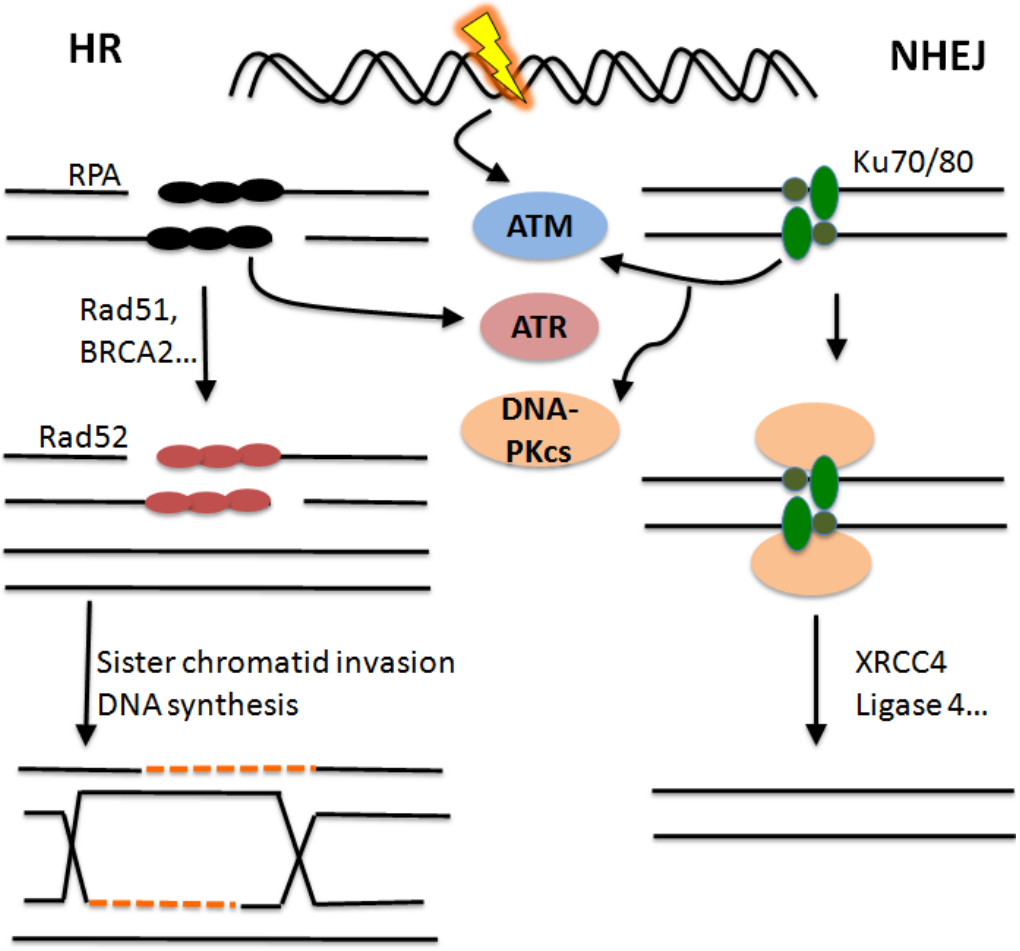

Figure 5. DSBs repair. Different types of damage can generate DSBs, which are repaired through different cellular mechanisms. The most simple repair mechanism, although not always the most reliable one, is NHEJ (B). DSBs can also be repaired through HR, that involves the recognition of sister chromatids (A). Fundamental steps of each route are represented.

6.2. Cell cycle checkpoints

The cell cycle is a sequence of events divided in different phases that are separated by transitions under a very strict control. Two of these phases are fundamental: the DNA synthesis phase - or S -, and mitosis -or M phase-. DNA is replicated during S phase and chromosomal segregation and division of the two daughter cells take place during M phase. In between these two phases we find G1–after M - and G2–after S phase-. During G1 and G phases cells get ready for the essential parts of the cell cycle. When no continuous proliferation is required, cells enter in a quiescent state that is called G0. Complex control mechanisms ensure the generation of a single and exact copy of genetic material and its equal distribution between the two daughter cells. Cells cannot enter a new phase until they meet all

the requirements that define the end of the previous one. The precise and tidy progression through the cell cycle is granted by the sequential activation of Cyclin dependent kinases (CDKs) (Morgan, 1997). Each of the cell cycle checkpoints will be reviewed in greater detail in the next section (B) of this introduction.

6.3. Senescence and apoptosis

Senescence was described over 50 year ago in human cells (HAYFLICK, 1965). They observed that human fibroblasts in serial culture entered an irreversible cell cycle arrest after 50-70 divisions. Later on, p53 was proven to mediate in this type of arrest together with the $p16^{INK4a}$/RB axis (Kuilman et al., 2010). Nowadays, senescence is considered a stress response implicated in cancer protection and ageing (Collado et al., 2007). One of the causes of this permanent cell cycle arrest is telomere shortening. Dysfunctional telomeres are recognized as DSBs and are able to trigger the DDR (d'Adda di Fagagna et al., 2003). Other forms of permanent damage that can induce premature senescence include oxidate stress in culture (Parrinello et al., 2003) and genotoxic agents (Campisi and d'Adda di Fagagna, 2007). Later it was discovered that oncogene activation could also lead to an irreversible and premature stop, similar to proliferation induced senescence, *in vitro* (Serrano et al., 1997). This arrest is activated by p53 and mediated through $p19^{ARF}$. Today we know that senescence also appears in human and murine tumors (Collado and Serrano, 2005). Consistently, some oncogenes, like RAS, CDC6, STAT5 or Cycline E trigger a DDR associated to DNA hiperreplication, which finally activates senescence (Bartkova et al., 2006; Di Micco et al., 2006). Finally, it has been proven that the activation of ATR is able to promote senescence even in the absence of damage (Toledo et al., 2008).

Apoptosis is a death mechanism actively executed by the cell. It is determined by the sensitivity of each particular cell type and by the intensity of the DDR, which is proportional to the number and severity of the lesions. When a certain amount of damage is reached, p53 is activated by the DDR and it induces the expression of genes implicated in cellular death, like Puma, Noxa and Bax (Nakano and Vousden, 2001). These genes cooperate in mitochondrial membrane permeabilization and cytochrome C release into the cytosol (Willis et al., 2007). All these events lead to the formation of a proapoptotic protein complex until finally,

caspase enzymes are activated. Caspases inactivate proteins that protect living cells from apoptosis, directly disassemble and degrade cell structures such us DNA, conducting to cell death.

Why certain cell types undergo apoptosis and other senescence in response to DNA damage is still a matter of debate (Campisi, 2007). For instance, whereas DNA damage in fibroblasts promotes senescence, low amounts of DNA breaks in lymphocytes are able to activate apoptosis.

6.4. Differentiation

Different genomic instability mouse models have showm a decrease in stem cell population (Murga et al., 2009; Ruzankina et al., 2007). This effect was considered to be due to the main effects of the DDR: senescence and apoptosis (Ruzankina et al., 2008). However, an in vivo study of the effects of the DDR in melanocytes unveiled that activation of the DDR can also lead to stem cell differentiation (Inomata et al., 2009). This differentiation implies a decrease in the stem cell pool and entails hair graying in animals. Hence, the DDR maintains the good quality of the stem cell pool through the differentiation of damaged stem cells.

7. Biological implications of the DDR

7.1. DDR and ageing

Ageing can be accelerated by different DDR-activating stimuli like telomere shortening, oxidative stress, genotoxic agents or replication stress. Being this last one the least understood cause. As mentioned, replication stress refers to any problem that might happen in the replication fork and that implies the accumulation of long ssDNA strands or even DSBs (López-Contreras and Fernandez-Capetillo, 2010). Moreover, aged tissues and stem cells show an active DDR (Rossi et al., 2007). Thus, DNA damage accumulation has been related to senescence and ageing (Garinis et al., 2008).

The relationship of ATR with aging barrier is still not well understood. Partial ATR loss is known to generate a progeroid phenotype (Murga et al., 2009) associated to high RS levels during embryonic stage. An interesting hypothesis is that the conditions during embryonic development have an important effect in the

life's expectancy of the adult organism (Fernandez-Capetillo, 2010). In addition, full ATR loss in adult tissues leads to the elimination of cells and the faster mobilization of stem cell pools, with the consequent development of ageing phenotypes (Ruzankina et al., 2007). Nevertheless, both hypotheses point towards a relationship between RS and ageing. At the cellular level, both the loss of ATR (Murga et al., 2009) or its activation (Toledo et al., 2008) lead to senescence. All of the above suggest a connection between ATR, RS and ageing.

7.2. DDR and cancer

One of the main hallmarks of a cancer cell is the development of genomic instability, which generates random mutations including chromosomal rearrangements. Eventually, some genetic changes might be able to promote cellular transformation (Hanahan and Weinberg, 2011). Also, the two main classical strategies for cancer treatment, chemotherapy and radiation, are based on the generation of DNA damage (Toledo et al., 2011a). When exposed to radiation or chemotherapy, both cancer and healthy cells will incorporate some damage in their DNA. However, cancer cells are particularly sensitive to DNA damage inducing agents due to their faster replication rates.

In this context, the relationship between the DDR and DNA repair with tumor development has been deeply investigated. On the one hand, the DDR has been proposed as an anticancer barrier in the first steps of tumor development (Halazonetis et al., 2008). During these initial stages oncogenes would promote uncontrolled proliferation; leading to RS, which will activate the DDR machinery (Bartkova et al., 2005; Gorgoulis et al., 2005). On the other hand, defects in HR and NHEJ lead to the accumulation of chromosomal aberrations. In fact, most human syndromes associated to defects in the signaling or repair of DNA damage are characterized by the appearance of cancer. Many of these syndromes have been reproduced in murine models. Sometimes, the loss of only one allele is sufficient to drive tumorogenesis, which classifies these genes as haploinsufficient tumor suppressors. ATR and CHK1 are both haploinsufficient genes, given than mice with a single ATR or CHK1 allele are prone to tumor development (Brown and Baltimore, 2000; Liu et al., 2000). This phenotype is seriously aggravated in a genetic background with other mutations that are also procancerous (Lam et al.,

2004). Paradoxically, even though ATR or CHK1 loss predisposes to cancer onset, CHK1 inhibitors have been proposed as a cancer treatment (Tao and Lin, 2006). This apparent contradiction can be explained analyzing CHK1 levels: whereas half of CHK1 predisposes to the accumulation of mutations and, therefore, tumor development, the complete inhibition of CHK1 is too toxic for replicating cells. For instance, CHK1 inhibition is especially toxic for p53 null cells, which accumulate higher levels of RS (Koniaras et al., 2001). This is of particular interest, since p53 is one of the most frequently mutated tumor suppressors in human cancer, and p53 deficient tumors are very often resistant to the available therapy. In agreement with this model, mice with low ATR levels do not develop cancer. Moreover, p53 elimination in this model not only does no lead to tumor development, but is extremely synthetic lethal (Murga et al., 2009; Ruzankina et al., 2007). This is the basis for a personalized use of ATR or CHK1 inhibitors, through directing their use to tumors with high levels of RS. This phenomenon is due to the role of CHK1 in suppressing RS, so that ATR or CHK1 inhibitors are particularly toxic for cells suffering from RS.

B. The cell cycle

The transitions through the different stages of the cell cycle are strongly regulated by the activity of Cyclin-dependent kinases (CDKs). CDKs are activated by Cyclins and inhibited by CDK inhibitors (CKIs) or inhibitory tyrosine phosphorylations (Guardavaccaro and Pagano, 2006). Briefly, four main CDKs are involved in the regulation of cell cycle; CDK2, CDK4 and CDK6 during interphase, and CDK1 during mitosis. When the DDR is activated, it can limit the activity of CDKs and therefore, prevent the progression of the cell cycle into the next phase (G1/S and G2/M checkpoints). Additionally, the DDR can also slow down replication (intra-S checkpoint), although this is not a full stop and cells progress with damage from S into G2 phase and finally stop at the G2/M checkpoint (Abraham, 2001).

1. Cell cycle checkpoints

The DDR reaches the CDKs through effectors that limit CDK activity: First, the main proteins from the DDR network that regulate checkpoints are the checkpoint kinases CHK1 and CHK2, direct substrates of ATR and ATM correspondingly (Bartek and Lukas, 2003). Checkpoint kinases act by regulating CDK inhibitory effectors such as Cdc25a, Wee1 or p53. Cdc25a is a phosphatase that controls CDK1 and CDK2 activities. In response to DNA damage, CHK1 phosphorylates Cdc25a inducing its degradation (Liu et al., 2000), and thus, inhibition of CDK activity (Boutros et al., 2006). Finally, a key mediator of cellular responses to DNA damage is p53. p53 is quickly stabilized upon DNA damage by a range of post translational modifications. ATM, ATR, CHK1 and CHK2 are all able to phosphorylate p53 contributing to its stabilization (Canman et al., 1998). Besides its apoptotic targets, p53 has several transcriptional targets that contribute to checkpoint onset, the most known being p21 (CDKN2A), a central regulator of the G1/S checkpoint.

1.1. G1/S Checkpoint

To avoid that a damaged cell enters S phase, the DDR can stop the cell cycle by two main effectors that function in parallel: CDC25A and p53 (Lukas and Bartek, 2004). Although phosphorylation of both substrates happens simultaneously, the cascade triggered by CDC25A is faster. This is because it does not require new protein synthesis (Mailand et al., 2000). Nevertheless, full checkpoint implementation is carried out by p53 accumulation. In response to DNA damage, CDC25A is phosphorylated. CDC25A phosphorylation marks the protein for its degradation in the proteasome, what avoids that CDC25A dephosphorylates and activates CDK2. The slower response implies p53 activation and stabilization. This happens through several mechanisms, such as p53 phosphorylation in its serine 15 by ATM (Tibbetts et al., 1999) and in its serine 20 and treonine 18 by CHK2 (Hirao et al., 2000; Shieh et al., 1997). Moreover, ATM interacts with the negative regulator of p53, MDM2 (Shieh et al., 1997) . Finally, p53 can also be acetylated (Dornan et al., 2003), what increases p53 transactivation potential in response to DNA damage (Dumaz and Meek, 1999). One of the transcriptional targets of p53 is p21, which is accumulated hours after the detection of damage. p21 inhibits G1-S transition by limiting to Cyclin E/CDK2 (Sherr and Roberts, 1999).

1.2. Intra-S Checkpoint

This checkpoint consists in a transitory delay in cell cycle progression (Abraham, 2001) through the prevention of replication origin firing. This is achieved by CDC25 degradation, after its phosphorylation by ATR/CHK1 and CDK2 inhibition (Bartek et al., 2004). SMC1 phosphorylation by ATM has also been suggested as an activator of the intra-S checkpoint (Kitagawa et al., 2004). The intra-S checkpoint helps stabilizating replication forks through a not yet elucidated mechanism. Through the activation of this checkpoint cells avoid replication fork collapse and the consequent DSBs that arise from it (Bartek et al., 2004).

1.3. G2/M Checkpoint

G2 arrest avoids the entrance in mitosis of cells that present DNA damage. The main target of this checkpoint is Cyclin B/CDK1. DDR phosphorylation of CDC25A by CHK1 and CHK2 avoids Cyclin B/CDK1 activation (Donzelli and Draetta, 2003). Once again, the p53/p21 axis is in charge of the maintenance of this checkpoint (Taylor and Stark, 2001). The DDR activates this checkpoint mostly through ATR/CHK1, although ATM/CHK2 can also participate in the G2/M checkpoint.

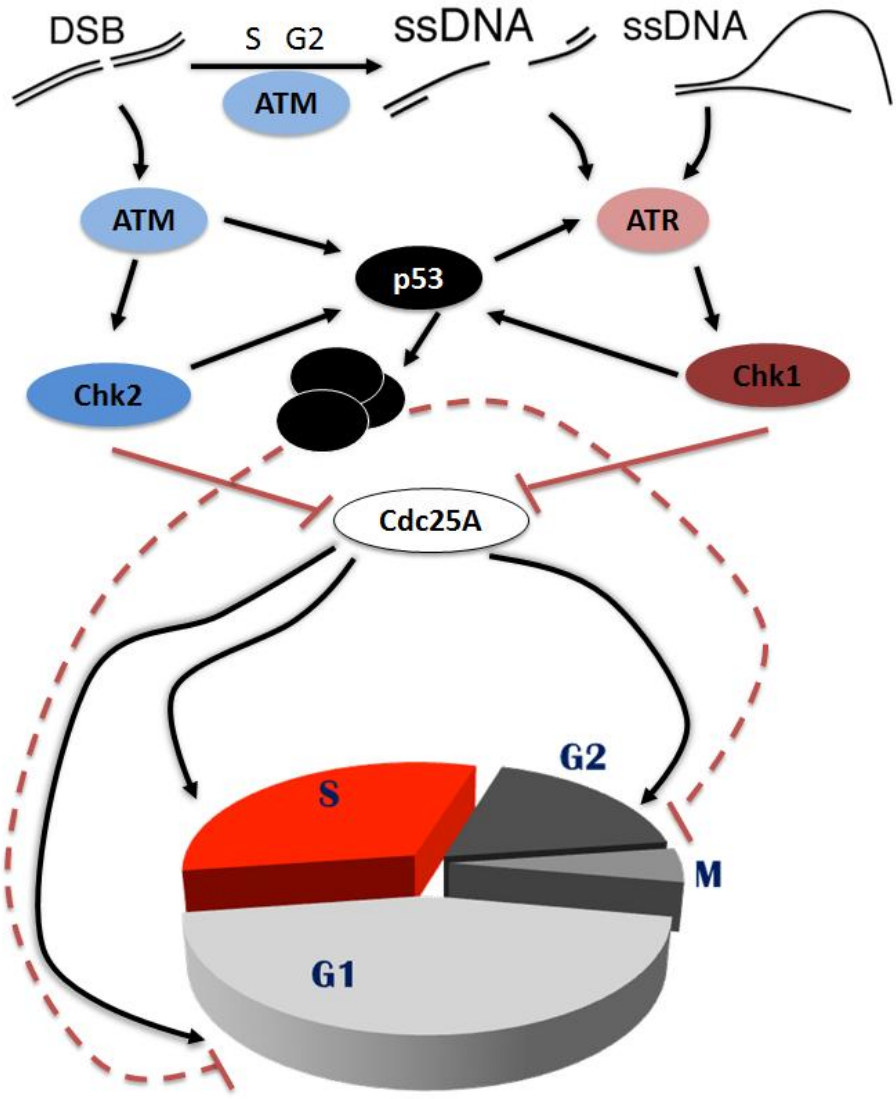

Figure 6. Checkpoints in the DDR. Representation of the transitory control of the cell cycle by ATM and ATR kinases. During G1 phase cell cycle arrest is activated by ATM/CHK2 kinase. On the other hand, during G2 phase ATM activates the ATR/CHK1 pathway.

2. Relevant cell cycle regulators

The advance through the different stages of the cell cycle is very tightly regulated by CDKs and CDKis (Guardavaccaro and Pagano, 2006). There are different effectors that can regulate CDK activity. In the following pages I will describe in more detail some of these regulators that are of special interest for this thesis.

2.1. Retinoblastoma

Retinoblastoma was the first tumor suppressor cloned, now more than 25 year ago. It controls whether the cell is prepared to initiate DNA replication and divide (Classon and Harlow, 2002). RB elimination promotes uncontrolled proliferation and tumor development.

The first studies of RB focused on its function at the G1-S transition, where it inhibits E2F. This perspective offered a clear explanation of RB tumor suppressor function (Hatakeyama and Weinberg, 1995). Different from most cell cycle proteins, RB is not usually degraded upon inactivation. On the contrary, RB persists until mitosis, when it is dephosphorylated in order to allow the start of a new ell cycle (Kolupaeva and Janssens, 2013). RB presence during the whole cell cycle allows its reactivation by dephosphorylation in response to intra S or G2/M checkpoints. In addition to this, new studies show that other post-transcriptional modifications, such us acetylation or methylation, can also modulate RB activity (Munro et al., 2012).

Human RB has three main domains: a central –pocket- domain flanked by two linker sequences than bind an amino-terminal domain and a carboxi-terminal one (Dick and Rubin, 2013). The pocket domain is highly conserved; it binds E2F and viral oncoproteins. The linker sequences contain CDK-dependent phosphorylation sites that have a critical role for RB regulation (Burke et al., 2012).

The vast majority of human sporadic cancers carry mutations in RB or in its pathway. Some of these mutations affect RB regulators suchs us Cyclin D, CDK4 or $p16^{INK4a}$ (Sherr, 1996). RB knock- out mice are embryonic lethal; they die at mid-gestation (E. 12-15) due to neurogenesis, erythropoyesis and lens development defects (Vooijs and Berns, 1999). RB heterozygous mice develop tumors in which

the RB wildtype (WT) allele is lost. Moreover, many models of tissue specific deletion of RB have been shown to lead to cancer, particularly when combined with p53 deletion (Vooijs and Berns, 1999).

RB can be inhibited by some proteins of viral origin (DeCaprio et al., 1988) such us the large T antigen of the simian virus 40. DNA viruses can code for a variety of oncoproteins with no cellular homolog. Polyomaviruses are a type of DNA virus that can cause different type of diseases in animals. Among them, SV40 is a polyomavirus from rhesus macaque (*Macaca mulata*) origin than contains two coding regions. In the first one, it contains two proteins: Large-T antigen and small-t antigen (White and Khalili, 2006). Large-T antigen is able to regulate important cellular signaling pathways to boost cell cycle progression. As a result of this enhance in proliferation, virus-infected cells transform. In fact, the Large-T antigen is able to interfere with the functions of the two main tumor suppressors: p53 (Lane and Crawford, 1979) and RB (DeCaprio et al., 1988) among many other proteins. It preferentially binds to hypophosphorylated RB (Ludlow et al., 1989), displacing E2F (Chittenden et al., 1991). Different forms of the SV40 Large-T antigen have been used to study RB and p53 roles and to immortalize culture cells. A mutant T-antigen composed of only the 121 N-terminal aminoacids retains the capacity to interact with RB but not p53 (Figure 7). This small version of the T-large antigen, called T-121, is able to avoid the inhibition of growth by cell contact (Tevethia et al., 1997b), to immortalize cells *in vitro* and to cause tumors *in vivo* (Tevethia et al., 1997a). Of note, T121 also binds to p107 and p130, the two other proteins of the Retinoblastoma family, together with RB (Stubdal et al., 1996), that also contribute to the regulation of the cell cycle in certain tissues.

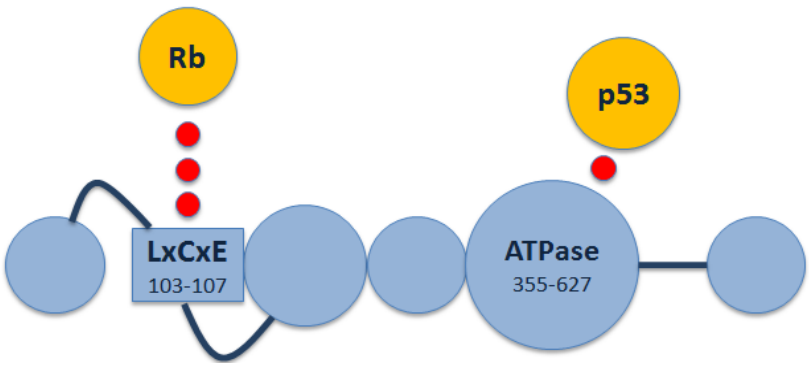

Figure 7. T-Large antigen domain structure Protein domains of the T-Large antigen (blue) and their corresponding aminoacids are indicated. Note the Rb-protein binding region (LXCXE) and the ATPase region containing the p53 binding site (ATPase). RB and p53 are depicted in orange with their corresponding interactions with T antigen (red dots). Modified from (Sáenz Robles and Pipas, 2009)

2.2. INK4a/ARF

The INK4 locus was extensively studied at the end of last century. Two different research lines claimed a new tumor suppressor gene in chromosome 9, which was named $p16^{INK4a}$. Later on, homologs of the gene were found at the same locus and designated $p15^{INK4b}$ (Quelle et al., 1995a), $p18^{INK4c}$ (Guan et al., 1994)and $p19^{INK4d}$ (Guan et al., 1996). Later, an alternative transcript from the INK4a locus was found. $p16^{INK4a}$ is composed by three exons: INK4a-1α, INK4-2 and INK4-3. The alternative transcript starts with exon INK4a1β, which is transcribed from a different promoter than exon INK4a-1α. Then, exon INK4a1β becomes spliced to the same second and third exons INK4-2 and INK4-3. Altogether, exon INK4a1β, INK4-2 and INK4-3 form an alternative transcript named $p14^{ARF}$ in human or $p19^{ARF}$ in mouse (Mao et al., 1995).

$p16^{INK4a}$ inhibits the kinase activities of CDK4 and CDK6. They directly bind to CDK4 and CDK6, acting as competitive inhibitors and preventing the association of CDKs with the corresponding Cyclin. Therefore $p16^{Inka4}$ overexpression leads to a G1 arrest (Guan et al., 1996; Hirai et al., 1995; Serrano et al., 1993). $p16^{INK4a}$ expression in primary tissues is generally low or undetectable. However, it is more easily detected in cells in culture. For instance, $p16^{INK4a}$ expression could not be detected in mouse embryos, but its transcription was activated the moment embryonic fibroblasts were cultivated (Zindy et al., 1997). This fact suggested that $p16^{INK4a}$ could be part of the mechanism that limits the lifespan of proliferative cells, namely proliferation-induced senescence (HAYFLICK, 1965). Further evidence came with the demonstration that $p16^{INK4a}$ accumulates with increasing numbers of population doublings (Hara et al., 1996). Apart from proliferation-induced senescence, there is another scenario in which $p16^{INK4a}$ levels are increased: a RB depleted background. The excess of $p16^{INK4a}$ in RB negative cells was already reported in the original publication by Serrano et al. (Serrano et al., 1993). Nullizigous mice for $p16^{INK4a}$ show certain tumor susceptibility and carcinogen hypersensitivity. Moreover embryo fibroblasts (MEF) derived from them show an increase rate of immortalization compared to wildtype, but they proliferate normally and undergo proliferative senescence (Krimpenfort et al., 2001; Sharpless et al., 2001). Thus, $p19^{ARF}$ could be compensating the lack of

p16^{INK4a} in MEFs, what suggests a predominant role for p19^{ARF} over p16^{INK4a} in this murine cell type (Sharpless et al., 2004).

In contrast to p16^{INK4a}, p19^{ARF} is widely expressed in primary tissues and it does not bind to CDKs, although its ectopic expression does lead to cell cycle arrest (Quelle et al., 1995b). The effects of p19^{ARF} are dependent on p53 status and lead to both G1 and G2 phase arrest (Kamijo et al., 1997). p19^{ARF} appears to protect p53 from degradation by directly binding MDM2 (Pomerantz et al., 1998). Accordingly, p19^{ARF} levels are higher in p53-negative cells (Kamijo et al., 1997). Nullizigus mice for p19^{ARF} develop spontaneous tumors, are susceptible to chemical carcinogens and their fibroblasts bypass proliferative senescence (Kamijo et al., 1997). Hence, and in contrast to the human situation, p19^{ARF} seems to be the key tumor suppressor at the INK4a/ARF locus in mice.

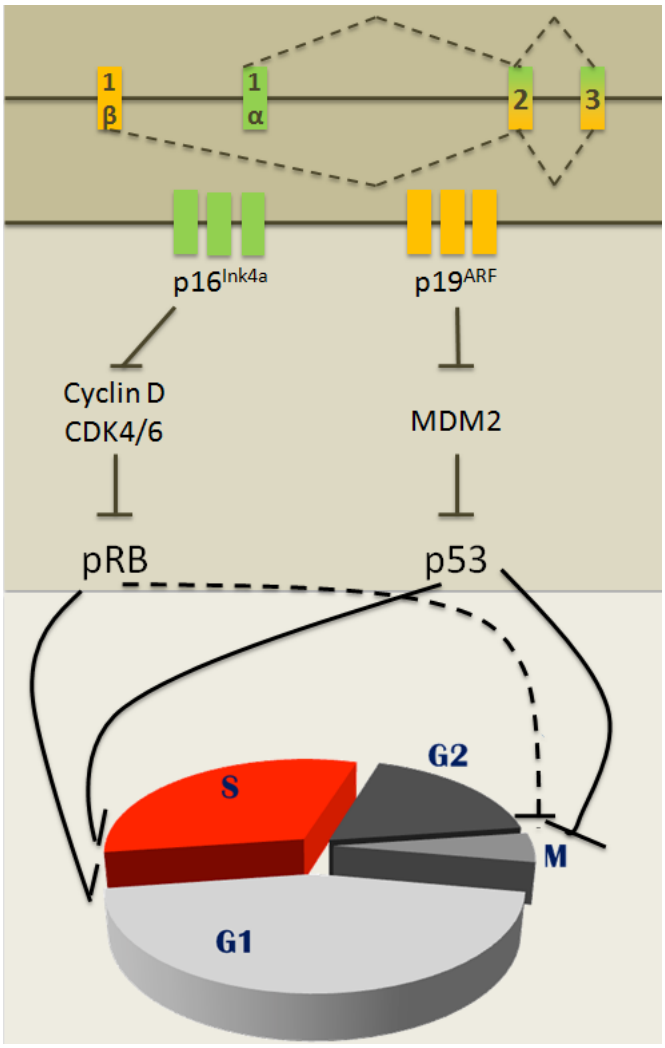

Figure 9. The INK4a/ARF locus and CC regulation. The INK4a/ARF locus encodes p16^{INK4a} and its relatives, as well as p19^{ARF}. The genetic locus is depicted as a grey line with exons indicated by colored boxes (green for p16^{INK4a} and yellow for p19^{ARF}). p16^{INK4a} is a member of the Cyclin dependent kinase inhibitors that binds to inactive CDK4/6, resulting in RB indirect activation. p19^{ARF} inhibits MDM2, resulting in p53 stabilization. Modified from (Kim and Sharpless, 2006).

Not long after its discovery, the locus encoding $p16^{INK4a}$ was found to be mutated in a wide range of cancer types (Kamb et al., 1994). Nevertheless, the locus also encodes $p19^{ARF}$, which is an activator of p53. Altogether, this makes the INK4a/ARF locus a key player in cancer, since it can activate the two main tumor suppressor pathways in our cells: $p16^{INK4a}$/RB and $p19^{ARF}$/p53. In human cells, however, $p16^{INK4a}$ seems to be the main contributor to tumor suppression. Moreover, cancer-associated mutations imply either the entire locus, or $p16^{INK4a}$ alone (Quelle et al., 1995b). The relevance of the whole INK4a/ARF locus is evidenced by its frequent loss in human cancer (Sherr, 2012). Deletion of the whole INK4a/ARF locus renders viable, but highly tumor prone mice that are very sensitive to carcinogenic treatments. INK4a/ARF depleted MEF proliferate rapidly and bypass proliferation induced senescence (Serrano et al., 1996). Nevertheless, $p16^{INK4a}$ is considered the main contributor of the locus to tumor suppression in humans (Kim and Sharpless, 2006). In fact, it has been found to have alterations in a wide variety of human cancers (Table 1) (Ruas and Peters, 1998).

	Cancer type	Deletion (%)	Mutation (%)
Hematological Malignancies	T- ALL	57,9	7
	Early pre B-ALL	21.9	0
	Mixed ALL	18,6	0
	ATL	14	6,4
	T-NHL	6,5	0
	CML-BC-L	24	0
Solid tumors	Bile duct	ND	56
	Bladder SCC	49,2	6,8
	Bladder TCC	29,2	2,1
	Glioma III and IV	34,9	2,7
	Esophagus	1,8	13,7
	Head and neck	15,1	6,7
	Mesothelioma	21,6	0
	NSCLC	11,5	8,7
	Melanoma	11,3	9,6
	NPC	35	0
	Ovary	7,8	2,9
	Pancreas	20,6	27,3
	Skin	ND	14,3

Table 1. The table represents the percentage of tumors that have sustained either homozygous deletions or mutations Abbreviations are used as follows: ALL, acute lymphoblastic leukemia; ATL Adult T-cell leukemia; NHL, Non-Hodgkin's lymphoma; CML, chronic myeloid leukemia; SCC, squamous cell carcinoma; TCC, transitorial cell carcinoma; NSCLC, non-small cell lung cancer; NPC, nasopharyngeal carcinoma. Only the most representative cases are depicted. Modified from (Ruas and Peters, 1998).

One of the cancer types in which $p16^{INK4a}$ is frequently mutated is melanoma. It is widely known that exposure to UV radiation increases the risk of melanoma due to the induction of DNA damage. Together, these two facts suggest an important role for $p16^{INK4a}$ in the response to the DNA damage induced by UV radiation. In fact, the depletion of $p16^{INK4a}$, $p19^{ARF}$ or the whole locus has been proven to cause a defect in the repair of DNA damaged with UV radiation (Sarkar-Agrawal et al., 2004). Moreover, $p16^{INK4a}$ null cells are hypersensitive to UV radiation (Al-Mohanna et al., 2004). Finally, $p16^{INK4a}$ has been shown to stabilize p21 and induce p53 in response to the exposure to UV radiation (Al-Mohanna et al., 2007). However, several studies failed to show a role for $p16^{INK4a}$ or $p19^{ARF}$ in the acute response to ionizing radiation (Efeyan et al., 2006; Kamijo et al., 1999), which suggested that this locus was not linked to the DDR.

2.3. p53

p53 is probably the most important tumor suppressor in pluricelullar organisms. In addition, it is the main regulator in the response to different cellular insults such us DNA damage. p53 is considered to prevent tumor onset by executing the effects of the DDR, and it is present in premalignant and malignant cells (Bartkova et al., 2005; Gorgoulis et al., 2005). DNA Damage was the first type of cellular insult associated to p53; this was the reason David Lane called this tumor suppressor "the guardian of the genome" over twenty years ago (Lane, 1992). p53 response to DNA damage has been deeply investigated; DNA damage activates the DDR proteins ATM, ATR, CHK1 and CHK2, which phosphorylate p53 (Chao et al., 2000; Kurz and Lees-Miller, 2004). This signaling cascade is hiperactivated in human tumors, suggesting that cancer and DNA Damage are intrinsically associated (Bartkova et al., 2005; Gorgoulis et al., 2005).

Oncogenes are also able to activate p53 (Serrano et al., 1997) through $p19^{ARF}$. $p19^{ARF}$ inhibits the degradation of p53, allowing p53 activation. Once it has been activated, p53 triggers a transcriptional program that results in different types of cellular responses that vary depending on the cellular type and the degree and duration of the activation. In the end, p53 activation will lead to cell cycle arrest, mainly through p21 regulation, and to apoptosis through the activation of genes such us Puma, Noxa or Bax.

The great importance of p53 in cancer has been widely proven. p53 nullizigous mice show a high cancer predisposition (Donehower et al., 1992; Jacks et al., 1994).

Objectives

OBJECTIVES

1. To investigate the potential role of the INK4a/ARF and RB pathways in the onset of replication stress induced senescence.

    a. To explore if the INK4a/ARF locus is activated in response to replication stress.

    b. To investigate whether the products of the INK4a/ARF locus; $p16^{INK4a}$ and $p19^{ARF}$, regulate cell cycle arrest in response to replication stress.

    c. To investigate whether the inactivation of the RB pathway allows the growth of cells with replication stress.

2. To investigate the impact of INK4a/ARF mutations in the context of reduced ATR activity.

    a. To study how INK4a/ARF deletion affects the symptoms of ATR-Seckel Syndrome in mice.

    b. To investigate whether the sensitivity of tumoral cells to ATR inhibitors is dependent on the status of the INK4a/ARF locus.

3. To explore whether the cell lethality of ATR deficient cells can be rescued by the inactivation of RB proteins.

Materials and methods

MATERIALS AND METHODS

1. Mice

1.1. Maintenance and genotyping of mice

All animals used during the development of this thesis were kept in the Animal facility of the Spanish National Cancer Research Center, according with Spanish animal protection law (RD1201/2005) and the European directive (86/609/CEE) established to regulate the standards of animal care.

In order to genotype the animals, DNA was extracted from small tail pieces that were digested during 12 hours at 55°C with the following lysis buffer: NaCl 100 mM, Tris-HCl pH 8 20 mM, EDTA10 mM, SDS 0.5% and proteinase K (Roche) 400 µg/ml. Cellular lysates were treated with a saturated NaCl solution and DNA was precipitated with isopropanol, washed with ethanol and resuspended in distillated water.

PCRs of this work were performed according to these two protocols:

A) In a 10 µl final volume reaction: 200 µM de dNTPs, 1.5 mM de $MgCl_2$, 1 µl reaction buffer 10X, 0.15 µl Taq polymerase (Platinum Taq, Invitrogen), 0,5 µM of each oligonucleotide and 100 ng of genomic DNA.

B) In a 10 µl final volume reaction: 200 µM de dNTPs, 1.5 mM de $MgCl_2$, 5 µl reaction buffer 2x (MasterAmp, Buffer F) 10X, 0.15 µl Taq polymerase (Platinum Taq, Invitrogen), 0,5 µM of each oligonucleotide and 100 ng of genomic DNA.

1.1.1. ATR Seckel

The oligonucleotides used amplify a region close to the vector's site of integration. They amplify a band of 500bp in the wild type allele or 300bp in the knocked in one. The PCR was performed according to protocol A, and the sequences of the oligonucleotides are:

Seckel 3'E8: GGAATAAATCCATGGAAGTGAGAGCAT

Seckel neo: TCCTCGTGCTTTACGGTATCGCC

Seckel 5'In7-8: CACTGGCCTCACAGACTTCAGCATG

1.1.2. INK4a/ARF KO and p16^{INK4a} KO

INK4a/ARF KO and INK4a KO alleles were genotyped by Transnetyx (Transnetyx, Inc).

1.1.3. p19^{ARF} KO

Two separate reactions were used to genotype ARF mutant mice. The nucleotides used to amplify the wild type (ARF1bis and ARF2) allele render a 457 bp band. The mutant allele maintains a neomycin site, when amplified with the chosen oligonucleotides (Neo-2 and ARF2) it gives a 250 bp band. The PCR was performed according to protocol B, and the nucleotides are:

ARF1 bis: TCTCACCTCGCTTGTCACAGTG

ARF2: TTGAGGAGGACCGTGAAGCCG

Neo-2: ACCACACTGCTCGACATTGGG

1.1.4. RB lox/lox UbCreERT-2

RB conditional knock out mice were genotyped with a single reaction. The nucleotides amplify a 680bp band in the case of a wild type allele, 750bp band in the case of the allele had integrated two lox sites and a 300bp in the knock out one. The PCR was performed according to protocol B, and the nucleotides are:

pRB18 Rev: GGCGTGTGCCATCAATG

pRB19 Fwd: AACTCAAGGGAGACCTG

Cre recombinase was amplified following PCR protocol A. The nucleotides chosen render a band of 400bp:

Cre1: CGATGCAACGAGTGATGAGGTTC

Cre2: GCACGTTCACCGGCATCAAC

1.2. Survival curve

At least 15 mice (male and female) from every genotype of interest were maintained for each survival analysis. Age and possible cause of death were annotated. Survival times were analyzed with GraphPrism and a Mantel-Cox test was performed in order to find out whether differences are statically significative.

1.3. Immunohistochemistry

Tissue samples and embryos were embedded in formalin to their later process at CNIO's Pathology Unit. 2,5mm tissue sections were treated with citrate and stained with the antibody of interest. Hematoxilin and Eosin were used to counterstain.

1.4. Body imaging analysis – Densitometry

Densitometry measurements were performed by CNIO's Imaging Unit, where they use DEXA equipment from GE. Pictures were analysed by PixiMus DeXa system from GE and a high resolution software that allows the stimation of bone density as well as fat accumulation. To perform these measurements animals were anesthesiased using non invasive techniques.

1.5. T121 conditional mice generation

Open biosystems gene targeting kit (cat num. MES4758) was used to generate inducible genetically- modified embryonic stem cells (ESC) that recombine T121 in a by specific site (Beard et al., 2006). The recombination took place in a particular type of ESC, called KH2 that includes a tetracycline-inducible system, (Urlinger et al., 2000), to regulate gene expression both *in vitro* and *in vivo*.

In brief, T121 was cloned in a pBS31 vector, which allows T121 introduction downstream of the tetO minimal CMV promoter. Sequencing was used to confirm the integrity of the construction that was later

electroporated in KH2 ESC by CNIO's transgenic's Unit personnell. In the presence of FLPe recombinase pBS31 will insert T121 genomic sequence at the frt site along with a promoter and an ATG initiation codon upstream and in frame with the hygromycin resistance gene thereby conferring hygromycin resistance to the correctly targeted cell.

Targeted ESC were selected by hygromycing treatment and the integration in different clones was later confirmmed by Southern blot, using the probe specified in the kit (Open biosystems; cat num. MES4758). Last, one of the selected clones was microinjeted in mouse morula to generate chimeric mice. The electroporation and microinjection were done twice and none of the chimeric mice produce were viable or fertile.

1.5.1. Southern blot

Aproximately 15ug of DNA were digested with the SpeI (New England Biolabs) enzyme for 12h at 37°C in a solution containing digestion buffer (NEB Tango) , BSA 0,1mg/ml and espermidine 2,5mM. Digested material was separated by electrophoresis in a 0,8% agarose gel at low voltage during enough time to adecuately separate DNA. After this, the gel was incubated in a 0,25M HCl solution for 15 minutes in order to denaturalize the DNA. The gel was washed with destilated water and treated with a second denaturalizing solution containing NaOH 0,4M and 0,6M NaCl. After a second washing the gel was immersed in a 0,5M Tris-HCl pH 7,5, 1,5M NaCl neutralizing solution during 30 minutes. All treatments were done at room temperature with gentle shaking. DNA was transferred by diffusion in SSC10x to a positively charged nylon membrane (Hybond XL, Amersham, Buckinghanshire, UK) during at least 12 hours. After this, ultraviolet light was used to enhace covalent linkage of DNA to the membrane (Stratalinker, Stratagene, Agilent Technologies, Santa Clara, CA). Membrane was prehibridated with a hibridating solution (0,25m sodic phosphate pH7,2, 1mM EDTA, 1%BSA, 7%SDS) at 65°C for a minimum of 2hours. During this time radiactive labeling of the probe was performed. 20ng of the adequate probe (Open biosystems; cat num. MES4758) are dissolved in a final volume of 45ul TE. The probe was denaturalized at 99°C and amplified according to the

instructions of the Random Prime System (Stratagene) with 50μCi of [32P] dCTP and it was purified by filtration in a Sephadex G-50 Column (ProbeQuant GE Healthcare). The DNA that had been transfered to the membrane was hibridated to the probe at 65°C overnight in a hibridating solution containing 0,05mg/ml salmon sperm (Invitrogen). Once the hibridation was over the membrane was washed in SSC 2x during 10 minutes, then it was washed again during 30 minutes in a SSC 2x, 1%SDS containing solution and finaly during another 15 minutes in SSC 0,2x, 0,1%SDS. After the washing steps the membrane was exposed in a cassette with a PhosphorImager detection screen during the convinient time and it was developed with a Typhoon TRIO scaner (GE Healthcare).

2. Molecular and cell biology

2.1. Cell biology

Unless otherwise specified, all cell lines were cultured in DMEM media (4.5 g/L Glucose; L- Glutamine) (Lonza, Switzerland) with 10% -20% of inactivated fetal bovine serum (FBS) (South American Origin, Lonza) and a mix of penicillin and streptomycin (Gibco, Invitrogen, Life Technologies, Carlsbad, CA). All cell types used are of murine origin: MEFs, B cells, pancreatic carcinoma cell lines, ESC, an iPSCs. They were kept in incubators at 37 °C and 5% C02, except for MEFs, that were maintain in hypoxia incubators, at 37 °C and 5% C02 and 5%O2.

2. 1. 1. Production of MEFs (mouse embryonic fibroblasts)

Female and male mice of the desired genotype were mated until vaginal plugs were visible. At 13.5 day of gestation the female was sacrificed and embryos were extracted. In sterile conditions, the fetal liver was removed, as well as a little piece of head from which DNA was obtained for genotyping. The remaining embryo was cut with a sterile blade and incubated 10 minutes in 1ml trypsin 0,25%, EDTA (Gibco). The resulting mix was pipetted up and down in order to dissolve all aggregates and finally the trypsin was neutralized with 9ml DMEM media (4.5 g/L glucose, L-Glutamine (Gibco) supplemented with 10 - 15% inactivated (30 minutes at 55°C) fetal bovine

serum (FBS) and 1% penicillin/streptomycin (Gibco, Invitrogen, Life Technologies, Carlsbad, CA). The total 10ml were transferred to a p100 plate that was kept in hypoxia incubators (5% O2). The media was changed the following day in order to eliminate dead and blood cells.

2. 1. 2. Isolation of splenic B lymphocytes

Splenectomy was performed in mice at age 6 to 10 weeks. Whole spleens were squeeze in washing buffer: PBS 1x with 1% bovine seroalbumin (BSA Fraction V, Roche). Supernatants were treated with a hypotonic solution (ACK Lysing Buffer, Lonza) during 5 minutes, after which the solution was inactivated with washing buffer. Cellular aggregates were later eliminated with a 40μm filter and the lysate was centrifuged 5 minutes at 350g. Sediment was suspended in 900μl of washing solution to which 80μl of anti-CD19 antibody conjugated magnetic beads Mouse CD19 Micro Beads, Miltenyi Biotech, Germany) were added. The mix was incubated at 4°C during 15minutes. After this time, cells were washed, suspended in 1ml washing solution and transfer to a separating column (MS Columns, Miltenyi) standing in a magnetic scaffold (OctoMACS separator, Miltenyi). Cells linked to the anti-CD19 beads are separated due to their attraction to the magnetic field. After washing the column, these cells were eluted outside of the magnetic field. Primary B lymphocytes were maintain in culture (1x 106 cells/ml) in RPMI media (Euroclone) supplemented with 10% FBS, 1%penicillin/streptomycin (Gibco, Invitrogen), glutamine 2mM (Gibco, Invitrogen), non essential aminoacids (Lonza), sodium pyruvate Gibco, Invitrogen), b-mercaptoetanol 50 mM (Gibco, Invitrogen) and HEPES 10 mM (Lonza). 25mg/ml LPS (LPS, Sigma-Aldrich, St. Louis, MO) were added to stimulate cells.

2.1.3. Proliferation curve

1,25 x 10^5 MEFs were seeded in a 35mm plate, after 2 days cells were counted and 1,25 x10^5 MEFs were seeded again (3T3 protocol) (Todaro and Green, 1963). The increase in population doublings (PDLs) was calculated applying the formula PDLs = log (nf / n0) / log2, where n0 is the initial cell number and nf is the final cell number in each passage.

2.1.4. shRNA infections

For the production or retrovirus, each retroviral vector (T121, shINK4a/ARF, shp16^{INK4a}, shp19^{ARF}, shp53, shpRB, shpBabe) was cotransfected in 293T cells with the retrovirus packaging vector pCL-ECO (ratio1.5:1). The transfection vehicle was lipofectamine 2000 (Invitrogen). 48 hours after the transfection, the viral containing supernatants were collected, filtered and supplemented with 8µg/ml de polybrene. MEFs were incubated with the viral supernatants for 8 hours. In the cases when secondary infections were needed, new fresh supernatants were added in the same way. 48 hours after infection cells were selected with 2 µg/ml puromycin during at least 48 hours. shRNAs were validated in previous studies (Dickins et al., 2007; Li et al., 2009)

2.1.5. β-gal staining

At 17.5 day of gestation the female was sacrificed and embryos were extracted. A little piece of tail was cut for genotyping purposes and the rest of the embryo was fixed in 2% formaldehyde, 0,2% glutaraldehyde in PBS for 45 minutes. After this, it was washed twice with PBS and incubated overnight in a staining solution at 37°C in a shaking incubator.

The cells were fixed directly in the cell culture plate, washed and stained with the very same solution, according to the instructions of the Senescence β-Galactosidase Staining Kit (Cell Signaling). Pictures were later taken with a bright field microscope connected to a digital camera.

2.1.6. Cell cycle and G2/M checkpoint measurements

$5x10^5$- 10^6 cells were harvested as usual; they were fixed with cold PBS 70% ethanol. After this, cells were permeabilized with 0,25%TritonX-100 in PBS during15 minute in ice. After centrifugation, cells were incubated with 0,75ug of the primary antibody that detects Histone 3 phosphorylation in serine 10 (Upstate 06-570) -an epigenetic mark of mitosis entrance- dissolved in BSA 1% in PBS (blocking solution) for 3 hours at room temperature. Next, cells were washed with this solution and incubated for 30 minutes at room temperature with a 1/200 dilution of the secondary antibody conjugated to Alexa 488. Finally, cells were washed and resuspended in blocking solution with 10ug/ml of propidium iodide and 100ug/ml RNase. After an overnight incubation at 4°C or 30 minutes one at room temperature, cell cycle and phosphorylation were analyzed in a BD FACSCanto II cytometer using a laser of 488 nm and a 530/30-Blue detector to measure Alexa488 and a 585/42-Blue detector for propidium iodide. Data were analyzed with the program Flowjo.

2.2. Molecular Biology

2.2.1. Western blot

The following primary antibodies were used: Anti-Actin (Sigma A5441), anti γH2AX (Millipore), anti-ATR (Serotec AHP386), anti-$p16^{INK4a}$ (M-156 Santa Cruz), anti-$p19^{ARF}$ (Santa Cruz 32748), anti-caspase3 ((R&D Systems); Histone 3 phosphorylation in serine 10 (Upstate 06-570)

Cells were lysate by incubation a minimum of 15 minutes in ice with RIPA solution (Tris-HCl pH 7.4 50 mM, NP-40 1%, Na-deoxycolate 0.25%, NaCl 150 mM and EDTA 1 mM) supplemented with protease (Sigma-Aldrich) and phosphatase (Sigma-Aldrich). After this, lysates were centrifuged at 16000 g to eliminate cell debris. Bradford method was used to determine protein concentration. Extracts were denaturalized by heat – 5minutes incubation at 99°C- and separated in gradient gels 4-12% SDS-PAGE (Tris-Acetate Nupage Novex, Invitrogen). Proteins were later wet- transferred to nitrocellulose membranes (Hybond ECL Nitrocellulose, Amersham). Once the

transference has been done, membranes are incubated for 30 minutes at room temperature in blocking solution (5% skimmed milk (Central Lechera Asturiana) in TBS Tween20 0.1% -TBS-T). Membranes are later incubated overnight at 4°C with primary antibodies diluted in blocking solution. After washing the membranes 3 times with TBS-T, they are incubated during 1 hour at room temperature with secondary antibodies that are already conjugated to a fluorophore. Proteins are visualized with Li-cor scanner that allows digital acquisition for quantitative analysis.

2.2.2. High throughput microscopy (HTM)

Cells were cultivated in 96 wells plates with a flat crystal bottom (Greiner Bio-One). After applying the corresponding treatments – when needed- to the cells, they were fixed with 2% paraformaldehyde in PBS at room temperature during 5 minutes. After this, they were permeabilized with a solution containing 0,1% sodium citrate and 0,1% Triton X-100 during 5 minutes. After washing three times with 0,25% BSA, 0,1%Tween20 in PBS, cells were incubated in blocking solution (2,5%BSA, 0,1%Tween20, 10% goat serum) for 30 minutes. Incubation with the corresponding primary antibody diluted in blocking solution took place overnight at 4°C and, after washing 3 times the secondary antibody conjugated to a fluorophore was added. This secondary antibody was also washed and, finally, nuclei were stained with a DAPI containing solution.

When Edu (5-ethynyl 2'-deoxyuridin) staining was required, cells were treated with Edu and after 30minutes to 1 hour, depending on the cellular replication rate, cells were fixed with 4% paraformaldehyde in PBS at room temperature during 5 minutes. After this, Click-iT Edu Cell proliferation Assay kit (Life Technologies) was used to stain the incorporated nucleoside.

Images were acquired automatically with the robotic microscope BD Pathway 855 BioImager (Beckton Dickinson) at room temperature and using a dry objective with a magnification of 20x or 40x. The analysis of the acquired images and quantification of the fluorescence signal was performed with AttoVision software (Beckton Dickinson). This method allows

acquisition of images in an automatic way and their later analysis is described below.

The robotic microscope takes pictures from different fields in each well, in the different fluorescence channels depending on a programmable mATRix. Once the pictures have been acquired, the analysis software allows the delimitation of regions of interest that are identified as individual objects according to certain visual parameters. In our case, each nucleus is recognized by the difference in the intensity of DNA staining with DAPI between the nuclei itself and the background. This way, we define a stencil of objects that allows us to filtrate the pictures captured in each channel, being able to analyze different parameters inside these objects. For instance, we can analyze the fluorescence intensity of different channels inside the nucleus. A list with all the objects (i.e. nuclei) and analyzed parameters (i.e. Fluorescence intensity) is generated and all these data can be represented directly in a graph, linking every object to a point in the graph. More complex analysis, like the identification of other objects inside the ones that have already been defined, can also be done.

In our case, we use HTM to measure gH2AX or EdU intensity in DAPI stained nuclei.

2.2.3. RNA extraction and analysis

Cells were harvested as usual Absolutely RNA Nano or Microprep Kits were used to isolate RNA. Samples harvested at different time points were stored in lysis buffer at -80°C and the extraction protocol was performed at the same time in all samples.

The extraction protocol consists of a column chromatography and includes a DNase treatment step. After the extraction, samples were stored at -80°C.

$p16^{INK4a}$ and $p19^{ARF}$ RNA levels were measured by a quantitative reverse transcription polymerase chance reaction. To this aim, 0,4 µg of RNA were used in a mix reaction according to the instructions of SuperScript III

Platinum kit (Invitrogen) that contains Sybr Green. The curve was normalized to the corresponding GAPDH levels of each sample. The oligonucleotides used were:

GAPDH Rev:	CATGATGGCCATGAGGTCCACCAC
GAPDH Fwd:	GCCACCCAGAAGACTGTGGATGGC
$p16^{INK4a}$ Rev:	TTGAGCAGAAGAGCTGCTACGT
$p16^{INK4}$a Fwd:	CGTACCCCGATTCAGTGAT
$p19^{ARF}$ Rev:	TTGAGCAGAAGAGCTGCTACGT
$p19^{ARF}$ Fwd:	GCCGCACCGAATCCT

Results

RESULTS

1. Rescue of senescence on ATR-Seckel MEF

1.1. T121 recues senescence on ATR-Seckel MEF

Mouse Embryonic Fibroblasts (MEF) are a very useful tool to study the consequences of genetic alterations in a *in vitro* setting. However, one of the technical disadvantages of cultivating primary cells is that they eventually stop proliferating and enter senescence. This is particularly true in the case of ATR-Seckel MEF, that stop proliferating at approximately 5 passages. Moreover, and in contrast to WT MEF, ATR-Seckel MEF do not spontaneously immortalize and we also failed to immortalize them by p53 deletion or by the addition of MYC or RAS/E1A oncogenes. Hence, to understand whether immortalization of cells with reduced ATR levels was at all possible became a key objective in our laboratory.

MEF can be immortalized by serial passage, when a stochastic genetic event – either p53 or P19ARF loss (Collado et al., 2007; Kamijo et al., 1997)- occurs. ATR-Seckel MEF spontaneous immortalization occurs very rarely and only if ATR levels are regained (Murga et al., 2009). Another classical way to immortalize MEF is the addition of certain oncogenes. One of the most common oncoprotein used is the large T antigen; an oncoprotein contained in the SV40 polyomavirus. This oncoprotein is able to control very important signaling pathways in order to promote cellular proliferation, what will induce transformation of virus-infected cells. More precisely, the T-large antigen is able to bind and inactivate the two major tumor suppressor RB and p53 (Colby and Shenk, 1982; Jat and Sharp, 1986; Todaro et al., 1966). Given that loss of p53, not only does not rescue, but rather aggravates the phenotypes of ATR-Seckel MEF (Murga et al., 2009), we decided to explore the impact of the expression of a fragment of the large T antigen (T121, the first 121 aa), which inactivates the whole retinoblastoma family of proteins (RB, p107, p130), but does not affect p53 (Chen et al., 1992). In contrast with p53 deletion, expression of T121 fully rescued the growth of ATR-Seckel MEF, this being the first case in which ATR-Seckel MEF could be immortalized (Figure 1).

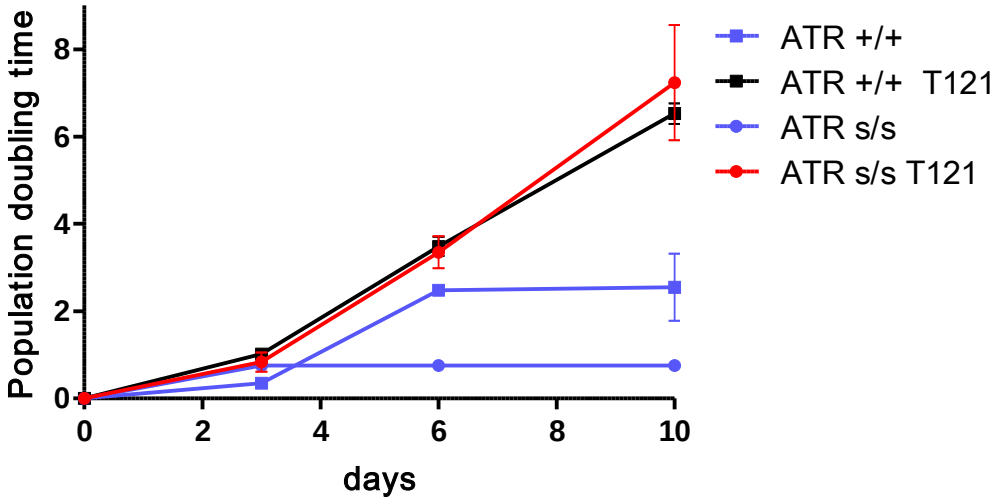

Figure 1. T121 recues senescence on ATR-Seckel MEF Growth curve of $ATR^{+/+}$ and $ATR^{s/s}$ MEF that have been infected with a control retrovirus or a retrovirus expressing T121 oncoprotein. Error bars indicate standard deviation (n=3).

1.1.1. T121 infected ATR-Seckel MEF maintain low ATR and high RS levels

ATR- Seckel MEF immortalization by T121 expression could be due to different reasons, such us a regain in ATR protein levels, the disappearance of the RS characteristic of ATR-Seckel MEF or, more interestingly, the capacity of ATR hypomorphic cells to proliferate indefinitely while they still accumulate high levels of RS. We decided to investigate these options.

First, growing cultures of T121 infected ATR-Seckel MEF show ATR levels equivalent to ATR-Seckel cells (Figure 2B). The fact that T121 expressing ATR-Seckel MEF maintain low ATR levels, suggested that these MEF could be growing in the presence of RS. In mammalian cells, RS leads to phosphorylation of the histone H2A variant H2AX, which has been previously be proven to be a very rigorous RS marker when measured by high throughput microscopy (HTM) (Murga et al., 2011; Toledo et al., 2011b). As shown below, growing cultures of T121 ATR-Seckel MEF are able to grow with substantial levels of RS (Figure 2A). Of note, T121 infected ATR-Seckel MEF show slightly lower RS levels than ATR-Seckel MEF infected with a control retrovirus. We believe that this small difference might be due to the death of T121-infected cells accumulating high amounts of RS, since very high levels of basal cell death are observed in culture.

Consistent with high RS levels, T121-expressing ATR-Seckel MEF also present an accumulation of cells in G2, which is a typical outcome of RS and also found on ATR-Seckel MEF (Murga et al., 2009) (Figure 2C). In summary, T121 infection allows the growth of ATR- Seckel MEF in the presence of high doses of RS.

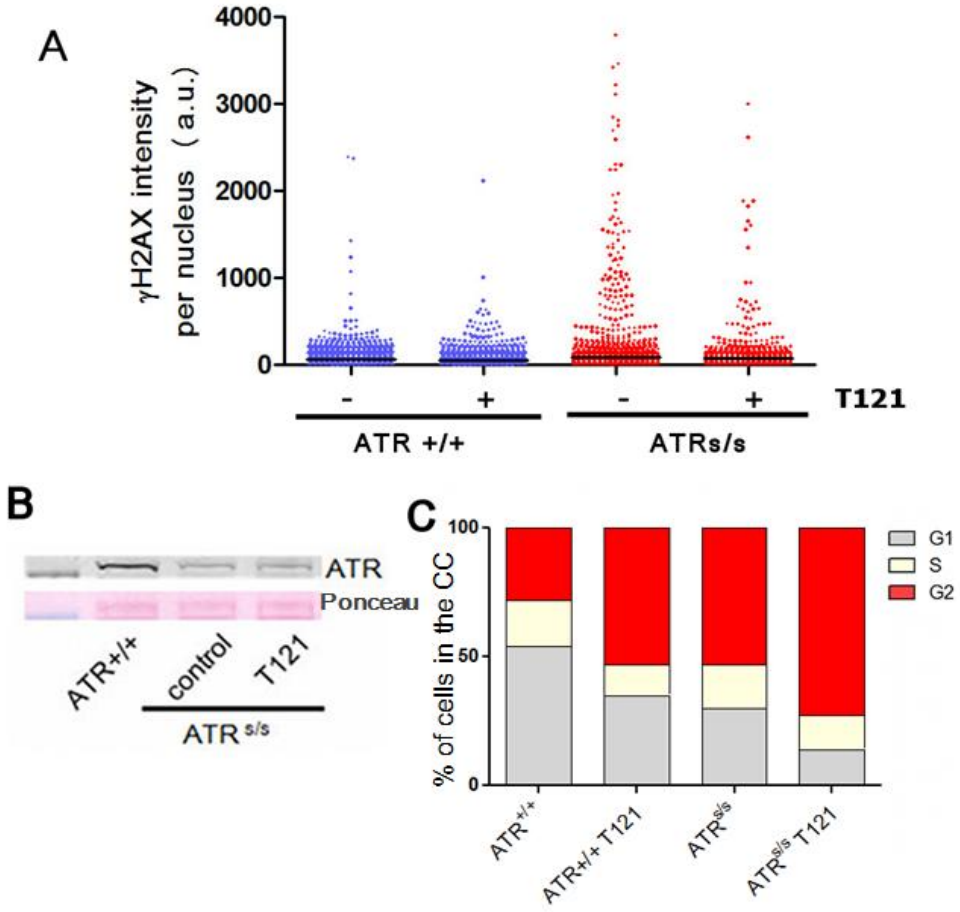

Figure 2. T121 ATR$^{s/s}$ MEF maintain low ATR levels, high RS dosis and G2 arrest (A) HTM mediated quantification of the nuclear levels of γH2AX in ATR$^{+/+}$ and ATR$^{s/s}$ infected with a control retrovirus or with a retrovirus containing T121. (B) Western Blot analysis of ATR levels in wt MEF as well as in ATR-Seckel MEF that had been infected with a control retrovirus or with a retrovirus expressing T121. (C) Graphical representation of the percentage (%) of cells in each phase of the cell cycle. Consistent with the activation of the RS Checkpoint, T121 ATR$^{s/s}$ MEF show a greater accumulation in G2 compared to their wildtype equivalent T121 ATR$^{+/+}$ MEF.

1.2. INK4a/ARF downregulation rescues senescence on ATR-Seckel MEF

Given that RB inactivation by T121 rescued the growth of ATR-Seckel MEF we decided to explore the consequences of the inactivation of other genes related to the RB pathway. Earlier studies in MEF showed that loss of the INK4a/ARF locus, which encodes for a regulator of RB, leads to the bypass of proliferation

induced senescence (Kamijo et al., 1997; Serrano et al., 1997). Thus, we decided to explore the impact of losing INK4a/ARF on ATR mutant cells. First, ATR-Seckel MEF were infected with retroviruses expressing short hairpin RNAs (shRNAs) targeting both products of the INK4a/ARF locus. These shRNAs had previously been validated in different studies (Dickins et al., 2007; Li et al., 2009). In contrast to MEF that had been infected with a control retrovirus, INK4a/ARF depletion rescued the proliferation of ATR-Seckel MEF (Figure 3). Of note, INK4a or ARF depletion (or genetic elimination, see below) alone, did not immortalize ATR-Seckel MEF.

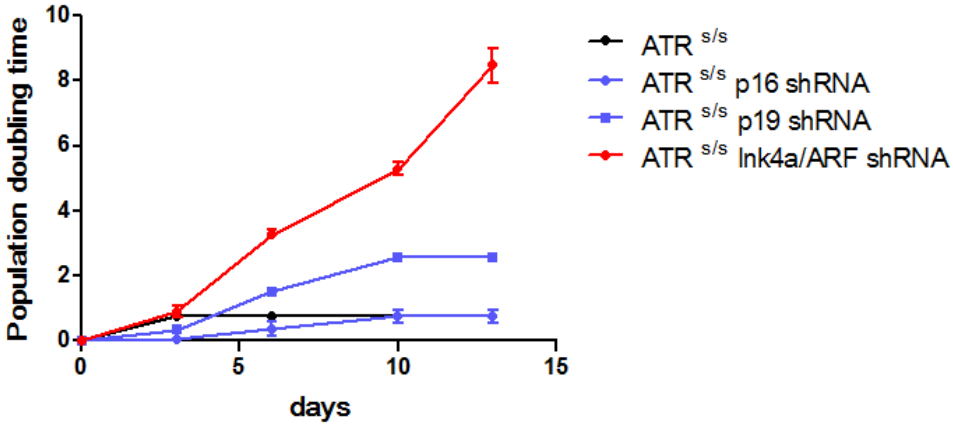

Figure 3. INK4a/ARF downregulation rescues senescence on ATR-Seckel MEF Growth curve of ATR $^{s/s}$ MEF that have been infected with a control retrovirus, or a retrovirus expressing shRNAs against $p16^{INK4a}$, $p19^{ARF}$ or the whole locus INK4a/ARF. Error bars indicate standard deviation (n=3).

1.2.1. INK4a/ARF depleted ATR-Seckel MEF maintain low ATR and high RS levels

We then tested whether INK4a/ARF depletion had an impact on ATR levels, and found that growing cultures of ATR-Seckel MEF depleted for INK4a/ARF presented the same reduction of ATR as the one seen in ATR-Seckel cells (Figure 4B). Additionally, shINK4a/ARF infected ATR-Seckel MEF kept high levels of RS (Figure 4A) and maintained the characteristic G2 accumulation associated to RS (Figure 4C). Of note, the infection with the retrovirus expressing INK4a/ARF shRNA increases G2 accumulation per se, what might be due to the loosening of the G1-S checkpoint associated to INK4a/ARF downregulation. These results are similar to what we found when ATR-Seckel MEF where infected with T121.

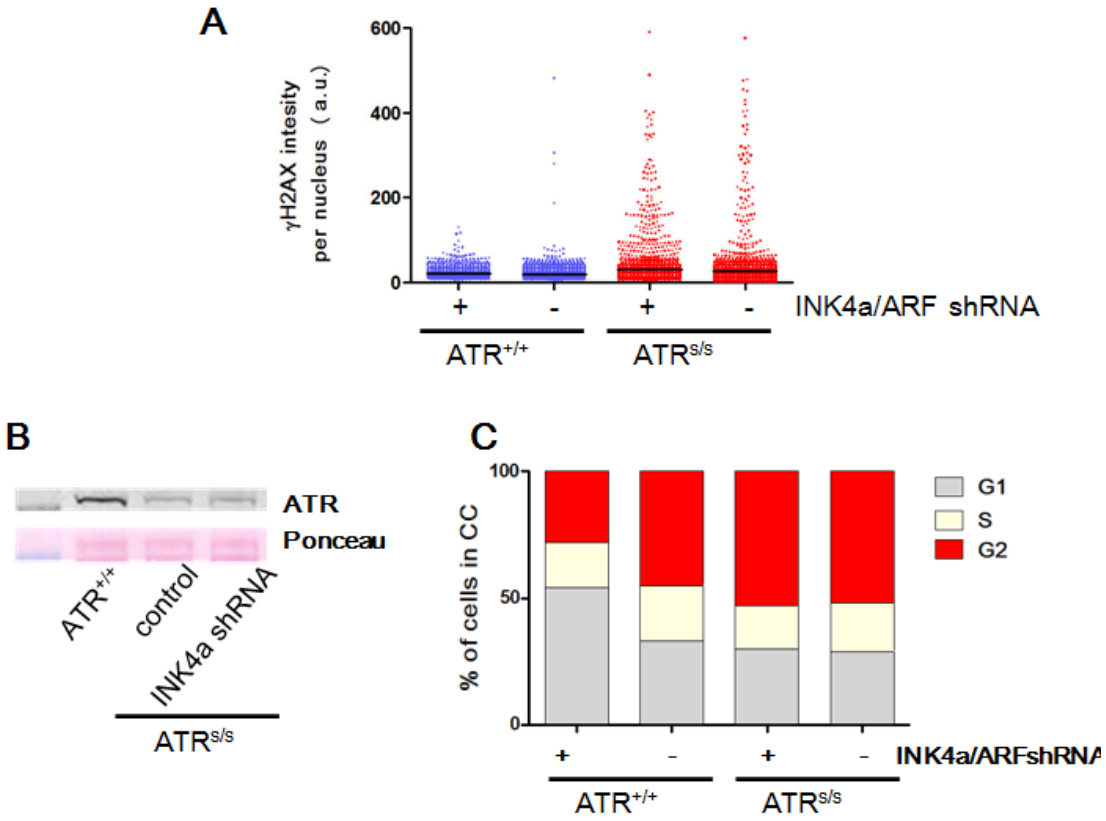

Figure 4. shRNA INK4a/ARF infected ATR $^{s/s}$ MEF maintain low ATR levels, high RS doses and G2 arrest (A) HTM mediated quantification of the nuclear levels of γH2AX in ATR $^{+/+}$ and ATR $^{s/s}$ infected with a control retrovirus or with a retrovirus containing a shRNA against INK4a/ARF. (B) Western Blot analysis of ATR levels in wt MEF as well as in ATR- Seckel MEF that had been infected with a control retrovirus or with a retrovirus expressing shINK4a/ARF or T121. (C) Graphical representation of the percentage (%) of cells in each phase of the cell cycle. In this case, it seems like the infection with the shRNA against INK4a/ARF increases G2 accumulation by itself.

2. INK4a/ARF regulates replication stress induced senescence

2.1. p16^{INK4a} and p19^{ARF} are expressed upon RS

In the following experiments we tried to understand the relationship between RS, the expression of the products of the INK4a/ARF locus and the onset of senescence. p16^{INK4a} and p19^{ARF} expression was measured by high throughput microscopy (HTM). Given the low expression and size of the two proteins, that usually makes their biochemical detection difficult, and in order to facilitate a precise quantification of p16^{INK4a} and p19^{ARF} levels, we decided to optimize a HTM assay that would allow us to quantify the expression of both proteins in every cell. We validated the specificity of the assay by including INK4a/ARF deficient MEF as a negative control for both signals (Figure 5A).

2.1.1. p16^{INK4a} and p19^{ARF} are expressed upon HU and ATRi induced RS

First, wild type (WT) MEF were treated with two RS inducing agents: hydroxyurea (HU) –an inhibitor of the ribonucleotide reductase that leads to nucleotide deficiency and, therefore, RS– and an ATR inhibitor (ATRi) recently developed in our group (Toledo et al., 2011b). As shown bellow, a persistent exposure to low doses of HU or ATRi led to the accumulation of cells with high levels of p16^{INK4a} and p19^{ARF} (Figure 5A), showing that a chronic exposure to replication stress boosts de expression of the whole INK4a/ARF locus.

Moreover, and consistent with this, protein and mRNA levels of p16^{INK4a} and p19^{ARF} were also induced by replication stress inducing agents, as shown in the quantitative retrotranscriptase polymerase change reaction (QRT-PCR) data (Figure 5C) and Western blot depicted below (Figure 5B).

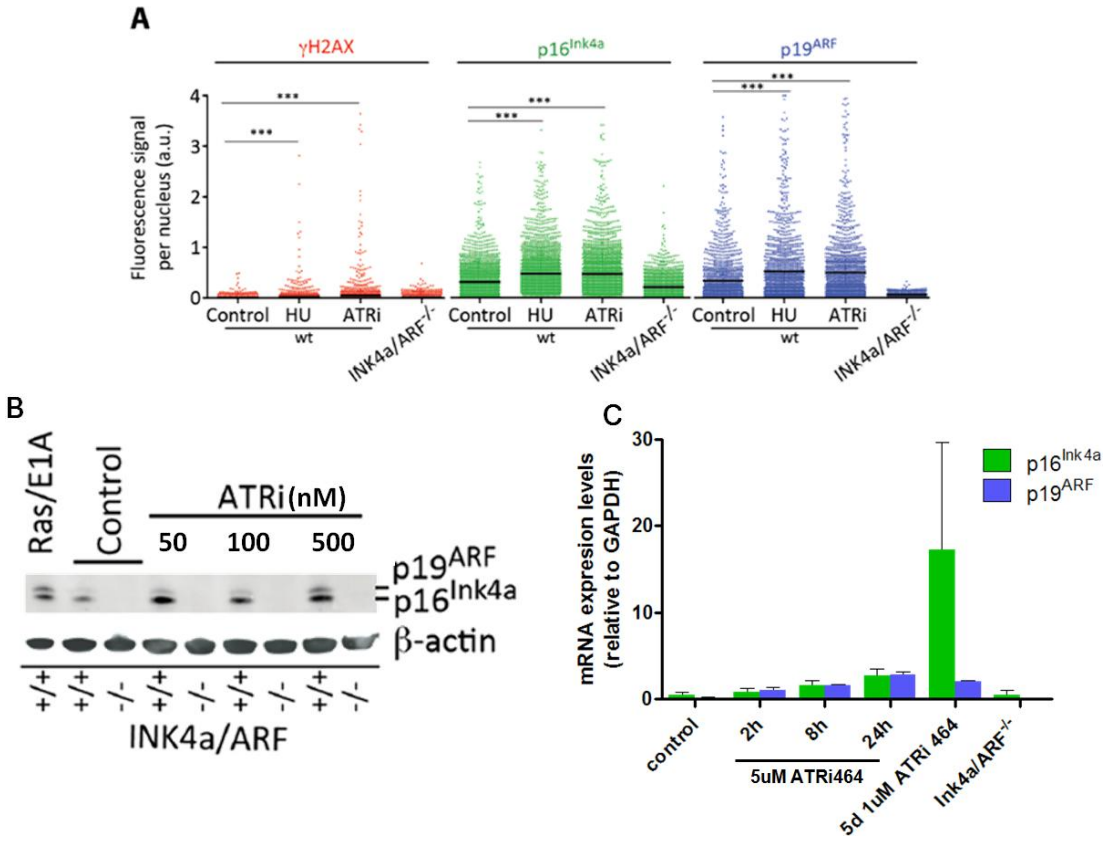

Figure 5. p16^{INK4a} and p19^{ARF} are expressed upon HU and ATRi induced RS (A) Quantification via HTM of γH2AX, p16^{INK4a} and p19^{ARF} nuclear levels in wt MEF exposed to HU (0,5mM) and ATRi (1μM) for 5 days. INK4a/ARF $^{-/-}$ MEF were included as a negative control. (B) Western Blot analysis of p16^{INK4a} and p19^{ARF} in WT MEF that had been exposed to ATRi for 5 days (50, 100 and 500nM). MEF infected with a retrovirus expressing RASV12 and E1A oncogenes were used as a positive control of INK4a/ARF activation. INK4ka/ARF$^{-/-}$ MEF were included as a negative control. (C) qRT-PCR analysis of the mRNA levels of p16^{INK4a} and p19^{ARF} in wt MEF treated with ATRi 464 5nM during 2, 8 and 24 hours and with ATRi464 1nM during 5 days. INK4a/ARF$^{-/-}$ MEF were included as a negative control. mRNA levels were normalized to the expression of GAPDH in each case. Error bars indicate standard deviation (n=3).

2.1.2. p16^{INK4a} and p19^{ARF} are expressed upon endogenous RS

We next measured p16^{INK4a} and p19^{ARF} levels in ATR-Seckel MEF which, as mentioned before, show high constitutive levels of RS. Consistent with the data obtained with HU or ATRi, the levels of both INK4a/ARF gene products were increased in these cells (Figure 6A).

Given that we had used a HTM platform to measure p16^{INK4a} and γH2AX protein levels, we were able very accurately measure p16^{INK4a} and γH2AX in each individual cell. Therefore, we decided to plot the intensity of both markers to seek for any kind of correlation between p16^{INK4a} expression and γH2AX levels. Importantly, we found that p16^{INK4a} and γH2AX fluorescence intensity correlated on a cell-by-cell basis on ATR-Seckel mutant cells, further supporting that RS induces the expression of the INK4a/ARF locus (Figure 6B).

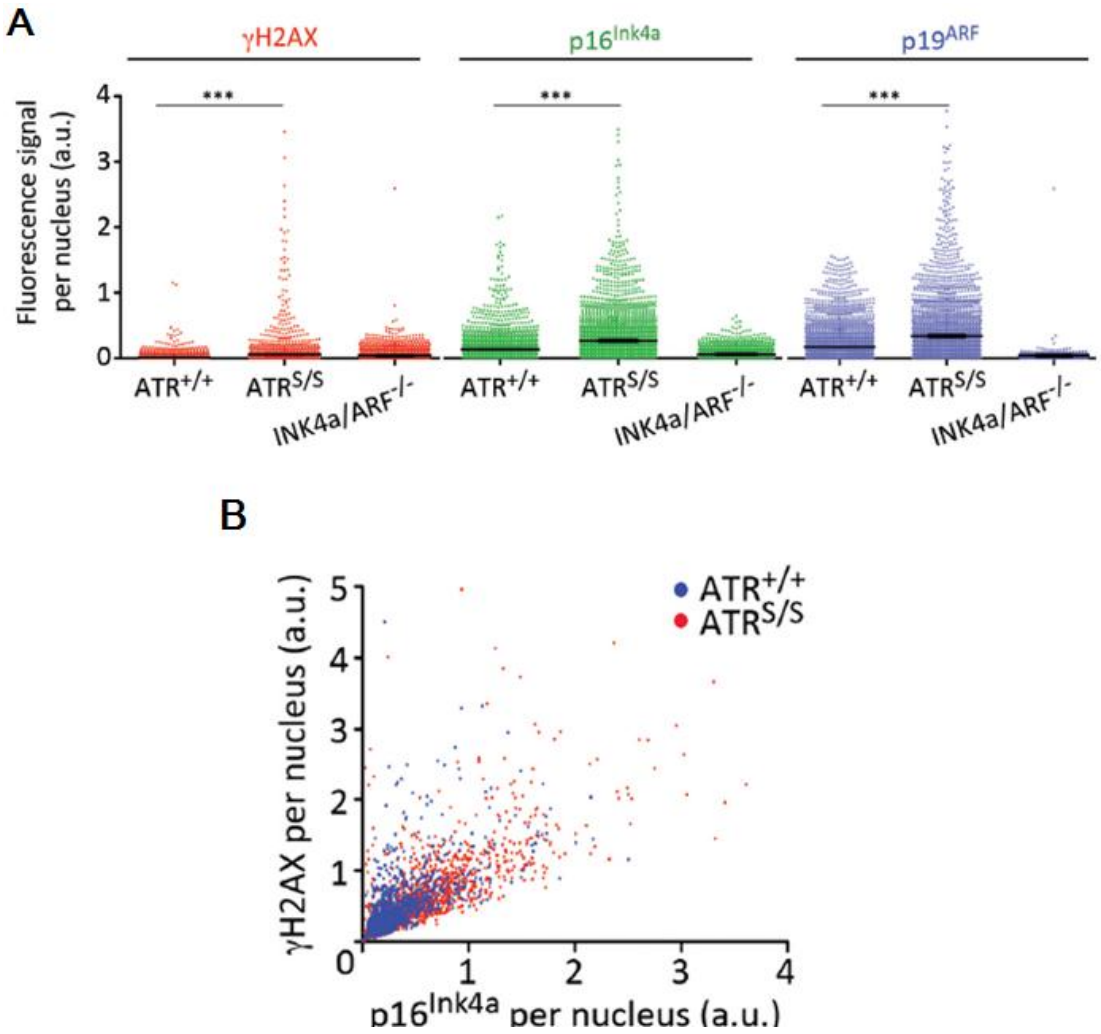

Figure 6. INK4a/ARF activation in response to endogenous RS (A) HTM mediated quantification of the nuclear levels of γH2AX, $p16^{INK4a}$ and $p19^{ARF}$ in $ATR^{+/+}$ and $ATR^{s/s}$ MEF. INK4a/$ARF^{-/-}$ MEF were included as a negative control. (B) 2D-plot showing the direct correlation between nuclear γH2AX and $p16^{INK4a}$ levels found in $ATR^{+/+}$ and $ATR^{s/s}$ MEF. In both cases (A and B), at least 2000 nuclei were quantified per condition *** p<0,001.

2.2. INK4a/ARF deletion rescues RS- induced senescence

ATR inhibitors are being explored in cancer therapy, particularly for tumors with high rates of RS (Toledo et al., 2011a). Considering that loss of INK4a/ARF is a common event in human cancer, our previous results raised the question of whether those tumors might not be sensitive to ATR inhibition. In order to explore this possibility in depth, we developed the next set of experiments.

2.2.1. INK4a/ARF-/- MEF are resistant to RS- induced senescence

Given that INK4a/ARF depletion was able to bypass RS-induced senescence in ATR-Seckel MEF, we decide to investigate whether INK4a/ARF depleted cells are overall more resistant to RS. To this end, we cultivated INK4a/ARF$^{-/-}$ and WT MEFs with RS-inducing agents. Precisely, the two cell types were exposed to low but chronic doses of HU and ATRi. In agreement with what has been explained hitherto, INK4a/ARF deficient MEF were able to maintain growth in the presence of the same ATRi or HU doses that induce senescence on WT MEF (Figure 7A). This piece of evidence proves that INK4a/ARF ablation is able to bypass senescence, not only when it is induced by proliferation or oncogenes, but also when it is activated by RS. Senescence-associated β-galactosidase activity was used to confirm senescence in each independent culture (Dimri et al., 1995) (Figure 7B).

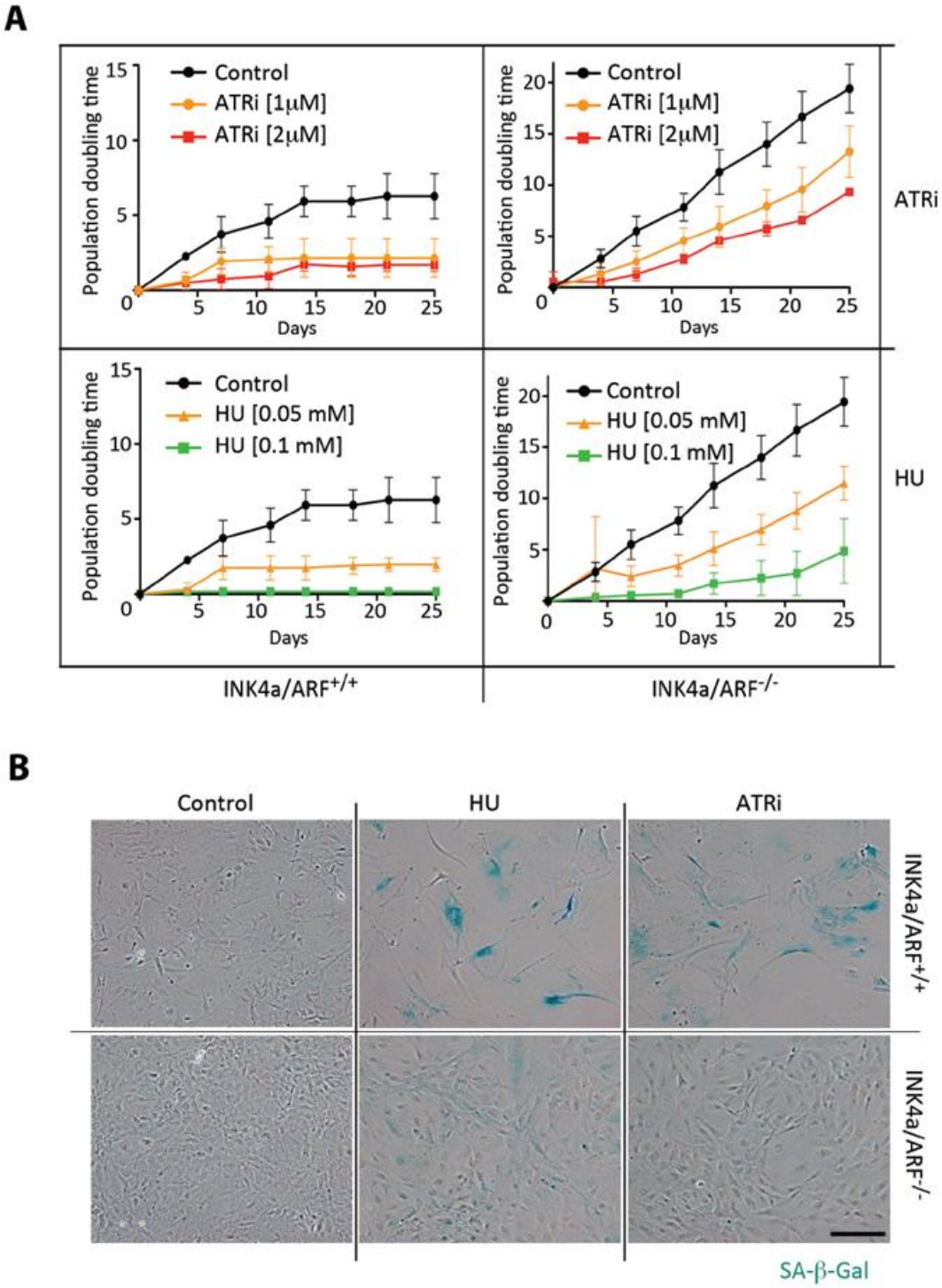

Figure 7. INK4a/ARF $^{-/-}$ MEF are resistant to RS- induced senescence (A) Proliferation curves of INK4a/ARF$^{-/-}$ and INK4a/ARF$^{+/+}$ MEF chronically exposed to HU or ATRi at the doses indicated above. Error bars indicate standard deviation (n=3) (B) Images of the senescence-associated beta-galactosidase activity in In4a/ARF$^{+/+}$ and INK4a/ARF$^{-/-}$ cultures exposed to HU (0,1mM) or ATRi (2μM) for 15 days. Images of untreated cells are included as controls.

2.2.2. ATR $^{s/s}$ INK4a/ARF$^{-/-}$ MEF bypass RS- induced senescence

To explore the impact of INK4a/ARF loss in a scenario in which ATR activity is challenged, we generated INK4a/ARF$^{-/-}$ MEF in an ATR-Seckel background. Similar to our previous findings with shRNAs, INK4a/ARF ablation fully rescued the growth of ATR-Seckel MEF (Figure 8A). As before, INK4a/ARF deletion was able to rescue cell proliferation without restoring ATR protein levels (Figure 8B) or RS (Figure 8C-E).

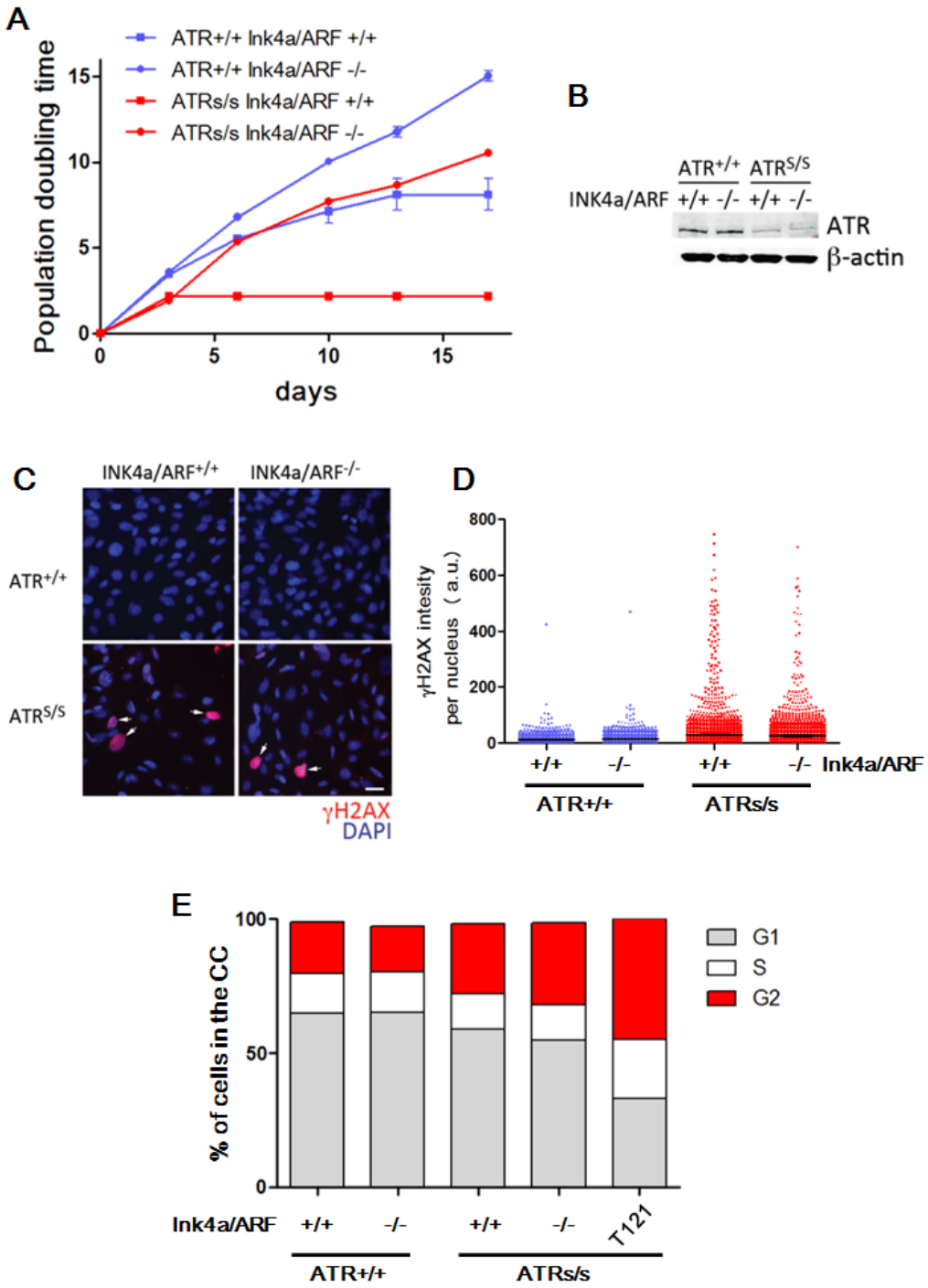

Figure 8. ATR- Seckel INK4a/ARF -/- MEF bypass RS- induced senescence (A) Proliferation curves of ATR$^{+/+}$ INK4a/ARF$^{+/+}$, ATR$^{+/+}$ INK4a/ARF$^{-/-}$, ATR$^{s/s}$ INK4a/ARF$^{+/+}$ and ATR$^{s/s}$ INK4a/ARF$^{-/-}$ MEF. Error bars indicate standard deviation (n=3) (B) Western Blot analysis of ATR levels in ATR$^{+/+}$ INK4a/ARF$^{+/+}$, ATR$^{+/+}$ INK4a/ARF$^{-/-}$, ATR$^{s/s}$ INK4a/ARF$^{+/+}$ and ATR$^{s/s}$ INK4a/ARF$^{-/-}$ MEF. (C) Representative examples of the γH2AX staining used for HTM in the next image. DAPI (blue) was used to stain DNA. Scale bar (white) indicates 10μm (D). Quantification via HTM of γH2AX levels per nucleus on ATR$^{+/+}$ INK4a/ARF$^{+/+}$, ATR$^{+/+}$ INK4a/ARF$^{-/-}$, ATRs/s INK4a/ARF$^{+/+}$ and ATR$^{s/s}$ INK4a/ARF$^{-/-}$ MEF. (E). Graphical representation of the percentage (%) of cells in each phase of the cell cycle.

Once again, and consistent with our shRNA experiments, this rescue was only true for the deletion of the whole locus, but not for the ablation of $p16^{INK4a}$ or $p19^{ARF}$ independently (Figure 9 A, B). In either case, deletion of $p16^{INK4a}$ or $p19^{ARF}$ did not affect ATR protein levels (Figure 9 C, D), and double-mutant cells kept high levels of RS (Figure 9 E, F).

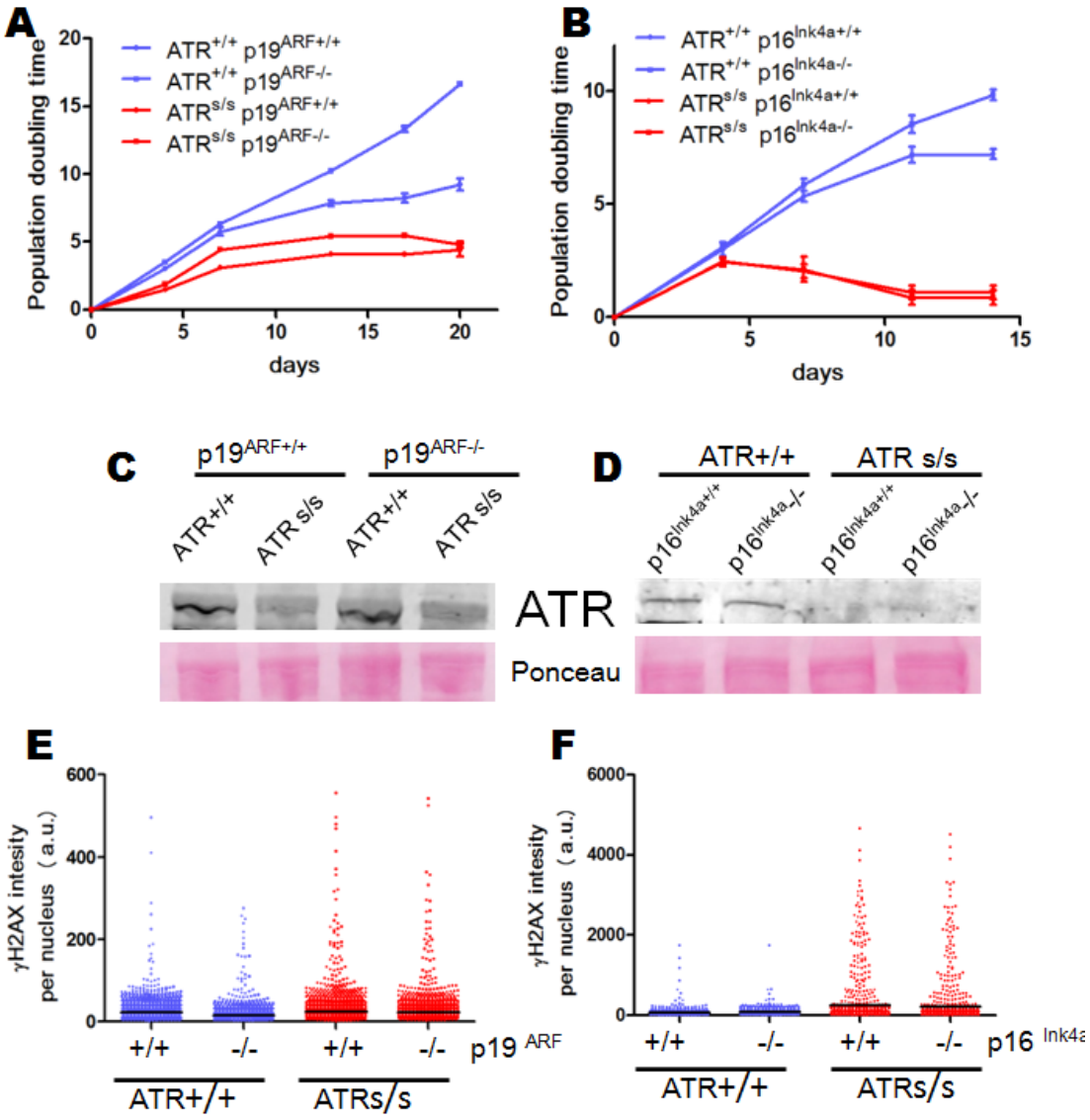

Figure 9. ATR- Seckel p16 $^{INK4a-/-}$ or ATR-Seckel p19 $^{ARF-/-}$ MEF are not resistant to endogenous RS- induced senescence In this figure we characterise $ATR^{+/+}$ $P19^{ARF+/+}$, $ATR^{+/+}$ $p19^{ARF-/-}$, $ATR^{s/s}$ $p19^{ARF+/+}$ and ATR $^{s/s}$ $p19^{ARF-/-}$ MEF and ATR $^{+/+}$ p16 $^{INK4a+/+}$, $ATR^{+/+}$ p16 $^{INK4a-/-}$, ATR $^{s/s}$ p16 $^{INK4a+/+}$ and ATR $^{s/s}$ p16 $^{INK4a-/-}$ MEF. (A and B) Proliferation curves. Error bars indicate standard deviation (n=3). (C and D) Western Blot analysis of ATR levels. (E and F) Quantification via HTM of γH2AX levels in every nucleus.

3. INK4a/ARF has a limited role on ATR-Seckel *in vivo* phenotypes

3.1. INK4a/ARF depletion does not rescue the ageing phenotype of ATR-Seckel mice

To learn about the impact of INK4a/ARF loss on limited ATR activity *in vivo*, ATR-Seckel; INK4a/ARF$^{-/-}$ mice were generated. INK4a/ARF$^{-/-}$ mice are viable and fertile, however, they have an increased susceptibility to the development of cancer (Serrano et al., 1996). ATR-Seckel mice die within 4-6 months due to a pleotropic progeroid disease. Surprisingly, despite the rescue of MEF senescence, INK4a/ARF deletion did not have any effect on the lifespan of ATR- Seckel mice (Figure 10B). Moreover, the loss of INK4a/ARF did not significantly rescue the sub-Mendelian ratios at which ATR-Seckel mice are born (Figure 10A).

A

540 total mice	INK4a/ARF	Expected ratio(%)	Real ratio(%)
ATR$^{s/s}$ 86mice	+/+	25	26
	+/-	50	42
	-/-	25	32

B

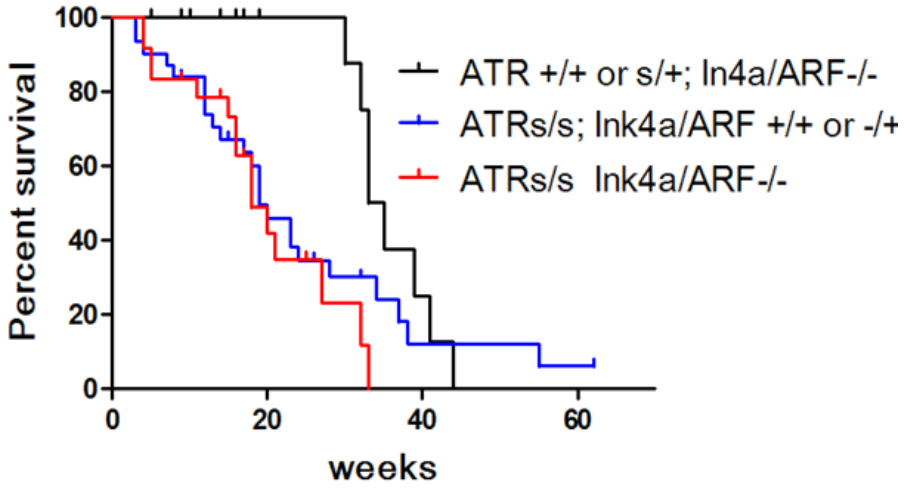

Figure 10. INK4a / ARF depletion does not rescue early death onset or low birth ratio of ATR-Seckel mice (A) Birth ratio of ATR-Seckel mice born from several matings between two ATR$^{s/+}$; INK4a/ARF$^{-/+}$ mice. 540 total births were analyzed. Birth ratios (%) are relative to ATR-Seckel mice only. (B) Kaplan - Mejer analysis of the lifespan of ATR$^{s/s}$ INK4a / ARF$^{+/+}$ or ATR$^{s/s}$ Innk4a / ARF$^{+/-}$, ATR$^{s/s}$ INK4a / ARF$^{-/-}$ and ATR$^{+/+}$ or ATR$^{s/+}$ INK4a/ARF$^{-/-}$ mice.

We also analyzed in detail whether INK4/ARF loss affected any of the phenotypes that are found on ATR-Seckel mice. ATR-Seckel mice present a dramatic retardation of intrauterine development, which results in a low size and weight at birth. This growth retardation is already evident at the embryonic state, when mice are born and it continues during adulthood, when the differences among littermates increase. No noticeable differences were detectable between the overall appearance of $ATR^{s/s}$ and $ATR^{s/s}$; INK4a/ARF $^{-/-}$ mice (Figure 11).

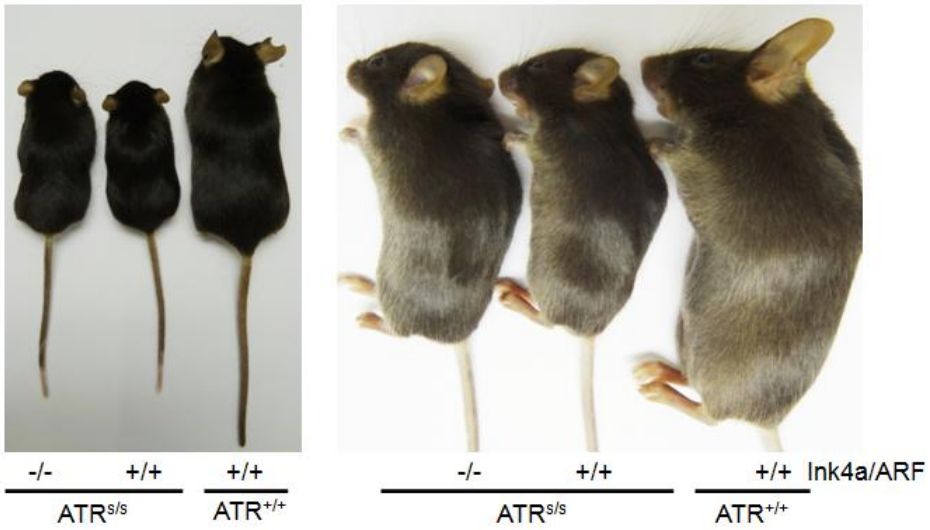

Figure 11. INK4a/ARF depletion does not rescue ATR-Seckel mice phenotypes Representative pictures of $ATR^{s/s}$ INK4a/$ARF^{+/+}$, $ATR^{s/s}$ INK4a/$ARF^{-/-}$ and $ATR^{+/+}$ INK4a/ARF $^{+/+}$ mice.

Besides their overall phenotype, ATR-Seckel mice present a numbef or ageing phenotypes at an early age such as hair graying, cachexia, kyphosis, and accumulation of adipose tissue in their bone marrow or low bone mineral density. Other ageing symptoms that we have detected in these mice include increased polyploidy in liver, reduced hair follicle density and thinner epidermis, as well as severe pancitopenia. Once again, we were unable to find any obvious differences between $ATR^{s/s}$ and $ATR^{s/s}$; INK4a/$ARF^{-/-}$ mice on any of these phenotypes (Figure 12).

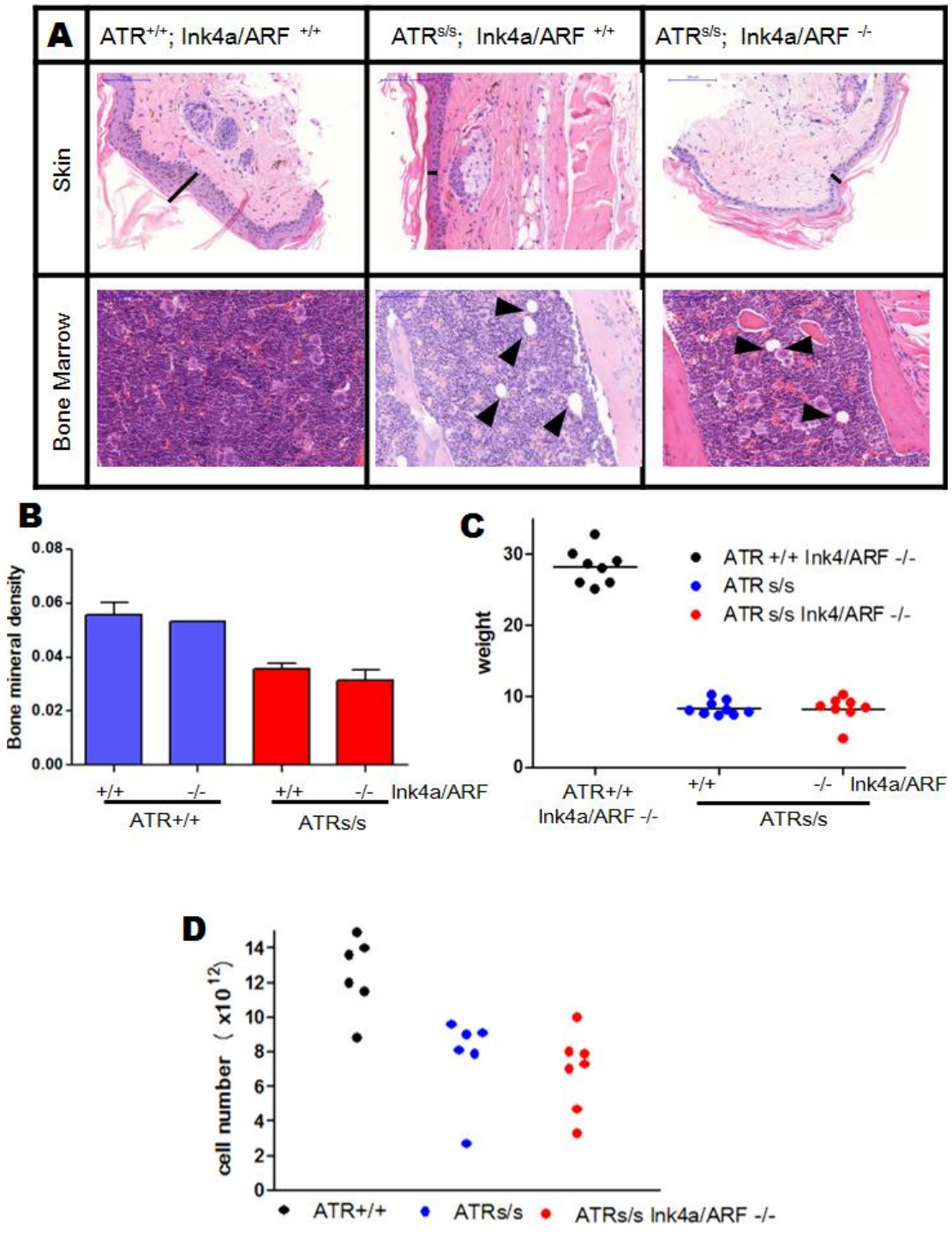

Figura 12. INK4a/ARF depletion does not rescue ATR-Seckel mice phenotypes (A) Hematoxilin & Eosin stained sections of paraffin embedded blocks of skin and bone marrow of ATR$^{+/+}$ INK4a/ARF$^{-/-}$, ATR$^{s/s}$ INK4a/ARF$^{+/+}$ and ATR$^{s/s}$ INK4a/ARF$^{-/-}$ and mice. All ATR- Seckel mice show very thin skin (black line) and fat deposits in their bone marrow (signaled with arrows) (B) Bone mineral density was lower in ATR-Seckel mice, without guarding any relationship with INK4a status. (C) Body weight of ATR-Seckel mice was lower with independence of INK4a/ARF status. (D) Red Blood Cell count was diminis in ATR$^{s/s}$ independently from INK4a/ARF status.

Finally, we tested the effects of the individual deletion of $p16^{INK4a}$ or $p19^{ARF}$ on an ATR-Seckel background. Surprisingly, deletion of either gene not only failed to rescue the ATR mutant phenotype but rather led to an aggravation of the disease . First, the birth ratios of ATR-Seckel animals deleted for either $p16^{INK4a}$ or $p19^{ARF}$ are extremely low (Figure 13 A and B). Given that ATR-Seckel mice lacking $p16^{INK4a}$ or $p19^{ARF}$ are born with such a low frequency, we mated $ATR^{s/+}$ mice nullizigous for either $p16^{INK4a}$ or $p19^{ARF}$ in order to obtain enough ATR-Seckel mice lacking each of the components of the INK4a/ARF locus. However, this did not help significantly in the case of $p19^{ARF}$, where we only obtained two $ATR^{s/s}$ $p19^{ARF-/-}$ mice, which even if they were markedly progeroid, was an insufficient number to analyze their ageing curve (Figure 13). As for the case of $p16^{INK4a}$, $ATR^{s/s}$ $p16^{INK4a-/-}$ mice show a very marked progeroid phenotype and die significantly earlier than $ATR^{s/s}$ $p16^{INK4a+/+}$ littermates (Figure 13C).

In summary, and even though the senescence of ATR-Seckel MEF is rescued by the loss of INK4a/ARF, the phenotype is of ATR-Seckel mice is not altered by the depletion of the whole INK4a/ARF locus. On the contrary, the severity of the symptoms is exacerbated by the individual elimination of either $p16^{INK4a}$ or $p19^{ARF}$. One possibility to explain this paradox could result by the compensatory expression at the locys. When $p16^{INK4a}$ is not present, more $p19^{ARF}$ is expressed, and vice versa, leading to an increased cell cycle arrest in each case which could worsen the phenotype (Baker et al., 2008).

A

191 total mice	$p16^{IN}_{K4a}$	Expected ratio(%)	Real ratio(%)
$ATR^{s/s}$ 10mice	+/+	25	65
	+/-	50	34
	-/-	25	1

B

199 total mice	$P19^{ARF}$	Expected ratio(%)	Real ratio(%)
$ATR^{s/s}$ 34 mice	+/+	25	40
	+/-	50	56
	-/-	25	4

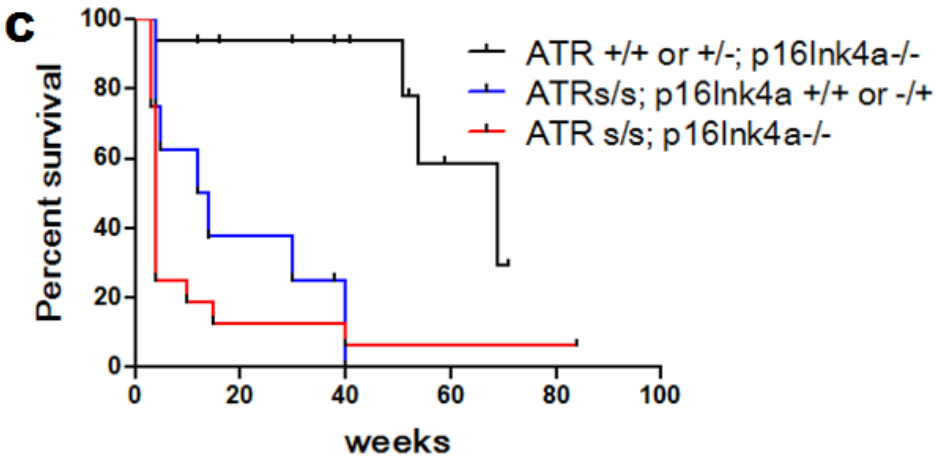

Figura 13. Individual deletion of $p16^{INK4a}$ or $p19^{ARF}$ worsens ATR-Seckel mice phenotype (A) Birth ratio of ATR-Seckel mice born from several matings between two $ATR^{s/+}$; $p16^{INK4a+/-}$ mice. 191 births were analyzed. Birth ratios (%) are relative to ATR-Seckel mice only. (B) Birth ratio of the different genotypes born from several matings between two $ATR^{s/+}$; $p19^{ARF+/-}$ mice. 191 births were analyzed. Birth ratios (%) are relative to ATR-Seckel mice only. (C) Kaplan- Mejer analysis of the lifespan of $ATR^{s/s}$ $p16^{INK4a+/+}$ or $ATR^{s/s}$ $p16^{INK4a+/-}$, $ATR^{s/s}$ $p16^{INK4a-/-}$ and $ATR^{+/+}$ or $ATR^{s/+}$ $p16^{INK4a-/-}$ mice. Kaplan- Mejer analysis of $ATR^{s/s}$ $p19^{ARF-/-}$ mice was not possible given that we only two mice with this genotype were born.

3.1.1. Cancer and ATR Seckel mice

Low levels of ATR have been shown to be synthetic lethal with mutations that promote cancer, such as p53 ablation (Murga et al., 2009) or MYC overexpression (Murga et al., 2011). Given that T121 expression or INK4a/ARF deletion were able to immortalize ATR-Seckel MEF the question was whether any of these conditions could also promote cancer in an ATR-Seckel background. Whereas ATR-Seckel;INK4A/ARF double mutant mice die prematurely due to the progeroid disease, we should notice that after more than 5 years of research with the ATR-Seckel strain, the only tumor ever found on these animals was a fibrosarcoma found at 12 weeks of age on an INK4/ARF deleted ATR-Seckel mouse. The reason

of not finding more tumors could be related to senescence playing a less important role than cell death *in vivo* on the ATR-Seckel phenotype. Given that most of the cell death in ATR-Seckel mice occurs during embryonic development, we propose that a better model to study the interaction between INK4a/ARF and ATR in cancer could be associated to the targeting of ATR on adult INK4A/ARF mice (Gilad et al 2012).

3.2. ATR-Seckel phenotype is more related to cell death than to senescence.

As mentioned, one possible way to explain the reduced impact of INK4a/ARF loss on the ATR-Seckel phenotypes, despite its impact on MEF senescence is that, *in vivo*, cell death rather than senescence is the main determinant of the progeroid disease. Several facts support this hypothesis. On one hand, we were unable to find any evidences of senescence in ATR-Seckel embryos, in contrast to what is observed on a mouse BRCA1 mutant model (delta-11) which was previously reported to show senescence during development (Cao et al., 2003) (Figure 14A). On the other hand, and as reported before (López-Contreras et al., 2012; Murga et al., 2009), ATR-Seckel embryos showed substantial amounts of apoptosis – measured by immunochemistry against activated caspase 3-, which correlate with the severity of the progeroid phenotype in adult mice.

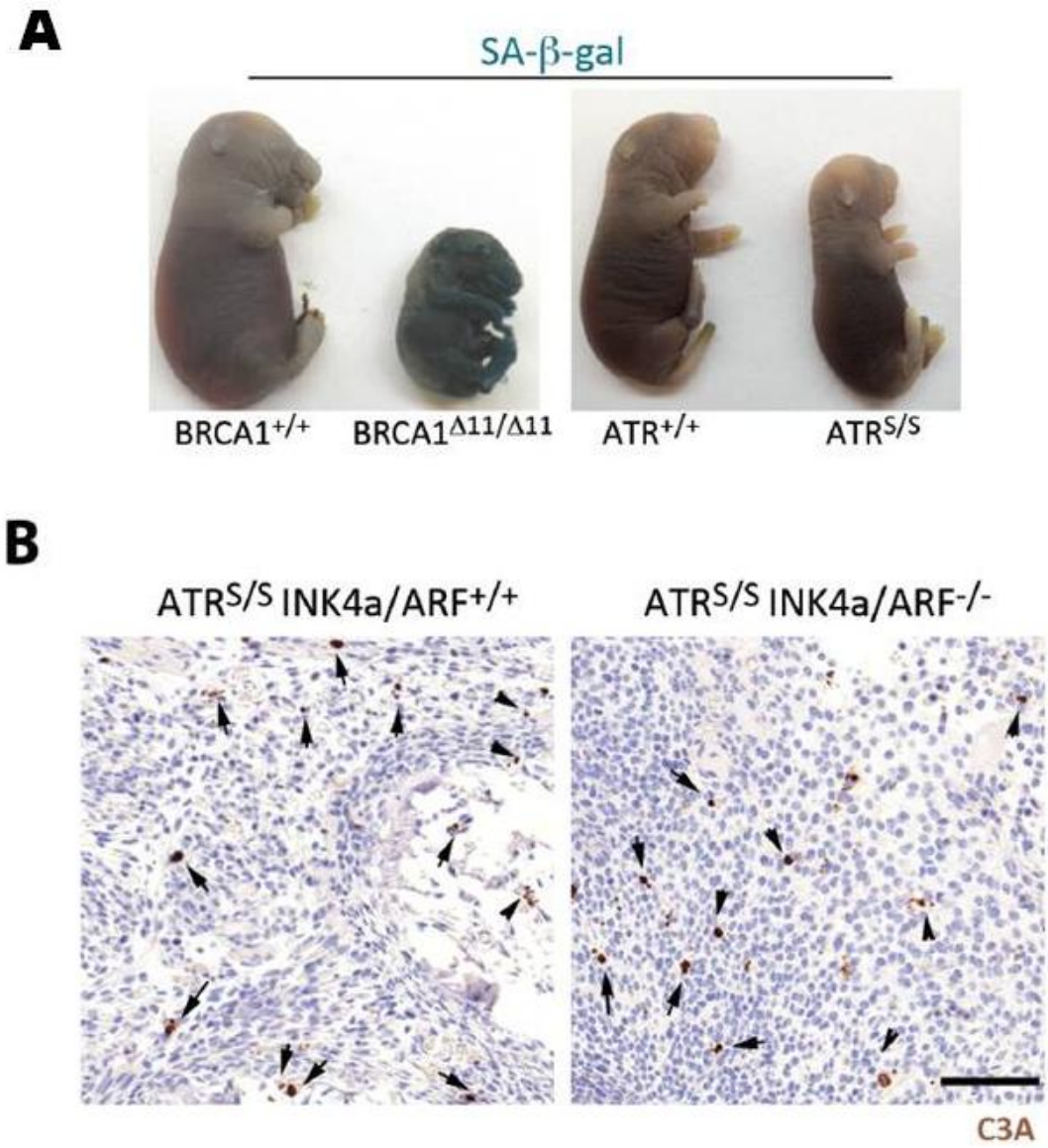

Figure 14. Evidences of apoptosis- but not senescence- on ATR-Seckel embryos (A) Images of $ATR^{+/+}$ and $ATR^{s/s}$ littermate embryos (right panel) in which whole mount SA-b-galactosidase activity was measured (blue) as a senescence marker. Littermate embryos of a BRCA1 mutant mouse model (delta-11), which have been previously reported to show senescence during embryonic development (Brown and Baltimore, 2000) were included as a positive control (left panel). (B) As it had already been reported (Murga et al., 2009), ATR- Seckel embryos show considerable levels of apoptosis. This was measured by immunohistochemistry against activated caspase 3 (brown, indicated with black arrows). The staining was also present on $ATR^{s/s}$ INK4a/$ARF^{-/-}$ littermate embryos. Scale bar indicates 50μm.

3.3. INK4a/ARF depletion rescues senescence but not cell death

As stated in point 2.2.2., the loss of INK4a/ARF rescues senescence in ATR-Seckel MEF. To explore whether INK4a/ARF deletion is also able to rescue RS-induced cell death we exposed INK4a/ARF$^{-/-}$ cells that are more prone to cell death than to senescence – such as splenocytes- to ATR inhibitors. WT and INK4/ARF null splenocytes were exposed to increasing doses of ATRi. After 24 hours of treatment, we calculated the percentage of dead cells by measuring the frequency of cells with a subG1 DNA content based on the intensity of propidium iodide through flow cytometry. In contrast to its impact on senescence, INK4a/ARF failed to modify the cytotoxic effects of ATRi (Figure 15A). Likewise, an *in vivo* treatment of mice with 5mg/kg of the CHK1 inhibitor UCN-01 leads to RS and apoptosis in the thymus – measured by γH2AX and activated caspase 3 immunochemistry respectively - independently of INK4a/ARF status (Figure 15B). All the above suggest that cell death rather than senescence is the main outcome to a limited ATR function *in vivo*.

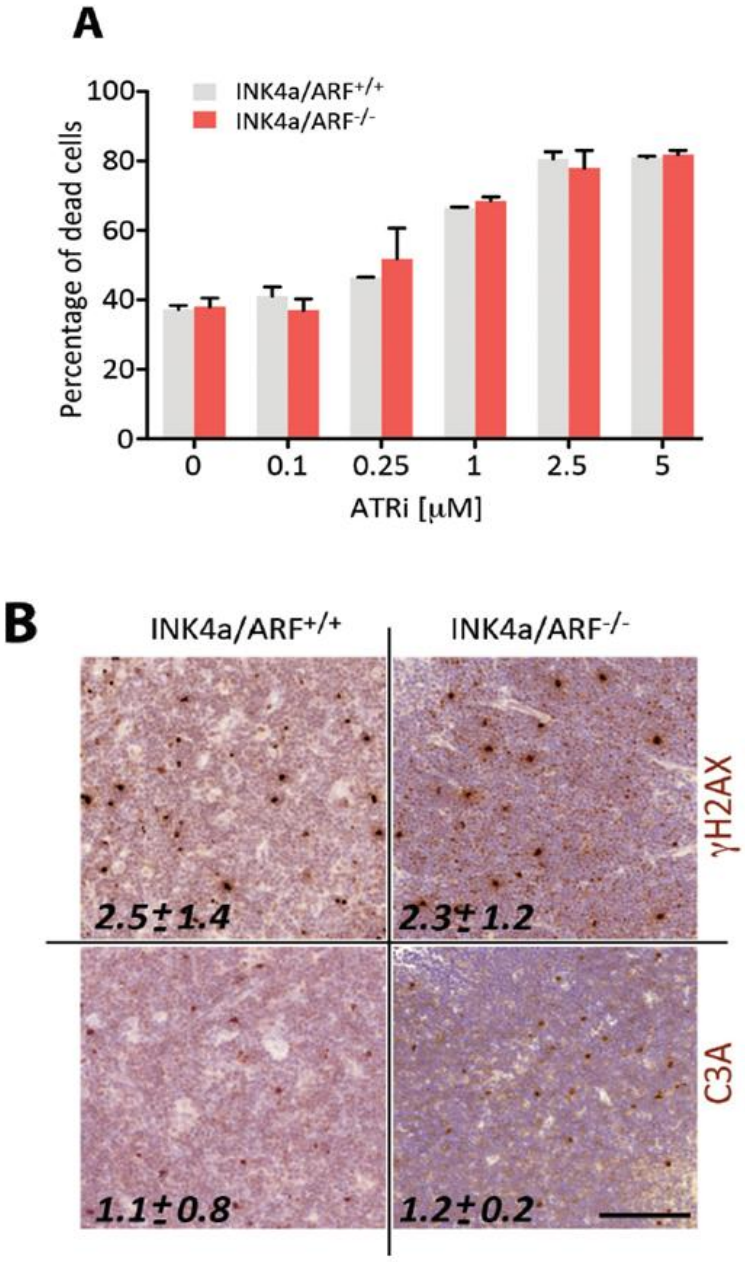

Figure15. INK4a/ARF ablation does not modify the cytotoxic response to ATRi (A) INK4a/ARF$^{+/+}$ and INK4a/ARF$^{-/-}$ splenocytes were stimulated for 48 hours with lipopolysaccharide (10μg/ml) and exposed to ATRi for 24h at the indicated doses. The percentage of dead cells was calculated by measuring the frequency of cells with a subG1 DNA content based on the intensity of propidium iodide observe in a citometer. Error bars indicate standard deviation (n=3). (B) INK4a/ARF$^{+/+}$ and INK4a/ARF$^{-/-}$ mice were injected intraperitoneally with the CHK1i UCN01 (5mg/kg). The images show the immunochemistry analysis of γH2AX and caspase 3 in each case.

4. Contribution of INK4a/ARF to the RS- response in a cancer context

The small impact of INK4a/ARF ablation on ATR-Seckel mice, contrasts with its role in the regulation of RS-induced senescence in MEF. To explain this disparity we propose that INK4a/ARF dependent senescence would be the result of a persistent exposure to limited amounts of RS, such as in the case of MEF exposed to low doses of HU or ATRi for long times. On the contrary, high amounts of RS would inevitably lead to cytotoxicity since cells would not be able to divide in the presence of regions that have not been replicated. This would be of particular importance in the case of ATR inhibitors, where the drugs would not only alter

DNA replication, but also abrogate the checkpoint activity that limits the entry into mitosis with unreplicated DNA. In agreement with the proposed model, and even if INK4a/ARF$^{-/-}$ MEF grow more when chronically exposed to low doses of ATRi, they show the same sensitivity as WT MEF to acute, high ATRi doses (Figure16). This piece of data suggested that the cytotocity of ATR inhibitors in cancer would not be modified by mutations in the INK4a/ARF locus.

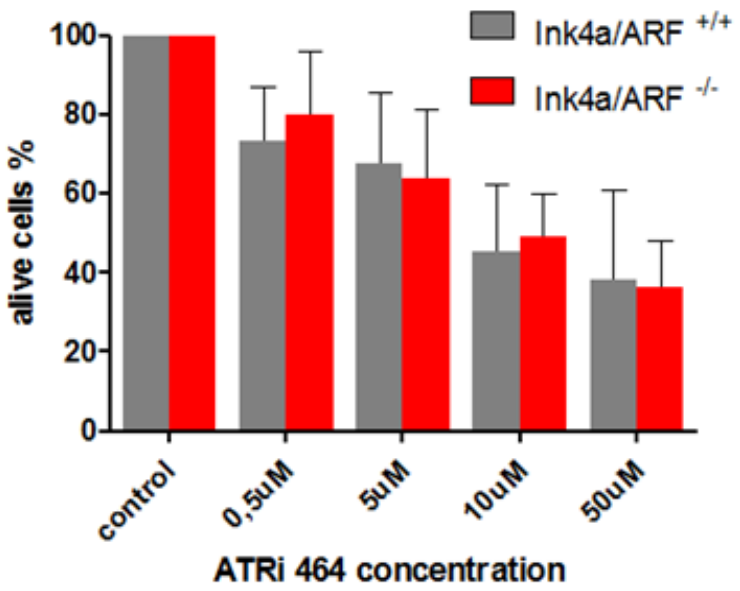

Figure16. INK4a/ARF$^{-/-}$ cells are sensitive to acute ATRi treatments INK4a/ARF$^{+/+}$ and INK4a/ARF$^{-/-}$ MEF were exposed to ATRi at the indicated doses for 24h, and the number of cells alive was counted. Numbers indicate the percentage of cells alive after the treatment (relative to untreated ones). Error bars indicate standard deviation (n=3)

4.1. INK4a/ARF or p53 status does not limit the toxicity of ATR or CHK1 inhibitors in cancer cell lines.

To further support the model presented in the previous point, we tested the toxicity of ATRi and UCN-01 in pancreatic ductal adenocarcinoma (PDAC) cell lines established from a mouse model of K-RASV12- induced tumorigenesis (Guerra et al., 2003) which were wt, p53$^{-/-}$ or INK4a/ARF$^{-/-}$. Importantly, ATRi was cytotoxic for all of the lines regardless of their p53 or INK4a/ARF status (Figure 17).

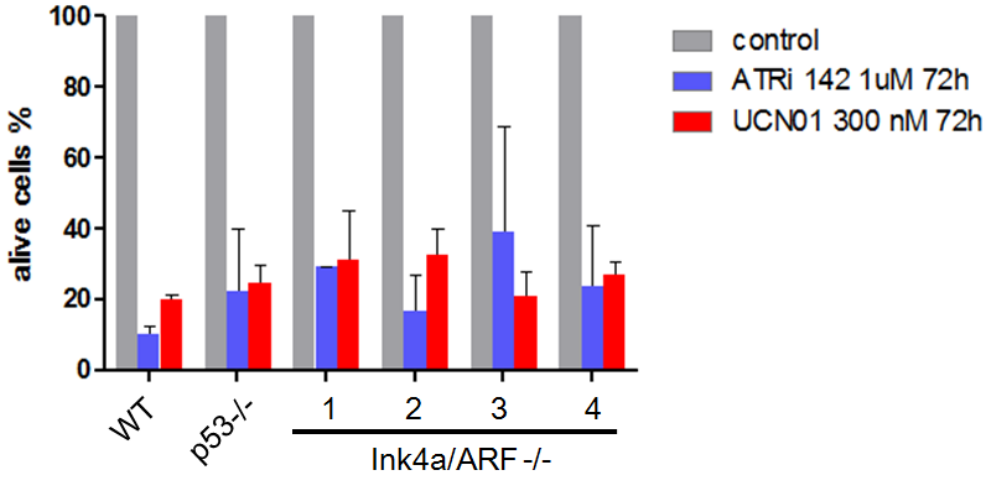

Figure 17. INK4a/ARF or p53 status does not limit the toxicity of ATR or CHK1 inhibitors in cancer cell lines To test our hypothesis in a cancer model system that is as genetically homogeneous as possible, we used murine pancreatic adenocarcinoma cell lines from a mouse model of K-RASV12- induced tumorogenesis (Guerra et al., 2003), which were wt, $p53^{-/-}$ or $INK4a/ARF^{-/-}$. Cells from these cancer cell lines were exposed to ATRi (10μM) or CHK1i (UCN-01, 300nM) for 24h, and the number of alive cells was counted. Numbers indicate the percentage of alive cells after the treatment (relative to untreated ones). The indexed numbers indicate several independent INK4a/ARF deficient lines. Error bars represent standard deviation (n=3).

4.2. Human cancer cell lines with high number of CNVs tend to present low $p16^{INK4a}$ levels

Notwithstanding of its limited influence on the ATR-Seckel phenotype, the RS-induced senescence pathway discovered during this thesis might still play an important role in cancer, where cells could be exposed to low but persistent doses of RS that could promote senescence rather than cell death. For instance, this might be happening in response to oncogenes. In fact, oncogene-induced senescence was already associated with increased $p16^{INK4a}$ levels since its discovery (Serrano et al., 1997). Moreover, more recent works suggested that oncogene-induced senescence could be secondary to the accumulation of oncogene-induced RS (Bartkova et al., 2006; Di Micco et al., 2006), which would in turn be the origin of the activated DNA Damage Response that is observed in cancer (Bartkova et al., 2005; Gorgoulis et al., 2005; Halazonetis et al., 2008). Taking this into account, the results exposed here could indicate that one of the roles of INK4a/ARF in the context of cancer could be to limit the expansion of cells suffering from oncogene-induced RS. In agreement with this idea, INK4a/ARF

ablation facilitates MEF transformation with oncogenes (Li et al., 2009; Serrano et al., 1997).

To investigate this hypothesis on a large dataset of human tumors, we took advantage of the Cancer Cell Line Encyclopedia (CCLE) project (Garnett et al., 2012) that contains genomewide gene expression and copy number variant (CNV) data for 947 human cancer cell lines of different origin. Given that CNVs are the result of a persistent exposure to RS (Arlt et al., 2012), we assessed the connection between the expression of $p16^{INK4a}$ and the total number of CNVs of every cell line in the CCLE. To this aim, the expression levels of $p16^{INK4a}$ and the total number of CNV events of the 947 human cancer cell lines were plotted against each other. Interestingly, we found an inverse correlation between the two parameters, which was particularly evident on the cell lines with the highest number of CNV (P:0,0023). In other words: human cancer cells with high number of CNVs tend to present low levels of $p16^{INK4a}$ (Figure 18). Unfortunately, we were unable to run the same type of analysis for $p19^{ARF}$, since no unique probes for this product were available in the CCLE dataset. Nonetheless, $p16^{INK4a}$ is considered the main contributor of the INK4a/ARF locus to tumor suppression in humans (Kim and Sharpless, 2006). Consistent with this key role of $p16^{INK4a}$, it is worth mentioning that its tumor suppressor activity is linked to the RB pathway. In this context, the only other condition -besides INK4a/ARF deletion- that rescues the growth of ATR-Seckel MEF is the inhibition of RB through the expression of T121 (point 1.1.).

Consistent with ATR expression being constitutive, we failed to found a similar correlation between ATR levels and the number of CNVs, what strengths the validity of the inverse correlation found between $p16^{INK4a}$ levels and the number of CNVs. Additionally, and on the contrary to $p16^{INK4a}$, there was an opposite trend between p53 expression and CNVs (cells with high CNV levels tend to present a high p53 expression). However, tumors with high p53 levels are frequently associated to mutant p53 versions (Muller and Vousden, 2013). In summary, there was a specific trend to present low levels of $p16^{INK4a}$ expression among tumors that accumulate high levels of CNV, which are known to be the outcome to RS.

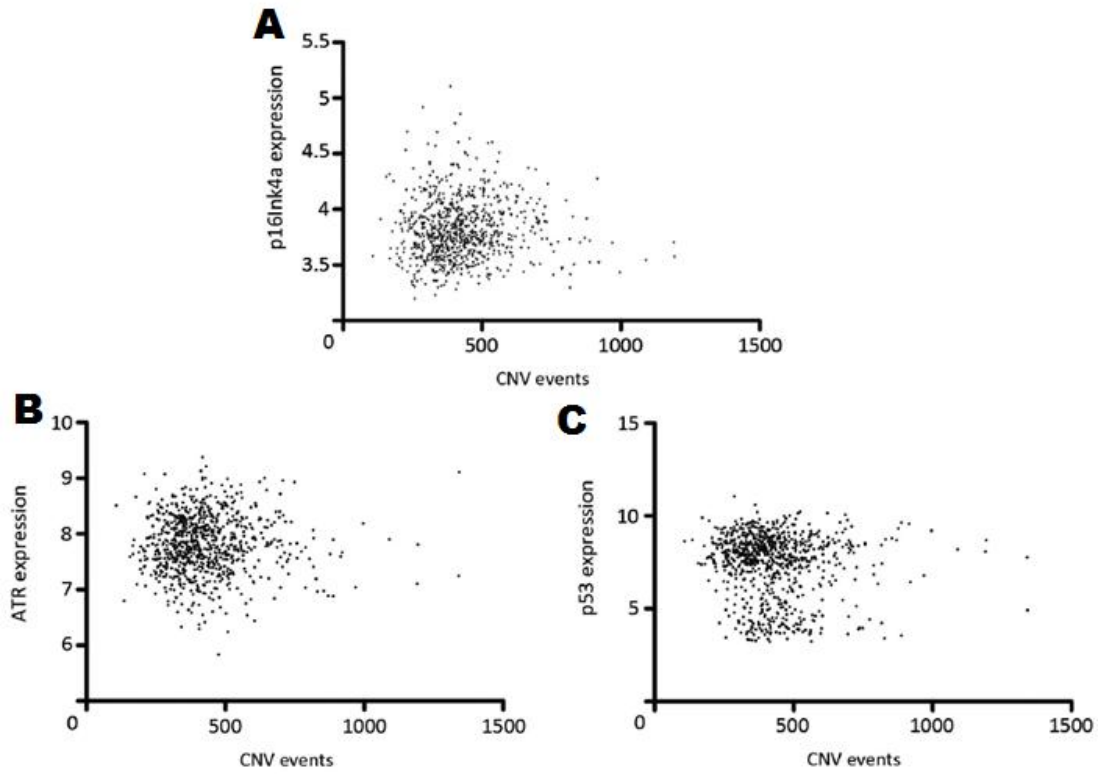

Figure 18. Correlation between p6^{INK4a} mRNA levels and CNV events in human cancer cell lines The expression levels of p16^{INK4a} and the total number of CNV events for 947 human cancer cell lines were obtained from the publicly accessible Cancer Cell Line Encyclopedia (CCLE) repository (Garnett et al., 2012) and plotted against each other. The boomerang shape of the distribution suggests that there is an inverse correlation between p16^{INK4a} and the number of CNVs. To judge the statistical significance of the observation the levels of p16^{INK4a} were compared between the top outliers (3% of the cell lines showing the highest number of CNV) and the rest of the cell lines, which proved that cells with the highest number of CNV present lower p16^{INK4a} levels. (t-test ,P= 0,0023) In agreement with ATR expression being constitutive, we failed to observe a similar boomerang shame distribution with ATR. Interestingly, and in contrast to p16^{INK4a}, cells with high CNV levels tend to present high levels of p53 expression.

5. Exploring the relationship between ATR and RB

Given that T121 expression allowed the immortalization of ATR-Seckel MEF and that T121 oncoprotein inhibits RB we decide to explore both the outcomes of T121 expression and RB deletion in an ATR-Seckel background. To this aim, we tried to generate a conditional T121 transgenic mouse model, which would be used for crossing it into ATR-Seckel. Additionally, RB conditional knockout mice were obtained for a similar purpose.

First of all, we decided to generate a new T121 transgenic mouse model that would allow us to control T121 antigen expression with a tetracycline inducible system. Our hypothesis was that these mice could develop widespread tumors upon T121 induction, which we could then study on the context of reduced ATR levels. To this end, we used a site-specific recombination strategy that enables the integration of the transgene at a specific locus that is widely expressed (collagenase), and under the control of the Tet-O promoter (Beard et al., 2006; Urlinger et al., 2000) (Figure 18).

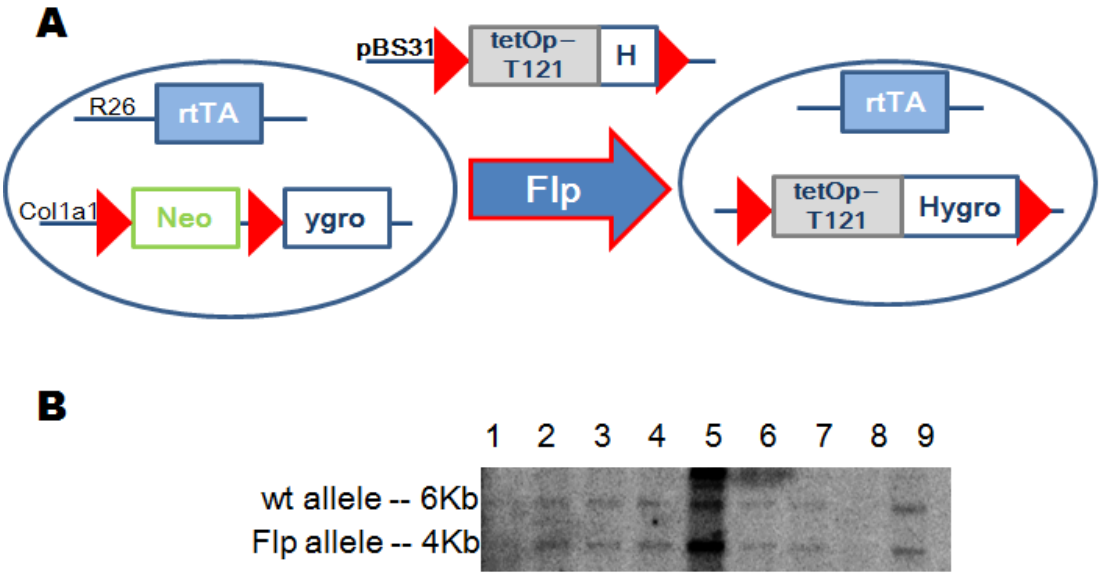

Figure 19. Generation of a T121 conditional mouse model (A) Strategy followed to introduce T121 in the Col1a1 locus, under the control of a tetracycline induced Operon, in cells that already contain a tretraCyclin transactivator domain (rtTA). (B) Southern blot of the hygromicin resistant clones, most of them had incorporated the Flp allele that contains T121.

Unfortunately, even if we were able to find many embryonic stem (ES) cells with a proper integration of the system, aggregation of these ES cells that had incorporated the transgene invariably gave rise to unviable chimeras that would either die perinatally or be hermaphrodite and, therefore sterile. We tried the strategy twice with two different clones of ES cells from independent electroporations, but we chimeras always found the same problem. We think that this might be due the Tet system being slightly leaky, which might be fatal in the context of proteins such as T121 during embryonic development. In fact, T121 expression has previously been shown to cause severe brain defects in mice (Sáenz

Robles and Pipas, 2009; Xiao et al., 2002). Moreover, when we tested the specificity of our system, we observed certain T121 expression in cells that carried the T121 transgene in control conditions. This expression was increased by 30 fold in the presence of doxicycline (Figure 20A). Using these ES cells, we tested the impact of T121 expression on RS. To this end, we treated WT and $T121^{TG}$ ES that expressed T121 under the Tet-O promoter with tetracycline and subsequently exposed them to the CHK1 inhibitor UCN01. The expression of T121 increased proliferation and therefore RS levels in both, UCN01 treated and untreated, cells (Figure 20B). Thus, T121 expression leads to RS in murine ES.

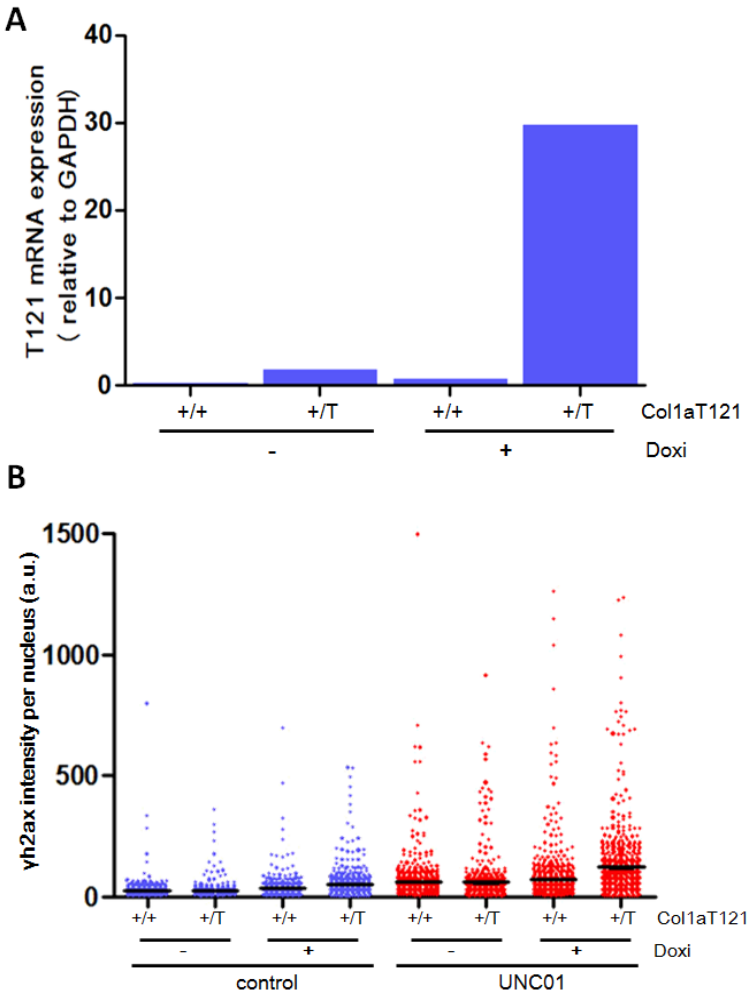

Figure 20. T121 expression increases RS (A) qRT-PCR analysis of the mRNA levels of T121 in WT and T121 inducible SC in control media and treated with doxicyclin. Only doxicyclin treated transgenic ESC show T121 expression. mRNA levels were normalized to the expression of GAPDH in each case. (B)Quantification via HTM of γH2AX levels in every nucleus found on Col1aT121 $^{+/+}$ and Col1aT121 $^{+/T}$ ESC treated with doxicyclin compared to control ones in a neutral situation or boosting replication stress levels with a 8 h treatment of CHK1i (UCN-01 300nM).

Following a different approach, we decided to generate a conditional knock out model for RB in an ATR-Seckel background. To this aim, we mated ATR-Seckel mice with mice carrying a $pRB^{lox/lox}$ allele and a tamoxifen-inducible UbCreERT-2 transgenic allele. Using these mice, RB can be depleted in adult mice upon tamoxifen treatment therefore circumbenting the problem of embryonic lethality. We tested different administration strategies and patterns. However, this approach faced serious technical challenges that precluded the development of the experiment. Experimental mice $ATR^{s/s}$; $pRB^{lox/lox}$; UbCreERT-$2^{T/+}$ were born at a very low ratio and, when exposed to the different tamoxifen treatments, very often died due to the treatment (i.e. intratracheal gauge). In addition to this, we were able to see very limited phenotypes linked to RB deletion in adult mice. RB $^{lox/lox}$; UbCreERT-2 $^{T/+}$ mice fed with tamoxifen diet during 4 weeks showed very mild phenotypes, the most striking being an increase in the mitotic index of the liver. Taking all this into consideration, we decided to discontinue the experiment.

Regardless of being unable to do the experiment *in vivo*, we decided to exploit this pipeline to respond to a very basic question on RS-research; namely whereas cellular life is at all possible in the absence of ATR. ATR knockout mice are early embryonic lethal, and full deletion of ATR also leads to cell death in adult mice (Brown and Baltimore, 2000; Ruzankina et al., 2007) . However, based on our previous results, we decided to explore whether the expression of T121 was able to rescue the lethality of ATR null cells. To this end, we employed ATR conditional KO MEF ($ATR^{lox/lox}$). $ATR^{lox/lox}$ MEF were first immortalized with T121. Subsequently, immortalized $ATR^{lox/lox}$ MEF were infected with a Cre recombinase expressing retrovirus carrying the puromycin resistance gene, to delete ATR. In this context, all puromycin resistant cells –the ones in which ATR had been eliminated- died within a few days (Figure 21). Thus, even if T121 is able to fully rescue senescence on ATR hypomorphic cells, it cannot rescue the viability of ATR nullyzygous cells.

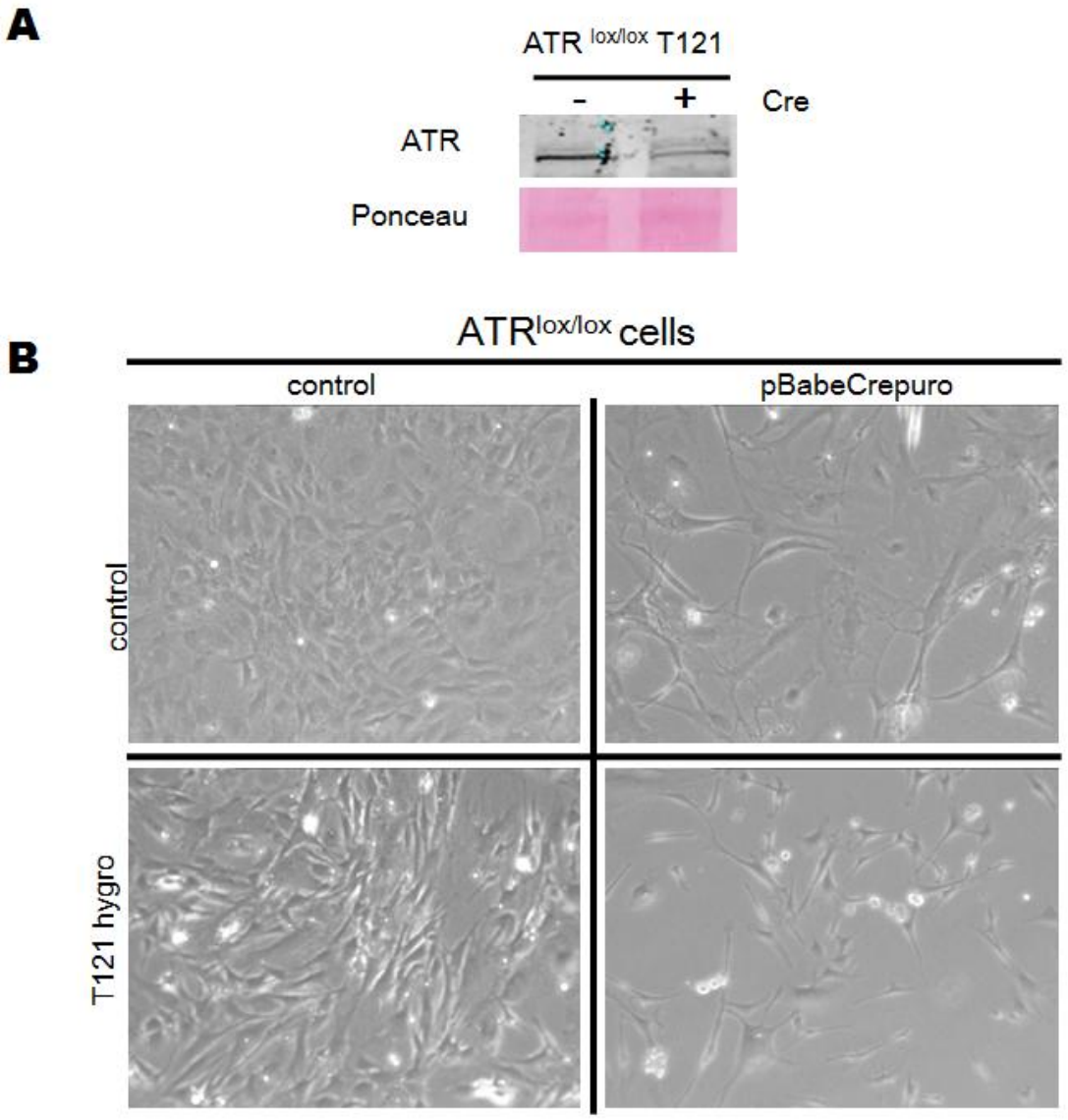

Figure 21. ATR is essential for life (A) WB analysis of ATR levels in ATR$^{lox/lox}$ T121 expressing cells infected with a control retrovirus or with a retrovirus expressing Cre recombinase. (B) Representative examples of the ATR$^{lox/lox}$ and ATR$^{lox/lox}$ T121 cells after the infection with a Cre expressing retrovirus.

Discussion

DISCUSSION

RS is a type of DNA damage reflective of the accumulation ssDNA during replication, which triggers a cellular response coordinated by the essential protein ATR. As proof of the physiological impact of RS, ATR-hypomorph mice (ATR-Seckel) present a progeroid disease to which they succumb early in life. Likewise, ATR-Seckel MEFs are unable to sustain proliferation due to the activation of RS induced senescence. Moreover, previous works from our group revealed that the severity of the ATR-Seckel mutation is aggravated through the loss of tumor suppressors like p53, or the gain of oncogenes like MYC, both of which further induce RS (Murga et al., 2009; Murga et al., 2011). Based on this synthetic lethal interaction with precancerous mutations, the use ATR or CHK1 inhibitors in cancer therapy might be particularly efficient in the context of mutations that drive RS. However, we have recently found that T121 expression or INK4a/ARF deletion can rescue growth on ATR-Seckel MEF, which raised the concern that the potential efficacy of ATR or CHK1 inhibitors for cancer therapy might be limited in tumors with deficiencies in these pathways. In this context, the aim of this PhD was to explore the genetic interactions between ATR and INK4a/ARF or RB *in vitro* and *in vivo*, and to investigate whether these mutations might limit the cytotoxicity of ATR and CHK1 inhibitors.

1. The INK4a/ARF locus and genome maintenance

As it was explained in greater detail in the introduction, $p16^{INK4a}$ was initially described as a CDK-interacting protein (Xiong et al., 1993), able to inhibit CDK4 and CDK6 (Serrano et al., 1993). The locus also encoded another transcript called $p19^{ARF}$ that uses a different first exon and that shares the last 2 exons with $p16^{INK4a}$, but in a different reading frame (Quelle et al., 1995b). Of note, $p19^{ARF}$ is not a CDK inhibitor but rather an activator of p53. All this taken into consideration, the INK4a/ARF locus is a key player in cancer: it activates the two core tumor suppressor pathways: $p16^{INK4a}$/RB and $p19^{ARF}$/p53. In human cells, however, $p16^{INK4a}$ seems to be the main contributor to tumor suppression. In fact, cancer-associated mutations imply either the whole locus, or $p16^{INK4a}$ alone (Quelle et al., 1995b).

Early studies unlinked INK4a/ARF from the response to DNA damage (Efeyan et al., 2006; Kamijo et al., 1999; Stott et al., 1998). In contrast, oncogenic stress was proven to activate p53 through the INK4a/ARF locus, suggesting that this was the only context where p53 activation would depend on $p19^{ARF}$ (Serrano et al., 1997). Accordingly, $p19^{ARF}$ deficiency does not alter the apoptosis induced by the activation of p53 in response to ionizing radiation *in vivo*. However, $p19^{ARF}$ is essential for p53 activation in response to oncogenic stress (Efeyan et al., 2006). In spite of all this, a number of reports also suggested a possible involvement of the locus in the response to DNA damage, (Al-Mohanna et al., 2007; Al-Mohanna et al., 2004; Lau et al., 2007; Sarkar-Agrawal et al., 2004). One option to reconcile these observations is to consider that oncogenic stress could relate to chronic and low amounts of RS, which would constitute a source of persistent DNA Damage (Bartkova et al., 2005; Di Micco et al., 2006; Gorgoulis et al., 2005). Thus, it is possible that while INK4a/ARF does not play an active role in the acute response to DNA breaks, it might otherwise be important in the context of a chronic exposure to other sources of DNA damage such as replication stress.

Consistent with the previous proposal, we show that low, but persistent amounts of RS can induce the expression of $p16^{INK4}$ and $p19^{ARF}$ as well as INK4a/ARF-dependent senescence. From the two products of the locus, a number of our observations point to $p16^{INK4a}$ as the main contributor to this phenomenon. First, while INK4a/ARF deletion rescues the senescence of ATR-Seckel MEF, p53 ablation aggravates the phenotype of ATR-Seckel MEF and mice (Murga et al., 2009). Second, a fragment of the large T antigen (aa. 1-121), which inactivates RB but not p53, also rescues senescence on ATR-Seckel MEF. Third, our qRT-PCR data show a greater increase in $p16^{INK4a}$ than in $p19^{ARF}$ expression upon treatment with reagents that induce RS. In agreement with this, it was earlier reported in our laboratory that RB is activated by a continuous exposure to doxorubicin, a drug that damages DNA during replication. Moreover, this activation was required to maintain a prolonged cell cycle arrest on the damaged cells (Cuadrado et al., 2009). Finally, our meta-analysis of the Cancer Cell Line Encyclopedia data shows that $p16^{INK4a}$ levels are exceptionally low on cancer cells with a high number of Copy Number Variations. Interestingly, we failed to observe a similar correlation between CNVs and the levels of ATR, which is the main kinase orchestrating the

response to RS but is expressed constitutively. In contrast to $p16^{INK4a}$, cells with high CNV levels tend to present high levels of p53 expression. However, most tumors presenting high levels of p53 are associated with inactive mutant forms of the protein (Muller and Vousden, 2013). Thus, these pieces of data suggest that the relationship between $p16^{INK4a}$ low levels and a high number of CNV is not casual.

In summary, we propose that the $p16^{INK4a}$/RB pathway has a key role in genome maintenance, through the restriction of the proliferation of cells that are a chronically exposed to low amounts of RS. The working model would then be summarized as follows. Upon the activation of oncogenes, tumor cells undergo promiscuous and uncontrolled proliferation, giving rise to a persistent exposure to RS. The INK4a/ARF locus would then be activated upon the continuous presence of RS. Upon expression of $p16^{INK4a}$ and $p19^{ARF}$, $p16^{INK4a}$/RB and $p19^{ARF}$/p53 pathways will restrain the growth of the (pre)malignant cells through the activation of senescence. However, in cells with a mutated INK4a/ARF locus this barrier would be abrogated. As a result, premalignant cells would be able to grow even in the presence of high levels of RS. Given that RS stands for ssDNA, which is a source of CNV and chromosomal rearrangements, growing in the presence of constant RS may eventually lead to genome configurations that favor cancer progression.

1.1. INK4a/ARF locus activation

At this point, it remains to be understood how a persistent exposure to low doses of RS leads to the activation of the INK4a/ARF locus. However, different reasons suggest that this activation is not dependent on ATR. First, the activation of INK4a/ARF in response to RS requires several days, which contrasts with the immediate activation of the ATR dependent RS-response. Second, ATR inhibition or hypomorphism lead to increased levels of $p16^{INK4a}$ and $p19^{ARF}$. Finally, it was previously shown in our group that a chronic activation of ATR is able to induce senescence in an INK4a/ARF independent manner (Toledo et al., 2008). This last piece of evidence formally proves that ATR-induced senescence is not dependent on INK4a/ARF. If not ATR, how a chronic exposure to RS ends up activating the locus still is an open question. We should note, though, that understanding how oncogenes trigger the activation of the INK4a/ARF locus remains unsolved after several years since its discovery.

Expression of the INK4a/ARF locus can be induced by several means. First, a wide range of harmful stimuli, such us UV light, ROS, IR, chemotherapy or nucleolar stress have been reported to induce $p16^{INK4a}$ and/or $p19^{ARF}$ expression both, *in vitro* and *in vivo* (Sherr, 2012). The nucleolus is a non-membrane bound structure found in the nucleus of cells, which transcribes and assembles ribosomal RNA (rRNA). A number of cytotoxic compounds, abnormal metabolic conditions, and physical insults can alter nucleolar structure and function. In this scenario, $p19^{ARF}$ protein binds MDM2, leading to p53 activation and cell-cycle arrest. Moreover, $p19^{ARF}$ has also been proposed to function independently of p53 to attenuate ribosome trafficking to the cytoplasm (Sherr, 2006). If or how RS can promote nucleolar stress remains unresolved. However, given that the rDNA is one of the biggest repeats of the human genome it should be preferentially affected by RS.

Second, a number of cancer-related proteins are known to induce the expression of the different components of the INK4a/ARF locus. The RAF–MEK–ERK kinase cascade is one of the best understood pathways that affect INK4a/ARF expression. This signaling pathway is of great importance given the high frequency of homozygous INK4a/ARF deletions in melanoma, the majority of which harbor mutations in the RAF–MEK–ERK signal transduction cascade (Sherr, 2012). In the case of MYC, it has been proposed that it may bind directly the INK4a/ARF promoter (Gil and Peters, 2006). The inverse correlation between $p16^{INK4a}$ expression and RB status in human cancer cell lines raised the question of whether the locus could also be regulated by members of the E2F family, that regulate the cell cycle progression. In fact, both the $p16^{INK4a}$ and $p19^{ARF}$ promoters harbor putative E2F binding sites (Sherr, 2012). However, and at least in MEF, oncogenes are capable of activating INK4a/ARF expression regardless of E2F1 or E2F2 (Palmero et al., 2002). Finally, it is known that members of the AP1 family of transcription factors can also regulate the INK4a/ARF locus, although it is difficult to present a simple picture of their activities given the fact that they are extremely pleotropic (Gil and Peters, 2006).

On the other hand, repressors of the expression of INK4a/ARF have also been identified. For example, the polycomb group (PcG) genes (BMI-1, Cbx7,

Mel18) have been shown to repress the expression of the entire locus. BMI-1 is required for stem cell maintenance, and Bmi1 deficiency in mice has been associated with a failure in the maintenance of the renewal of stem cells. Importantly, this phenotype can be partially rescued by INK4a/ARF deficiency (Molofsky et al., 2005).

Finally, although DNA replication and transcription are generally considered independent processes, coupling of the processes has been described in yeast. A molecular connection between DNA replication and the transcription of the INK4a/ARF locus has also been reported (Gonzalez et al., 2006).

Whether the activation of the INK4a/ARF locus in response to RS involves any of the mechanisms summarized above is still not known. At this point, we can only rule out that this induction is mediated directly by the ATR kinase. Still, the finding of RS-induced senescence reveals an important role for the INK4a/locus in limiting the growth of cells harboring RS, as it could frequently occur in cancer.

2. The DDR and cancer

Genome integrity maintenance is an essential part of DNA metabolism. In healthy cells, DNA lesions activate a number of responses that lead to DNA repair or the elimination of the cell if damage is irreversible. Tumoral cells, in contrast, present high levels of chromosomal aberrations. These aberrations come together with the deregulation of several pathways. In fact, several alterations of the DDR machinery have been found in different types of cancer. For instance, mutations in BRCA1 and BRCA2 genes, involved in HR, were the first ones to be found associated with familiar cases of breast and ovarian cancer.

The DDR-associated checkpoint response has been proposed to work as an anticancer barrier during the initial stages of cancer development (Bartkova et al., 2006; Halazonetis et al., 2008). However, besides its checkpoint role, it is also possible that its tumor suppressive role might derive from deficiencies in DNA repair. For instance, germ-line mutations in HR-repair genes BRCA1 and BRCA2 confer a high risk of breast cancer development. In these cases of familiar breast cancer, where DNA repair deficiencies seem to be the driver of tumorigenesis, mutations on ATM and CHK2 have also been reported.

Regardless of its repair function, the current model suggests that it is the activation of p53-dependent checkpoint and/or apoptotic responses what would be the key role of the DDR as a cancer barrier. In fact, activation of the DDR has been reported in early cancer lesions, where it could be protecting against tumor expansion (Bartkova et al., 2005; Gorgoulis et al., 2005). For instance, phosphorylations of γH2AX, CHK2 or ATM have been reported in precancerous lesions, suggesting the presence of an activated DDR. In this scenario, the subsequent inactivation of components of the DDR may allow the malignant progression of the initial lesion. The prevalence of p53 and, to a lower extent, p53 mutations in advanced stages of cancer seems to favor this view (Halazonetis et al., 2008). Moreover, the absence of a proficient DDR will increase mutation rates and therefore further promote tumor plasticity. In what regards specifically to RS, it is worth mentioning that CNV are a hallmark of cancer cells that arise as a result of RS (Arlt et al., 2012), and which can also facilitate tumor evolution.

Oncogene-induced DNA damage is thought to occur through the generation of RS. However, how oncogenes generate RS is still not known. Several, non exclusive, options have been proposed so far. First, oncogene expression may lead to promiscuous S entry leading to an imbalanced replication process and RS. Second, the unrestrained proliferation induced by oncogenes can lead to the exhaustion of dNTP pools, which is another source of RS (Bester et al., 2011). Finally, oncogene expression has also been associated with increased firing of replication origins which can impair replication fork progression and promote breakage at replication forks (Jones et al., 2012).

2.1. ATR and cancer

Accordingly to the previous point, and given that RS leads to ATR/CHK1 activation, this pathway may play an important role in suppressing RS in cancer. To explore the impact of ATR in cancer, ATR-Seckel mice, which have severely reduced ATR levels and an extremely low cancer frequency, have been previously used in combination with different cancer models.

The first model to be considered was the p53 deficiency. It had been previously reported that other progeria models, different from ATR-Seckel mice,

showed an alleviation of their ageing phenotypes upon p53 loss (Rodier et al., 2007). However, loss of p53 aggravated the phenotype of both, ATR-Seckel cells and mice. At the cellular level, p53 deletion in ATR-Seckel MEF gave rise to a dramatic loss in cellular viability as well as an increase RS. In mice, the absence of p53 dramatically decreased the birth ratios of ATR-Seckel mice and led to an exacerbation of the progeroid phenotype on the few mice that were born. This synthetic lethality was associated to an increase in RS and cell death levels during embryonic development (Murga et al., 2009). The explanation to this paradox derives from the role of p53 in restricting S phase entry. In the absence of p53, the more promiscuous S phase entry would be particularly deleterious in the context of a limited ATR pathway. If this model were to be correct, we predicted that ATR hypomorphism should also be synthetic lethal with other mutations that promote S phase entry.

To investigate this possibility, Eμ-MYC mice were crossed with ATR-Seckel mice. Eμ-MYC mice die prematurely due to the development of B-cell lymphomas that show abundant levels of DNA damage. Once again, the presence of the MYC transgene significantly limited the viability of ATR-Seckel mice. This observation revealed that MYC overexpression was having an effect on embryonic development in the context of reduced ATR levels. Accordingly, Eμ-MYC showed an increased number of apoptotic cells on ATR mutant embryos, accompanied by a higher incidence of cells presenting RS. This generalized synthetic lethal interaction between MYC and ATR was unexpected given that MYC overexpression was thought to be restricted to B cells in the Eμ-MYC model. However, widespread overexpression of the oncogen was found in transgenic embryos. Besides its impact on the number of ATR-Seckel mice that were born, and even though Em-MYC transgenic ATR-Seckel mice die at about the same age as Eμ-MYC, lymphomas were never observed on Eμ-MYC mice that were hypomorphic for ATR (Murga et al., 2011). Most importantly, established Eμ-MYC lymphomas were shown to be very sensitive to CHK1 inhibitors, due to the accumulation of very high doses of RS. We now know that ATR inhibitors are also very efficient as a therapy for MYC induced lymphomas in mice (Murga et al, unpublished).

These series of evidences suggested a model in which a limited activity of ATR is synthetic lethal with mutations that promote RS. The implications of such a model are of great importance, especially in designing a rational use of ATR and CHK1 inhibitors in the clinic, by directing their use to tumors presenting high levels of RS.

2.1.1. ATR and fragile site stability

Common fragile sites (CFS) are specific and conserved regions of the genome that are normally not broken (also known as "silenced"), but that tend to be break (what is known as "express") spontaneously, and even more in the presence of RS. Importantly, these sites have been shown to be involved in chromosome rearrangements – such us copy number variations (CNVs)- in cancer cells (Casper et al., 2002). The reasons of CFS breakage are still not clear. They tend to occur at heterochromatic, late-replicating areas of the genome, which show a low density of replication origins (Hellman et al., 2000; Le Beau et al., 1998), or that arise due to conflicts between replication and transcription, which is more frequent at large genes (Helmrich et al., 2011). Notweorthy, ATR was the first gene to be known that plays a role in the suppression of CFS expression (Casper et al., 2002).

CFS are also associated with CNVs. As mentioned, RS can drive the formation of CNVs, preferentially at CFS (Arlt et al., 2011a). In fact, it has been shown that hydroxyurea (HU) and aphidicolin (APH) –two RS-inducing agents- produce CNVs that overlap with fragile sites in MEF (Arlt et al., 2009; Arlt et al., 2011b). Although HU and APH use different mechanisms to induce RS, both give rise to CNVs with similar frequency, size and distribution; identical to many normal and pathogenic CNVs. Altogether, these data strongly suggest a common mechanism mediated by RS for the formation of CNVs (Arlt et al., 2012). Moreover, hotspots with a high frequency of CNVs were detected and mapped, and they match with the location of chromosomal fragile sites (Arlt et al., 2011b).

Finally, it is worth mentioning that besides CFS, a new class of Early Replicating Fragile Sites (ERFS) has been recently discovered (Barlow et al Cell 2013). Importanly, ERFS are also associated with recurrent hotspots of genome rearrangements in cancer, and their stability is also maintained by the action of

ATR. Altogether, ATR plays a critical role in the suppression of RS, which can contribute to cancer through the generation of chromosomal rearrangements such as CNV, which show a preference to occur at spontaneous fragile sites.

2.2. ATR inhibitors as a potencial anticancer therapy

Although ATR or CHK1 heterozigosity predispose to tumor onset, ATR (Toledo et al., 2011b) and CHK1 (Tao and Lin, 2006) inhibitors have been proposed for cancer treatment. This paradox is explained by the analysis of CHK1 and ATR levels. Whereas half of their normal amounts will generate small doses of RS that can facilitate tumor evolution, a stronger inhibition of ATR and CHK1 will generate so much RS that it would be toxic for fast replicating tumoral cells. This would be particularly true for tumors with mutations that generate high loads of RS, which would be particularly sensitive to ATR or CHK1 inhibitors (Figure1).

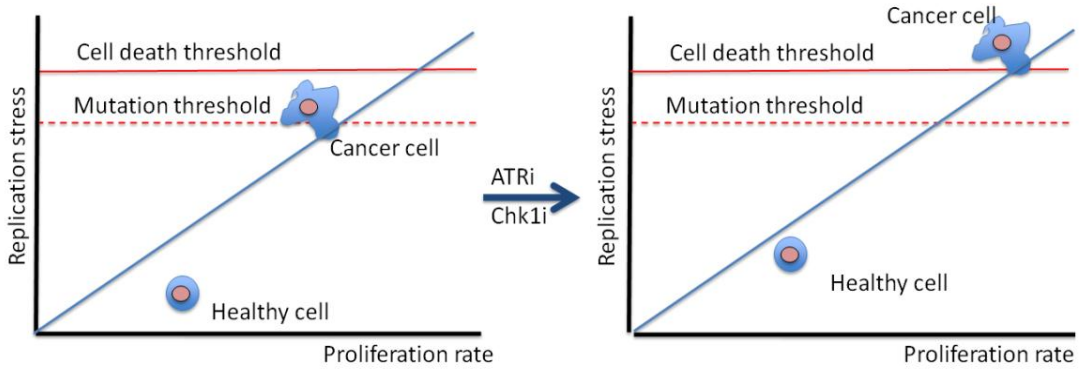

Figure 1. Rationale for ATRi and CHK1i use in cancer treatment Promiscuous proliferation increases cellular RS, which is a hallmark of tumoral cells. If we inhibit the ATR/CHK1 pathway, RS repair will be impaired. This will constitute a major problem to cancer cells that already show high RS levels. When we treat these cells with ATRi or CHK1i, RS accumulation will reach toxic levels, leading to cell death. Healthy cells, on the other hand will not be affected since they are usually not replicating or doing it at a slow pace.

2.3.1. How do ATR and CHK1 inhibitors kill cancer cells?

The main mechanism through which we propose that ATRi may kill cancer cells is the accumulation of lethal amounts of ssDNA. This is particularly risky given that ATR and CHK1 also control the G2/M checkpoint. Hence, ATR or CHK1 inhibitors would lead to the entry into mitosis of cells that have not completed replication, leading to mitotic catastrophe and a kind of cell death that would be very difficult to suppress. In addition to this problem, recent data have suggested that unreplicated regions of the genome might be cleaved by structure-specific endonucleases like Mus81 or Gen1, which would be also activated in mitosis. Accordingly, it has been reported that the use of CHK1i or ATRi abrogates the G2/M checkpoint and leads to the presence of micronuclei or completely fragmented nuclei in cell exposed to IR (Figure2). However, the relative contribution of these two pathways (segregation defects or nuclease cleavage) to the mitotic catastrophe observed in response to ATR/CHK1 inhibitors is yet not known. A screening to find possible resistance mechanisms to ATR and CHK1 inhibitors is currently being performed in our group with the help of haploid mammalian cells.

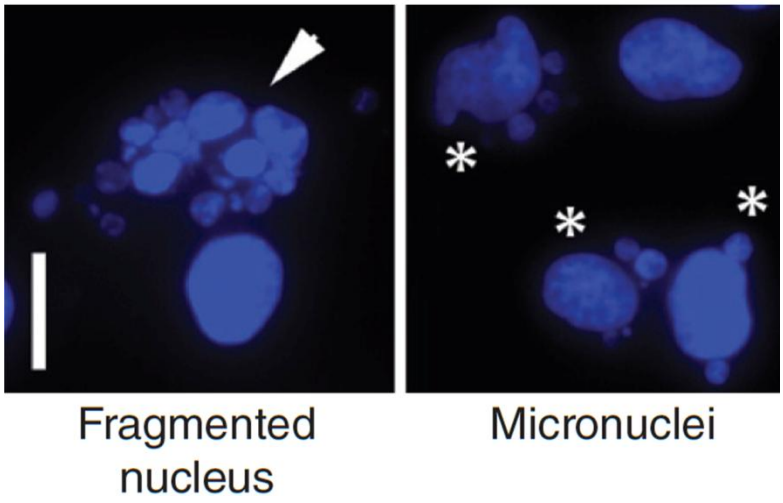

Figure 2. Typical aberrations observed in IR treated cells in the presence of ATRi
Asterisks and arrows highlight cells with aberrations. Scale bar 5 (μm).

2.3.2. Are INK4a/ARF or RB mutated tumors good candidates for their treatment with ATR or CHK1 inhibitors?

In contrast to p53 depletion or MYC overexpression, INK4a/ARF deletion or T121 expression rescue RS-induced senescence on ATR-Seckel MEF. In sight of these data, we wondered whether ATR and CHK1 inhibitors might not be effective in tumors carrying mutations in the RB or INK4a/ARF locus, which are very frequent in cancer. However, MEF are particularly prone to senescence and most replicating cells tend to die in response to DNA damage. In agreement with this, and in contrast to the senescence phenotype in MEF, we failed to observe any difference in the sensitivity to ATRi of WT and INK4a/ARF$^{-/-}$ splenocytes. Moreover, we decided to check the sensitivity to ATRi of different cancer cell lines derived from mouse pancreatic tumors, getting a similar result. ATRi were toxic for these cells regardless of their p53 or INK4a/ARF status. In addition to this, ATR-Seckel; INK4a/ARF $^{-/-}$ mice recapitulate all the obvious phenotypes of ATR-Seckel mice, which we believe is due to cell-death, rather than senescence, being the main determinant of the Seckel pathologies (Figure 2). Altogether, these observations suggest the efficacy of ATR or CHK1 inhibitors would not be limited by INK4a/ARF or RB status.

In summary, this work has revealed a new role for the INK4a/ARF locus in limiting the expansion of cells suffering from RS, which places this tumor suppressor locus as a key player in the maintenance of genomic integrity during tumor evolution.

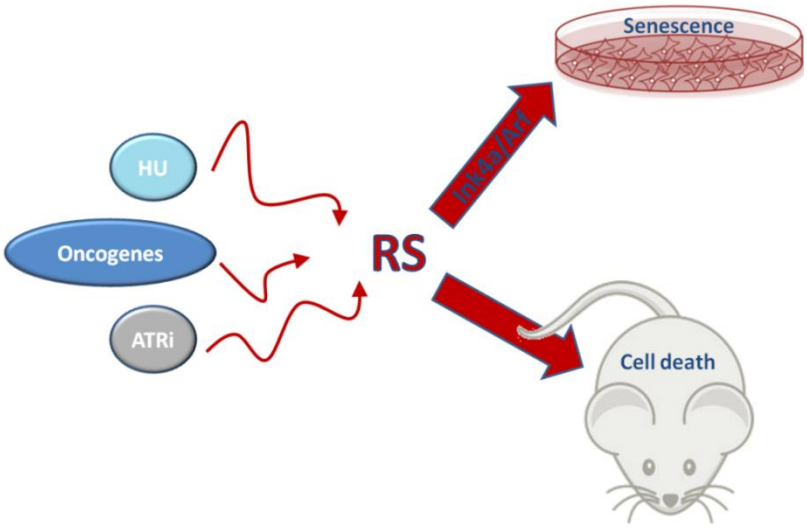

Figure 2. Our working model

INK4a/ARF limits the expansion of cells with RS, by inducing cellular senescence. RS can be induced by drugs such as HU or ATRi or by different oncogenes. The accumulation of RS will lead to two different outcomes. On the one hand, it will activate a senescence pathway, dependent on INK4a/ARF, through an unknown mechanism. On the other hand, high RS doses will give rise to cell death through apoptosis and mitotic catastrophe. Of the two consequences of RS accumulation, senescence will be of importance in certain cell types – i.e. MEF- ; whereas cell death will define the in vivo phenotype of the mouse.

3. ATR is essential at the cellular level

In contrast to ATM, ATR is essential at the single cell level (Brown and Baltimore, 2000). Based on our results on ATR-Seckel MEF senescence, we tried to rescue the lethality associated to ATR nullizigosity through the expression of T121. Whereas this strategy did not work, we are currently exploring other possibilities that could rescue ATR deficiency in our laboratory. In *S.cervisiae,* deletion of the ATR orthologue MEC1 is also lethal. However, even though MEC1 is an essential gene, the lethality can be rescued by increasing dNTP levels (Desany et al., 1998). Moreover, MEC1 could also be rescued by the concomitant deletion of Sml1 (Suppresor of Mec Lethality 1), a negative regulator of the ribonucleotide reductase (RNR). The role of this pathway in mammalians remains largely unexplored. However, we already know that ATR-Seckel MEF show a decrease in RS levels and a partial growth rescue when extra nucleosides are added to the media. Moreover, the median lifespan of ATR-Seckel mice can be doubled when crossed with a transgenic mouse model of the RNR complex (Lopez-Contreras *et al* unpublished data). Hence, preliminary evidence suggests that the essential role of ATR in mammals can also be related to its role in promoting dNTP biosynthesis.

Conclusions

CONCLUSIONS

1. A chronic exposure to RS activates the expression of $p16^{INK4a}$ and $p19^{ARF.}$

2. Deletion of INK4a/ARF facilitates the growth of MEF that are chronically exposed to low doses of RS-inducing agents such as ATRi or HU.

3. INK4a/ARF depletion or genetic deletion rescues senescence on ATR-Seckel MEF, without rescuing ATR levels or presence of RS on these cells. However, depletion or deletion of $p16^{INK4a}$ or $p19^{ARF}$ alone is not sufficient to rescue growth on ATR mutant MEF.

4. Deletion of INK4a/ARF does not have an impact on viability or lifespan of ATR-Seckel mice.

5. ATR-Seckel embryos present widespread apoptosis but no clear signs of senescence, which suggests a limited role of senescence on the Seckel phenotype.

6. INK4a/ARF deletion does not modify the cytotoxic effects of ATR inhibitors *in vitro* or CHK1i administration *in vivo*.

7. Cancer cell lines with a high number of CNVs present low levels of $p16^{INK4a}$.

8. Expression of the T121 fragment from the SV40 large T antigen, which inhibits retinoblastoma but not p53, is able to immortalize ATR-Seckel MEF.

9. Expression of T121 is not able to rescue the viability of ATR nullizygous cells.

Bibliography

Abraham, R. T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev *15*, 2177-2196.

Al-Mohanna, M. A., Al-Khalaf, H. H., Al-Yousef, N., and Aboussekhra, A. (2007). The p16INK4a tumor suppressor controls p21WAF1 induction in response to ultraviolet light. Nucleic Acids Res *35*, 223-233.

Al-Mohanna, M. A., Manogaran, P. S., Al-Mukhalafi, Z., A Al-Hussein, K., and Aboussekhra, A. (2004). The tumor suppressor p16(INK4a) gene is a regulator of apoptosis induced by ultraviolet light and cisplatin. Oncogene *23*, 201-212.

Arlt, M. F., Mulle, J. G., Schaibley, V. M., Ragland, R. L., Durkin, S. G., Warren, S. T., and Glover, T. W. (2009). Replication stress induces genome-wide copy number changes in human cells that resemble polymorphic and pathogenic variants. Am J Hum Genet *84*, 339-350.

Arlt, M. F., Ozdemir, A. C., Birkeland, S. R., Lyons, R. H., Glover, T. W., and Wilson, T. E. (2011a). Comparison of constitutional and replication stress-induced genome structural variation by SNP array and mate-pair sequencing. Genetics *187*, 675-683.

Arlt, M. F., Ozdemir, A. C., Birkeland, S. R., Wilson, T. E., and Glover, T. W. (2011b). Hydroxyurea induces de novo copy number variants in human cells. Proc Natl Acad Sci U S A *108*, 17360-17365.

Arlt, M. F., Wilson, T. E., and Glover, T. W. (2012). Replication stress and mechanisms of CNV formation. Curr Opin Genet Dev *22*, 204-210.

Baker, D. J., Perez-Terzic, C., Jin, F., Pitel, K. S., Pitel, K., Niederländer, N. J., Jeganathan, K., Yamada, S., Reyes, S., Rowe, L.*, et al.* (2008). Opposing roles for p16Ink4a and p19Arf in senescence and ageing caused by BubR1 insufficiency. Nat Cell Biol *10*, 825-836.

Bakkenist, C. J., and Kastan, M. B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature *421*, 499-506.

Bakkenist, C. J., and Kastan, M. B. (2004). Phosphatases join kinases in DNA-damage response pathways. Trends Cell Biol *14*, 339-341.

Bartek, J., Bartkova, J., and Lukas, J. (2007). DNA damage signalling guards against activated oncogenes and tumour progression. Oncogene *26*, 7773-7779.

Bartek, J., Lukas, C., and Lukas, J. (2004). Checking on DNA damage in S phase. Nat Rev Mol Cell Biol *5*, 792-804.

Bartek, J., and Lukas, J. (2003). Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell *3*, 421-429.

Bartkova, J., Horejsí, Z., Koed, K., Krämer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J. M., Lukas, C.*, et al.* (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature *434*, 864-870.

Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L. V., Kolettas, E., Niforou, K., Zoumpourlis, V. C.*, et al.* (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature *444*, 633-637.

Beard, C., Hochedlinger, K., Plath, K., Wutz, A., and Jaenisch, R. (2006). Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. Genesis *44*, 23-28.

Beck, H., Nähse-Kumpf, V., Larsen, M. S., O'Hanlon, K. A., Patzke, S., Holmberg, C., Mejlvang, J., Groth, A., Nielsen, O., Syljuåsen, R. G., and Sørensen, C. S. (2012). Cyclin-dependent kinase suppression by WEE1 kinase protects the genome through control of replication initiation and nucleotide consumption. Mol Cell Biol *32*, 4226-4236.

Becker-Catania, S. G., and Gatti, R. A. (2001). Ataxia-telangiectasia. Adv Exp Med Biol *495*, 191-198.

Benson, F. E., Stasiak, A., and West, S. C. (1994). Purification and characterization of the human Rad51 protein, an analogue of E. coli RecA. EMBO J *13*, 5764-5771.

Bermudez, V. P., Lindsey-Boltz, L. A., Cesare, A. J., Maniwa, Y., Griffith, J. D., Hurwitz, J., and Sancar, A. (2003). Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex in vitro. Proc Natl Acad Sci U S A *100*, 1633-1638.

Bester, A. C., Roniger, M., Oren, Y. S., Im, M. M., Sarni, D., Chaoat, M., Bensimon, A., Zamir, G., Shewach, D. S., and Kerem, B. (2011). Nucleotide deficiency promotes genomic instability in early stages of cancer development. Cell *145*, 435-446.

Biedermann, K. A., Sun, J. R., Giaccia, A. J., Tosto, L. M., and Brown, J. M. (1991). scid mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. Proc Natl Acad Sci U S A *88*, 1394-1397.

Boutros, R., Dozier, C., and Ducommun, B. (2006). The when and wheres of CDC25 phosphatases. Curr Opin Cell Biol *18*, 185-191.

Brown, E. J., and Baltimore, D. (2000). ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev *14*, 397-402.

Buckley, R. H. (2004). The multiple causes of human SCID. J Clin Invest *114*, 1409-1411.

Burke, J. R., Hura, G. L., and Rubin, S. M. (2012). Structures of inactive retinoblastoma protein reveal multiple mechanisms for cell cycle control. Genes Dev *26*, 1156-1166.

Byun, T. S., Pacek, M., Yee, M. C., Walter, J. C., and Cimprich, K. A. (2005). Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes Dev *19*, 1040-1052.

Bzymek, M., Thayer, N. H., Oh, S. D., Kleckner, N., and Hunter, N. (2010). Double Holliday junctions are intermediates of DNA break repair. Nature *464*, 937-941.

Callén, E., Jankovic, M., Wong, N., Zha, S., Chen, H. T., Difilippantonio, S., Di Virgilio, M., Heidkamp, G., Alt, F. W., Nussenzweig, A., and Nussenzweig, M. (2009). Essential role for DNA-PKcs in DNA double-strand break repair and apoptosis in ATM-deficient lymphocytes. Mol Cell *34*, 285-297.

Campisi, J. (2007). Aging and cancer cell biology, 2007. Aging Cell *6*, 261-263.

Campisi, J., and d'Adda di Fagagna, F. (2007). Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol *8*, 729-740.

Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science *281*, 1677-1679.

Cao, L., Li, W., Kim, S., Brodie, S. G., and Deng, C. X. (2003). Senescence, aging, and malignant transformation mediated by p53 in mice lacking the Brca1 full-length isoform. Genes Dev *17*, 201-213.

Casper, A. M., Nghiem, P., Arlt, M. F., and Glover, T. W. (2002). ATR regulates fragile site stability. Cell *111*, 779-789.

Celeste, A., Fernandez-Capetillo, O., Kruhlak, M. J., Pilch, D. R., Staudt, D. W., Lee, A., Bonner, R. F., Bonner, W. M., and Nussenzweig, A. (2003). Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat Cell Biol *5*, 675-679.

Celeste, A., Petersen, S., Romanienko, P. J., Fernandez-Capetillo, O., Chen, H. T., Sedelnikova, O. A., Reina-San-Martin, B., Coppola, V., Meffre, E., Difilippantonio, M. J.*, et al.* (2002). Genomic instability in mice lacking histone H2AX. Science *296*, 922-927.

Cimprich, K. A., and Cortez, D. (2008). ATR: an essential regulator of genome integrity. Nat Rev Mol Cell Biol *9*, 616-627.

Cimprich, K. A., Shin, T. B., Keith, C. T., and Schreiber, S. L. (1996). cDNA cloning and gene mapping of a candidate human cell cycle checkpoint protein. Proc Natl Acad Sci U S A *93*, 2850-2855.

Classon, M., and Harlow, E. (2002). The retinoblastoma tumour suppressor in development and cancer. Nat Rev Cancer *2*, 910-917.

Colby, W. W., and Shenk, T. (1982). Fragments of the simian virus 40 transforming gene facilitate transformation of rat embryo cells. Proc Natl Acad Sci U S A *79*, 5189-5193.

Collado, M., Blasco, M. A., and Serrano, M. (2007). Cellular senescence in cancer and aging. Cell *130*, 223-233.

Collado, M., and Serrano, M. (2005). The senescent side of tumor suppression. Cell Cycle *4*, 1722-1724.

Collis, S. J., DeWeese, T. L., Jeggo, P. A., and Parker, A. R. (2005). The life and death of DNA-PK. Oncogene *24*, 949-961.

Constantinou, A., Chen, X. B., McGowan, C. H., and West, S. C. (2002). Holliday junction resolution in human cells: two junction endonucleases with distinct substrate specificities. EMBO J *21*, 5577-5585.

Cortez, D., Guntuku, S., Qin, J., and Elledge, S. J. (2001). ATR and ATRIP: partners in checkpoint signaling. Science *294*, 1713-1716.

Cuadrado, M., Gutierrez-Martinez, P., Swat, A., Nebreda, A. R., and Fernandez-Capetillo, O. (2009). p27Kip1 stabilization is essential for the maintenance of cell cycle arrest in response to DNA damage. Cancer Res *69*, 8726-8732.

Chao, C., Saito, S., Kang, J., Anderson, C. W., Appella, E., and Xu, Y. (2000). p53 transcriptional activity is essential for p53-dependent apoptosis following DNA damage. EMBO J *19*, 4967-4975.

Chen, J., Tobin, G. J., Pipas, J. M., and Van Dyke, T. (1992). T-antigen mutant activities in vivo: roles of p53 and pRB binding in tumorigenesis of the choroid plexus. Oncogene *7*, 1167-1175.

Chittenden, T., Livingston, D. M., and Kaelin, W. G. (1991). RB associates with an E2F-like, sequence-specific DNA-binding protein. Cold Spring Harb Symp Quant Biol *56*, 187-195.

d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. Nature *426*, 194-198.

D'Arpa, P., Beardmore, C., and Liu, L. F. (1990). Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. Cancer Res *50*, 6919-6924.

Daniel, J. A., Pellegrini, M., Lee, J. H., Paull, T. T., Feigenbaum, L., and Nussenzweig, A. (2008). Multiple autophosphorylation sites are dispensable for murine ATM activation in vivo. J Cell Biol *183*, 777-783.

De Bont, R., and van Larebeke, N. (2004). Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis *19*, 169-185.

DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E., and Livingston, D. M. (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell *54*, 275-283.

Desany, B. A., Alcasabas, A. A., Bachant, J. B., and Elledge, S. J. (1998). Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev *12*, 2956-2970.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre', M., Nuciforo, P. G., Bensimon, A., *et al.* (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638-642.

Dick, F. A., and Rubin, S. M. (2013). Molecular mechanisms underlying RB protein function. Nat Rev Mol Cell Biol *14*, 297-306.

Dickins, R. A., McJunkin, K., Hernando, E., Premsrirut, P. K., Krizhanovsky, V., Burgess, D. J., Kim, S. Y., Cordon-Cardo, C., Zender, L., Hannon, G. J., and Lowe, S. W. (2007). Tissue-specific and reversible RNA interference in transgenic mice. Nat Genet *39*, 914-921.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., and Pereira-Smith, O. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A *92*, 9363-9367.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Butel, J. S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-221.

Donzelli, M., and Draetta, G. F. (2003). Regulating mammalian checkpoints through Cdc25 inactivation. EMBO Rep *4*, 671-677.

Dornan, D., Shimizu, H., Burch, L., Smith, A. J., and Hupp, T. R. (2003). The proline repeat domain of p53 binds directly to the transcriptional coactivator p300 and

allosterically controls DNA-dependent acetylation of p53. Mol Cell Biol *23*, 8846-8861.

Dudley, D. D., Chaudhuri, J., Bassing, C. H., and Alt, F. W. (2005). Mechanism and control of V(D)J recombination versus class switch recombination: similarities and differences. Adv Immunol *86*, 43-112.

Dumaz, N., and Meek, D. W. (1999). Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. EMBO J *18*, 7002-7010.

Duursma, A. M., Driscoll, R., Elias, J. E., and Cimprich, K. A. (2013). A Role for the MRN Complex in ATR Activation via TOPBP1 Recruitment. Mol Cell *50*, 116-122.

Efeyan, A., Garcia-Cao, I., Herranz, D., Velasco-Miguel, S., and Serrano, M. (2006). Tumour biology: Policing of oncogene activity by p53. Nature *443*, 159.

Fernandez-Capetillo, O. (2010). Intrauterine programming of ageing. EMBO Rep *11*, 32-36.

Fernandez-Capetillo, O., Chen, H. T., Celeste, A., Ward, I., Romanienko, P. J., Morales, J. C., Naka, K., Xia, Z., Camerini-Otero, R. D., Motoyama, N.*, et al.* (2002). DNA damage-induced G2-M checkpoint activation by histone H2AX and 53BP1. Nat Cell Biol *4*, 993-997.

Fernandez-Capetillo, O., and Nussenzweig, A. (2008). ATM breaks into heterochromatin. Mol Cell *31*, 303-304.

Fu, D., Calvo, J. A., and Samson, L. D. (2012). Balancing repair and tolerance of DNA damage caused by alkylating agents. Nat Rev Cancer *12*, 104-120.

Garinis, G. A., van der Horst, G. T., Vijg, J., and Hoeijmakers, J. H. (2008). DNA damage and ageing: new-age ideas for an age-old problem. Nat Cell Biol *10*, 1241-1247.

Garnett, M. J., Edelman, E. J., Heidorn, S. J., Greenman, C. D., Dastur, A., Lau, K. W., Greninger, P., Thompson, I. R., Luo, X., Soares, J.*, et al.* (2012). Systematic identification of genomic markers of drug sensitivity in cancer cells. Nature *483*, 570-575.

Gil, J., and Peters, G. (2006). Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nat Rev Mol Cell Biol *7*, 667-677.

Gonzalez, S., Klatt, P., Delgado, S., Conde, E., Lopez-Rios, F., Sanchez-Cespedes, M., Mendez, J., Antequera, F., and Serrano, M. (2006). Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. Nature *440*, 702-706.

Goodarzi, A. A., Noon, A. T., Deckbar, D., Ziv, Y., Shiloh, Y., Löbrich, M., and Jeggo, P. A. (2008). ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. Mol Cell *31*, 167-177.

Gorgoulis, V. G., Vassiliou, L. V., Karakaidos, P., Zacharatos, P., Kotsinas, A., Liloglou, T., Venere, M., Ditullio, R. A., Kastrinakis, N. G., Levy, B.*, et al.* (2005). Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature *434*, 907-913.

Gorrini, C., Squatrito, M., Luise, C., Syed, N., Perna, D., Wark, L., Martinato, F., Sardella, D., Verrecchia, A., Bennett, S.*, et al.* (2007). Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. Nature *448*, 1063-1067.

Gottlieb, T. M., and Jackson, S. P. (1993). The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. Cell *72*, 131-142.

Guan, K. L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. (1994). Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. Genes Dev *8*, 2939-2952.

Guan, K. L., Jenkins, C. W., Li, Y., O'Keefe, C. L., Noh, S., Wu, X., Zariwala, M., Matera, A. G., and Xiong, Y. (1996). Isolation and characterization of p19INK4d, a p16-related inhibitor specific to CDK6 and CDK4. Mol Biol Cell *7*, 57-70.

Guardavaccaro, D., and Pagano, M. (2006). Stabilizers and destabilizers controlling cell cycle oscillators. Mol Cell *22*, 1-4.

Günes, C., and Rudolph, K. L. (2013). The role of telomeres in stem cells and cancer. Cell *152*, 390-393.

Guerra, C., Mijimolle, N., Dhawahir, A., Dubus, P., Barradas, M., Serrano, M., Campuzano, V., and Barbacid, M. (2003). Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context. Cancer Cell *4*, 111-120.

Halazonetis, T. D., Gorgoulis, V. G., and Bartek, J. (2008). An oncogene-induced DNA damage model for cancer development. Science *319*, 1352-1355.

Han, D., Williams, E., and Cadenas, E. (2001). Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. Biochem J *353*, 411-416.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S., and Peters, G. (1996). Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. Mol Cell Biol *16*, 859-867.

Harper, J. W., and Elledge, S. J. (2007). The DNA damage response: ten years after. Mol Cell *28*, 739-745.

Hatakeyama, M., and Weinberg, R. A. (1995). The role of RB in cell cycle control. Prog Cell Cycle Res *1*, 9-19.

HAYFLICK, L. (1965). THE LIMITED IN VITRO LIFETIME OF HUMAN DIPLOID CELL STRAINS. Exp Cell Res *37*, 614-636.

Hefferin, M. L., and Tomkinson, A. E. (2005). Mechanism of DNA double-strand break repair by non-homologous end joining. DNA Repair (Amst) *4*, 639-648.

Helmrich, A., Ballarino, M., and Tora, L. (2011). Collisions between replication and transcription complexes cause common fragile site instability at the longest human genes. Mol Cell *44*, 966-977.

Hellman, A., Rahat, A., Scherer, S. W., Darvasi, A., Tsui, L. C., and Kerem, B. (2000). Replication delay along FRA7H, a common fragile site on human chromosome 7, leads to chromosomal instability. Mol Cell Biol *20*, 4420-4427.

Hiom, K. (2000). Homologous recombination. Curr Biol *10*, R359-361.

Hirai, H., Roussel, M. F., Kato, J. Y., Ashmun, R. A., and Sherr, C. J. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol Cell Biol *15*, 2672-2681.

Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J., and Mak, T. W. (2000). DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science *287*, 1824-1827.

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. Nature *411*, 366-374.

Inomata, K., Aoto, T., Binh, N. T., Okamoto, N., Tanimura, S., Wakayama, T., Iseki, S., Hara, E., Masunaga, T., Shimizu, H., and Nishimura, E. K. (2009). Genotoxic stress abrogates renewal of melanocyte stem cells by triggering their differentiation. Cell *137*, 1088-1099.

Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T., and Weinberg, R. A. (1994). Tumor spectrum analysis in p53-mutant mice. Curr Biol *4*, 1-7.

Jackson, S. P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature *461*, 1071-1078.

Jat, P. S., and Sharp, P. A. (1986). Large T antigens of simian virus 40 and polyomavirus efficiently establish primary fibroblasts. J Virol *59*, 746-750.

Jiang, Y., Rabbi, M., Kim, M., Ke, C., Lee, W., Clark, R. L., Mieczkowski, P. A., and Marszalek, P. E. (2009). UVA generates pyrimidine dimers in DNA directly. Biophys J *96*, 1151-1158.

Jones, R. M., Mortusewicz, O., Afzal, I., Lorvellec, M., García, P., Helleday, T., and Petermann, E. (2012). Increased replication initiation and conflicts with transcription underlie Cyclin E-induced replication stress. Oncogene.

Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., Johnson, B. E., and Skolnick, M. H. (1994). A cell cycle regulator potentially involved in genesis of many tumor types. Science *264*, 436-440.

Kamijo, T., van de Kamp, E., Chong, M. J., Zindy, F., Diehl, J. A., Sherr, C. J., and McKinnon, P. J. (1999). Loss of the ARF tumor suppressor reverses premature replicative arrest but not radiation hypersensitivity arising from disabled atm function. Cancer Res *59*, 2464-2469.

Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosveld, G., and Sherr, C. J. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell *91*, 649-659.

Kastan, M. B., and Bartek, J. (2004). Cell-cycle checkpoints and cancer. Nature *432*, 316-323.

Kim, W. Y., and Sharpless, N. E. (2006). The regulation of INK4/ARF in cancer and aging. Cell *127*, 265-275.

Kitagawa, R., Bakkenist, C. J., McKinnon, P. J., and Kastan, M. B. (2004). Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway. Genes Dev *18*, 1423-1438.

Kolupaeva, V., and Janssens, V. (2013). PP1 and PP2A phosphatases--cooperating partners in modulating retinoblastoma protein activation. FEBS J *280*, 627-643.

Koniaras, K., Cuddihy, A. R., Christopoulos, H., Hogg, A., and O'Connell, M. J. (2001). Inhibition of Chk1-dependent G2 DNA damage checkpoint radiosensitizes p53 mutant human cells. Oncogene *20*, 7453-7463.

Koster, D. A., Crut, A., Shuman, S., Bjornsti, M. A., and Dekker, N. H. (2010). Cellular strategies for regulating DNA supercoiling: a single-molecule perspective. Cell *142*, 519-530.

Krimpenfort, P., Quon, K. C., Mooi, W. J., Loonstra, A., and Berns, A. (2001). Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. Nature *413*, 83-86.

Kruhlak, M. J., Celeste, A., Dellaire, G., Fernandez-Capetillo, O., Müller, W. G., McNally, J. G., Bazett-Jones, D. P., and Nussenzweig, A. (2006a). Changes in chromatin structure and mobility in living cells at sites of DNA double-strand breaks. J Cell Biol *172*, 823-834.

Kruhlak, M. J., Celeste, A., and Nussenzweig, A. (2006b). Spatio-temporal dynamics of chromatin containing DNA breaks. Cell Cycle *5*, 1910-1912.

Kuilman, T., Michaloglou, C., Mooi, W. J., and Peeper, D. S. (2010). The essence of senescence. Genes Dev *24*, 2463-2479.

Kumagai, A., Lee, J., Yoo, H. Y., and Dunphy, W. G. (2006). TopBP1 activates the ATR-ATRIP complex. Cell *124*, 943-955.

Kurz, E. U., and Lees-Miller, S. P. (2004). DNA damage-induced activation of ATM and ATM-dependent signaling pathways. DNA Repair (Amst) *3*, 889-900.

Lam, M. H., Liu, Q., Elledge, S. J., and Rosen, J. M. (2004). Chk1 is haploinsufficient for multiple functions critical to tumor suppression. Cancer Cell *6*, 45-59.

Lane, D. P. (1992). Worrying about p53. Curr Biol *2*, 581-583.

Lane, D. P., and Crawford, L. V. (1979). T antigen is bound to a host protein in SV40-transformed cells. Nature *278*, 261-263.

Lau, W. M., Ho, T. H., and Hui, K. M. (2007). p16INK4A-silencing augments DNA damage-induced apoptosis in cervical cancer cells. Oncogene *26*, 6050-6060.

Lavin, M. F. (2008). Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. Nat Rev Mol Cell Biol *9*, 759-769.

Le Beau, M. M., Rassool, F. V., Neilly, M. E., Espinosa, R., Glover, T. W., Smith, D. I., and McKeithan, T. W. (1998). Replication of a common fragile site, FRA3B, occurs late in S phase and is delayed further upon induction: implications for the mechanism of fragile site induction. Hum Mol Genet *7*, 755-761.

Lee, J. H., and Paull, T. T. (2005). ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Science *308*, 551-554.

Li, H., Collado, M., Villasante, A., Strati, K., Ortega, S., Cañamero, M., Blasco, M. A., and Serrano, M. (2009). The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature *460*, 1136-1139.

Lindahl, T., and Barnes, D. E. (2000). Repair of endogenous DNA damage. Cold Spring Harb Symp Quant Biol *65*, 127-133.

Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A.*, et al.* (2000). Chk1 is an essential kinase that is regulated

by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev *14*, 1448-1459.

López-Contreras, A. J., and Fernandez-Capetillo, O. (2010). The ATR barrier to replication-born DNA damage. DNA Repair (Amst) *9*, 1249-1255.

López-Contreras, A. J., Gutierrez-Martinez, P., Specks, J., Rodrigo-Perez, S., and Fernandez-Capetillo, O. (2012). An extra allele of Chk1 limits oncogene-induced replicative stress and promotes transformation. J Exp Med *209*, 455-461.

Ludérus, M. E., van Steensel, B., Chong, L., Sibon, O. C., Cremers, F. F., and de Lange, T. (1996). Structure, subnuclear distribution, and nuclear matrix association of the mammalian telomeric complex. J Cell Biol *135*, 867-881.

Ludlow, J. W., DeCaprio, J. A., Huang, C. M., Lee, W. H., Paucha, E., and Livingston, D. M. (1989). SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell *56*, 57-65.

Lukas, J., and Bartek, J. (2004). Cell division: the heart of the cycle. Nature *432*, 564-567.

Mahaney, B. L., Meek, K., and Lees-Miller, S. P. (2009). Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. Biochem J *417*, 639-650.

Mailand, N., Bekker-Jensen, S., Bartek, J., and Lukas, J. (2006). Destruction of Claspin by SCFbetaTrCP restrains Chk1 activation and facilitates recovery from genotoxic stress. Mol Cell *23*, 307-318.

Mailand, N., Falck, J., Lukas, C., Syljuâsen, R. G., Welcker, M., Bartek, J., and Lukas, J. (2000). Rapid destruction of human Cdc25A in response to DNA damage. Science *288*, 1425-1429.

Mao, L., Merlo, A., Bedi, G., Shapiro, G. I., Edwards, C. D., Rollins, B. J., and Sidransky, D. (1995). A novel p16INK4A transcript. Cancer Res *55*, 2995-2997.

Martens, U. M., Brass, V., Engelhardt, M., Glaser, S., Waller, C. F., Lange, W., Schmoor, C., Poon, S. S., and Landsdorp, P. M. (2000). Measurement of telomere length in haematopoietic cells using in situ hybridization techniques. Biochem Soc Trans *28*, 245-250.

Matsuoka, S., Ballif, B. A., Smogorzewska, A., McDonald, E. R., Hurov, K. E., Luo, J., Bakalarski, C. E., Zhao, Z., Solimini, N., Lerenthal, Y., *et al.* (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science *316*, 1160-1166.

Matsuoka, S., Huang, M., and Elledge, S. J. (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science *282*, 1893-1897.

Molofsky, A. V., He, S., Bydon, M., Morrison, S. J., and Pardal, R. (2005). Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. Genes Dev *19*, 1432-1437.

Morgan, D. O. (1997). Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol *13*, 261-291.

Muller, P. A., and Vousden, K. H. (2013). p53 mutations in cancer. Nat Cell Biol *15*, 2-8.

Munro, S., Carr, S. M., and La Thangue, N. B. (2012). Diversity within the pRb pathway: is there a code of conduct? Oncogene *31*, 4343-4352.

Murga, M., Bunting, S., Montaña, M. F., Soria, R., Mulero, F., Cañamero, M., Lee, Y., McKinnon, P. J., Nussenzweig, A., and Fernandez-Capetillo, O. (2009). A mouse model of ATR-Seckel shows embryonic replicative stress and accelerated aging. Nat Genet *41*, 891-898.

Murga, M., Campaner, S., Lopez-Contreras, A. J., Toledo, L. I., Soria, R., Montaña, M. F., D'Artista, L., Schleker, T., Guerra, C., Garcia, E.*, et al.* (2011). Exploiting oncogene-induced replicative stress for the selective killing of Myc-driven tumors. Nat Struct Mol Biol *18*, 1331-1335.

Nakano, K., and Vousden, K. H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell *7*, 683-694.

O'Driscoll, M., Ruiz-Perez, V. L., Woods, C. G., Jeggo, P. A., and Goodship, J. A. (2003). A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. Nat Genet *33*, 497-501.

Palmero, I., Murga, M., Zubiaga, A., and Serrano, M. (2002). Activation of ARF by oncogenic stress in mouse fibroblasts is independent of E2F1 and E2F2. Oncogene *21*, 2939-2947.

Pandita, T. K., Lieberman, H. B., Lim, D. S., Dhar, S., Zheng, W., Taya, Y., and Kastan, M. B. (2000). Ionizing radiation activates the ATM kinase throughout the cell cycle. Oncogene *19*, 1386-1391.

Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S., and Campisi, J. (2003). Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. Nat Cell Biol *5*, 741-747.

Paulsen, R. D., and Cimprich, K. A. (2007). The ATR pathway: fine-tuning the fork. DNA Repair (Amst) *6*, 953-966.

Petrini, J. H., and Stracker, T. H. (2003). The cellular response to DNA double-strand breaks: defining the sensors and mediators. Trends Cell Biol *13*, 458-462.

Poli, J., Tsaponina, O., Crabbé, L., Keszthelyi, A., Pantesco, V., Chabes, A., Lengronne, A., and Pasero, P. (2012). dNTP pools determine fork progression and origin usage under replication stress. EMBO J *31*, 883-894.

Pomerantz, J., Schreiber-Agus, N., Liégeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W.*, et al.* (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell *92*, 713-723.

Quelle, D. E., Ashmun, R. A., Hannon, G. J., Rehberger, P. A., Trono, D., Richter, K. H., Walker, C., Beach, D., Sherr, C. J., and Serrano, M. (1995a). Cloning and characterization of murine p16INK4a and p15INK4b genes. Oncogene *11*, 635-645.

Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. (1995b). Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell *83*, 993-1000.

Raynard, S., Niu, H., and Sung, P. (2008). DNA double-strand break processing: the beginning of the end. Genes Dev *22*, 2903-2907.

Richardson, C., and Jasin, M. (2000). Coupled homologous and nonhomologous repair of a double-strand break preserves genomic integrity in mammalian cells. Mol Cell Biol *20*, 9068-9075.

Rodier, F., Campisi, J., and Bhaumik, D. (2007). Two faces of p53: aging and tumor suppression. Nucleic Acids Res *35*, 7475-7484.

Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., and Bonner, W. M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem *273*, 5858-5868.

Rossi, D. J., Bryder, D., Seita, J., Nussenzweig, A., Hoeijmakers, J., and Weissman, I. L. (2007). Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. Nature *447*, 725-729.

Ruas, M., and Peters, G. (1998). The p16INK4a/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta *1378*, F115-177.

Ruzankina, Y., Asare, A., and Brown, E. J. (2008). Replicative stress, stem cells and aging. Mech Ageing Dev *129*, 460-466.

Ruzankina, Y., Pinzon-Guzman, C., Asare, A., Ong, T., Pontano, L., Cotsarelis, G., Zediak, V. P., Velez, M., Bhandoola, A., and Brown, E. J. (2007). Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. Cell Stem Cell *1*, 113-126.

Sáenz Robles, M. T., and Pipas, J. M. (2009). T antigen transgenic mouse models. Semin Cancer Biol *19*, 229-235.

Sarkar-Agrawal, P., Vergilis, I., Sharpless, N. E., DePinho, R. A., and Rünger, T. M. (2004). Impaired processing of DNA photoproducts and ultraviolet hypermutability with loss of p16INK4a or p19ARF. J Natl Cancer Inst *96*, 1790-1793.

Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., Sfez, S.*, et al.* (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science *268*, 1749-1753.

Serrano, M., Hannon, G. J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature *366*, 704-707.

Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R. A. (1996). Role of the INK4a locus in tumor suppression and cell mortality. Cell *85*, 27-37.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Sharpless, N. E., Bardeesy, N., Lee, K. H., Carrasco, D., Castrillon, D. H., Aguirre, A. J., Wu, E. A., Horner, J. W., and DePinho, R. A. (2001). Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. Nature *413*, 86-91.

Sharpless, N. E., Ramsey, M. R., Balasubramanian, P., Castrillon, D. H., and DePinho, R. A. (2004). The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. Oncogene *23*, 379-385.

Sherr, C. J. (1996). Cancer cell cycles. Science *274*, 1672-1677.

Sherr, C. J. (2006). Divorcing ARF and p53: an unsettled case. Nat Rev Cancer *6*, 663-673.

Sherr, C. J. (2012). Ink4-Arf Locus in Cancer and Aging. Wiley Interdiscip Rev Dev Biol *1*, 731-741.

Sherr, C. J., and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev *13*, 1501-1512.

Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell *91*, 325-334.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer *3*, 155-168.

Shiloh, Y., and Kastan, M. B. (2001). ATM: genome stability, neuronal development, and cancer cross paths. Adv Cancer Res *83*, 209-254.

Smith, G. C., and Jackson, S. P. (1999). The DNA-dependent protein kinase. Genes Dev *13*, 916-934.

Smits, V. A., Reaper, P. M., and Jackson, S. P. (2006). Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. Curr Biol *16*, 150-159.

Sørensen, C. S., Syljuåsen, R. G., Falck, J., Schroeder, T., Rönnstrand, L., Khanna, K. K., Zhou, B. B., Bartek, J., and Lukas, J. (2003). Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A. Cancer Cell *3*, 247-258.

Stamatoyannopoulos, J. A., Adzhubei, I., Thurman, R. E., Kryukov, G. V., Mirkin, S. M., and Sunyaev, S. R. (2009). Human mutation rate associated with DNA replication timing. Nat Genet *41*, 393-395.

Stewart, G. S., Wang, B., Bignell, C. R., Taylor, A. M., and Elledge, S. J. (2003). MDC1 is a mediator of the mammalian DNA damage checkpoint. Nature *421*, 961-966.

Stiff, T., O'Driscoll, M., Rief, N., Iwabuchi, K., Löbrich, M., and Jeggo, P. A. (2004). ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. Cancer Res *64*, 2390-2396.

Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. (1998). The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. EMBO J *17*, 5001-5014.

Stubdal, H., Zalvide, J., and DeCaprio, J. A. (1996). Simian virus 40 large T antigen alters the phosphorylation state of the RB-related proteins p130 and p107. J Virol *70*, 2781-2788.

Svendsen, J. M., and Harper, J. W. (2010). GEN1/Yen1 and the SLX4 complex: Solutions to the problem of Holliday junction resolution. Genes Dev *24*, 521-536.

Syljuåsen, R. G., Sørensen, C. S., Hansen, L. T., Fugger, K., Lundin, C., Johansson, F., Helleday, T., Sehested, M., Lukas, J., and Bartek, J. (2005). Inhibition of human Chk1 causes increased initiation of DNA replication, phosphorylation of ATR targets, and DNA breakage. Mol Cell Biol *25*, 3553-3562.

Tao, Z. F., and Lin, N. H. (2006). Chk1 inhibitors for novel cancer treatment. Anticancer Agents Med Chem *6*, 377-388.

Taylor, W. R., and Stark, G. R. (2001). Regulation of the G2/M transition by p53. Oncogene *20*, 1803-1815.

Tevethia, M. J., Bonneau, R. H., Griffith, J. W., and Mylin, L. (1997a). A simian virus 40 large T-antigen segment containing amino acids 1 to 127 and expressed under the control of the rat elastase-1 promoter produces pancreatic acinar carcinomas in transgenic mice. J Virol *71*, 8157-8166.

Tevethia, M. J., Lacko, H. A., Kierstead, T. D., and Thompson, D. L. (1997b). Adding an Rb-binding site to an N-terminally truncated simian virus 40 T antigen restores growth to high cell density, and the T common region in trans provides anchorage-independent growth and rapid growth in low serum concentrations. J Virol *71*, 1888-1896.

Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev *13*, 152-157.

Todaro, G. J., Green, H., and Swift, M. R. (1966). Susceptibility of human diploid fibroblast strains to transformation by SV40 virus. Science *153*, 1252-1254.

Toledo, L. I., Murga, M., and Fernandez-Capetillo, O. (2011a). Targeting ATR and Chk1 kinases for cancer treatment: a new model for new (and old) drugs. Mol Oncol *5*, 368-373.

Toledo, L. I., Murga, M., Gutierrez-Martinez, P., Soria, R., and Fernandez-Capetillo, O. (2008). ATR signaling can drive cells into senescence in the absence of DNA breaks. Genes Dev *22*, 297-302.

Toledo, L. I., Murga, M., Zur, R., Soria, R., Rodriguez, A., Martinez, S., Oyarzabal, J., Pastor, J., Bischoff, J. R., and Fernandez-Capetillo, O. (2011b). A cell-based screen identifies ATR inhibitors with synthetic lethal properties for cancer-associated mutations. Nat Struct Mol Biol *18*, 721-727.

Urlinger, S., Baron, U., Thellmann, M., Hasan, M. T., Bujard, H., and Hillen, W. (2000). Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc Natl Acad Sci U S A *97*, 7963-7968.

Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L., and Shiloh, Y. (2003). Requirement of the MRN complex for ATM activation by DNA damage. EMBO J *22*, 5612-5621.

van Attikum, H., and Gasser, S. M. (2009). Crosstalk between histone modifications during the DNA damage response. Trends Cell Biol *19*, 207-217.

Vooijs, M., and Berns, A. (1999). Developmental defects and tumor predisposition in Rb mutant mice. Oncogene *18*, 5293-5303.

Vorechovský, I., Luo, L., Lindblom, A., Negrini, M., Webster, A. D., Croce, C. M., and Hammarström, L. (1996). ATM mutations in cancer families. Cancer Res *56*, 4130-4133.

Walter, J., and Newport, J. (2000). Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. Mol Cell *5*, 617-627.

Welcsh, P. L., Owens, K. N., and King, M. C. (2000). Insights into the functions of BRCA1 and BRCA2. Trends Genet *16*, 69-74.

White, M. K., and Khalili, K. (2006). Interaction of retinoblastoma protein family members with large T-antigen of primate polyomaviruses. Oncogene *25*, 5286-5293.

Willis, S. N., Fletcher, J. I., Kaufmann, T., van Delft, M. F., Chen, L., Czabotar, P. E., Ierino, H., Lee, E. F., Fairlie, W. D., Bouillet, P., *et al.* (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science *315*, 856-859.

Wu, L., and Hickson, I. D. (2003). The Bloom's syndrome helicase suppresses crossing over during homologous recombination. Nature *426*, 870-874.

Xiao, A., Wu, H., Pandolfi, P. P., Louis, D. N., and Van Dyke, T. (2002). Astrocyte inactivation of the pRb pathway predisposes mice to malignant astrocytoma development that is accelerated by PTEN mutation. Cancer Cell *1*, 157-168.

Xiong, Y., Zhang, H., and Beach, D. (1993). Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. Genes Dev *7*, 1572-1583.

Zachos, G., and Gillespie, D. A. (2007). Exercising restraints: role of Chk1 in regulating the onset and progression of unperturbed mitosis in vertebrate cells. Cell Cycle *6*, 810-813.

Zhao, H., and Piwnica-Worms, H. (2001). ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol *21*, 4129-4139.

Zickler, D., and Kleckner, N. (1998). The leptotene-zygotene transition of meiosis. Annu Rev Genet *32*, 619-697.

Zindy, F., Quelle, D. E., Roussel, M. F., and Sherr, C. J. (1997). Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. Oncogene *15*, 203-211.

Zou, L., Cortez, D., and Elledge, S. J. (2002). Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev *16*, 198-208.

Zou, L., and Elledge, S. J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science *300*, 1542-1548.

Annex