FACULTAD DE CIENCIAS

DEPARTAMENTO DE BIOLOGÍA MOLECULAR

ATR INHIBITORS AS A THERAPEUTIC APPROACH TO TREAT TUMOURS WITH HIGH LEVELS OF REPLICATION STRESS

DOCTORAL THESIS

ISABEL MORGADO PALACÍN

MADRID, 2017

Departamento de Biología Molecular
Facultad de Ciencias
Universidad Autónoma de Madrid

ATR inhibitors as a therapeutic approach to treat tumours with high levels of replication stress

Isabel Morgado Palacín
BSc, Biochemistry

The work presented in this Doctoral Thesis has been carried out at the Genomic Instability Group in the Spanish National Cancer Research Centre (CNIO, Madrid), under the direction and supervision of Dr. Oscar Fernández-Capetillo Ruiz.

Madrid, 2017

May 5th 2017

To whom it might concern:

I, Dr. **Oscar Fernández-Capetillo Ruiz,** group leader of the Genomic Instability Group at the Spanish National Cancer Research Centre (CNIO) and Professor of Cancer Therapy at the Department of Medical Biochemistry and Biophysics (Karolinska Institutet)

CERTIFY:

That the Doctoral Thesis titled **"ATR inhibitors as a therapeutic approach to treat tumours with high levels of replication stress"** developed by **Mrs Isabel Morgado Palacín, BSc, MSc** was carried out under my direction in the Spanish National Cancer Research Centre, and that I authorize its presentation to the tribunal. This Doctoral Thesis meets all the requirements to obtain the degree of **Doctor of Philosophy (PhD) in Molecular Biology** and, with the aforementioned objective, it will be defended at the Universidad Autónoma de Madrid.

Yours sincerely,

Oscar Fernández-Capetillo Ruiz, Ph.D.

ViceDirector of Tranlational Research
Director of Innovation
Genomic Instability Group
Spanish National Cancer Research Centre (CNIO)
C/Melchor Fernández Almagro, 3
E-28029, Madrid (Spain)
Phone: +0034-91 732 80 00

Professor of Cancer Therapy
Division of Genome Biology
MBB
Karolinska Institutet
Solnavägen, 1
SE-171 77, Stockholm (Sweden)
Phone: +46-8-524 800 00

A mi padre, mi madre y mi hermana Lucía

A mis tíos José Luis y Santos

A Guillermo

INDEX

RESUMEN

A lo largo de los últimos años, se ha dedicado un gran esfuerzo en dirigir la terapia contra el cáncer hacia el desarrollo de compuestos quimioterapéuticos enfocados en atacar las enzimas encargadas de reparar el DNA. Con este fin, la aproximación terapéutica actual consiste en aprovecharse de una debilidad específica de las células tumorales para eliminarlas de forma selectiva, este concepto se conoce como letalidad sintética y es el modelo clave para el tratamiento del cáncer. En esta Tesis Doctoral aportamos evidencias del potencial terapéutico de bloquear la quinasa ATR en tumores con altos niveles de estrés replicativo (RS, por sus siglas en inglés), un tipo de daño específico del DNA inducido por oncogenes y suprimido por las enzimas ATR y CHK1. Basándonos en estudios previos de nuestro laboratorio, hemos establecido la hipótesis de que los tumores con altos niveles de RS pueden identificarse en base a altos niveles de expresión de CHK1. Siguiendo esta lógica, hemos identificado un subtipo de leucemia mieloide aguda (AML) y el tumor sólido Sarcoma de Ewing, los cuales responden favorablemente al tratamiento con inhibidores de ATR.

Nos hemos centrado en el subtipo de AML iniciado a partir de reorganizaciones cromosómicas del oncogén *MLL* (AML-MLL). Las células tumorales de AML-MLL son resistentes a las terapias genotóxicas actuales debido a que presentan una respuesta atenuada del supresor tumoral p53, lo que implica un pronóstico desfavorable para esta enfermedad. En esta tesis, demostramos que la inhibición *in vitro* de ATR provoca la eliminación de células de AML^{MLL} de ratón, independientemente de la respuesta de p53 que presenten. Además, la inhibición de ATR genera una actividad anti-tumoral *in vivo* en un modelo de AML^{MLL} en ratones inmuno-suprimidos y en xenotransplantes de una línea celular humana de AML-MLL. Cuando el RS es persistente conlleva la rotura de la horquilla de replicación y la generación de roturas de doble cadena en el DNA. En relación a esto, describimos que la inhibición de ATM, una quinasa relacionada con ATR que responde al daño por roturas de doble cadena en el DNA, también mejora la supervivencia de los ratones con AML^{MLL}.

El Sarcoma de Ewing es un tumor pediátrico originado por una translocación cromosómica, mayoritariamente EWS/FLI. Este tumor puede remitir mediante cirugía y radiación en un estadio temprano; sin embargo, cuando se disemina por metástasis no tiene cura. Varios estudios han demostrado que el Sarcoma de Ewing es altamente sensible al daño en el DNA por lo que el tratamiento actual implica agentes genotóxicos. Sin embargo, se desconoce la razón por la cual estos tumores responden a dicha terapia. En este trabajo demostramos que el Sarcoma de Ewing presenta altos niveles endógenos de RS, lo cual le hace particularmente dependiente de la actividad de ATR. Asimismo, dos inhibidores independientes de ATR evaluados como agentes terapéuticos únicos demuestran eficacia *in vitro* en líneas celulares de Sarcoma de Ewing y eficacia *in vivo* en xenotransplantes de este tumor. La expresión de las translocaciones cromosómicas EWS/FLI o EWS/ERG es suficiente para sensibilizar a otras líneas celulares de distinta naturaleza al Sarcoma de Ewing a los inhibidores de ATR. En resumen, esta Tesis Doctoral confirma la utilidad de los niveles de expresión de CHK1 como un biomarcador de tumores con altos niveles de RS y aporta dos ejemplos preclínicos de la eficacia de los inhibidores de ATR en dichos tumores.

ABSTRACT

Over the past years, among the general chemotherapeutic approaches for cancer treatment, multiple efforts have focused on targeting DNA repair enzymes. In this regard some approaches aim to exploit cancer-specific defects to selective kill tumour cells; a concept known as synthetic lethality that has arisen as a key model for the treatment of cancer. In this Doctoral Thesis, we provide evidence of the therapeutic potential of targeting the ATR kinase for the treatment of cancers bearing high levels of replication stress (RS), a specific type of DNA damage which is induced by oncogenes and suppressed by ATR and CHK1. Based on previous work from our laboratory, we hypothesised that tumours with high levels of RS could be identified on the basis of expressing high levels of CHK1. Following this rationale, we here identified a liquid tumour (subtype of acute myeloid leukaemia (AML)) and a solid tumour (Ewing Sarcoma), as two cancer types that respond well to a treatment with ATR inhibitors.

In what regards to AML, we focused on the subtype that is initiated by chromosomal rearrangements of the *MLL* oncogene (AML-MLL). AML-MLL tumour cells are resistant to current genotoxic therapies because of an attenuated response by p53, which confers a bad prognosis to this disease. Here, we show that *in vitro* ATR inhibition induces the death of mouse AML^{MLL} cells independently of p53. More importantly, ATR inhibition presents anti-tumour activity *in vivo* in an immunocompetent allograft mouse model of AML^{MLL} and in xenografts of a human AML-MLL cell line. When RS is persistent it leads to the breakage of replication forks and the generation of DNA double strand breaks. Along these lines, we also found that inhibition of ATM, an ATR-related kinase that responds to DNA double strand breaks, also promoted the survival of the AML^{MLL} mice.

Regarding Ewing Sarcoma, these are paediatric bone tumours that arise from a driver translocation, most frequently EWS/FLI1. While the tumour can be cured by surgery and radiation in its earliest stages, metastatic disease has no cure. Several studies have shown a high sensitivity of Ewing Sarcoma to DNA damage, and current treatment involves genotoxic agents. However, the basis for the sensitivity to these therapies remains unknown. We here show that Ewing Sarcomas suffer from high endogenous levels of RS, rendering them particularly dependent to the ATR kinase. Accordingly, two independent ATR inhibitors show *in vitro* toxicity in Ewing Sarcoma cell lines as well as *in vivo* efficacy in Ewing Sarcoma xenografts as single agents. Expression of EWS/FLI1 or EWS/ERG oncogenic translocations suffices to confer sensitivity to ATR inhibitors in non-Ewing Sarcoma cell lines. Collectively, this Doctoral Thesis confirms the usefulness of CHK1 levels as a biomarker of tumours with high levels of RS, and provides two preclinical examples of the efficacy of ATR inhibitors in cancer therapy.

ABBREVIATIONS

AAD	ATR Activation Domain
ABL1	Abelson murine leukaemia viral oncogene homolog 1
ACK	Ammonium-chloride-potassium
ADP	Adenosine diphosphate
AF4	ALL1-fused gene from chromosome 4 (ALL1; Acute Lymphoblastic Leukaemia 1, also known as MLL)
AF9	ALL1-fused gene from chromosome 9 (ALL1; Acute Lymphoblastic Leukaemia 1, also known as MLL)
ALT	Alternative Lengthening Telomere
alt-NHEJ	alternative NHEJ
AML	Acute Myeloid Leukaemia
AML1	Acute Myeloid Leukaemia 1
Arg	Arginine
ATCC	American Type Culture Collection
ATM	Ataxia Telangiectasia Mutated
ATMi	ATM inhibitor
ATMi^{Pr}	ATM inhibitor prevention protocol
ATMiTh	ATM inhibitor therapy protocol
ATR	Ataxia Telangiectasia and Rad3 related
ATRi	ATR inhibitor
ATRIP	ATR Interacting Protein
ATRi^{Pr}	ATR inhibitor prevention protocol
ATRiTh	ATR inhibitor therapy protocol
AURKA	Aurora Kinase A
BARD1	BRCA1-associated RING domain protein 1
Bax	Bcl-2-associated X protein (Bcl-2; B-cell lymphoma 2)
BET	Bromodomain Extra-Terminal
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
BRD4	Bromodomain-containing protein 4
BrdU	Bromodeoxyuridine
Brn3	Class IV (Pit1-Oct1-Unc86)-domain-containing transcription factor
CBP/p300	CREB-Binding Protein / p300 (CREB; cAMP responsive element binding protein 1 and protein 300 kDa)
CCT2	Chaperonin Containing T-complex polypeptide 1 subunit 2b
CDC25A	Cell division cycle 25
CDC45	Cell Division Cycle protein 45
CDK1	Cyclin Dependent Kinase 1
CDK2	Cyclin Dependent Kinase 2
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
Chr.	Chromosome
CldU	Chlorodeoxyuridine
COOH	C-terminal

DAPI	4´,6´-diamidino-2-phenylindole
DBD	DNA binding domain
DC	Dendritic Cell
DDK	DBF4-dependent kinase (DBF4; DumbBell Forming protein 4)
DDR	DNA Damage Response
DMEM	Dulbecco´s Minimum Essential Media
DMSO	Dimethyl sulfoxide
DNA-PKcs	DNA Protein Kinase catalytic subunit
dNTP	Deoxynucleotide triphosphate
DOT1L	Disruptor of telomeric silencing 1-like
DOX	Doxycycline
DSB	Double-Strand Break
dsDNA	double-stranded DNA
E1A	Adenovirus early region 1A
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl 2´-deoxyuridin
ENL	Eleven Nineteen Leukaemia
ER	Estrogen Receptor
ERG	ETS-Related Gene
esiRNA	Endonuclease-prepared Small Interfering RNAs
ETAA1	Ewing Tumour-Associated Antigen 1
ETO	Eight Twenty One
ETS	E26 Transformation-Specific
ETV1	ETS Translocation Variant 1
ETV4	ETS Translocation Variant 4
EWS	Ewing Sarcoma breakpoint region 1
EWSR1	Ewing Sarcoma breakpoint region 1
FACS	Fluorescence Activated Cell Sorting
FAT	FRAP, ATM and TRRAP domain
FATC	FAT C-terminal domain
FBS	Foetal Bovine Serum
FET	FUS, EWSR1 and TAF15 family of DNA- and RNA-binding proteins
FEV	Fifth Ewing Variant
FLI1	Friend Leukaemia Integration 1
Flp-In™	Flippase recombinase-mediated integration
FOXO1	Forkhead box O
FRAP	FKBP-12-Rapamycin Associated Protein; also known as mTOR
FRT	Flippase Recombination Target
FUS	Fused in Sarcoma
g	times gravity
GFP	Green Fluorescent Protein
GLO	“glow-type” luminescent signal of CellTiter-Glo® Luminescent Cell Viability Assay
H3K79	Histone 3 Lysine 79
HCS	High-Content Screening

HEAT	Huntingtin, Elongation factor 3, a subunit of protein phosphatase 2A and Target of rapamycin kinase 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFF	Human Foreskin Fibroblast
HOXA	Homeobox protein Hox-A
HR	Homologous Recombination
H-RAS	Harvey sarcoma virus- RAS oncogene
HRP	Horseradish peroxidase
Hrs	hours
HSCs	Hematopoietic Stem Cells
HU	Hydroxyurea
HUS1	Hydroxyurea sensitive 1
IdU	Iododeoxyuridine
IGFR-1	Insulin-like Growth Factor 1 Receptor
IHC	Immunohistochemistry
IRES	Internal Ribosome Entry Site
IVIS	In Vivo Imaging System
JQ1	In honour of chemist Jun Qi, PhD, who synthesized the original compound
KMT2A	Histone-lysine N-methyltransferase 2A; also known as MLL
KAP1	KRAB-associated protein 1 (KRAB; Krüppel associated box)
LD50	Lethal Dose, median
LDS	Lithium Dodecyl Sulfate
LSCs	Leukaemic Stem Cells
MDM2	Mouse Double Minute 2 homolog
MEF	Mouse Embryonic Fibroblasts
MEIS1	Myeloid Ecotropic viral Integration Site 1 Homolog
mIL-3	mouse interleukin-3
mIL-6	murine interleukin-6
MKI67	Marker of proliferation Ki-67 (The name is derived from the city of origin of the original antibody against this marker (Kiel, Germany) and the number of the original clone in the 96-well plate).
MKI67IP	Nucleolar protein interacting with the forkhead-associated domain of MKI67
MLL	Mixed Lineage Leukaemia
MRE11	Meiotic recombination 11 homolog 1
MRN	MRE11-RAD50-NBS1 complex
mRNA	messenger-RNA
mTOR	mechanistic Target Of Rapamycin
MYC	Myelocytomatosis oncogene
NB	Neuroblastoma
NBAD	Nucleic Acid Binding Domain
NBS1	Nijmegen breakage syndrome 1
NCS	Neocarzinostatin
neo	Neomycin-resistance

NH_2	N-teminal
NHEJ	non-homologous end-joining
NK	Natural Killer
NLS	Nuclear Localization Signal
NMP	Nmethyl-2-pyrrolidone
Noxa	Latin for *damage*
NP-40	Nonident-P40
N-RAS	Neuroblastoma- RAS oncogene
N-RAS^{G12D}	Glycine substitution (missense) by Aspartic acid at position 12 in N-RAS
NRG	Non-obese diabetic Rag1 (Recombination Activating Gene 1) null and IL2rg (interleukin 2 receptor, gamma chain) null mice (NOD.Cg-*Rag1^{tm1Mom} Il2rg^{tm1Wjl}*)
NUP155	Nucleoporin 155
OCT4	Octamer-binding transcription factor 4
OHT	4-hydroxy-tamoxifen
p16^{INK4a}/pRB	protein 16 inhibiting cycling dependent kinase 4 / Retinoblastoma protein
p53	Tumour suppressor protein 53
PAGE	PolyAcrylamide Gel Electrophoresis
PARP1	poly (ADP-ribose) polymerase 1
PARPi	PARP inhibitor
PARPs	poly (ADP-ribose) polymerases
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEG	PolyEthylene Glycol
Pen/Strep	Penicillin/Streptomycin
PFA	Paraformaldehyde
PI	Propidium Iodide
PI3Ks	Phosphatidylinositol-3-kinases
PIKK	Phosphatidylinositol-3-kinase related kinases
PLT	Platelets
PRMT1	Protein arginine methyltransferase 1
Puma	p53 upregulated modulator of apoptosis
RAD1	RADiation sensitive 1
RAD17	RADiation sensitive 17
RAD50	RADiation sensitive 50
RAD9	RADiation sensitive 9
RAS	RAt Sarcoma oncogene
RBC	Red Blood Cells
RCF	Replication factor C
RGG	Arginine-Glycine-Glycine- rich
RMA	Robust Multichip Average
RMS	Rhabdomyosarcoma
RPA	Replication Protein A
RPMI	Roswell Park Memorial Institute medium
RRM	RNA-Recognition Motif

rRNA	ribosomal RNA
RS	Replication Stress
SCID	Severe Combined Immuno-Deficient
SDS	Sodium Dodecyl Sulfate
SFRS3	Serine and Arginine Rich Splicing Factor 3
shRNA	short hairpin RNA
SMC1	Structural Maintenance of Chromosomes 1
SMG-1	serine/threonine-protein kinase
SSA	Single-Strand Annealing
ssDNA	single-stranded DNA
STAG	streptavidin-tagged
SYGQ	serine-tyrosine-glycine-glutamine domain
TAD	Transcription Activation Domain
TAF15	TATA-Box Binding Protein Associated Factor 15
Tet-ON	Tetracycline-Controlled Transcriptional Activation
TFIID	Transcription Factor II D
TINP1	TGFβ-Inducible Nuclear Protein 1 (TGFβ; Transforming Growth Factor beta)
TOPBP1	Topoisomerase II binding protein
TP53	p53 gene
TRRAP	Transformation/Transcription domain Associated Protein
UQ/Cre^{ERT2}	Cre^{ERT2} expressed from the ubiquitin promoter
v/v	volume/volume percentage
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cells
WT	Wild-Type
XTT	Yellow tetrazolium salt of Cell Proliferation Kit II
ZF	Zinc Finger motif
γH2AX or p-Ser139 H2AX	Phosphorylated form of histone H2A.X at serine 139 (ser139)
-/-	Knock-Out

INTRODUCTION

The *Edwin Smith Papyrus* is a part of Egyptian book on medicine that dates back to around 3000 BC and that contains the first reference in history about cancer. Remarkably, the writing says about the disease, “There is no treatment” (Breasted, 1930). Since then, and in particular over the last decades, due to the high incidence of cancer, multiple studies have been conducted with the purpose of elucidating the aetiology of cancer, which have contributed to the understanding that cancer is a highly heterogeneous disease and that genomic instability is one of its hallmarks (Hanahan and Weinberg, 2000; Halazonetis, 2008). Indeed, the pathways that preserve genomic stability are commonly mutated in cancer cells (Hanahan and Weinberg, 2011), and for this reason, the study of genomic instability has emerged as key to decipher the tumourigenesis process and to identify efficient therapeutic targets and strategies, thereby contributing to the notion that there can be effective treatments for cancer.

1. GENOMIC INSTABILITY AND CANCER

The maintenance of the stability of the genetic material is essential for the proper function and survival of all organisms. This is nonetheless a complicated task, since multiple endogenous and exogenous agents threaten DNA stability, and thus, DNA damage accumulation is very frequent and consequently a fundamental problem for life, as it can accelerate ageing (Garinis *et al.*, 2008) and contribute to cancer onset and progression (Hanahan and Weinberg, 2000). Therefore, and even though DNA lesions can have substantial evolutionary implications, being fundamental to provide variations in genetic material that enable the adaption required for evolution (Stamatoyannopoulos *et al.*, 2009), they also pose a severe threat to cell viability as they can interfere with essential cellular processes (*e.g.* transcription and DNA replication), thereby potentially leading to genomic instability (Hanahan and Weinberg, 2000). DNA replication is arguably one of the processes most dramatically affected by the presence of lesions in DNA. It is an essential process for life because the genetic information contained in the DNA needs to be faithfully copied and transmitted to the daughter cells every cell cycle. However, the DNA replication machinery can be severely blocked when encountering these challenges and this hampers the faithful copy of the DNA and cell viability. For all these reasons, multiple mechanisms have emerged through evolution to detect, signal and repair DNA damage, and they are collectively termed as the DNA damage response (DDR) (Kastan and Bartek, 2004; Harper and Elledge, 2007; Ciccia and Elledge, 2010).

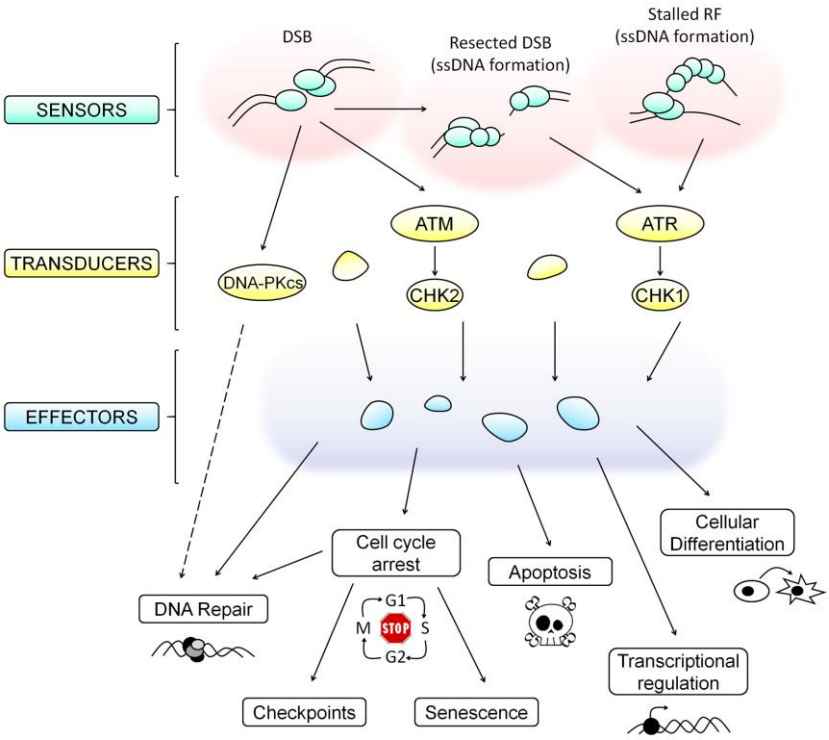

Figure 1. The DNA Damage Response (DDR). The DDR has a hierarchical organization whereby different types of damage are initially recognized by sensor proteins that recruit transducers to trigger and amplify the signal. Mediators guide the signal until it reaches effectors that are in charge of executing the appropriate response. The DDR activation results in multiple effects: activation of DNA repair mechanisms, regulation of transcription, cellular differentiation, and cell cycle arrest either in a transitory way (through checkpoints) or in an irreversible manner by the onset of senescence. Depending on the type and amount of damage, the DDR can also lead to apoptosis (Adapted from Zhou and Elledge, 2000).

1.1. DNA Damage Response

The DDR is a complex network that detects, signals and repairs DNA lesions to protect the integrity of the genetic material (Figure 1). The DDR functions through a signalling-based transduction cascade involving post-translation modifications (such as phosphorylation, acetylation, ubiquitination and sumoylation) that activates specific checkpoints in different phases of the cell cycle and promotes DNA repair while delaying cell cycle progression until chromosomes are repaired (Harper and Elledge, 2007). In addition to checkpoint activation, the DDR leads to the induction of transcriptional programs and DNA repair pathways. If the DNA damage becomes intolerable because full repair cannot be ensured or because DNA damage persists, the DDR is able to promote apoptosis or to induce cell cycle-cell arrest leading damaged cells to an irreversible quiescent state known as senescence (Figure 1) (Harper and Elledge, 2007).

Apoptosis and senescence are two strong anti-tumour barriers that are activated in the earliest steps of malignant transformation (Bartkova *et al.*, 2005; Gorgoulis *et al*., 2005). The DDR can trigger these processes manly through the activation of the tumour suppressor protein 53 (p53), encoded by *TP53*, which induces the expression of genes implicated in apoptosis, like Puma (p53 upregulated modulator of apoptosis), Noxa (latin for *damage*) and Bax (Bcl-2-associated X protein) (Miyashita and Reed, 1995; Oda, 2000; Nakano and

Vousden, 2001); and that can also mediate senescence together with the $p16^{INK4a}$/pRB (protein 16 inhibiting cycling dependent kinase 4 / Retinoblastoma protein) axis (Kuilman *et al.*, 2010). Tumour cells progressively accumulate mutations that activate oncogenes and inactivate of tumour suppressors leading to unrestrained DNA replication and replication stress as a consequence (Bartkova *et al.*, 2005). Moreover, further mutations that partially alter other processes concerning the DDR such as damage signalling or DNA repair leading, also contribute to malignant transformation. For all these reasons, the DDR is unambiguously considered to play an essential role in preventing tumourigenesis, rendering the understanding of the processes that involve the DDR as essential in order to address the aetiology of cancer. The initial signalling events that trigger the DDR are arguably the most essential to preserve genomic stability, and the members of the family of phosphatidylinositol-3-kinase related kinases (PIKK) are central players to this process.

1.2. The family of phosphatidylinositol-3-kinase related kinases

The DDR is organised in a hierarchical fashion whereby specific lesions in the DNA are recognised by distinct protein sensors, which lead to the activation of the effector kinases that ultimately coordinate the repair process by modulating a wide range of effectors (Figure 1). The DDR starts through the activation of one of the members of the PIKK family of protein that includes: ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), DNA protein kinase catalytic subunit (DNA-PKcs), serine/threonine-protein kinase (SMG-1) and mechanistic target of rapamycin (mTOR) (Lovejoy and Cortez, 2009).

ATM, ATR and DNA-PKcs are the main effectors for signalling DNA damage and promoting its repair (Berti and Vindigni, 2016) (Figure 1). ATM and DNA-PKcs are mainly activated by DNA double-strand breaks (DSBs) throughout the cell cycle (Gottlieb and Jackson, 1993; Pandita *et al.*, 2000) (Figure 1). DSBs are highly cytotoxic lesions that are repaired by several conserved pathways including non-homologous end-joining (NHEJ), alternative NHEJ (alt-NHEJ), homologous recombination (HR) and single-strand annealing (SSA) (the latter considered by some as a subpathway of HR) (Ciccia and Elledge, 2010). NHEJ and HR are the major pathways to repair DSBs and they diverge in their requirement for limited or extensive resection of the ends respectively (Hoeijmakers, 2001). NHEJ is generally considered as an error-prone pathway since it implies direct binding and ligation of the two broken ends of the DSB, requiring little or no processing of the ends, at the expense of causing possible local microdeletions, but is essential during G1 phase of the cell cycle. Conversely, HR is generally error-free but it can only take place during S and G2 phases as it relies on the use of an homologous chromatid for recombination. The role of DNA-PKcs seems to be limited to the repair of DSBs through NHEJ (Weterings and Van Gent, 2004),

and although DNA-PKcs is arguably the main kinase promoting DSBs repair, 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 and thus facilitate DNA repair in these regions (Goodarzi *et al.*, 2008).

In contrast to DNA-PKcs and ATM, ATR responds to the accumulation of long stretches of single-stranded DNA (ssDNA) (Figure 1), and, consequently, its kinase activity remains restricted to the S and G2 phases of the cell cycle (Zou and Elledge, 2003). ATR and its major target, the kinase checkpoint kinase 1 (CHK1), mainly prevent a specific DNA damage known as replication stress (RS), which is indeed characterised by long stretches of ssDNA. Remarkably, in clear contrast to DNA-PKcs, whose activity is limited to lesion site, (Collis *et al.*, 2005), ATR and also ATM have major roles in global –rather than local- signalling of DNA damage through the phosphorylation of thousands of substrates including their specific targets CHK2 and CHK1 respectively (Matsuoka *et al.*, 2007). Although DNA-PKcs- and ATM-mediated damage signalling is essential to genomic stability, in this doctoral thesis we have mainly focused our studies in the ATR-mediated signalling of RS due to its outstanding potential as a therapeutic target for cancer (Lecona and Fernández-Capetillo, 2014).

1.3. ATR and CHK1 as main effectors of the Replication Stress Response

RS is characterized by the presence of high amounts of ssDNA at the replication fork, caused mainly by DNA replication stalling which, if persistent, can give rise to DSBs. Multiple problems, such as low dNTP (deoxynucleotide triphosphate) levels, polymerase inhibition or DNA alterations, can block replication forks and thus perturb DNA replication, being those the main sources of RS. Remarkably, RS unavoidably arises every time a cell replicates its genetic material when replication forks stall in response to DNA alterations and, in mammalian cells, ATR suppresses RS and limits the presence of ssDNA through the phosphorylation and activation of a wide set of targets including CHK1, thereby initiating the RS response (Cimprich and Cortez, 2008; López-Contreras and Fernandez-Capetillo, 2010). While the main role ATR is facilitating replication under stress conditions (Hurley and Bunz, 2007) it is also key to maintain and repair replication forks every S phase, being essential for cell division and survival. A fundamental aspect of ATR biology is its recruitment and activation, which requires not only the generation of ssDNA but also the interaction with additional proteins.

1.3.1. ATR recruitment and activation

The understanding of ATR structural organisation has been relevant to elucidate its interaction with other proteins (Figure 2A). Likewise most of PIKK members, ATR harbours an N-terminal region formed by HEAT (Huntingtin, Elongation factor 3, a subunit of protein phosphatase 2A and Target of rapamycin kinase 1) repeats variable in length followed by a FAT (FRAP, ATM and TRRAP) domain and a C-terminal region formed by the catalytic core and the FATC (FAT C-terminal) domain (Baretić and Williams, 2014) (Figure 2A). The N-terminal region is essential for ATR activity since it constitutes the interaction domain for its partner ATRIP (ATR interacting protein) and the FATC domain mediates the binding to TOPBP1 (topoisomerase II binding protein), which allosterically activates ATR (Figure 2A) (Mordes *et al.*, 2008).

As aforementioned, the signal that triggers the ATR pathway relies is, in all cases, ssDNA. The presence of ssDNA is highly dangerous in a cell and, for this reason, ssDNA is quickly protected by the ssDNA binding protein complex RPA (replication protein A) that directly interacts with and recruits the ATR partner, ATRIP (Walter and Newport, 2000) (Figure 2B). The interaction between ATR and ATRIP is critical for ATR function as, in fact, ATR is always present as an ATR-ATRIP heterodimer complex (Zou and Elledge, 2003). The formation of ssDNA-RPA patches also promotes the loading on the ssDNA and double-stranded DNA (dsDNA) junction of the two key mediators of ATR activation, RAD17-RCF (checkpoint protein Radiation sensitive protein 17 and replication factor C) complex and 9-1-1 (RAD9, 9, HUS1 and RAD1) clamp, which in turn recruit of TOPBP1 (Figure 2B). The activation of ATR-ATRIP is stronger in the presence of DNA structures that combine

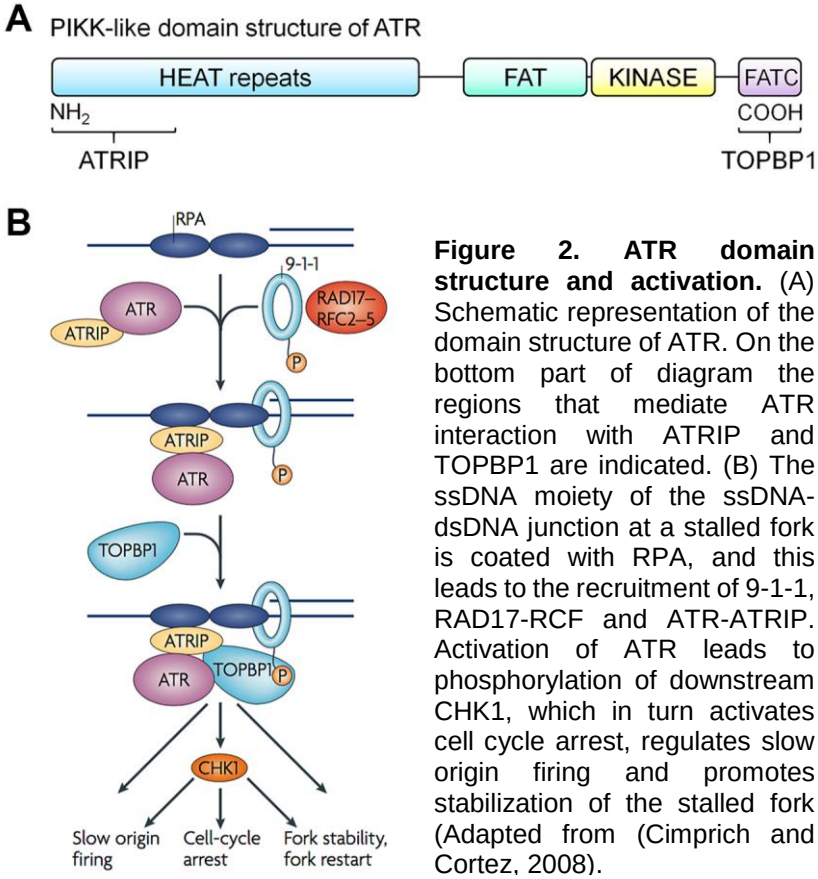

Figure 2. ATR domain structure and activation. (A) Schematic representation of the domain structure of ATR. On the bottom part of diagram the regions that mediate ATR interaction with ATRIP and TOPBP1 are indicated. (B) The ssDNA moiety of the ssDNA-dsDNA junction at a stalled fork is coated with RPA, and this leads to the recruitment of 9-1-1, RAD17-RCF and ATR-ATRIP. Activation of ATR leads to phosphorylation of downstream CHK1, which in turn activates cell cycle arrest, regulates slow origin firing and promotes stabilization of the stalled fork (Adapted from (Cimprich and Cortez, 2008).

ssDNA stretches and dsDNA, resembling stalled replication forks (Kumagai *et al*., 2004). Ultimately, the activity of ATR is unleashed by its interaction with TOPBP1, which acts as the key allosteric activator factor of ATR-ATRIP complex (Figure 2B) (Kumagai *et al.*, 2006). More recently, the RPA binding-protein ETAA1 (Ewing tumour-associated antigen 1) has been identified as an additional activator of ATR activity that operates independently of TOPBP1, although its relevance for ATR activation remains unclear (Haahr *et al.*, 2016).

The MRE11-RAD50-NBS1 (MRN) complex also takes part in the regulation ATR-ATRIP activation as it cooperates with 9-1-1 clamp to recruit TOPB1 to stalled replication forks. The MRN complex also plays a relevant role in DSB repair where it initiates the ATM mediated response to these lesions (Petrini and Stracker, 2003; Lee and Paull, 2005). Of note, the response to DSBs also can lead to the generation of ssDNA stretches through the resection of the broken end, thereby leading to ATR activation. Thus, while the main role of ATR is in responding to RS, it has also a less prominent function in the DSB-response.

1.3.2. ATR- and CHK1-mediated signalling

CHK1 is one of the key transducers of the ATR checkpoint response (Figure 2B). Like ATR, CHK1 is also essential for coordinating DNA replication and the cell cycle in the presence of RS. In contrast to many other substrates that can be phosphorylated by ATR directly, CHK1 phosphorylation demands the presence of CLASPIN, which serves as a scaffold adaptor between ATR-ATRIP and CHK1 (Liu *et al.*, 2006).

The main functions of ATR/CHK1 signalling are arresting the cell cycle to prevent progression into mitosis with unreplicated DNA and limiting the number of active origins of replication to prevent RPA exhaustion (Smits and Gillespie, 2015). These functions are in part achieved thanks to CHK1-mediated phosphorylation of CDC25A (cell division cycle 25) and of the mitotic CDK (CDK1; Cyclin Dependent Kinase 1), which prevents their activation thereby inhibiting the entry into mitosis (Peng *et al.*, 1997; Mailand, 2000; Lee *et al*., 2001; Chen *et al*., 2003), and also thanks to the capacity of ATR/CHK1 to block origin firing (Figure 2B). It is well established that during DNA replication initiation only a subset of origins that have been licensed are actually fired, while a number of origins remain “dormant” (Blow *et al*., 2011), and are only fired to help complete the replication of their region when a replication fork encounters a problem that stops its progression (Ge *et al*., 2007; Ibarra *et al*., 2008). In the presence of RS or DNA damage, the strong activation of CHK1 leads to inhibition of the S-phase CDK (CDK2; Cyclin Dependent Kinase 2) (Petermann *et al*., 2010), thereby blocking the loading of CDC45 (cell division cycle protein 45) on chromatin and in turn blocking origin firing, given that CDC45 loading is required for the formation of an active helicase at origins

(Boos *et al.*, 2011; Guo *et al.*, 2015). Moreover, CHK1 signalling reduces the activity of the DDK (DBF4-dependent kinase), which is also necessary for CDC45 loading (Heffernan *et al.*, 2007; Hills and Diffley, 2014).

ATR activity is especially critical in early S phase, since the high number of active origins can lead to an accumulation of excessive levels of RS, thereby exhausting the pool of RPA and lead to the presence of naked molecules of ssDNA, which seems to be the signal for the nucleolytic degradation of stalled replication forks (Toledo *et al.*, 2013). ATR-ATRIP also stabilises the fork structure by recruiting factors that protect it from degradation and promote its restart when the problems are fixed (Dungrawala *et al.*, 2015; Berti and Vindigni, 2016).

In summary, ATR and CHK1 are necessary to sustain DNA replication through controlling the amount of origins that are fired, by stabilizing stalled forks, and ultimately by preventing the entry into mitosis with a non-replicated genome.

1.4. Replication Stress in cancer

Oncogene activation leads to unrestrained proliferation (López-Contreras and Fernandez-Capetillo, 2010), which is an incessant source of RS that contributes to transformation and tumour progression in a decisive way. Oncogenic events frequently promote a promiscuous S-phase entry, leading cells to initiate DNA replication before they are ready and have all the proper metabolites to undergo this reaction, leading to RS and DNA damage. The persistent accumulation of RS ultimately generates DNA breaks and activates the ATM/p53 DDR, which constitutes the basis of the “oncogene-induced DNA damage” model of cancer progression (Halazonetis, 2008). As mentioned previously, the DDR acts as a barrier during the initial steps of tumour transformation, by activating apoptosis and senescence pathways in response to oncogene-induced DNA damage (Bartkova *et al.*, 2005; Gorgoulis *et al*., 2005; Nuciforo *et al.*, 2007). While oncogene activation can generate RS through multiple mechanisms, all of them are signalled through the ATR pathway, therefore placing ATR as a key guardian of genome stability during carcinogenesis.

The important role of ATR in suppressing RS leads to tumours becoming “addicted” to a proficient ATR response, in order to be able to cope with high levels of RS. Accordingly, targeting the RS response can be exploited for selectively eliminating tumours harbouring elevated levels of oncogene-induced RS (Figure 3). The first example of this idea came from the use of a mouse model of the Seckel Syndrome (Murga *et al.*, 2011). One of the variants of this disease is characterized by a mutation in the ATR gene that alters the splicing of its

mRNA thereby reducing the levels of mRNA and functional ATR protein to only 5% (O'Driscoll *et al.*, 2003). While patients can survive with 5% of normal levels of ATR, they display several developmental problems, including features of segmental progeria and severe dwarfism (Seckel, 1960). The human ATR-Seckel mutation was used to generate a viable humanized mouse model that recapitulates the human ATR-deficient-Seckel syndrome and that suffers from high levels of RS during embryonic development and from premature aging in adults (Murga *et al.*, 2009). While ATR was classically considered a "tumour suppressor", these studies showed that ATR-Seckel mice were in contrast tumour protected (Murga *et al.*, 2009), raising the idea that low levels of ATR could prevent tumorigenesis. In fact, ATR-Seckel mice were found to be resistant to the development of tumours imitated by MYC (myelocytomatosis oncogene) or MLL-ENL [(Mixed Lineage Leukaemia) – (Eleven Nineteen Leukaemia)] oncogenes, or by the loss of p53 (Murga *et al.*, 2009, 2011; Schoppy *et al.*, 2012).

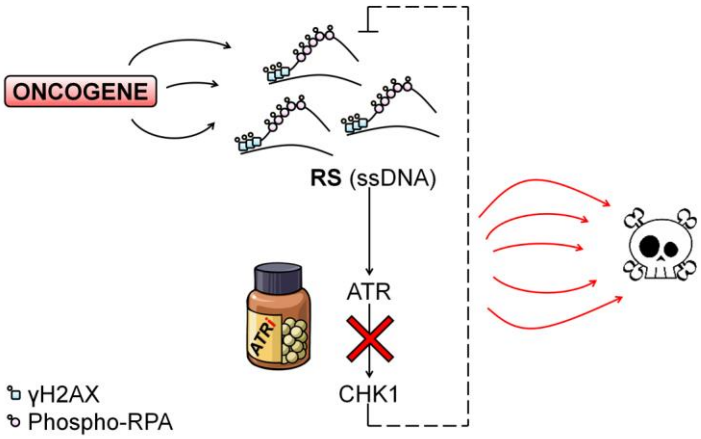

Figure 3. Targeting the Replicative Stress Response for cancer therapy. Elevated RS levels favour malignant transformation and tumorigenesis rendering tumour cells more dependent on a proficient RS response. Targeting RS response using ATR inhibitors (ATRi) results in excessive RS levels that leads cells to collapse and death (Murga *et al.*, 2009; Schoppy *et al.*, 2012).

Collectively, these studies provided the first genetic evidences to support the idea that certain cancers become highly dependent on a proficient ATR pathway, and thus that targeting ATR could become a therapeutic strategy for the treatment of such tumours.

1.5. ATR inhibitors

Given that genomic instability is widespread in cancer cells (Boveri, 1914), it is now well established that targeting genomic instability offers an opportunity to develop treatments that preferentially kill cancer cells. This idea gained momentum with the development of therapies such as inhibitors of poly (adenosine diphosphate (ADP) -ribose) polymerases (PARPs), which are highly toxic for cells with mutations in *BRCA1/2* (breast cancer 1 / breast cancer 2) that are thus defective in DNA repair by HR (Bryant *et al.*, 2005; Farmer *et al.*, 2005). Remarkably, exploiting the presence of high endogenous levels of DNA damage in tumours arose an alternative therapeutic strategy to targeting a specific mutation of the

tumour cells, and this can be achieved by inhibiting the DDR kinases involved in signalling for the repair of DNA damage (Figure 3).

One recurrent finding in cancer is the presence of RS, which, if persistent, leads to DSBs that initiate genomic rearrangements in cancer cells (Bartkova *et al.*, 2005; Gorgoulis *et al.*, 2005; Hanahan and Weinberg, 2011). Thus, the presence of RS renders cancer cells particularly dependent on a proficient RS response for survival and in fact, the expression of ATR and CHK1 is frequently upregulated in cancer (Lecona and Fernández-Capetillo, 2014). Consequently, targeting RS response kinases ATR and CHK1 is preferentially toxic for cells with altered DNA replication that depend on their action to prevent entry into mitosis with unreplicated DNA, causing widespread DNA damage (Figure 3) (Ruiz *et al.*, 2016). Accordingly, ATR or CHK1 inhibitors have exhibited efficacy in hematopoietic tumours, mostly in *in vitro* settings (Murga *et al.*, 2011; Schoppy *et al.*, 2012; Cottini *et al.*, 2015; Derenzini *et al.*, 2015; Sarmento *et al.*, 2015; Kwok *et al.*, 2016).

Independent strategies have been used to develop selective ATR inhibitors (Charrier *et al.*, 2011; Reaper *et al.*, 2011; Toledo *et al.*, 2011; Foote *et al.*, 2013). Given that ATR and CHK1 activity is restricted to the S and G2 phases of the cell cycle, one of these strategies conveyed the development of an *in vitro* cellular system in which ATR is specifically activated at will, in the absence of actual DNA damage (Toledo *et al.*, 2008). The system relied on a fusion of the ATR activation domain (AAD) of TOPBP1, which is sufficient to trigger the activation of ATR-ATRIP (Kumagai *et al.*, 2006) and a fragment of the estrogen receptor (ER) that, in response to an inert derivative of tamoxifen (4-hydroxy-tamoxifen; OHT), is translocated from the cytoplasm to the nucleus and promotes generalized ATR activation in the absence of real damage (Toledo *et al.*, 2008). This system enabled the identification of specific inhibitors of the ATR pathway by a High-Content Imaging-based screening that started from a library of compounds that had previously shown activity to ATR-related PI3Ks (phosphatidylinositol-3-kinases) (Toledo *et al.*, 2011).

Although persistent RS or DNA damage activates the tumour suppressor p53 which triggers cell cycle arrest and apoptosis (Muller and Vousden, 2014), cell death caused by low ATR activity is not only p53-independent, but also enhanced by p53 deficiency (Murga *et al.*, 2009; Ruzankina *et al.*, 2009). Likewise, the toxicity of chemical inhibitors of ATR is higher in cells lacking p53 (Reaper *et al.*, 2011; Toledo *et al.*, 2011; Kwok *et al.*, 2016). This p53-independent cell killing by ATR inhibitors is linked to their capacity to induce the accumulation of RS and premature mitotic entry, activities that are unrelated to p53 function (Buisson *et al.*, 2015; Ruiz *et al.*, 2016). Therefore, ATR inhibitors offer an alternative for the

elimination of p53-deficient tumours, which is relevant since a wide range of current chemotherapeutic agents induce tumour cell death through a p53-dependent pathway.

Additionally, many of the agents used in genotoxic chemotherapy, including antifolates, nucleotide analogues, topoisomerase inhibitors, or platinum derivatives, are potent inducers of RS. DNA replication was one of the first targets in the development of modern chemotherapy. While ATR inhibitors are, in way, a modified version of this strategy, they provide a key advantage in that, in addition to inducing RS, they also promote mitotic entry, thereby leading to major segregation problems (Ruiz *et al.*, 2016).

Overall, these increasing evidence indicate that the inhibition of ATR, and also of CHK1, are potentially effective therapeutic strategies especially for tumours with high levels of RS, and consequently, the identification of cancers presenting high levels of RS is essential to guide the use of ATR and CHK1 inhibitors as therapy (Toledo *et al.*, 2011).

1.6. High levels of CHK1 expression as biomarker of ATR inhibitor sensitivity

The presence of high levels of RS in cancer cells creates a pressure to acquire mutations that suppress RS and therefore facilitate their growth (Lecona and Fernández-Capetillo, 2014; Zeman and Cimprich, 2014; Dobbelstein and Sørensen, 2015). Consistently, certain tumours overexpress CHK1 to reduces the toxic effects of RS (Bartek *et al*., 2012; Sarmento *et al.*, 2015), and supporting this view, CHK1 overexpression increases the efficiency of transformation by oncogene RAS (rat sarcoma oncogene) and E1A (adenovirus early region 1A), by suppressing oncogene-induced RS (López-Contreras *et al.*, 2012; Schulze *et al.*, 2014).

Because ATR inhibition is preferentially toxic for cells experiencing RS (Figure 3), tumours with high endogenous levels of this stress could be promising targets for treatment with ATR inhibitors. On this basis, high levels of CHK1 expression could be a signature of tumours with high levels of RS and, therefore, may provide a biomarker for ATR inhibitors sensitivity (López-Contreras *et al.*, 2012). To gain further insight into this idea, we comparatively analysed the relative levels of CHK1 gene expression across different cancer types, using data extracted from the human Cancer Cell Line Encyclopaedia (Barretina *et al.*, 2012) (Figure 4). As a proof of the usefulness of this approach, CHK1 mRNA was most abundant in Burkitt lymphomas (Figure 4), which we previously showed to be highly dependent on ATR and CHK1 for their survival (Murga *et al.*, 2011). In addition to Burkitt lymphoma, CHK1 expression was also distinctively high in various lymphomas and leukaemias (Figure 4), which could be indicative that these types of tumours are particularly

prone to suffer from RS. Accordingly, a number of studies have shown a good efficacy of ATR or CHK1 inhibitors in hematopoietic tumours (Murga *et al.*, 2011; Schoppy *et al.*, 2012; Cottini *et al.*, 2015; Derenzini *et al.*, 2015; Sarmento *et al.*, 2015; Kwok *et al.*, 2016).

For the purpose of this thesis, we decided to select one example of a liquid tumour and a solid tumour where ATR inhibitors could show efficacy. To this end, we selected Acute Myeloid Leukaemia (AML) and Ewing Sarcoma, two tumour types that display high levels of CHK1 expression, which currently lack a curative treatment and that often affect children or adolescents.

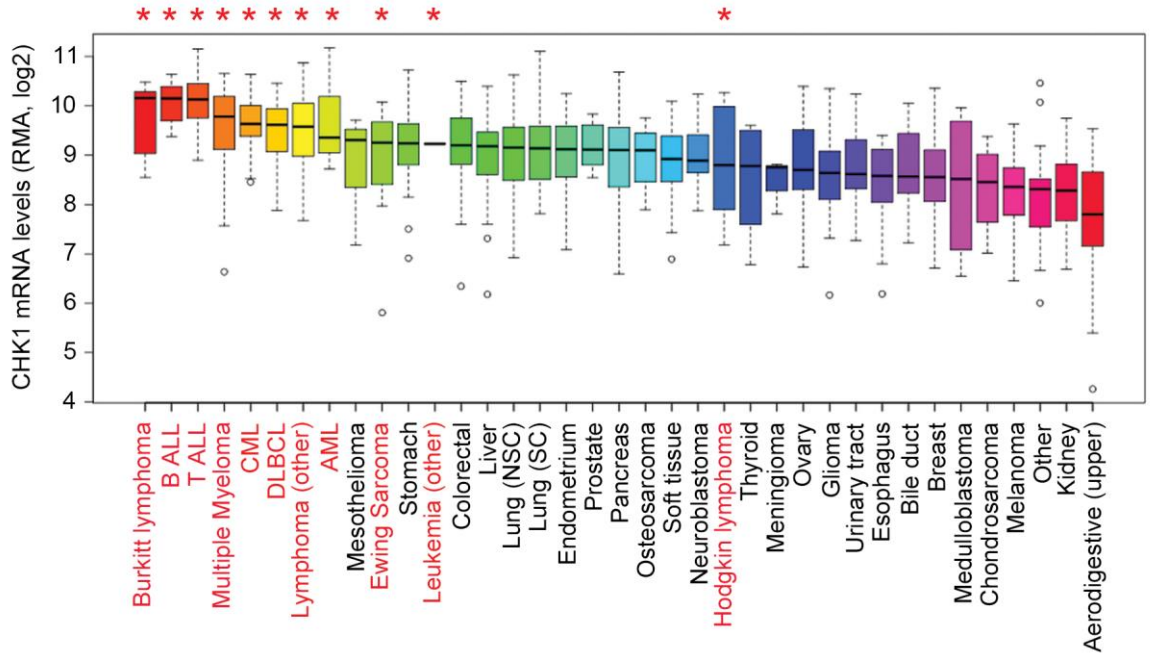

Figure 4. CHK1 mRNA levels in human cancer cell lines. The expression levels of CHK1 mRNA in a large variety of human cancer cell lines was extracted from the Cancer Cell Line Encyclopaedia (www.broadinstitute.org/ccle/home). Leukaemias, lymphomas and Ewing Sarcoma are marked in red, and with an asterisk on top of the panel. Expression is shown as RMA (Robust Multichip Average) on a $\log_2$ scale.

2. ACUTE MYELOID LEUKAEMIA

AML is a highly aggressive and heterogeneous tumour from the hematopoietic system; characterized by the abnormal proliferation of a clonal population of undifferentiated myeloid progenitor cells, which results in impaired haematopoiesis, bone marrow failure and eventually in lower levels of differentiated red blood cells, platelets and white blood cells (Figure 5A) (Döhner *et al*., 2015). AML tumours have high metastatic potential since these abnormally proliferating myeloid progenitor cells are endowed with high capacity of infiltration, mainly within the bone marrow and peripheral blood, but also in other tissues. Remarkably, AML often arises in patients with an underlying haematological disorder or as

a consequence of prior cancer therapy (*e.g.*, exposure to topoisomerases II poisons, alkylating agents or radiation) (Sill *et al.*, 2011), although in most cases, AML arises as a *de novo* malignancy in healthy individuals.

AML occurs at all ages, but predominantly in older people (>60 years of age). Nevertheless, acute leukaemias, including AML and Acute Lymphoblastic Leukaemia, are overall the most common paediatric cancer (Puumala *et al.*, 2013). Remarkably, genomic rearrangements involving the Mixed Lineage Leukaemia (*MLL or KMT2A*) gene are particularly common in infants (ages < 12 months) with AML, and overall, cause very aggressive acute leukaemias that are associated with the poorest prognosis of any acute leukaemia subset. In fact, these *MLL* rearrangements predominately occur in paediatric patients, including 69%-79% of infant leukaemias (Mohan *et al.*, 2010; Meyer *et al.*, 2013), corresponding AML the 30-50% of those cases (Köller *et al.*, 1989; Pui *et al.*, 1994; Borkhardt *et al.*, 2002; Jansen *et al.*, 2007; Pieters *et al.*, 2007; Chen *et al.*, 2010). The *MLL* gene encodes for the histone-lysine N-methyltransferase 2 (Rao and Dou, 2015) and *MLL* rearrangements can fuse these gene in frame to different partner genes (Hess, 2004; Meyer *et al.*, 2013), being the most common

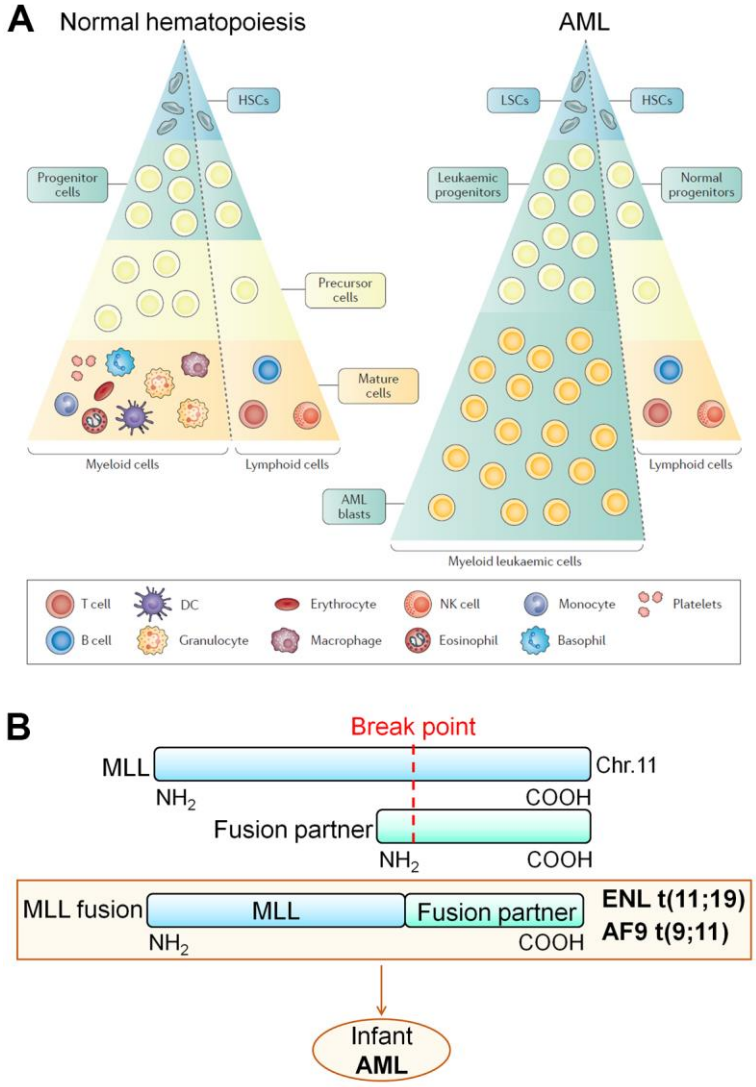

Figure 5. Altered haematopoiesis in AML and structure of MLL fusion proteins. (A) The general hierarchical structure of normal haematopoiesis and aberrant haematopoiesis observed in acute myeloid leukaemia (AML) are shown. HSCs, hematopoietic stem cells; LSCs, leukaemic stem cells; DC, dendritic cell; NK, natural killer (extracted from Khwaja *et al.*, 2016. (B) Graphic representation of structure of MLL fusion proteins generated by MLL translocations. A typical MLL fusion protein contains the N-terminus of MLL and the C-terminus of one of over 50 fusion partner genes, two of the most common chromosomal translocation found in AML are MLL-ENL ((11;19)(q23;p13.3)) and MLL-AF9 ((9;11)(p22;q23).

translocations with the *AF9* (ALL1-fused gene from chromosome 9) and *ENL* genes in the case of AML, thereby creating MLL fusion proteins that ablate the normal histone methyltransferase function of the MLL protein (Figure 5B).

2.1. Prognosis and therapy

Unfortunately, AML is typically associated with a rapid onset of symptoms attributable to bone marrow failure, such as anaemia and thrombocytopenia, and may be fatal within weeks or months if left untreated. In fact, in the case of patients diagnosed before 60 years of age, AML is curable in 35–40% of cases, whereas only 5–15% of those developing AML later involving *MLL* rearrangements are alive five years after diagnosis (Kosaka *et al.*, 2004; Pui *et al.*, 2004; Hilden *et al.*, 2006; Tomizawa *et al.*, 2007).

Patients with AML show a heterogeneous response to therapy. Refractory disease is common and the development of resistance and the high relapse rates with established therapies represents are the major cause of treatment failure (Tomizawa *et al.*, 2007; Döhner *et al*., 2015; Pigneux *et al.*, 2015). The backbone of the current therapy remains a combination of cytarabine- and anthracycline- base regimens combined with allogeneic stem cell transplantation to consolidate remission in those patients who are deemed to be at high risk of relapse (Döhner *et al.*, 2010). While reductions in leukaemic cells can be achieved initially with cytarabine and anthracycline chemotherapy in most patients, long-term outcomes have not improved significantly over the last three decades, suggesting the need to identify new and more effective therapeutic strategies. Consistently, a plethora of new agents, including those targeted at specific biochemical pathways and immunotherapeutic approaches, are now in trial based on improved understanding of disease pathophysiology (review in Khwaja *et al.*, 2016).

2.2. ATR inhibitor as a potential new therapeutic approach for AML-MLL?

Previous studies have established that an intact p53 network is a critical determinant of the effectiveness of chemotherapy in AML (Zuber *et al.*, 2009). In contrast to other oncogenic fusion proteins, cells from AML patients with MLL fusion proteins (AML-MLL) do not mount an effective p53 response and are therefore resistant to current genotoxic treatments (Zuber *et al.*, 2009). Consistent with this, *MLL* rearrangements and mutations in *TP53* gene rarely occur together in human AML (Megonigal *et al.*, 1998; Haferlach *et al.*, 2008). Therefore, alternative therapies are needed to overcome chemotherapy resistance associated with p53 dysfunction in AML-MLL. In addition to the need of a therapy that works on p53-deficient tumours, several lines of evidence suggested that targeting ATR could be particularly beneficial in AML-MLL. First, reduced amounts of ATR in mouse models inhibited

growth of AML driven by the MLL-ENL oncogene, which encodes a fusion of *KMT2A* (Histone-lysine N-methyltransferase 2A; also known as *MLL*) and the transcription activator *ENL* (Schoppy *et al.*, 2012). Second, inhibitors of ATR or its target CHK1 are toxic to human cells and mouse models of several lymphomas and leukaemias, including p53-deficient tumours (Murga *et al.*, 2011; Schoppy *et al.*, 2012; Cottini *et al.*, 2015; Derenzini *et al.*, 2015; Kwok *et al.*, 2015; Sarmento *et al.*, 2015). Moreover, the particular efficacy of RS response inhibitors in lymphoid tumours is consistent with a preferential role for the RS response in the untransformed lymphoid compartment, exemplified by the frequent presence of anaemia in mice suffering from RS (Murga *et al.*, 2009; Austin *et al.*, 2012; Farrés *et al.*, 2014; Flach *et al.*, 2014; Alvarez *et al.*, 2015). Finally, inhibition of ATR or inhibition of the related DDR kinase ATM predisposed primary stem cells infected with retroviruses expressing MLL-AF9, a fusion between KMT2A and the transcription activator AF9, toward differentiation *in vitro* (Santos *et al.*, 2014). On the basis of these data, in these Doctoral Thesis we have evaluated whether inhibition of ATR or ATM could have potential as a therapy for MLL-associated leukaemia.

3. EWING SARCOMA

Ewing Sarcoma, which was first identified by James Ewing in 1921 as a "diffuse endothelioma of bone" (Ewing, 1921), is an aggressive primary bone tumour that is the second most common cancer affecting preferentially children and adolescents. Ewing Sarcoma is characterised by a strong metastatic potential and consequently by an unfavourable prognosis (Ordonez *et al.*, 2009). Ewing Sarcoma current 5-year survival rate, which describes the number of patients alive 5 year after diagnosis of the disease, is of 70% in the case of patients with localised diseased, but is less than 20% for patients with metastatic or recurrent tumours (Bacci *et al.*, 2002; Burdach and Jürgens, 2002; Iwamoto, 2007). The standard care for patients suffering from Ewing Sarcoma is based on a multimodal therapy consisting in surgical resection in combination with local radiotherapy and chemotherapy (Bacci *et al.*, 1998; Burdach and Jürgens, 2002; Iwamoto, 2007; Subbiah *et al.*, 2009). Although this approach was demonstrated to clearly improve prognosis, several studies indicate that the efficacy of this conventional multimodal therapy has reached a plateau phase (Bacci *et al.*, 1998; Craft *et al.*, 1998), indicating the need for new therapeutic strategies.

Ewing Sarcoma tumours arise from a primitive cell derived either from either neural crest or mesenchymal stem cells, which are cells capable of differentiating to various tissue types. Cellular transformation in Ewing Sarcoma is triggered by chromosomal translocation

between the *EWSR1* (Ewing Sarcoma breakpoint region 1, also known as *EWS*) gene and one of the genes of the ETS (E26 transformation-specific) transcription factor family (*FLI1,* Friend Leukaemia Integration 1*; ERG,* ETS-Related Gene; *ETV1,* ETS Translocation Variant 1; *ETV4,* ETS Translocation Variant 4 or *FEV,* Fifth Ewing Variant) (Figure 6A) (Wang *et al.*, 2007). The *EWS/FLI1* fusion is the most common, being found in 85% of the cases, followed by *EWS/ERG* fusion that is found in 10% of cases (Figure 6A). Interestingly, irrespective of the ETS family member involved, all these translocations result in the generation of a chimeric aberrant transcription factor by rearrangement of the N-terminal transcriptional activation domain of the *EWSR1* gene and the C-terminal DNA-binding domain of the ETS gene (Figure 6B) (Ordonez *et al.*, 2009; Balamuth and Womer, 2010; Lessnick and Ladanyi, 2012; Paronetto, 2013). The *EWS/FLI1* translocation is the best characterized, and is thought to operate as an aberrant transcription activator (Ohno *et al.*, 1993) that can alter the expression of genes relevant for malignant transformation. For instance, the EWS/FLI1 protein product upregulates levels of AURKA (Aurora kinase A) (Wakahara *et al.*, 2008), whose overexpression is linked to tumourigenesis (Humme *et al.*, 2015). EWS/FLI1 was also demonstrated to transcriptionally repress FOXO1 (Forkhead box O), which regulates differentiation, proliferation, tumour suppression, autophagy, and cell death (Yang *et al.*, 2010; Niedan *et al.*, 2014).

Remarkably, to date, most of the research on Ewing Sarcoma aetiology is focused on the potential gain of functions of these translocation products such as EWS/FLI1. However, the possible contribution of the potential loss of function of the EWSR1 protein (coded by the *EWSR1* gene) to Ewing Sarcoma development is not well defined and might has been

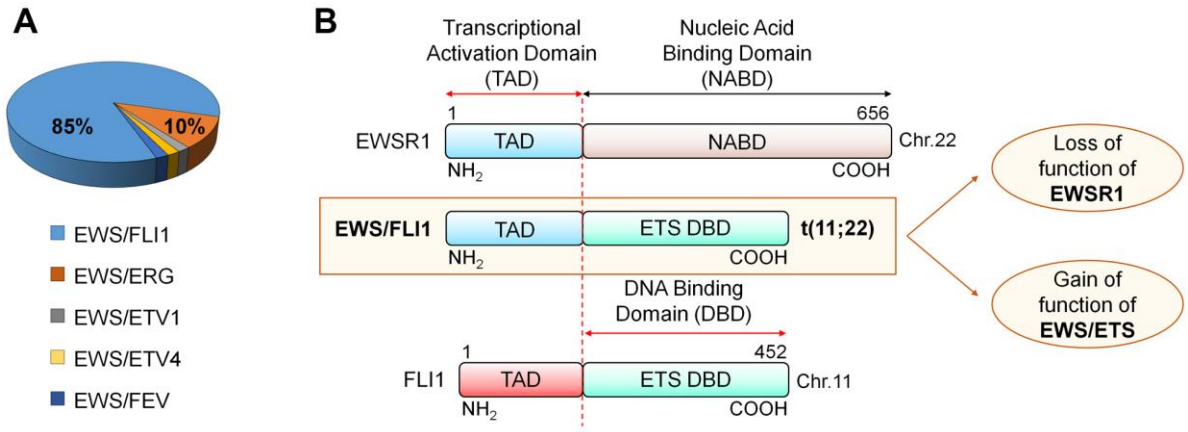

Figure 6. EWS/ETS fusions in Ewing Sarcoma. (A) Frequency of translocation of EWSR1 with the members of the ETS family**.** EWS/FLI fusions comprise 85% and EWS/ERG comprise 10% of all translocations in Ewing Sarcoma. Translocations involving other ETS family members such as ETV4, ETV1 and FEV are less common. (B) Graphical representation of all EWS/ETS translocation. The transcriptional activating domain (TAD) in the N-terminus of EWSR1 is fused to the C-terminal DNA binding domain (DBD) of the ETS family member. The resultant chimeric fusion protein functions as a potent oncogenic transcription factor responsible for tumorigenesis in Ewing Sarcoma (gain of function). It is unknown whether the chimeric fusion protein implies the loss of function of EWSR1.

possibly overlooked (Figure 6B). This could be due to the fact that the role of the EWSR1 protein itself is also not well characterised, and, although most studies suggest that it is related to transcription and splicing (Bertolotti *et al.*, 1998; Knoop and Baker, 2000), other evidence indicate that EWSR1 could also be involved in the DDR. In fact, EWSR1 ablation in mice recapitulates some of the phenotypes observed in ATR-Seckel mice (Li *et al.*, 2007). For all the above reasons, and in addition to the evaluation of ATR inhibitors as a potential new therapy for Ewing Sarcoma, in this work we have also investigated the role of EWSR1 in the response to DNA damage.

3.1. Structure and function of the EWSR1 protein

EWSR1 is a member of the FET family of DNA- and RNA-binding proteins that comprises FUS (Fused in Sarcoma), EWSR1 and TAF15 (TATA-Box Binding Protein Associated Factor 15) (Andersson *et al.*, 2008). Remarkably, all these genes are found rearranged with various transcription factor genes predominantly in sarcomas and in rare hematopoietic and epithelial cancers (Figure 7A) (Ladanyi and Gerald, 1994; Panagopoulos *et al.*, 1996; Labelle *et al.*, 1999; Thomsen *et al.*, 2013). In fact, the EWSR1 gene was first identified as a fusion gene generated by the chromosomal translocation with FLI1 ((11;22)(q24;q12)) in Ewing Sarcoma (Figure 6B) (Aurias *et al.*, 1983; Delattre *et al.*, 1992). FET proteins are ubiquity expressed and mainly localise to the nucleus (Rossow and Janknecht, 2001; Andersson *et al.*, 2008); although their localization is dynamically regulated because stress conditions trigger their accumulation in cytoplasmic stress granules involved in translational control (Andersson *et al.*, 2008). All FET members display a similar structure with a serine-tyrosine-glycine-glutamine (SYGQ) domain in the N-terminal, which constitutes the transcription activation domain (TAD), and a C-terminal domain with an RNA-recognition motif (RRM), a zinc finger motif (ZF), and three RNA-binding Arginine-Glycine-Glycine- (RGG-) rich domains (Figure 7B) (Morohoshi *et al.*, 1998). Several of these domains enable EWSR1 to bind different nucleic acid sequences and structures such as polyU and polyG sequences (Ohno *et al.*, 1994) DNA and RNA forming G-quadruplexes (Takahama *et al.*, 2011; Oyoshi and Kurokawa, 2012), and ssDNA (Paronetto *et al.*, 2011).

Although the function of EWSR1 is not fully understood, EWSR1 is known to be involved in several cellular processes and has a well-established role in transcription and splicing, possibly coupling these two processes. EWSR1 role in transcriptional regulation was suggested by virtue of its capacity to interact with different subunits of the transcription factor II D (TFIID) (Bertolotti *et al.*, 1998), its direct association with RNA polymerase II (Bertolotti *et al.*, 1996, 1998) and its interaction with activators and repressor of transcription such as OCT4 (Octamer-binding transcription factor 4), Brn3 (Class IV (Pit1-Oct1-Unc86)-

domain-containing transcription factor), and CBP/p300 (CREB-Binding Protein and p300) (Fujimura *et al.*, 2001; Thomas and Latchman, 2002; Araya *et al.*, 2003; Gascoyne *et al.*, 2004; Lee *et al.*, 2005). On the other hand, EWSR1 potential role in splicing was suggested based on EWSR1 capability to interact with components of the spliceosome (Knoop and Baker, 2000) and different splicing factors, some of which play a role in mRNA stability and translation (Yang, 2000; Chansky *et al.*, 2001; Dutertre *et al.*, 2010). Additionally, EWSR1 has been linked to microRNA processing (Sohn *et al.*, 2012) because of its identification as part of a complex associated with the ribonuclease DROSHA (Gregory *et al.*, 2004), which is required for microRNA biogenesis (Lee *et al.*, 2002).

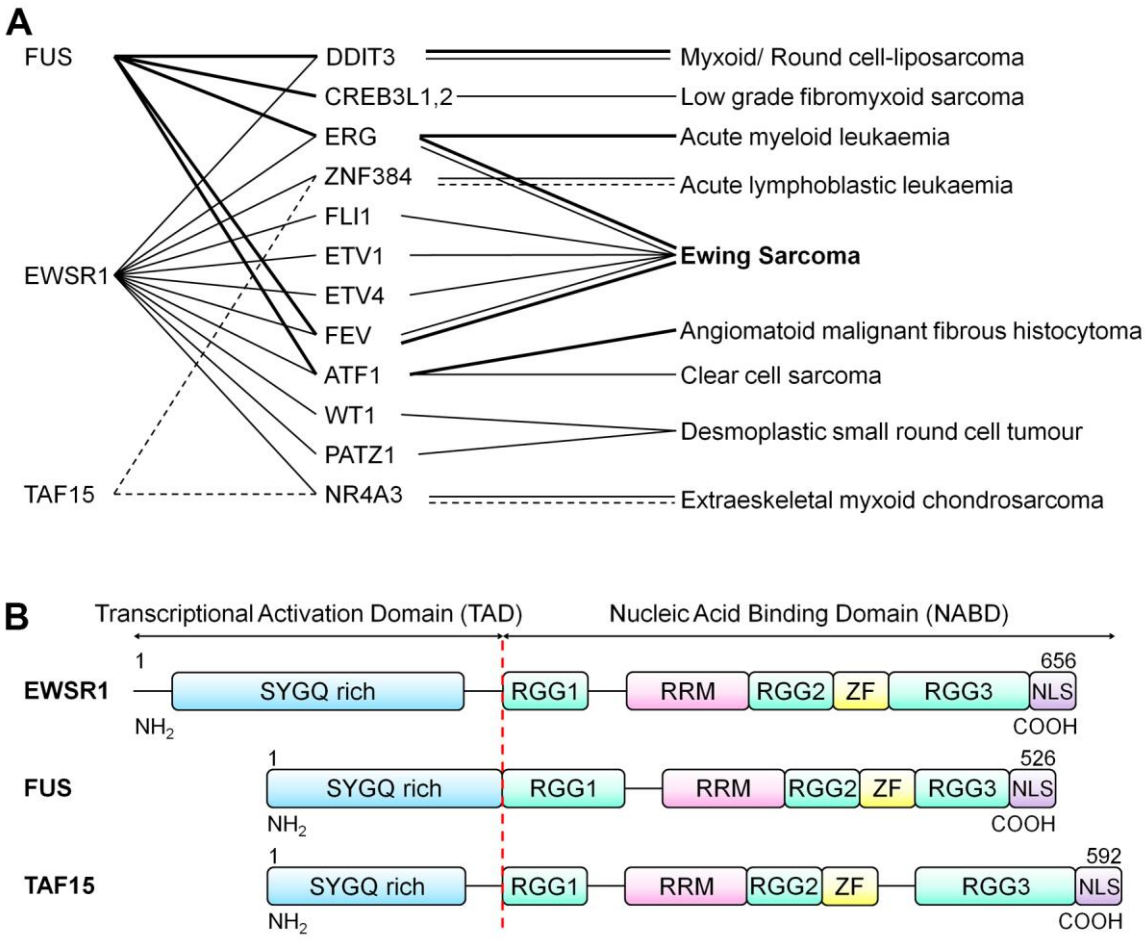

Figure 7. The FET family of genes (*FUS, EWSR1 and TAF15*) and their associated with cancer. (A) The N-terminal parts of FET genes are fused to genes encoding various transcription factors (middle column) and the fusion oncogenes generated are associated to different tumour types (right column). It should be noted that the figure is not complete, as more FET gene fusions are continuously discovered. (Extracted from Thomsen *et al.*, 2013). (B) Structural organisation of FET family proteins. From N-terminus to C-terminus: a transcriptional activation domain (TAD) containing multiple degenerate hexapeptide repeats (SYGQ rich; glycine, glutamine, serine, tyrosine) and a nucleic acid binding domain (NABD) containing three arginine/glycine rich domains (RGG regions; arginine/glycine/glycine), a RNA recognition motif (RRM), a Zinc finger (ZF) and a nuclear localization signal (NLS) (Adapted from Paronetto, 2013).

3.2. A possible role for EWSR1 in the DDR

Although EWSR1 function is thought to be mainly associated with transcription and splicing, several lines of evidence indicate that EWSR1 may also play a role in the DDR either indirectly, related to these transcription and splicing functions, or also directly. Of note and importantly, EWSR1 role in the DDR is supported by the fact that it becomes phosphorylated upon DNA damage, although the functional implication of this modification remains unknown (Klevernic *et al.*, 2009). Moreover, EWSR1 is involved in the DDR through the regulation of alternative mRNA splicing of genes involved in the activation of the DNA damage checkpoint and for DNA repair such as the p53 repressor MDM2 (mouse double minute 2 homolog), the tumour suppressor BRCA1, CHK2 (checkpoint kinase 2) and ABL1 (Abelson murine leukaemia viral oncogene homolog 1) (Dutertre *et al.*, 2010; Paronetto *et al.*, 2011). Finally, recent evidences suggest that EWSR1 could be directly involved in DNA repair, although its exact role is still obscure. For instance, all three member of the FET family were demonstrated to be recruited to laser microirradiation stripes in a PARP-dependent manner (Mastrocola *et al.*, 2013; Rulten *et al.*, 2014; Altmeyer *et al.*, 2015), and PARP1 (poly (ADP-ribose) polymerase 1) was shown to be a cofactor for EWS/FLI1 DNA binding (Brenner *et al.*, 2012). EWSR1 was also suggested to be involved in DNA repair reactions during HR based on its ability to interact with BARD1 (BRCA1-associated RING domain protein 1), a putative tumour suppressor component of the BRCA1/BARD1 complex (Spahn *et al.*, 2002). EWSR1 has been identified as a gene mediating resistance to DSBs inducing agents such ionizing radiation (Hurov *et al.*, 2010) and camptothecin, a topoisomerase I poison capable of trapping topoisomerase I-cleavage complexes in the DNA (O'Connell *et al.*, 2010). In any case, while all of the above suggest a role for EWSR1 in the DDR, the mechanism by which it participates remains unclear.

3.3. ATR inhibitor as a potential new therapeutic approach for Ewing Sarcoma?

Current Ewing Sarcoma treatment relies on a multidisciplinary approach consisting of intensive neoadjuvant and adjuvant chemotherapies with surgery and/or radiotherapy to limit possible metastatic processes. Interestingly, chemotherapy treatment is based in the administration of various combinations of drugs that include RS-inducing agents. These include vincristine (which interferes with correct chromosome segregation), actinomycin D (a transcription inhibitor) and agents that perturb DNA replication such as doxorubicin (capable of interfering with topoisomerase II), etoposide (a topoisomerase II poison), cyclophosphamide (an inducer of interstrand and intrastrand DNA crosslinks), and ifosfamide (an alkylating agent) (reviewed Gaspar *et al.*, 2015). More recently, PARP1 inhibitors has been described as a possible additional treatment for Ewing Sarcoma (Garnett *et al.*, 2012;

Vormoor and Curtin, 2014), and this is in agreement with the fact that Ewing Sarcoma models are highly sensitive to the PARP1 inhibitor Olaparib either alone or in combination with temozolomide (an alkylating agent) (Brenner *et al.*, 2012). However, single agent clinical trials have not been successful and combined chemotherapy of PARP inhibitors with DNA damaging drugs is still under investigation (Choy *et al.*, 2014; Vormoor and Curtin, 2014; Smith *et al.*, 2015). There are also ongoing trials aiming to use irinotecan (a topoisomerase I inhibitor) and temozolomide for patients with advanced Ewing Sarcoma (Wagner *et al.*, 2007; Casey *et al.*, 2009). Regardless of genotoxic chemotherapy, insulin-like growth factor 1 receptor (IGFR-1) antibodies are also being used to target Ewing Sarcoma with the purpose of rendering treatments much more specific and to reduce side effects (Olmos *et al.*, 2010; Toretsky and Gorlick, 2010). In spite of these multiple approaches, it is very important to remark that treatment efficacy for patients with metastatic and recurrent Ewing Sarcoma still remains dramatically poor. Therefore, a better understanding of Ewing Sarcoma biology is critical to understand identify novel therapies and also to unveil mechanisms of resistance. Remarkably, several lines of evidence led us to hypothesise that Ewing Sarcoma tumours might be suffering from RS, and that consequently, ATR inhibitors could be used as single chemotherapy agent for the treatment of these tumours. First, as mentioned earlier, several pieces of evidence indicate that EWSR1 may be involved either directly or indirectly in the DDR, which could render these tumours more dependent on ATR signalling for survival. This could be in line with the aforementioned fact that Ewing Sarcoma display increased levels of CHK1 mRNA expression, as shown by the data from the human Cancer Cell Line Encyclopaedia (Figure 4) (Barretina *et al.*, 2012). Second, EWSR1-deficient mice present increased DNA damage, anaemia and skeletal abnormalities (Li *et al.*, 2007; Cho *et al.*, 2011); paralleling the phenotype of mice with reduced ATR levels that accumulate substantial amounts of RS (Murga *et al.*, 2009). Finally, the EWS/FLI1 translocation product is a *bona fide* oncogene due to its capacity to transform mouse fibroblasts (May *et al.*, 1993) and, as mentioned earlier, oncogenes are a known source of RS (Halazonetis, 2008). Altogether, for all these reasons, we speculate that Ewing Sarcoma could be harbour distinctively high levels of RS and that, consequently, ATR inhibitors could use as single chemotherapy agents for the treatment of Ewing Sarcoma.

OBJECTIVES

During the course of this Doctoral Thesis we set the following objectives

1. To evaluate the toxicity of ATR inhibitors for MLL-translocation driven AML cells *in vitro*.

2. To investigate whether ATR-induced cell death in AML cells depends on p53 status.

3. To test the efficacy of ATR and ATM inhibitors in a preclinical allograft mouse model of MLL-translocation driven AML.

4. To determine the toxicity of ATR inhibitors for Ewing Sarcoma cells *in vitro*.

5. To test the efficacy of ATR inhibitors as single agents in a xenografts model of Ewing Sarcoma.

6. To investigate whether the loss of EWSR1-function and/or the expression of EWSR1-involving translocation lead to RS and confer sensitivity to ATR inhibitors.

7. To perform a proteomic analysis of EWSR1 interactors aiming to identify the biological pathways in which EWSR1 participates.

MATERIALS AND METHODS

1. MOUSE WORK

1.1. Generation of an inducible EWS/FLI1-CreER mouse model

Lox/STOP/lox-EWS/FLI1 transgenic mice (Lin *et al.*, 2008) were crossed with inducible CreER recombinase mice (EWS/FLI1^{ind}). Expression of Cre recombinase is induced by tamoxifen administration resulting in deletion of the STOP cassette and expression of the EWS/FLI1 translocation.

1.2. Mouse genotyping

DNA from tail preparations was used to determine the genotype of EWS/FLI1^{ind} mice as previously described in (Lin *et al.*, 2008). Primers for EWS/FLI1 spanning the junction site amplify a product of 567bp, and sequences are: EF2 forward 5´GACCGCCTATGCAACTTCTTATGG and EF2 reverse 5´ TGGGGCCGTTGCTCTGTATTCTTA. Primers for CreER spanning the junction site amplify a product of 350bp, and sequences are: CRE1 forward 5´CGATGCAACGAGTGATGAGGTTC y CRE2 reverse 5´GCACGTTCACCGGCATCAAC. This produced a product of 350 bases.

1.3. Transplantation and *in vivo* treatment studies

For tumour induction and treatment studies, 10^5 AML^{MLL} cells were transplanted by tail vein injection into 8- to 12-weeks-old immunocompetent albino recipient mice (C57BL/6/BrdCrHsd-Tyrc). For xenografts experiments, 1.4×10^5 MV4:11 cells or 10^6 A4573 cells were injected subcutaneously into the flanks of 8- to 10-weeks-old severe combined immuno-deficient (SCID) mice (CB17/lcr-Prkdc scid/Crl), and tumour growth was measured with a Vernier calliper. Tumour volumes (mm^3) in xenografts were calculated according to the following formula: (width x $(length)^2$)/2.

Mice were treated daily with ATRi [60 mg/kg; dissolved in 10% Nmethyl-2-pyrrolidone (NMP; 443778, Sigma- Aldrich), 90% polyethylene glycol 300 (PEG-300; 202371, Sigma-Aldrich)], ATMi [20 mg/kg; dissolved in 10% dimethyl sulfoxide (DMSO; Sigma), 90% Captisol (30%) (CYDEX)] or MSC253 [50 mg/kg; (kind gift from Merck KGaA, Darmstadt, Germany) dissolved in 10% NMP, 50% PEG-300 and 40% H_2O sterile], and the corresponding vehicles by oral gavage.

Health status of mice was monitored daily. Mice were maintained at the CNIO under standard housing conditions with free access to chow diet and water, in recommendation of the Federation of European Laboratory Animal Science Association. All mouse work was

performed in accordance with the Guidelines for Humane Endpoints for Animals Used in Biomedical Research and under the supervision of the Ethics Committee for Animal Research of the Instituto de Salud Carlos III.

1.4. Monitoring of leukaemias

To monitor tumour formation, AML^{MLL} transplanted mice were monitored every 3 to 4 days, starting 5 days after the injection of tumour cells, by bioluminescent imaging with an *in vivo* imaging system (IVIS) spectrum. Mice were intraperitoneally injected with 150 mg/kg of D-luciferin (Perkin-Elmer), anesthetized with isoflurane, and imaged for 30 seconds after 5 min after luciferin injection. For bioluminescence analysis of organs, mice were injected with D-luciferin and euthanized by CO_2, and organs were collected and imaged for 5 seconds. Blood samples were obtained from heart and collected in ethylenediaminetetraacetic acid (EDTA) -treated microtubes (Aquisel) and run on an Abacus Junior Vet haematology analyser (Diatron), which provides complete blood analyses, including counts of leukocytes and platelets. To measure the persistence of AML^{MLL} cells, we isolated bone marrow from vehicle, ATRi- or ATMi-treated animals by flushing femurs and tibias [Roswell Park Memorial Institute (RPMI)-1640 (EuroClone) medium supplemented with 10% (v/v) foetal bovine serum (FBS; Sigma-Aldrich) and 1% (v/v) penicillin/streptomycin (Pen/Strep; Life Technologies)]. Erythrocyte lysis was performed by treating bone marrow cells with a commercial ACK (ammonium-chloride-potassium) red lysis buffer (Lonza) for 5 minutes at room temperature. Cells were stained with c-KITAPC-H7 antibody (BD Biosciences) and analysed by fluorescence activated cell sorting (FACS) in FACSCanto II (BD Biosciences; FACSDiva software). Data were analysed with FlowJo (Tree Star) software.

1.5. Immunohistochemistry (IHCs)

Tissues were collected, fixed in formalin and embedded in paraffin/formalin blocks for subsequent processing. Consecutive 2.5 µM sections were treated with citrate for antigenic recovery and processed for immunohistochemistry with an antibody against p-Ser139 H2AX (05-636, Millipore) following standard procedures. IHCs were scanned and digitalized with a MIRAX system (Zeiss) for further analysis. p-Ser139 H2AX positive cells were automatically quantified from digitalized slides with AxioVision 4.6.3 software (Zeiss).

1.6. Statistical analyses

Prism 5.0 (GraphPad Software) was used to perform statistical analyses and representation of data. One on one comparisons of normal distributions were performed using unpaired t-tests. Two-way ANOVA test was used for comparison of growth kinetics of

treated tumours in xenografts experiments. Unless otherwise stated, all data points per condition are shown. Bar graphs represent the mean ± s.d.

2. CELLULAR BIOLOGY

2.1. Generation of primary Mouse Embryonic Fibroblasts (MEF) cell culture

For the generation of primary EWS/FLI1^{ind} MEFs, pregnant female mice were euthanized in a CO_2 chamber at 13.5 dpc. Each embryo was isolated inside a laminar airflow hood with a pair of forceps and tweezers, and extraembryonic layers and foetal liver were discarded. The embryo head was cut below the eye for genotyping. The remaining embryo tissue was transferred to a 35mm dish, chopped with a sterile blade and incubated for 10 minutes in 1 ml 0.25% trypsin-EDTA (Life Technologies). The resulting mixture was disaggregated by pipetting up and down and trypsin was neutralized with 9 ml cell culture media in Dulbecco´s Minimum Essential Media (DMEM; Lonza) supplemented with 15% (v/v) FBS (Sigma-Aldrich) and 1% (v/v) Pen/Strep (Life Technologies). Cell suspension was transferred to a 10cm dish and incubated under hypoxia conditions (3% O_2 and 5% CO_2) at 37ºC and medium was changed on the following day.

2.2. Generation of human 293T-Rex Flp-In^{TM} Tet-ON stable cell lines

Human stable cell lines 293T-REx Flp-In^{TM} Tet-ON cells (Life Technologies) (were Flp-In^{TM} is a Flippase recombinase-mediated integration and Tet-ON a tetracycline-controlled transcriptional activation) expressing EWSR1^{STAG}, EWS/FLI1^{STAG} or Empty^{STAG} (where STAG is a C-terminal streptavidin tag) were generated following the manufacturer´s instructions. Cells that integrated the gene of interest were selected with 400 µg/ml Hygromycin B (Calbiochem) for 7 days. Recombinant protein expression was induced by adding 200 ng/ml doxycycline to culture medium for 48 hours.

2.3. Cells and reagents

Mouse AML cells carrying the MLL-ENL translocation (plus IRES-GFP) and oncogenic N-RAS (luciferase-IRES-N-RAS^{G12D}), referred to in the text as AML^{MLL}, were developed as previously described (Zuber *et al.*, 2009). MV4:11, K562, HUVEC, U2OS, SAOS-2, 293T cells and Human Foreskin Fibroblast (HFF) were obtained from the American Type Culture Collection (ATCC) and 293T-REx Flp-In^{TM} Tet-ON were obtained from Life Technologies. Immortalized MEFs (EWSR1$^{-/-}$ and WT) were provided by Dr. Sean Bong Lee (National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), USA), AML^{ETO} cells were kindly provided by Scott A. Armstrong (Dana-Farber Cancer Institute, USA) and all Ewing

Sarcoma cell lines were kindly provided by Dr. Enrique de Alava (Instituto de Biomedicina de Sevilla (IBiS), Spain).

AML^{MLL}, MV4:11, K562, SAOS-2 and Ewing Sarcoma cell lines used in this study were cultured in RPMI-1640 (EuroClone) supplemented with 10% (v/v) FBS (Sigma-Aldrich) and 1% (v/v) Pen/Strep (Life Technologies). AML^{ETO} were culture in DMEM (Lonza) supplemented with 15% (v/v) FBS (Sigma-Aldrich), 20 ng/ml mouse Stem Cell Factor (mSCF; BioLegend), 10 ng/ml recombinant murine interleukin-6 (mIL-6; 216-16, PeproTech), 6 ng/ml recombinant mouse interleukin-3 (mIL-3; 403-ML-010, R&D systems) and 1% (v/v) Pen/Strep (Life Technologies). U2OS, 293T and 293T-REx Flp-In™ Tet-ON cells were cultured in DMEM (Lonza) supplemented with 10% (v/v) FBS (Sigma-Aldrich) and 1% (v/v) Pen/Strep (Life Technologies). HUVEC cells were cultured in a commercial kit of Endothelial Cell Growth Medium containing 2% (v/v) FBS and vascular endothelial growth factor (VEGF) for rapid proliferation (EMG-2 BulletKit; CC-3162, Lonza). Primary and immortalized MEFs and HFF were grown in DMEM (Lonza) supplemented with 15% (v/v) FBS (Sigma-Aldrich) and 1% (v/v) Pen/Strep (Life Technologies). For all experiments, primary MEF and HFF were used at a low passage (<3). All MEFs and HFF were grown in hypoxia (3% O_2 and 5% CO_2) to minimize exposure to reactive oxygen species while the rest of cells lines were cultured under standard conditions (20% O_2 and 5% CO_2).

Doxycycline (Pancreac Applichem), (Z)-4-Hydroxytamoxifen (OHT; Sigma-Aldrich), ATR inhibitor (AZ20; synthesized by GVK BIO), ATR inhibitor[2] (Toledo *et al.*, 2011), ATM inhibitor (AZD0156; AstraZeneca), PARP1 inhibitor (Olaparib; AstraZeneca), Neocarzinostatin (Sigma-Aldrich) and Hydroxyurea (HU; Sigma-Aldrich) were used as indicated.

2.4. Transfection and retroviral infection

The retroviral plasmid expressing a p53-targeting short hairpin RNA (pRetroSuper p53shRNA) was kindly provided by M. Barbacid (CNIO) and the retroviral pMYs-EWS/ERG expression plasmid was kindly provided by Takuro Nakamura (The Cancer Institute of JFCR). Transfections of plasmids and esiRNAs were performed with Lipofectamine® 2000 (Invitrogen) or Lipofectamine® RNAiMAX (Invitrogen) using OPTIMEM medium (Life Technologies) and following manufacturer´s instruction. Retrovirus for transduction were produced in 293T cells using the corresponding plasmid and the retrovirus packaging vector pCL-Eco (kindly provided by Manuel Serrano, CNIO) using standard methods.

2.5. Colonies survival assays

For clonogenic assays, 500 cells were seeded per well on six-well plates and drugs were added 24 hours later. After 10 days for human cells or 5 days for MEFs, cells were fixed and stained with methylene blue at 0.33% (w/v) in methanol, subsequently washed in water and air-dried. Images were taken with a Canon scanner.

2.6. Flow cytometry

To measure viability, cells were collected [with 0.05% Tripsyn-EDTA (Life Techologies) in the case of adherent cells], washed once with phosphate buffer saline (PBS) (pH 7.4), stained in a 4´,6´-diamidino-2-phenylindole (DAPI) solution [DAPI (0.2 mg/ml) in PBS], and analysed by flow cytometry in a FACSCanto II (Becton- Dickinson) machine. For cell cycle profiles, cells were collected, washed with PBS, and fixed in suspension in ice-cold 70% (v/v) ethanol in water. After washing in PBS, cells were stained in PBS containing propidium iodide (10 mg/ml) and ribonuclease A (0.5 mg/ml) and analysed in a Becton-Dickinson FACSCalibur machine. For DNA content and γH2AX analysis, p-Ser139 H2AX (Millipore) antibody was used as previously described (Ruiz *et al.*, 2016). Data was analysed with FACSDiva (BD Biosciences) and FlowJo (Tree Star) softwares.

2.7. DNA fibre analyses

Cells were pulse-labelled with 50 µM chlorodeoxyuridine (CldU) (20 min) followed by 250 mM iododeoxyuridine (IdU) (20 min). Labelled cells were collected, and DNA fibres were spread in buffer containing 0.5% (v/v) sodium dodecyl sulfate (SDS), 200 mM Tris-HCl (pH 7.4), and 50 mM EDTA. For immunodetection of labelled tracks, fibres were incubated with primary antibodies [for CldU, rat anti-BrdU (bromodeoxyuridine); for IdU, mouse anti-BrdU] for 1 hour at room temperature and developed with the corresponding secondary antibodies for 30 min at room temperature. Mouse anti–single-stranded DNA antibody was used to assess fibre integrity. Slides were examined with a Leica DM6000 B microscope, as described previously (Jacome *et al.*, 2015). The conversion factor used was 1 mm = 2.59 kb (Jackson and Pombo, 1998).

2.8. Endonuclease-prepared Small Interfering RNAs (esiRNAs) screen

Stable 293T-REx Flp-In^{TM} Tet-ON cells expressing EWS/FLI1^{STAG} (293$^{EWS/FLI1}$) were transfected with a custom-made esiRNA library (Table 1, Sigma-Aldrich). EWS/FLI1^{STAG} expression was induced 24 hours after transfection. 3 days after doxycycline induction (4 days after transfection), cells were processed and analysed through High-Throughput

microscopy.

2.9. Immunofluorescence and High-Throughput microscopy

Cells were seeded on μClear® bottom 96-well plates (Greiner Bio-One) pre-treated with 0.1% gelatine. For cell viability screening using the esiRNA library, cells were fixed with 4% paraformaldehyde (PFA) and DAPI was used to counterstain cells. For analysis of DNA replication by 5-Ethynyl 2´-deoxyuridin (EdU) incorporation, cells were treated with EdU for 30 min and fixed with 4% PFA in PBS at room temperature for 10 min. Then, cells were permeabilized with 0.1% Triton-X100 in PBS at room temperature for 15 min. The EdU staining was done using Click-It EdU Cell proliferation Assay Kit (Life Technologies) following manufacturer's instructions to stain the incorporated nucleoside. In all cases, images were automatically acquired from each well using an Opera High-Content Screening System (HCS OPERA, Perkin Elmer). A 20x water magnification lens was used and images were taken at non-saturating conditions. Images were segmented using the DAPI signal to generate masks matching cell nuclei from which the mean signals for the rest of the staining (EdU) were calculated. Data were represented with the use of the Prism software (GraphPad Software).

2.10. Cell viability assays

Cell Proliferation Kit II (XTT) (Roche) and CellTiter-Glo® Luminescent Cell Viability Assay (Promega) kits were used to calculate the median lethal dose (LD50) values or to asses cytotoxicity of Ewing Sarcoma lines, respectively.

3. MOLECULAR BIOLOGY AND BIOCHEMISTRY

3.1. DNA extraction

For genotyping, tails from pre-weaned mice or head fragment from embryos were lysed in 50 mM Tris-HCl (pH 8), 100 mM NaCl, 5 mM EDTA and 1% SDS (10%) containing 0.5% (v/v) proteinase K (Roche) at 55°C. Then, proteins were precipitated with 6 M NaCl and separated by centrifugation. DNA was precipitated by adding isopropanol to the supernatant and isolated through centrifugation. Finally, DNA was washed with 70% (v/v) ethanol and re-suspended in water.

3.2. Plasmid construction

For the construction of pcDNA5/FRT/TO-EWSR1^{STAG}, –EWS/FLI1^{STAG} or Empty^{STAG} inducible expression vectors (where FRT is a Flippase recombination target), the coding sequence of human variant 2 of EWS or EWS/FLI1 fusion type 1 (h–EWS or –EWS/FLI1)

was amplified by PCR (Polymerase Chain Reaction) from human cDNA and cloned into the pEXPR-IBA103 (Novagen) STAG expression vector at SacII/XhoI sites. From there, the EWSR1^{STAG}, EWS/FLI1^{STAG} or Empty^{STAG} sequence were PCR amplified adding AfII/NotI restriction sites for subsequent cloning into the pcDNA5/FRT/TO vector (Life Technologies).

3.3. Protein extraction and Western blotting

For total protein extract preparation, cells were collected, washed once with PBS, and lysed in urea buffer [8 M urea, 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and 50 mM Tris-HCl (pH 8.0)] for 30 minutes at 4°C with agitation or lysed in buffer 50 mM Tris-HCl (pH 7.4), 0.2% (v/v) Nonidet-P40, 200 mM NaCl, 50 MM Glicerophosphate and 1% (v/v) Tween-20, containing protease and phosphatase inhibitors (Sigma). After lysis, soluble protein extracts were separated by centrifugation. Bradford method was used to determine protein concentration. NuPAGE LDS (Life Technologies) loading buffer was added to protein extract and samples were incubated for 10 minutes at 70 ºC. Samples were resolved by SDS-polyacrylamide gel electrophoresis and analysed by standard western blotting techniques.

The following primary antibodies were used: p-Ser345 CHK1 (2348S, Cell Signaling), CHK1 (NCL, Novacastra), p-Ser4/Ser8 RPA32 (A300-245A, Bethyl), RPA32 (2208, Cell Signaling), p-Ser139 H2AX (05-636, Millipore), PRMT1 (A300-723A, Bethyl) p-SMC1 [Monoclonal Antibody Unit, Spanish National Cancer Centre (CNIO)], p-Ser824 KAP-1 (Bethyl), PARP1 (9542S, Cell Signaling), p53 (2524, Cell Signaling), CDK2 (sc-163, Santa Cruz), EWSR1 (sc-6533 and sc-28327, Santa Cruz), FLI1 (sc-356, Santa Cruz), TUBULIN (T9026, Sigma) and β-ACTIN (A5316, Sigma). Alexa Fluor 680– or Alexa Fluor 800–conjugated secondary antibodies (Life Technologies) were used for detection with a LI-COR Odyssey infrared imaging system (LI-COR Biosciences) or Horseradish peroxidase (HRP)-conjugated secondary antibodies were used for detection with SuperSignal™ West Pico Chemiluminescent Substrate HRP (34080, Fisher Scientific).

3.4. Subcellular fractionation

Cells were collected, washed with PBS and lysed in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.9), 10 mM potassium chloride (KCl) and 0.1 mM EDTA containing protease and phosphatase inhibitors for 10 min on ice. For complete disruption of the cytoplasmic membrane, 10% (v/v) of 1% Nonident-P40 (NP-40) was added and cells were incubated for 3 minutes at room temperature. Then, samples were vortexed for at least 10 seconds and cytoplasm was separated by centrifugation (2500g for 2 minutes

at 4ºC). The pellet containing the nuclei was washed once with lysis buffer in the absence of detergent. Extraction of nuclear proteins was performed by addition of 20 mM HEPES (pH 7.9), 600 mM NaCl, 1mM EDTA containing protease and phosphatase inhibitors to pelleted nuclei and vortexing for 10 min at 4ºC. The nuclei were sonicated briefly (until clear) and incubated at 4ºC for an additional 30 minutes followed by centrifugation at 16000g for 5 min at 4ºC. The cleared nuclear extract diluted to 1 mg/ml and the NaCl concentration was adjusted to 200 mM.

3.5. Protein purification by affinity chromatography

2 mg of total protein from nuclear extracts were loaded on a Bio-Spin disposable chromatography column (Bio-Rad) and incubated with Streptavidin-Tactin® MacroPrep® Resins (Iba) at 4°C for 1h with rotation. The column was washed twice with buffer W-astringent (100 mM Tris-HCl (pH 8), 1 mM EDTA, 350 mM NaCl) containing 0.1% NP-40 and once with buffer W (100 mM Tris-HCl (pH 8), 1 mM EDTA, 200 mM NaCl) without NP-40. EWSR1^{STAG} and associated proteins were eluted with 2 mM biotin in buffer W. Fractions containing EWSR1^{STAG} were analysed in a 4-12% SDS- polyacrylamide gel and protein staining with Imperial^{TM} Protein-Stain (Thermo Scientific). Selected fractions were analysed using liquid chromatography and mass spectometry by the CNIO Proteomics Unit.

3.6. Liquid Chromatography and mass spectometry (LC-MS/MS)

Samples were digested (Wisniewski *et al.*, 2009) and the resulting peptides were separated by reverse-phase chromatography using a nanoLC Ultra system (Eksigent), directly coupled with a LTQ-Orbitrap Velos instrument (Thermo Fisher Scientific) via nanoelectrospray source (ProxeonBiosystem). The peptides were directly electrosprayed into the mass spectrometer and mass spectra were acquired in a data-dependent manner, with an automatic switch between MS and MS/MS scans using a top 10 method with a threshold signal of 800 counts. Samples were analysed in duplicates. Raw files were analysed either by Proteome Discoverer (version 1.4.1.2) or by MaxQuant (Cox and Mann, 2008) (v1.4.1.2) against a forward-reverse concatenated database. The database contained human proteins (UniProtKB/Swiss-Prot 39,748 sequences, downloaded on January 2014) and a list of common contaminants. Minimal peptide length was set 6 amino acids and a maximum of two missed-cleavages were allowed. Only proteins with a fold-change above 1 (log2) were considered as regulated. Protein classification enrichment analysis (molecular function, biological process and protein class) was performed by STRING software.

Table of Endonuclease-prepared Small Interfering RNAs (esiRNAs) library					
Catalogue-#	ID	ID (Ensembl)	ID (RefSeq)	Gene name	Organism
EHU115631-1UG	HU-11563-1	ENSG00000182944	NM_005243 NM_013986	EWSR1	Homo sapiens
EHU115751-1UG	HU-11575-1	ENSG00000126457	NM_198318 NM_198319	PRMT1	Homo sapiens
EHU029581-1UG	HU-02958-1	ENSG00000011243		AKAP8L	Homo sapiens
EHU133931-1UG	HU-13393-1	ENSG00000092199	NM_001077442 NM_001077443 NM_004500 NM_031314	HNRNPC	Homo sapiens
EHU084371-1UG	HU-08437-1	ENSG00000159140	NM_032195 NM_138927	SON	Homo sapiens
EHU111261-1UG	HU-11126-1	ENSG00000183684	NM_005782	THOC4	Homo sapiens
EHU129271-1UG	HU-12927-1	ENSG00000108654	NM_004396	DDX5	Homo sapiens
EHU067811-1UG	HU-06781-1	ENSG00000109606	NM_001358	DHX15	Homo sapiens
EHU137681-1UG	HU-13768-1	ENSG00000164548	NM_013293	TRA2A	Homo sapiens
EHU010631-1UG	HU-01063-1	ENSG00000172660	NM_139215	TAF15	Homo sapiens
EHU135921-1UG	HU-13592-1	ENSG00000130254	NM_014649	SAFB2	Homo sapiens
EHU109681-1UG	HU-10968-1	ENSG00000135829	NM_001357	DHX9	Homo sapiens
EHU110901-1UG	HU-11090-1	ENSG00000108468	NM_006807	CBX1	Homo sapiens
EHU082261-1UG	HU-08226-1	ENSG00000171490	NM_015659	RSL1D1	Homo sapiens
EHU131531-1UG	HU-13153-1	ENSG00000115524	NM_001005526 NM_012433	SF3B1	Homo sapiens
EHU067331-1UG	HU-06733-1	ENSG00000130726	NM_005762	TRIM28	Homo sapiens
EHU114521-1UG	HU-11452-1	ENSG00000188529	NM_054016 NM_006625	FUSIP1	Homo sapiens
EHU091931-1UG	HU-09193-1	ENSG00000136527	NM_004593	SFRS10	Homo sapiens
EHU098771-1UG	HU-09877-1	ENSG00000099783	NM_005968 NM_031203	HNRNPM	Homo sapiens
EHU105811-1UG	HU-10581-1	ENSG00000162521	NM_005610	RBBP4	Homo sapiens
EHU116951-1UG	HU-11695-1	ENSG00000198563	NM_080598 NM_004640	BAT1	Homo sapiens
EHU116341-1UG	HU-11634-1	ENSG00000196313	NM_172020	POM121	Homo sapiens
EHU000631-1UG	HU-00063-1	ENSG00000196510	NM_016238	ANAPC7	Homo sapiens
EHU094111-1UG	HU-09411-1	ENSG00000153207	NM_015446	AHCTF1	Homo sapiens
EHU065631-1UG	HU-06563-1	ENSG00000131778	NM_004284	CHD1L	Homo sapiens
EHU074601-1UG	HU-07460-1	ENSG00000124160	NM_020967	NCOA5	Homo sapiens
EHU093901-1UG	HU-09390-1	ENSG00000083520	NM_014953	DIS3	Homo sapiens
EHU155011-1UG	HU-15501-1	ENSG00000153827	NM_004238	TRIP12	Homo sapiens
EHU134411-1UG	HU-13441-1	ENSG00000168872	NM_018332	DDX19A	Homo sapiens
EHU009861-1UG	HU-00986-1	ENSG00000112159	NM_014611	MDN1	Homo sapiens
EHU052241-1UG	HU-05224-1	ENSG00000069248	NM_018230	NUP133	Homo sapiens
EHU109431-1UG	HU-10943-1	ENSG00000164346	NM_014886	TINP1	Homo sapiens
EHU131601-1UG	HU-13160-1	ENSG00000155561	NM_015135	NUP205	Homo sapiens
EHU041941-1UG	HU-04194-1	ENSG00000067596	NM_004941	DHX8	Homo sapiens
EHU022481-1UG	HU-02248-1	ENSG00000113569	NM_153485 NM_004298	NUP155	Homo sapiens
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EHU124301-1UG	HU-12430-1	ENSG00000132128	NM_006369	LRRC41	Homo sapiens
EHU154691-1UG	HU-15469-1	ENSG00000120800	NM_014503	UTP20	Homo sapiens
EHU033461-1UG	HU-03346-1	ENSG00000115816	NM_005760	CEBPZ	Homo sapiens
EHU063801-1UG	HU-06380-1	ENSG00000130810	NM_020230	PPAN	Homo sapiens
EHU145651-1UG	HU-14565-1	ENSG00000100650	NM_001039465 NM_006925	SFRS5	Homo sapiens
EHU113551-1UG	HU-11355-1	ENSG00000124193	NM_006275	SFRS6	Homo sapiens

EHU049301-1UG	HU-04930-1	ENSG00000116754	NM_004768	SFRS11	Homo sapiens
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EHU014921-1UG	HU-01492-1	ENSG00000116350	NM_005626	SFRS4	Homo sapiens
EHU132201-1UG	HU-13220-1	ENSG00000136450	NM_001078166 NM_006924	SFRS1	Homo sapiens
EHU217661-1UG	HU-21766-1	ENSG00000180771	NM_032102	SRSF8	Homo sapiens
EHU074471-1UG	HU-07447-1	ENSG00000161547	NM_003016	SFRS2	Homo sapiens
EHU075361-1UG	HU-07536-1	ENSG00000115875	NM_001031684	SFRS7	Homo sapiens
EHU125071-1UG	HU-12507-1	ENSG00000112081	NM_003017	SFRS3	Homo sapiens
EHU062901-1UG	HU-06290-1	ENSG00000111642	NM_001273	CHD4	Homo sapiens
EHU107381-1UG	HU-10738-1	ENSG00000110367		DDX6	Homo sapiens
EHU143951-1UG	HU-14395-1	ENSG00000108406	NM_024612	DHX40	Homo sapiens
EHU083701-1UG	HU-08370-1	ENSG00000174953	NM_020865	DHX36	Homo sapiens
EHU008131-1UG	HU-00813-1	ENSG00000100201	NM_006386	DDX17	Homo sapiens
EHU131251-1UG	HU-13125-1	ENSG00000079785	NM_004939	DDX1	Homo sapiens
EHU091391-1UG	HU-09139-1	ENSG00000165732	NM_004728	DDX21	Homo sapiens
EHU053101-1UG	HU-05310-1	ENSG00000141141	NM_007010 NM_152300	DDX52	Homo sapiens
EHU157301-1UG	HU-15730-1	ENSG00000080603	NM_006662	SRCAP	Homo sapiens
EHU065401-1UG	HU-06540-1	ENSG00000144028	NM_014014	ASCC3L1	Homo sapiens
EHU012231-1UG	HU-01223-1	ENSG00000166226	NM_006431	CCT2	Homo sapiens
EHU036751-1UG	HU-03675-1	ENSG00000135624	NM_001009570	CCT7	Homo sapiens
EHU092111-1UG	HU-09211-1	ENSG00000089280	NM_004960	FUS	Homo sapiens
EHU074871-1UG	HU-07487-1	ENSG00000137776	NM_024755	SLTM	Homo sapiens
EHU065891-1UG	HU-06589-1	ENSG00000148773	NM_002417	MKI67	Homo sapiens
EHU130481-1UG	HU-13048-1	ENSG00000056097	NM_016107	ZFR	Homo sapiens
EHU064491-1UG	HU-06449-1	ENSG00000095319	NM_015354	NUP188	Homo sapiens
EHU049771-1UG	HU-04977-1	ENSG00000111581	NM_020401	NUP107	Homo sapiens
EHU087381-1UG	HU-08738-1	ENSG00000110713	NM_005387 NM_139132 NM_139131 NM_016320	NUP98	Homo sapiens
EHU108481-1UG	HU-10848-1	ENSG00000147274	NM_002139	RBMX	Homo sapiens
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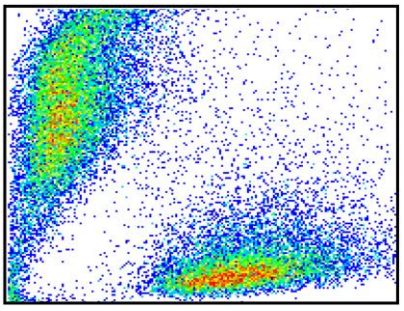
EHU013541-1UG	HU-01354-1	ENSG00000106344	NM_018077	RBM28	Homo sapiens
EHU006231-1UG	HU-00623-1	ENSG00000188739	NM_015014	RBM34	Homo sapiens
EHU014721-1UG	HU-01472-1	ENSG00000105202	NM_001436	FBL	Homo sapiens
EHU024481-1UG	HU-02448-1	ENSG00000184967	NM_024078	NOC4L	Homo sapiens
EHU027691-1UG	HU-02769-1	ENSG00000155438	NM_032390	MKI67IP	Homo sapiens
EHU019931-1UG	HU-01993-1	ENSG00000138160	NM_004523	KIF11	Homo sapiens
EHURLUC-1UG	RLUC			RLUC	

Table 1. Table of Endonuclease-prepared Small Interfering RNAs (esiRNAs) library. esiRNA library (Sigma-Aldrich) use for screening in human stable 293T-REx Flp-In™ Tet-ON cells expressing EWS/FLI1^{STAG} (293$^{EWS/FLI1}$)

RESULTS

CHAPTER 1

Targeting the kinase activities of ATR and ATM exhibits anti-tumoural activity in mouse models of *MLL*-rearranged AML



As mentioned in the introduction section, blood tumours present distinctively high endogenous levels of RS (Figure 4) (Cancer Cell Line Encyclopaedia dataset; Barretina *et al.*, 2012). Why hypothesised that this feature could render these tumours particularly sensitive to the inhibition of ATR, the major kinase that copes with and alleviates RS in cells in mammalian cells. From the different kinds of hematopoietic malignancies, we decided to focus on AML carrying MLL translocations for the following reasons. First, mouse genetic studies have shown that ATR abundance is particularly important for the viability of AML cells (Schoppy *et al.*, 2012). Second, ATR-dependent phosphorylation of MLL is involved in the response to RS (Liu *et al.*, 2010). Third, *in vitro* experiments showed that ATR inhibitors promote the differentiation of primary stem cells infected with MLL-AF9 (Santos *et al.*, 2014). Finally, based on the p53-independent cell death triggered by ATR inhibitors (Ruiz *et al.*, 2016), they could potentially overcome the limitation impinged by p53-deficient responses in MLL-driven AML to the current chemotherapy.

1. AML^{MLL} cells in culture are highly sensitive to ATR inhibitors

To test the efficacy of ATR inhibition in the treatment of AML *in vitro*, we used a previously described mouse cell line generated by transforming bone marrow cells with viruses expressing MLL-ENL and N-RAS^{G12D} (AML^{MLL}). These cells have an activating mutation in N-RAS (Neuroblastoma- RAS oncogene), which is also common in human AML-MLL patients (Zuber *et al.*, 2009), and they recapitulate the deficient p53 signalling and poor responses to conventional chemotherapy that are observed in the clinic with AML-MLL patients (Zuber *et al.*, 2009).

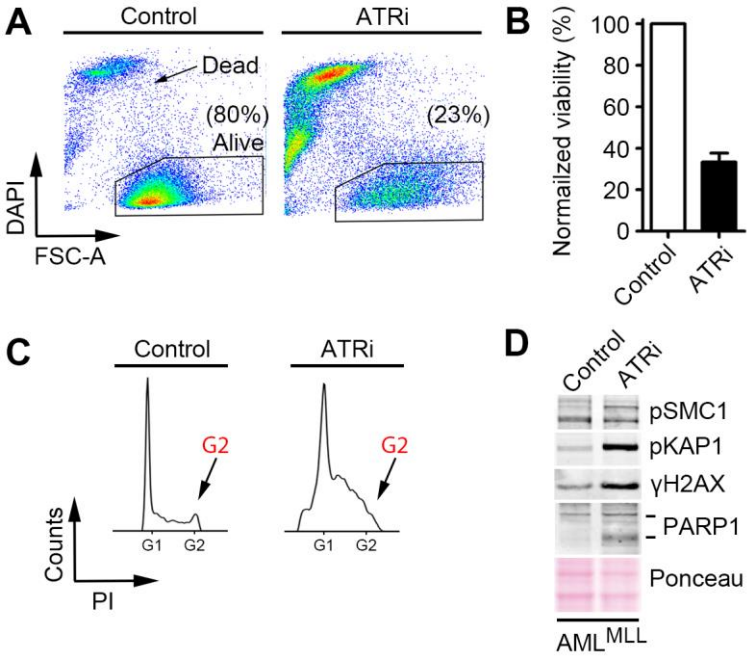


Figure 8. Toxicity of ATRi in AML^{MLL} cells in culture. (A) Fluorescence activated cell sorting (FACS) analysis showing the percentage of viable AML^{MLL} cells (identified by size and DAPI exclusion) either untreated or exposed to ATRi (10 µM, 24 hrs); FSC-A, forward scattered light. Data are representative of two independent experiments. (B) Quantification of alive cells shown in A. (C) FACS analysis of DNA content (propidium iodide (PI)) from the cultures used in (A) illustrating the depletion of G2 cells observed in response to ATRi. Data are representative of two independent experiments. (D) Western blot of SMC1, KAP1, and H2AX phosphorylation and poly(ADP-ribose) polymerase 1 (PARP1) cleavage products in AML^{MLL} cells exposed to ATRi (5 µM, 6 hrs). Data are representative of two independent experiments

We observed a reduction of the 75% of viable cells in culture after treatment of AML^{MLL} cells with the ATR inhibitor AZ20 (Foote *et al.*, 2013) (hereafter referred to as ATRi) for 24 hours (Figure 8A, B). ATR inhibitors result in cytotoxicity by forcing premature mitotic entry of cells from G2, the phase during which DSBs are generated (Ruiz *et al.*, 2016). Consistently, exposure to ATRi led to the disappearance of most AML^{MLL} cells from the G2 phase of the cell cycle (Figure 8C). ATRi treatment also resulted in the accumulation of DSBs, as indicated by the phosphorylation of the DDR targets KRAB-associated protein 1 (KAP1), structural maintenance of chromosomes 1 (SMC1), and histone H2AX (Figure 8D). Additionally, we also detected the apoptotic cleavage of PARP1 in AML^{MLL} cells exposed to ATRi, indicative of cytotoxicity (Figure 8D).

2. ATR inhibitors induce accumulation of replicative DNA damage

To determine the mechanism for the sensitivity of AML^{MLL} cells to ATRi, we analysed the RS response in these cells. We first ruled out a deficiency in activation and signalling through the RS pathway in AML^{MLL} cells by exposing them to the ribonucleotide reductase inhibitor hydroxyurea (HU), a well-known agent to induce RS (dNTP-depleting agent). HU administration stimulated the phosphorylation of the ATR targets CHK1 and RPA, as expected in cells with an intact RS response (Figure 9A).

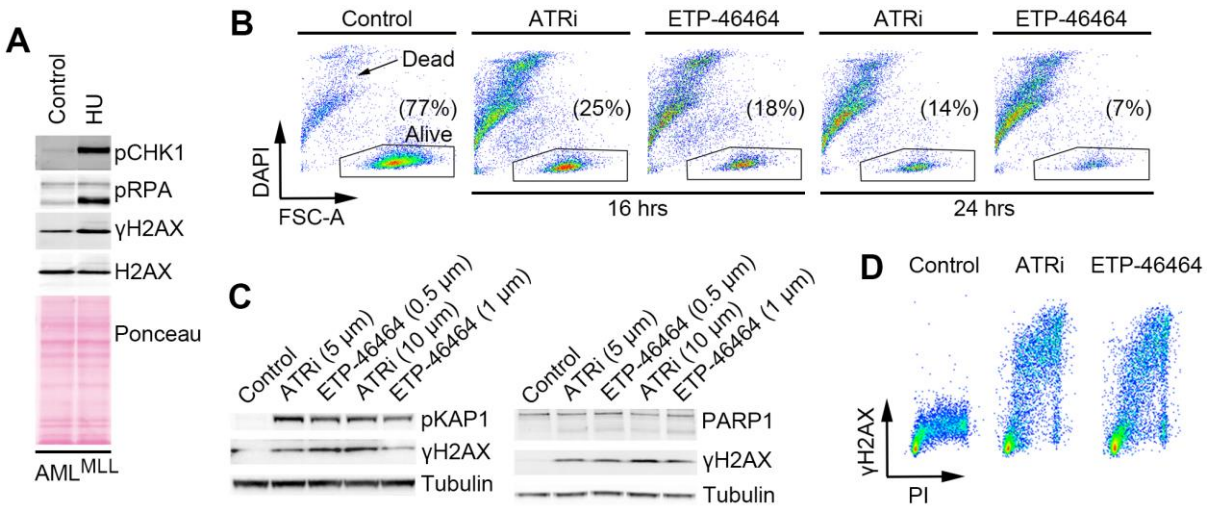


Figure 9. Toxicity, RS, and DNA breakage induced by two distinct ATR inhibitors in AML^{MLL} cells. (A) Western blot of CHK1, RPA, and H2AX phosphorylation in AML^{MLL} cells exposed to HU (2 mM, 2 hrs). Data are representative of two independent experiments. (B) FACS analysis showing the percentage of viable AML^{MLL} cells (identified by size and DAPI exclusion) either untreated or exposed to ATRi (10 μM) or to ETP-46464 (1 μM), an independent ATR inhibitor (Toledo *et al.*, 2011) for the indicated times. (C) Western blots showing markers of DNA breakage and cellular toxicity. The left Western blot shows KAP1 and H2AX phosphorylation in AML^{MLL} cells exposed to ATRi or ETP-46464 at the indicated conditions. The right blot shows the accumulation of PARP1 cleavage (lower band) products in cells with DNA damage indicated by H2AX phosphorylation. (D) FACS analysis of DNA content (PI) and H2AX phosphorylation in AML^{MLL} cells exposed to ATRi (10 μM, 6 hrs) or ETP-46464 (1 μM, 6 hrs), illustrating that the γH2AX signal detectable by Western blot is restricted to replicating cells.

A structurally different ATR inhibitor previously described by our group, ETP-46464 (Toledo *et al.*, 2011), induced similar toxicity in the viability of AML^{MLL} cells (Figure 9B) and accumulation of DNA damage markers like phosphorylated KAP1 and phosphorylated H2AX (γH2AX) (Figure 9C). ATRi and ETP-46464 induced a greater γH2AX signal in replicating cells, as measured by monitoring γH2AX phosphorylation of cells labelled with propidium iodide by flow cytometry (Figure 9D). Furthermore, both ATR inhibitors induced cleavage of PARP1, an indicator of apoptosis (Tallis *et al.*, 2014), which confirms the toxicity of these inhibitors under conditions that induced DNA damage (Figure 9C).

3. The toxicity of ATR inhibitors in AML^{MLL} cells in culture is p53-independent

As previously mentioned, a functional p53 pathway is necessary in AML for chemotherapy to be effective. However, AML^{MLL} cells are not able to efficiently activate p53 in response to chemotherapeutic agents (Zuber *et al.*, 2009). This suggests that ATR inhibition could be particularly beneficial to treat AML since its toxicity is even higher in cells lacking p53 (Murga *et al.*, 2009; Ruzankina *et al.*, 2009). In agreement with this fact, depletion of p53 with a retrovirus expressing a p53-targeting short hairpin RNA (shRNA) did not rescue AML^{MLL} cells from the toxic effects of ATRi (Figure 10, A to C). Furthermore, we confirmed in these cells that ATRi leads cells to premature mitotic entry from G2 (Figure 10C).

We also compared the response to ATRi in AML^{MLL} cells in an equivalent cell line that was generated by transforming bone marrow cells with viruses expressing the translocation between *AML1* (Acute Myeloid Leukaemia 1) and *ETO* (Eight Twenty One) genes (AML^{ETO}). This translocation models a p53-proficient type of AML with better prognosis than AML patients bearing the MLL-ENL translocation (Zuber *et al.*, 2009). ATRi was more toxic for AML^{MLL} than for AML^{ETO} cells as observed in both cell viability analyses by flow cytometry (Figure 10D) and luminescent assay (CellTiter-Glo®) (Figure 10F). The enhanced sensitivity of AML^{MLL} cells correlated with higher levels of DNA damage in replicating cells, as measured by monitoring γH2AX phosphorylation of cells labelled with propidium iodide by flow cytometry (Figure 10G). ATRi induced a greater γH2AX signal, indicating higher levels of damage in AML^{MLL} cells. Consistent with the flow cytometry data, AML^{MLL} and AML^{ETO} cells exhibited differences in the rate of DNA replication fork progression when analysed by stretched DNA fibre analyses. Whereas replication fork progression in AML^{MLL} cells was slower under basal conditions than that in AML^{ETO} cells (Figure 10H), exposure to ATRi had a bigger effect in reducing replication fork rates in AML^{MLL} cells, leading to an almost complete impairment of replication fork progression in these cells (Figure 10H).

In summary, MLL-driven AML cells exhibited an intrinsically higher sensitivity to ATR inhibitors than AML^{ETO} cells do, with ATRi inducing the accumulation of replicative DNA damage, activation of the DDR, and p53-independent death in these cells.

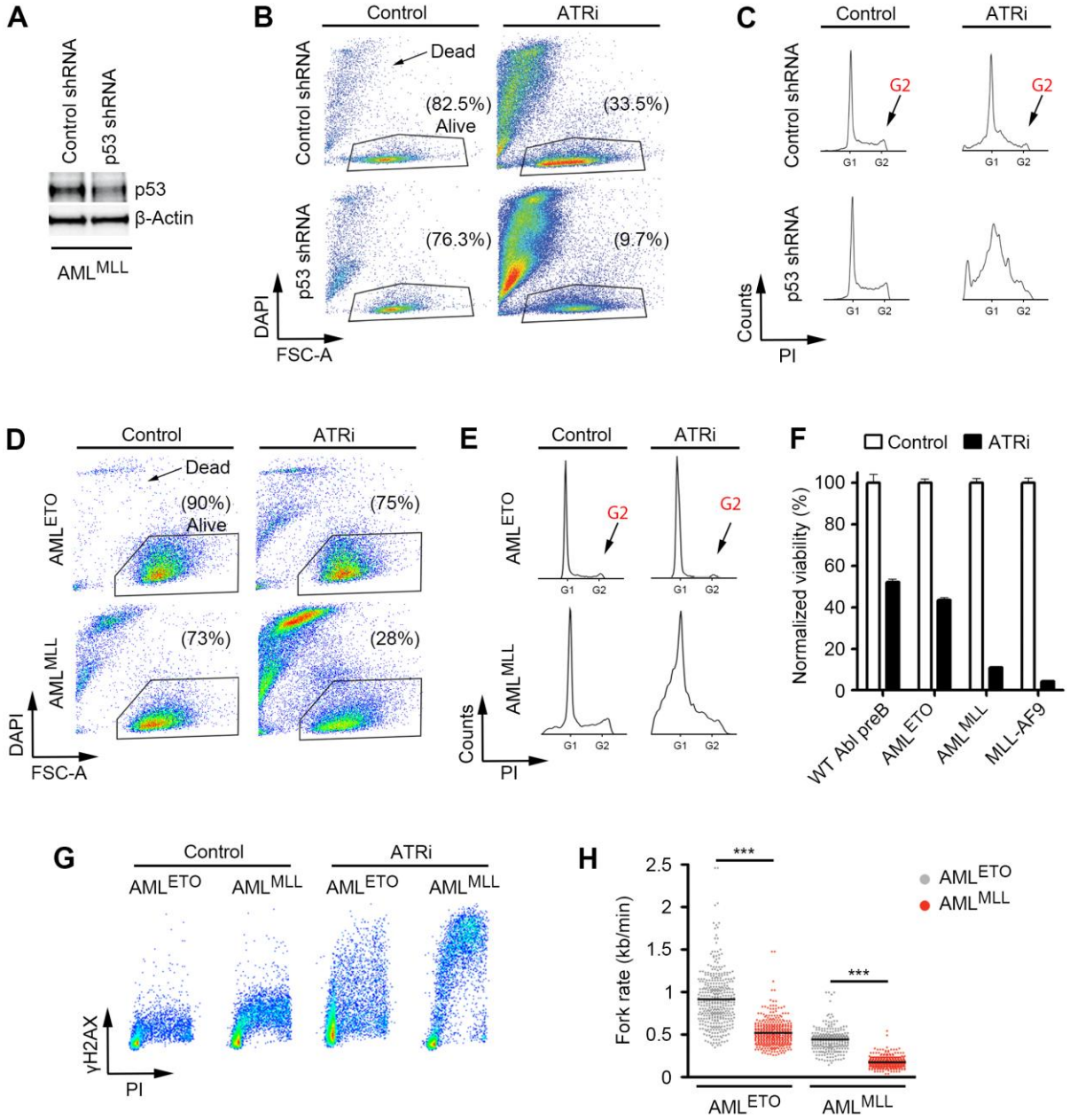


Figure 10. p53-independent toxicity of ATRi in AML^{MLL} cells in culture. (A) Western blot confirming the depletion of p53 in AML^{MLL} cells after infection with retroviruses expressing a p53-targeting shRNA. β-Actin was used as loading control. (B) FACS analysis showing the percentage of viable AML^{MLL} cells (identified by size and DAPI exclusion) from control or p53 shRNA–infected AML^{MLL} cells either untreated or exposed to ATRi (10 μM, 24 hrs). (C) FACS analysis of DNA content (PI) from the cultures used in (B) illustrating the depletion of G2 cells observed in response to ATRi. (D) FACS analysis showing the percentage of viable AML^{MLL} and AML^{ETO} cells either untreated or exposed to ATRi (3 μM, 16 hrs). (E) FACS analysis of DNA content (PI) from the cultures used in (D) illustrating the depletion of G2 cells observed in response to ATRi. (F) Luminescent cell viability assay (GLO) of AMLL^{MLL} cells and AML^{ETO} cells exposed (or not) to ATRi (1 μm, 24 hrs). (G) FACS analysis of DNA content (PI) and H2AX phosphorylation in AML^{MLL} and AML^{ETO} cells exposed to ATRi (10 μM, 5 hrs). (H) Fork rates were measured in stretched DNA fibres prepared from AML^{MLL} and AML^{ETO} cells exposed (or not) to ATRi (10 μM, 5 hrs). At least 200 tracks were measured per condition. ***P < 0.001 by two-tailed t test. (A), (B), (C), (D), (E), (F), (G) and (H) Data are representative of two independent experiments.

4. ATR inhibitors show efficacy as single agents in a mouse model of MLL-driven AML

To examine the *in vivo* efficacy of ATRi, we injected AML^{MLL} tumour cells, which also expressed both green fluorescent protein (GFP) and luciferase for tracking, into immunocompetent mice. Even in immunocompetent recipients, intravenous injection of AML^{MLL} cells results in a very aggressive form of AML that infiltrates multiple organs and kills mice in a few weeks (Zuber *et al.*, 2009). In contrast to previous studies (Zuber *et al.*, 2009; Zuber *et al.*, 2011), we did not irradiate recipient animals before transplant. Irradiation depletes the bone marrow of the recipient mice, facilitating the expansion of the transplanted tumour. Because the injection of AML^{MLL} tumour cells was able to induce tumourigenesis even in the absence of irradiation, we preferred to avoid this radiation treatment to further mimic the normal context of AML. Transplanted mice were then treated daily by oral gavage with ATRi and tumour development was followed through monitoring luciferase activity with an *in vivo* imaging system (IVIS). Finally, to test the efficacy of ATR inhibitors, we used two protocols as follows: a prevention protocol in which mice started receiving treatment on the day of the injection of AML^{MLL} cells ($ATRi^{Pr}$); and a therapy protocol in which mice started receiving treatment after tumours were detectable by IVIS ($ATRi^{Th}$), at day 13 after injection of AML^{MLL} cells.

AML^{MLL} cells rapidly expand in the absence of drug treatment, leading to a lethal disease in control animals with a median survival of 23 days (Figure 11A). Both therapy and prevention groups showed a marked response as measured by IVIS at day 18, being tumours not detectable at this time point in the $ATRi^{Pr}$ group (Figure 11B). IVIS on isolated organs confirmed that ATRi treatment dramatically limits tumour infiltration to organs, including liver, spleen, and lung (Figure 11C). The decreased tumour burden was also evident from visual analysis of spleen sizes (Figure 11D). We also observed increased levels of the white blood cells count in vehicle treated mice compared to ATRi treated and control healthy mice (Figure 11E). Moreover and in agreement with the observations made *in vitro* by exposure of cells to ATR inhibitors (Figure 10G), treatment with ATRi led to a widespread accumulation of γH2AX-positive cells in spleens from the treated mice (Figure 11F), indicating an accumulation of DNA damage in tumour cells after the treatment. The presence of tumour cells was measured in bone marrow cells isolated from all groups of mice by GFP fluorescence at day 23. GFP-positive cells were undetectable in the prevention group, and the therapy group exhibited a 17-fold decrease in the percentage of GFP-positive AML^{MLL} cells in bone marrow (Figure 11G). Consistent with this, videos of animals recorded at day 25 showed a clear improvement in the overall health of both ATRi-treated groups (movies 1 to 3).

Although all animals eventually succumbed to leukaemia, both prevention and therapy groups showed a significant increase in the median life span (vehicle, 23 days; ATRiTh, 33 days; ATRi^{Pr}, 45 days) (Figure 11A). Strikingly, at 40 days, all animals from the prevention group were alive, a time at which we decided to stop the treatment to explore potential curative effects of the therapy. Forty percent of these mice survived for more than 50 days, and one was alive for 117 days before succumbing to the disease.

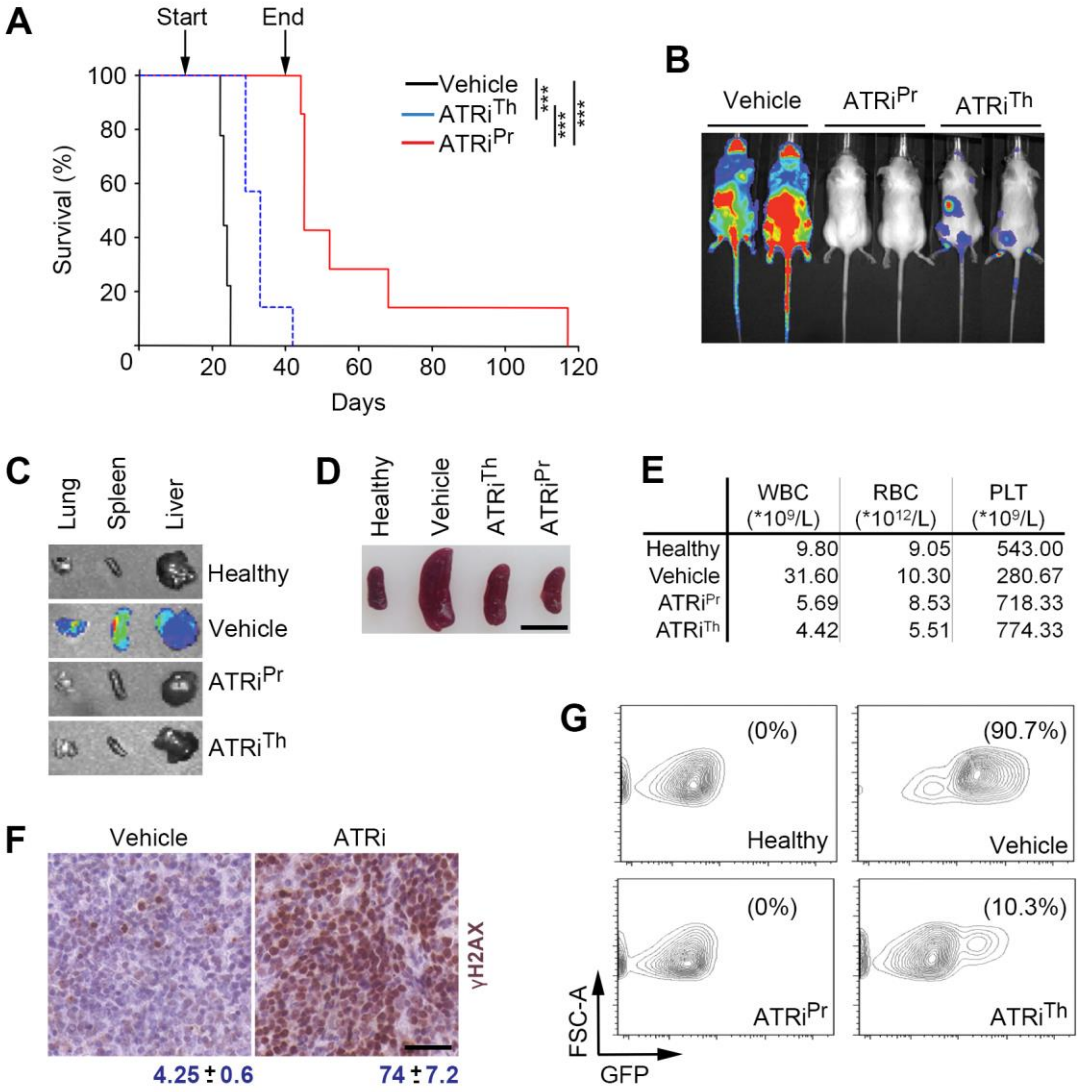


Figure 11. In vivo responses of AML^{MLL} to ATRi. (A) Kaplan-Meier curves of AML^{MLL} transplanted mice that were either treated with vehicle (n = 9), ATRi from day 1 (ATRi^{Pr}; n = 7), or ATRi from day 13 (ATRiTh; n = 7). Treatment on the prevention group stopped at day 40. P value was calculated with the Mantel-Cox log-rank test. ***P<0.001. (B) Representative IVIS of the luciferase signals observed on mice from the groups indicated on (A) on day 18. (C) Representative examples of the luciferase signal observed by IVIS on isolated organs from the indicated groups at day 23. (D) Picture of the spleen sizes observed at day 23 of the in vivo treatment experiment. Scale bar, 1 cm. (E) Blood analysis of mice from the groups indicated on (A), White Blood Cells (WBC), Red Blood Cells (RBC) and Platelets (PLT). (F) Representative images of γH2AX immunohistochemistry on spleens of AML^{MLL} transplanted mice treated with vehicle or ATRi (60 mg/kg, 11 days). Scale bar (black), 50 mm. Numbers indicate the percentage of γH2AX positive cells in each case (means ± SD). (G) FACS analysis from the bone marrow collected from mice at day 23 of the treatment experiment indicated in (A). GFP (x axis) is used to monitor the presence of AML^{MLL} cells. y axis indicates FSC-A. The percentage of live GFP positive cells detected in each case is indicated. Data are representative of two independent groups.

In summary, our data showed that ATR inhibition elicits anti-tumour responses when used as a single agent in *allografts* of mouse AML^{MLL} cells and provide an example of anti-tumour activity of this class of drugs in an immunocompetent model of cancer.

5. MLL-translocation driven human leukaemia cells are sensitive to ATR inhibitors in a *xenograft* mouse model

In order to test the inhibition of ATR as chemotherapy for AML, we used the human AML cell line MV4:11, which is driven by a translocation between *MLL* and *AF4* (ALL1-fused gene from chromosome 4) genes (Andersson *et al.*, 2005). MV4:11 cell line was originally isolated from a 10-years-old patient with biphenotypic B myelomonocytic leukaemia, a subtype of AML^{MLL}. First, we analysed *in vitro* the cell viability of this cell line and we

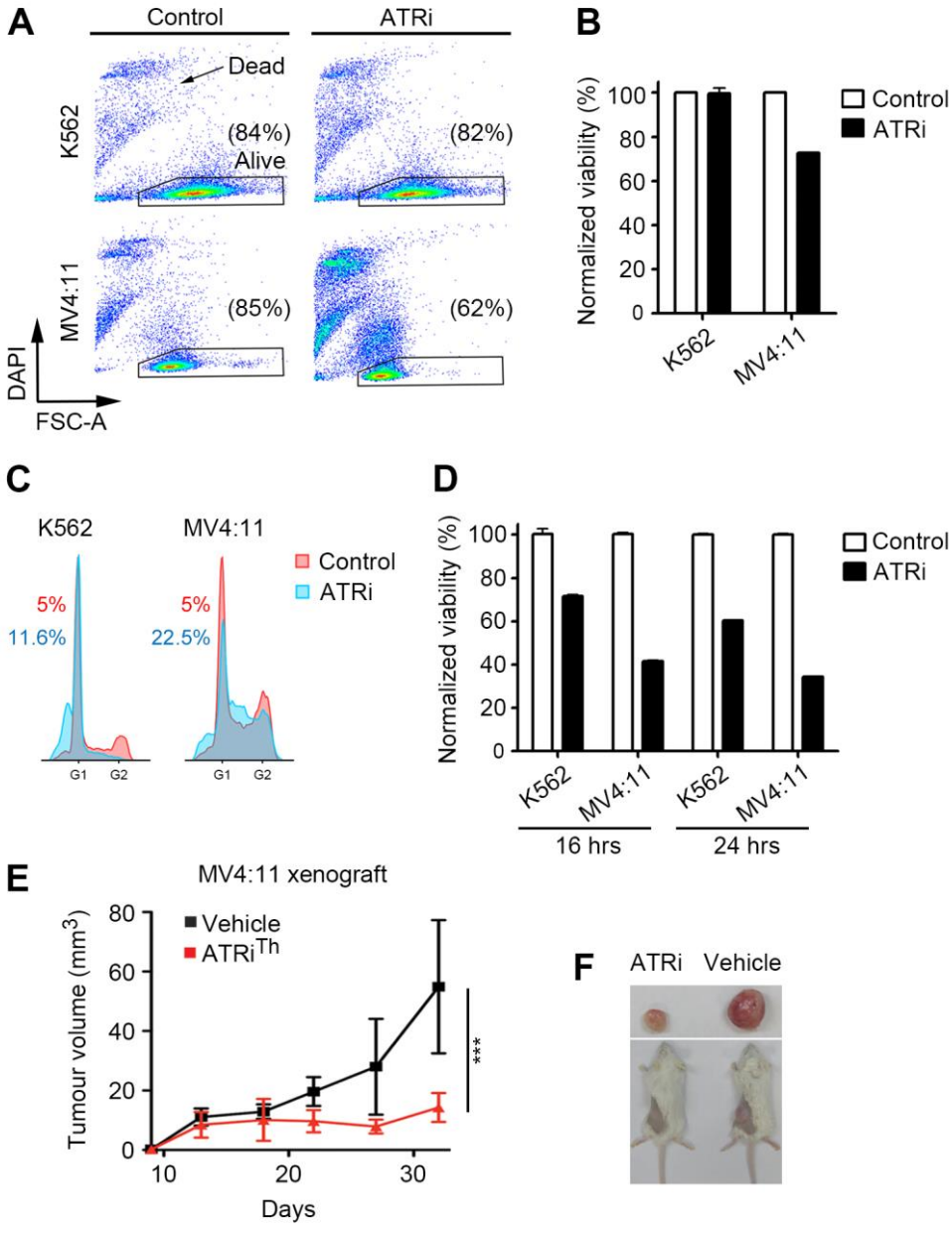


Figure 12. Toxicity of ATRi in AML^{MLL} cells from a patient (MV4:11). (A) FACS analysis showing the percentage of viable human MV4:11 cells compare to human K562 cells (identified by size and DAPI exclusion) either untreated or exposed to ATRi (10 µM, 16 hrs). (B) Quantification of alive cells shown in A. (C) DNA content (PI) analyses by flow cytometry illustrating, first, the toxicity of ATRi (10 µM, 24 hrs) on MV4:11 cells compared to K562 cells and, second, the depletion of G2 cells observed in response to ATRi. SubG1 population percentages are indicated. (D) Luminescent cell viability assay (GLO) of MV4:11 cells and K562 cells exposed (or not) to ATRi (10 µM) at indicated times. (E) Effect of ATRi as monotherapy on the growth of xenografts from the human MV4:11 cell line of MLL-driven AML. Treatment started when tumours became palpable, and eight animals were used per group. ***P < 0.001 by two-way analysis of variance. (F) Examples of the tumour sizes observed at endpoint from E.

confirmed that MV4.11 cells are more sensitive to ATRi treatment than K562 cells (derived from a chronic myelogenous leukaemia patient) (Figure 12A, B). Then, we monitored the cell cycle profile of non-treated and ATRi-treated MV4:11 and K562 cells and we observed the same toxic effect with MV4:11 cells presenting an increase in the percentage of cellular death (defined by the subG1 phase) (Figure 12C). Reduced cell viability measured by a luminescent assay (CellTiter-Glo®) confirmed our previous observations (Figure 12D). Concomitantly, we observed the disappearance of most MV4:11 cells from the G2 phase of the cell cycle after ATRi treatment (Figure 12C).

Finally, we tested *in vivo* the efficacy of ATRi in a *xenograft* model of the MV4:11 cell line. MV4:11 cells were implanted subcutaneously into the flanks of SCID mice. Treatment with ATRi started when tumours became palpable and was administered daily through oral gavage. We monitored the tumour growth and observed that ATRi therapy significantly limits MV4:11 *xenograft* growth (Figure 12E, F). Collectively, our data demonstrate that ATR inhibition elicits anti-tumour responses when used as a single agent in *xenografts* of a human AML cell line.

6. ATM inhibitors show efficacy as single agents in a mouse model of MLL-driven AML

Persistent RS leads to the breakage of replication forks and thus to DSBs that triggers an ATM-dependent DDR, suggesting an active role of ATM in limiting the toxicity of RS. Consistently, ATM deficiency is lethal in ATR-Seckel mice (ATR deficiency mouse model) which accumulate high levels of RS (Murga *et al.*, 2009). Moreover, previous data *in vitro* reveal that treatment of primary MLL-AF9 transformed cells with an ATM inhibitor led to an increased differentiation of these leukaemic cells, and the transformation of ATM knockout cells with MLL-AF9 results in poor growth (Santos *et al.*, 2014).

In order to determine if ATM inhibition is also effective for treatment of AML, we used the newly developed ATM inhibitor AZD0156 (referred to as ATMi hereafter) (Degorce *et al.*, 2016) (Figure 13A). First, a cell viability assay was performed in which we confirmed that AML^{MLL} cells are sensitive to a treatment with either ATRi or ATMi (Figure 13B). Once again, and consistent with the mechanism of action of ATR inhibitors, we observed the disappearance of most AML^{MLL} cells from the G2 phase of the cell cycle in response to this agent (Figure 13C).

As a first trial of the efficacy of a treatment with AMTi *in vivo*, we used the above *allograft* mouse model generated from intravenous injection of AML^{MLL} cells. Treatment started on the day of the injection of AML^{MLL} cells (prevention protocol). IVIS of mice at day

16 showed halted tumour progression in both ATRi and ATMi treated mice when compared to the vehicle treated group (Figure 13D). Importantly, the survival of AML^{MLL}-bearing mice was improved after either ATRi or ATMi treatment (Figure 13E).

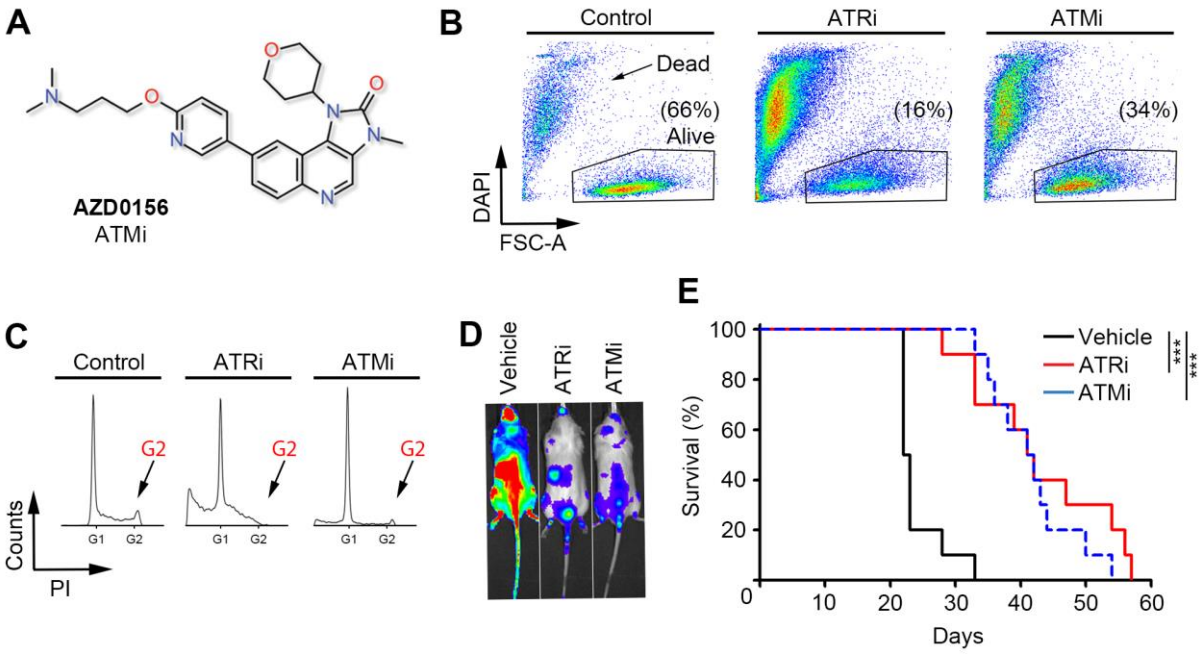


Figure 13. Toxicity of ATRi and ATMi in AML^{MLL} cells in culture. (A) Chemical structure of the ATM inhibitor AZD0156 (ATMi). (B) FACS analysis showing the percentage of viable AML^{MLL} cells (identified by size and DAPI exclusion) either untreated or exposed to ATRi (10 µM, 24 hrs) or to ATMi (10 µM, 24 hrs). (C) FACS analysis of DNA content (PI) from the cultures used in (B) illustrating the depletion of G2 cells observed in response to ATRi. (D) Representative IVIS of the luciferase signals observed on AML^{MLL} transplanted mice that were either treated with vehicle, ATRi (60 mg/Kg) or ATMi (20 mg/Kg) from day 1. IVIS imaging was conducted on day 16 of the experiment. (E) Kaplan-Meier curves of AML^{MLL} transplanted mice that were either treated with vehicle (n = 10), ATRi (n = 10), or ATMi (n = 10). Treatment on both groups stopped at day 54. P value was calculated with the Mantel-Cox log-rank test. ***P < 0.001.

To further determine whether ATM is required for MLL leukaemia *in vivo*, wild-type and ATM$^{-/-}$ AML^{MLL} tumours were generated by transforming bone marrow hematopoietic progenitor cells of both genotypes with retroviruses expressing MLL-AF9-IRES-neo (were neo is neomycin-resistance) and N-RAS^{G12D}-IRES-GFP. When injected into immunodeficient NRG (Rag1 null; IL2rg null) recipient mice, both ATM wild-type and ATM$^{-/-}$ AML^{MLL} cells caused lethal leukaemia with no difference in median survival (Figure 14A). Thus, even though loss of ATM activity inhibits growth *in vitro* (Santos *et al.*, 2014), we found that it did not have a detectable impact on the development of MLL-AF9 leukaemia *in vivo* in primary transplants (This part of the work was performed by the group of André Nussenzweig on the National Institutes of Health, USA).

A plausible explanation for the lack of effect on tumour progression when ATM is genetically depleted is that complete abrogation of ATM expression (ATM$^{-/-}$) might not resemble the phenotype triggered by attenuation of the kinase activity of ATM with chemical

inhibitors. For example, ATM kinase–inactivating mutations lead to embryonic lethality in mice, whereas ATM knockout mice are viable and show less genome instability than kinase-dead mutants (Daniel *et al.*, 2012; Yamamoto *et al.*, 2012). To determine the effects of ATM inhibition on MLL leukaemia, we used the ATM inhibitor and the *allografts* model of AML^{MLL}.

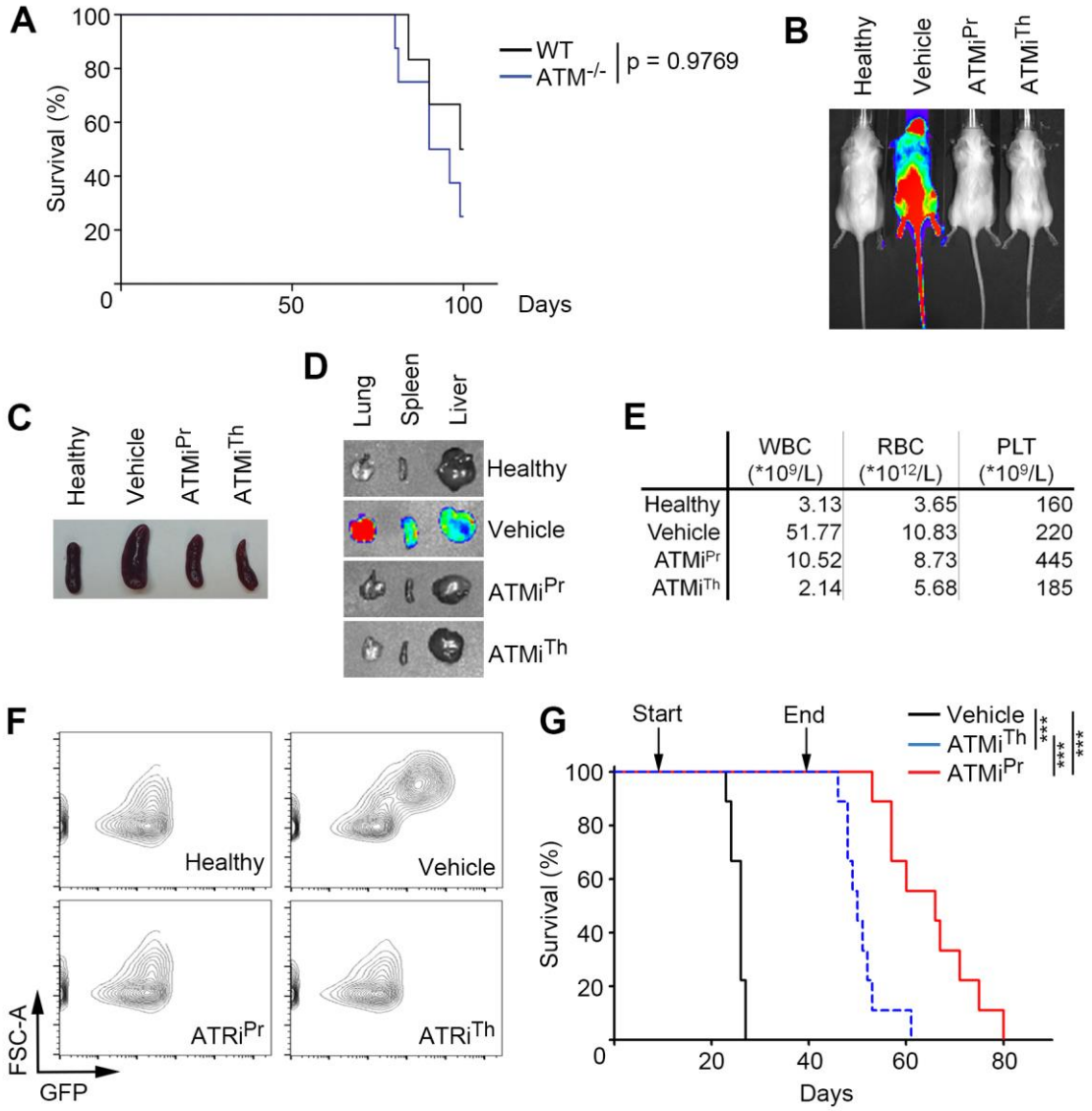


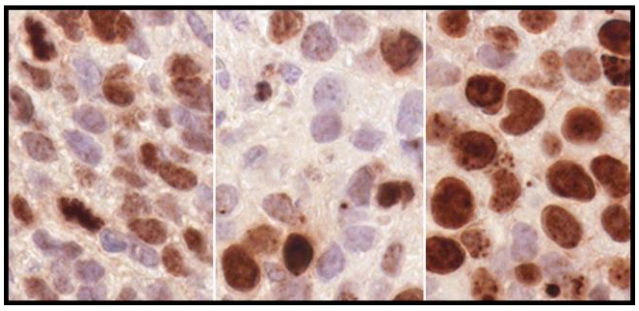
Figure 14. In vivo responses of AML^{MLL} to ATMi. (A) Kaplan-Meier curves of mice that were transplanted with ATM WT (n = 6) and ATM$^{-/-}$ (n = 8) bone marrow hematopoietic progenitors that had been infected with retroviruses expressing MLL-AF9-IRES-neo and N-RAS^{G12D}-IRES-GFP. P value was calculated with the Mantel-Cox log-rank test. (B) Representative IVIS of the luciferase signals observed on AML^{MLL} transplanted mice that were either treated with vehicle, ATMi from day 1 (ATMi^{Pr}), or ATMi from day 8 (ATMiTh). IVIS imaging was conducted on day 22 of the experiment. (C) Image of the spleen sizes observed at day 22 of the in vivo treatment experiment explained in (B). Scale bar, 1 cm. (D) Representative examples of the luciferase signal observed by IVIS on isolated organs from the indicated groups at day 22. (E) Blood analysis of mice from the groups indicated on (B), White Blood Cells (WBC), Red Blood Cells (RBC) and Platelets (PLT). (F) FACS analysis from the spleen collected from mice at day 23 of the treatment experiment indicated in (B). GFP (x axis) is used to monitor the presence of AML^{MLL} cells. y axis indicates FSC-A. (G) Kaplan-Meier curves of AML^{MLL} transplanted mice that were either treated with vehicle (n = 9), ATMi from day 1 (ATMi^{Pr}; n = 9), or ATMi from day 8 (ATMiTh; n = 9). Treatment on both groups stopped at day 40. P value was calculated with the Mantel-Cox log-rank test. ***P < 0.001.

In order to determine the efficacy of the ATMi, we followed the same prevention and therapy protocols that we used before to evaluate ATR inhibitors. We injected AML^{MLL} cells through the tail vein into the immunocompetent mice and treated them daily with the ATMi or with the vehicle. The untreated cohort behaved similarly to the previously shown experiments, with a median survival of 26 days. As with ATRi, the therapy with ATMi had a notable effect with either protocol, leading to reduced overall luciferase signal measured by IVIS (Figure 14B), smaller spleen size (Figure 14C), reduced organ infiltration (Figure 14D), normal levels of white blood cells count (Figure 14E), decrease in the percentage of GFP positive AML^{MLL} cells in the spleens (Figure 14F) and prolonged survival of AML^{MLL}-injected mice (vehicle, 23 days; $ATMi^{Th}$, 50 days; $ATMi^{Pr}$, 66 days) (Figure 14G). Videos of mice recorded on day 23 confirmed the improvement in the overall health of ATMi-treated mice (movies 4 to 6).

Collectively, these data indicate that ATR or ATM inhibition represent potential therapeutic strategies for the treatment of AML, especially MLL-driven leukaemias.

CHAPTER 2

Efficacy of ATR inhibitors as single agents in Ewing Sarcoma



1. Ewing Sarcoma presents high amount of replication stress

As previously mentioned, increased CHK1 expression and/or gene copy number gains have been observed in tumours with a high degree of genomic instability, which correlates with an increased sensitivity to ATR or CHK1 inhibition (Krajewska *et al.*, 2015; Sarmento *et al.*, 2015). We therefore reasoned that the presence of high CHK1 levels could be used to identify tumour types with elevated amounts of RS. Besides AML and after mesothelioma, Ewing Sarcoma is the solid tumour showing the highest levels of CHK1 mRNA from the Cancer Cell Line Encyclopaedia dataset (Figure 4) (Barretina *et al.*, 2012). In agreement with this, CHK1 protein levels were distinctively higher in a panel of Ewing Sarcoma lines compared to primary cells or other osteosarcomas (Figure 15A).

The presence of high CHK1 levels correlates with increased phosphorylation of histone H2AX in Ewing Sarcoma cell lines (Figure 15A), consistent with the presence of RS in these cells. Immunohistochemistry (IHC) of human tumour samples revealed the abundant presence of cells positive for γH2AX in Ewing Sarcomas, which was more abundant than other related tumours such as neuroblastoma or rhabdomyosarcoma (Figure 15B). Moreover, γH2AX showed a pan-nuclear distribution, which is the pattern found in tumours with high levels of RS (Murga *et al.*, 2011) and induced by ATR or CHK1 inhibitors (Syljuåsen *et al.*, 2005; Toledo *et al.*, 2011). Finally, and to directly evaluate DNA replication in Ewing Sarcoma cells, we analysed replication fork progression on isolated stretched DNA fibres. These experiments revealed that fork progression is significantly slower on any Ewing Sarcoma line tested (TC71,

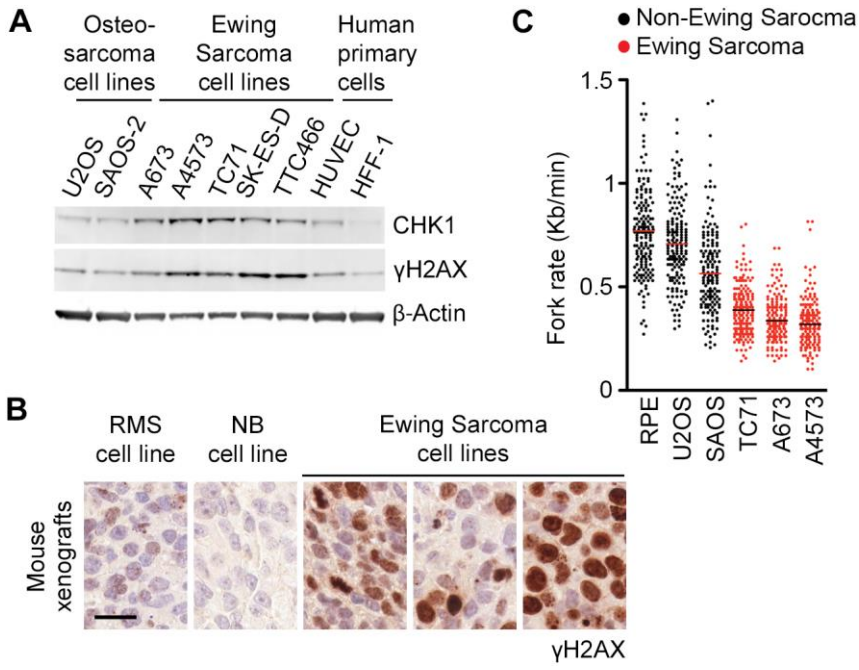


Figure 15. Increased RS levels in Ewing Sarcomas. (A) CHK1 and γH2AX levels evaluated by western blot on several Ewing Sarcoma lines, together with two osteosarcoma lines and two human primary cell types. (B) γH2AX IHC on mouse xenografts from three Ewing Sarcoma lines (A4573, A673 and TC71), and two independent xenografts from Ewing Sarcoma-related tumours (rhabdomyosarcoma (RMS); neuroblastoma (NB)). Scale bar (black) indicates 20 µm. (C) Fork rates were measured in stretched DNA fibres prepared from non-Ewing Sarcoma (RPE, U2OS, SAOS) and Ewing Sarcoma (TC71, A673 and A4573) cell lines. At least 200 tracks were measured per condition. ***P<0.001 by two-tailed t test.

A673 and A4573) than in human primary retinal pigmentum epithelial (RPE) cells or in U2OS and SAOS osteosarcoma cell lines (Figure 15C). All these data together reveal the presence of RS in Ewing Sarcoma suggesting that these tumours could be particularly responsive to ATR inhibitors.

2. Cells lines from Ewing Sarcoma patients are sensitive to ATR inhibitors *in vitro*

To determine the efficacy of ATR inhibitors on Ewing Sarcoma, we first calculated the median lethal dose (LD50) of these compounds *in vitro* (Figure 16A). Two independent ATR inhibitors (AZ20 [Foote *et al.*, 2013)] and ETP-46464 [Toledo *et al.*, 2011]) showed higher toxicity for Ewing Sarcoma cells than for human primary cells or non-Ewing Sarocma osteosarcomas, and significantly lower LD50 values than temozolamide, the chemotherapeutical drug currently used for treating Ewing Sarcoma, or the PARP inhibitor Olaparib. The use of PARP inhibitors has been described as a promising complementary treatment for Ewing Sarcoma (Garnett *et al.*, 2012). Moreover, the toxicity of ATR inhibitors correlated with the levels of CHK1 and γH2AX present on Ewing Sarcoma lines (see Figure 15A), consistent with the toxicity of these compounds being proportional to the levels of RS. Noteworthy, one of the cell lines from our panel was U2OS, a non-Ewing Sarcoma osteosarcoma cell line recently identified as being highly sensitive to ATR inhibitors due to its reliance on the alternative lengthening telomere (ALT) pathway for telomere maintenance (Flynn *et al.*, 2015). The toxicity of ATR inhibitors on all Ewing Sarcoma lines tested was higher (up to 20-fold) than on U2OS, indicating that ATR inhibitors are more efficient against tumour cells bearing high levels of RS. Clonogenic assays confirmed a greater impact of ATR inhibition on Ewing Sarcoma cells than on U2OS, since A473 cells gave rise to a reduced number of colonies compared to control cells upon treatment with an ATR inhibitor. In a similar manner, these cells lines are sensitive either to treatment with an ATR inhibitor or with a PARP inhibitor (Figure 16B, C). Together, these results support that ATR inhibitors are especially toxic for Ewing Sarcoma cells.

Next, we analysed the effects of ATR inhibition in Ewing Sarcoma cells. We confirmed by flow cytometry analyses of DNA content an increased toxicity of ATRi in Ewing Sarcoma lines, at doses at which no obvious impact of the inhibitor was observed on the cell cycle distribution of U2OS or SAOS-2 osteosarcoma cells (Figure 16D). The compound triggered apoptosis in Ewing Sarcoma cells, evidenced by the emergence of cells with a subG1 DNA content, as well as by the apoptotic cleavage of PARP1 (Figure 16E), indicative of cytotoxicity. In agreement with the main mechanism by which ATR inhibitors kill cells, which is forcing premature mitotic entry in cells suffering from RS (Ruiz *et al.*, 2016), Ewing Sarcoma cell lines ATR inhibition led to the accumulation of cells in the S/G2 phases of the

A

LD50 values (nM)		ETP-46464	AZ-20	Olaparib
Human primary cell lines	HFF-1	>10^4	2259	>10^5
	HFF-2	>10^4	3742	>10^5
	HuVEC	1276	2279	36860
Osteosarcoma cell lines	U2OS	407	1231	>10^5
	SAOS-2	1299	2420	7027
Ewing sarcoma cell lines	A673	33	297	2851
	A4573	30	222	1149
	TC-71	96	705	3410
	SK-ES-D	152	876	739
	TTC466	19	105	161

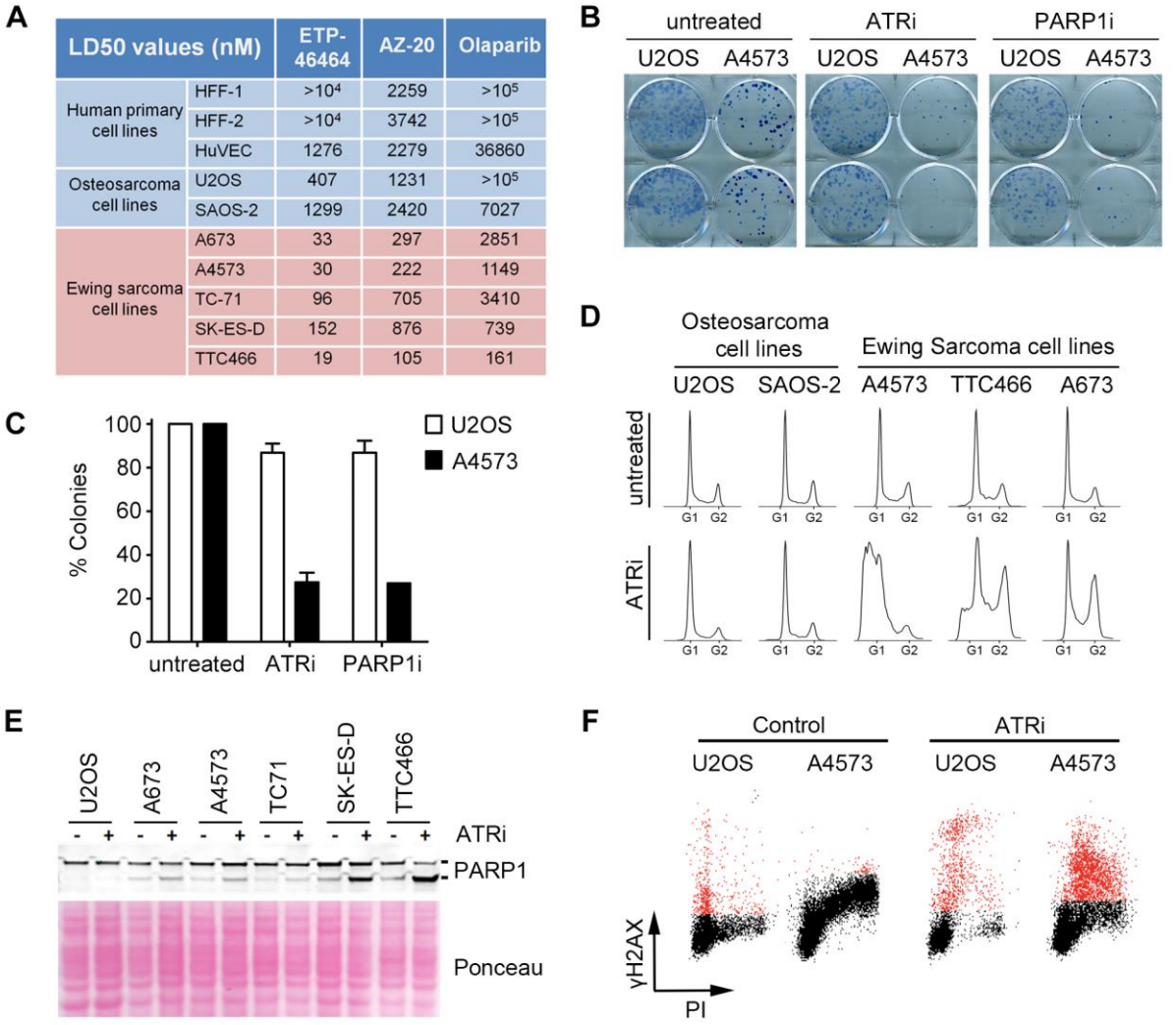


Figure 16. Sensitivity of Ewing Sarcoma to ATR inhibitors *in vitro*. (A) LD50 values of two independent inhibitors (ETP-46464 (Toledo *et al.*, 2011) and AZ20 (Foote *et al.*, 2013) and a PARP1 inhibitor (Olaparib, PARPi hereafter) on the same lines used in Figure 15A. The LD50 values for temozolomide, currently used in Ewing Sarcoma chemotherapy, were above 100 µM in all lines tested. (B) Clonogenic assays illustrating the differential effects of ATRi (100 nM; 16h) and PARPi (10 nM; 16 hrs) on U2OS and A4573 cells. (C) Quantification of the percentage of survivor colonies shown in A. (D) DNA content (PI) was assessed by flow cytometry on two non-Ewing Sarcoma osteosarcoma lines and three Ewing Sarcoma lines exposed to ATRi for 72 hrs (1 µm). (E) Western blot illustrating the cleavage of PARP1 on Ewing Sarcoma lines and U2OS upon a short exposure to ATRi (1 µM, 4 hrs). (F) FACS analysis of DNA content (PI) and H2AX phosphorylation in U2OS and A4573 cells exposed to ATRi (10 µM, 5 hrs), illustrating the increased levels of ATRi-induced RS (as measured by γH2AX in cells with an S-phase DNA content) in Ewing Sarcoma cells.

cell cycle. Moreover, flow cytometry analyses of H2AX phosphorylation together with DNA content revealed that ATR inhibition led to increased γH2AX levels specifically in S phase, and which were exacerbated in Ewing Sarcoma (A4573) cells compared to U2OS (Figure 16F). Thus, the sensitivity of Ewing Sarcoma to ATR inhibition correlates with an increased induction of RS by the compound in these cells.

3. The presence of EWSR1 translocations sensitises cells to ATR inhibitors

As previously mentioned, the most common translocations found in Ewing Sarcoma take place between EWSR1 protein and the transcription factor FLI1 (EWS/FLI1), counting for the 85% of cases. To determine whether the sensitivity towards ATR inhibitors observed in Ewing Sarcoma cells was not something particular of the chosen cell lines but rather a consequence of the initiating oncogenic translocation, we used a mouse transgenic line where EWS/FLI1 expression can be activated by induction of Cre recombinase upon

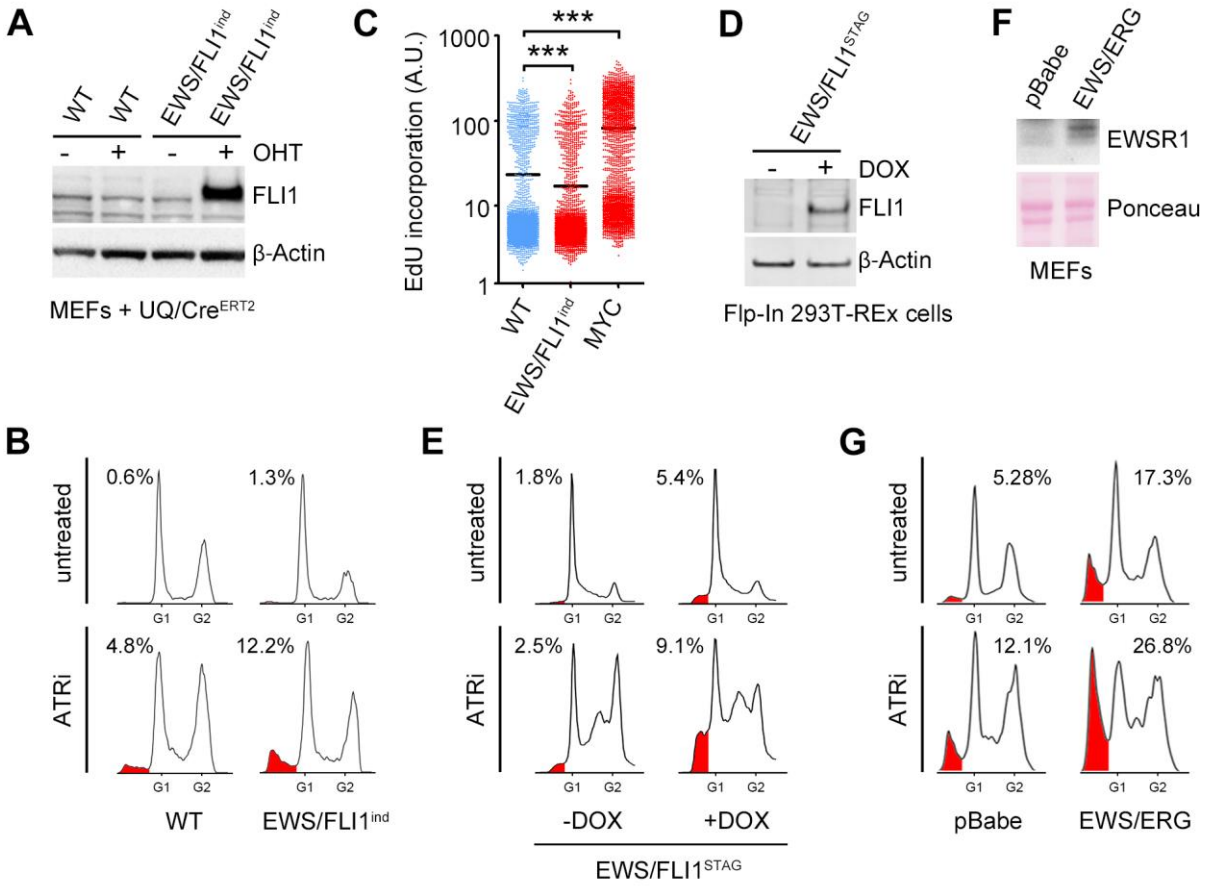


Figure 17. Expression of EWSR1 translocations sensitises cells to ATRi. (A) Western blot illustrating the expression of EWS/FLI1 (measured with a FLI1 antibody) that can be obtained in EWS/FLI1^{ind} MEF upon OHT-induced activation of a Cre^{ERT2} expressed from the ubiquitin promoter (UQ/Cre^{ERT2}) (Ruzankina *et al.*, 2007). OHT was added for 48 hrs at 1 µM. β-Actin was used as loading control. (B) DNA content (PI) analyses by flow cytometry illustrating the toxicity of ATRi (5 µM, 48 hrs) on WT and EWS/FLI1^{ind} MEFs harbouring UQ-Cre^{ERT2} exposed to OHT (1 µM, 48 hrs). SubG1 populations are shaded in red and their percentages are indicated. (C) DNA replication rates of WT and EWS/FLI1^{ind} MEF harbouring UQ-Cre^{ERT2} exposed to OHT, as well as of WT MEF infected with a retrovirus expressing the MYC oncogene were evaluated by quantifying the incorporation of EdU per nucleus by High Throghput Microscopy. (D) Western blot illustrating the expression of EWS/FLI1 (measured with FLI1 antibody) that can be obtained in Flp-In 293T-REx cells carrying a EWS/FLI1-STAG cDNA (EWS/FLI1^{STAG}) upon induction with doxycycline (DOX) (200 ng/ml, 48 hrs). β-Actin was used as loading control. (E) DNA content (PI) analyses by flow cytometry illustrating the toxicity of ATRi (1 µM, 24 hrs) on EWS/FLI1^{STAG} cells exposed or not to DOX (48 hrs). SubG1 populations are shaded in red and their percentages are indicated. (F) Western blot illustrating the expression of EWS/ERG (measured with an EWSR1 antibody) that can be obtained in MEF upon infection with a EWS/ERG expressing retrovirus (or empty vector; pBabe). Ponceau was used as loading control. (G) DNA content (PI) analyses by flow cytometry illustrating the toxicity of ATRi (5 µM, 48 hrs) on MEF infected with an EWS/ERG expressing retrovirus (or empty vector). SubG1 populations are shaded in red and their percentages are indicated.

tamoxifen administration (EWS/FLI1^{ind}) (Lin *et al.*, 2008) (Figure 17A). Supporting a causative role for the translocation, Cre induction was sufficient to sensitise EWS/FLI1^{ind} primary mouse embryonic fibroblasts (MEFs) to ATR inhibition, as observed by an increase in the percentage of apoptotic cells (SubG1 phase) in EWS/FLI1^{ind} MEFs compare to wild-type (WT) MEFs after ATRi treatment (Figure 17B). Interestingly, and in contrast to other oncogenes that sensitise tumour cells to limited ATR activity such as MYC (Murga *et al.*, 2011), EWS/FLI expression did not increase DNA replication rates as measured by EdU incorporation (Figure 17C).

To test the effect of EWS/FLI1 expression in human cells and independently of Cre activation, we generated a doxycycline inducible EWS/FLI1 expressing line in human Flp-In T-Rex 293 cells (293$^{EWS/FLI1}$) (Figure 17D). Similar to the observations in EWS/FLI1^{ind} MEFs, doxycycline exposure sensitised 293$^{EWS/FLI1}$ cells to ATRi, as shown by an increased percentage of dead cells in subG1 phases of cell cycle when expressing the EWS/FLI1 translocation (Figure 17D, E).

In addition to EWS/FLI1, expression of EWS/ERG, a different translocation found in Ewing Sarcoma patients, also sensitised cells to ATR inhibition in primary MEFs. Cells expressing the EWS/ERG translocation presented an increase in the cellular death as shown by the rising of the percentage of cells in subG1 phase (Figure 17F, G). Moreover, one of the ATRi-sensitive Ewing Sarcoma cell lines tested above (TTC466) carries an EWS/ERG translocation instead of EWS/FLI1. These data indicate that the presence of EWSR1-involving translocations sensitises human and mouse cells to ATR inhibitors.

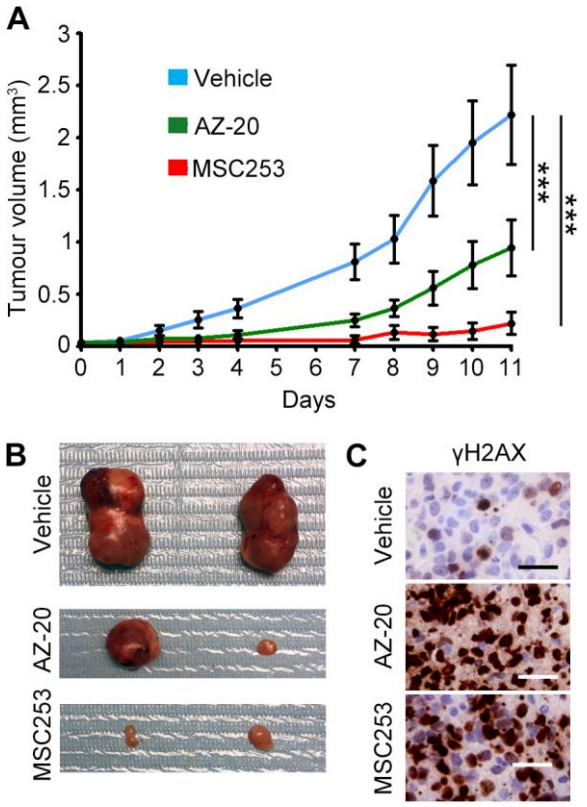


Figure 18. Efficacy of ATR inhibitors in Ewing Sarcoma xenografts as single agents. (A) Efficacy of AZ20 and an independent ATR inhibitor (MSC253) as monotherapy on the growth of Ewing Sarcoma xenografts (A4573). Treatment started when tumours became palpable. (B) Examples of the tumour sizes observed at endpoint from A. (C) γH2AX IHC on xenografts from A 48 hrs after starting the treatment. Scale bar indicates 30 µm. Error bars indicate s.d. ***$P < 0.001$.

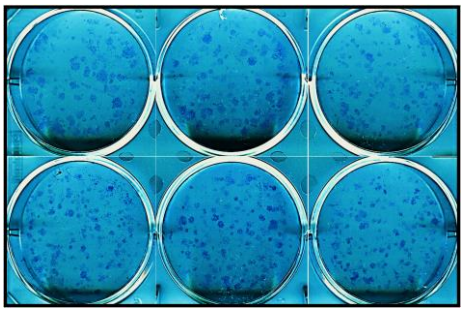
4. ATR inhibitors show efficacy as single agents in a *xenograft* mouse model of Ewing Sarcoma

Finally, to determine the efficacy of ATR inhibitors *in vivo*, we evaluated their

antitumour effects using a *xenograft* model in SCID mice. For that, we subcutaneously implanted the human A4573 Ewing Sarcoma cell lines into the flanks of these mice, treated them with ATR inhibitors starting when tumours became palpable and monitored tumour growth. Remarkably, oral administration of two independent ATR inhibitors reduced the growth of *xenografts* from A4573 Ewing Sarcoma cells (Figure 18A, B). Moreover, *xenografts* from mice treated with ATR inhibitors presented a generalized accumulation of pan-nuclear γH2AX-positive cells, consistent with the mechanism of action of ATR and CHK1 inhibitors (Murga *et al.*, 2011; Sarmento *et al.*, 2015) (Figure 18C). Of note, and whereas all current clinical trials using ATR inhibitors rely on combination therapies with additional genotoxic agents (https://clinicaltrials.gov/), both ATR inhibitors were used as single agents in these experiments. Taken together, these data identify ATR inhibitors as a potential therapy for Ewing Sarcoma.

CHAPTER 3

EWSR1 protein as a potential new player in the Replication Stress Response



1. EWSR1 ablation results in accumulation of replication stress and sensitises cells to ATR inhibitors

As mentioned in previous sections, although the role of EWSR1 is not fully understood, increasing evidence suggest that it may be involved in the DDR, either directly or indirectly. For instance, genetic ablation of *EWSR1* generates an alternative splicing of several genes that are involved in DNA repair and p53-mediated signalling pathways (Dutertre *et al.*, 2010; Paronetto *et al.*, 2011). The potential role of EWSR1 in the DDR is also supported by the phenotype of EWSR1 Knock-Out (EWSR1$^{-/-}$) mice, which in several aspects resembles that of ATR hypomorphic mice (Li *et al.*, 2007). However, the precise role of EWSR1 in the DDR is far from understood, and elucidating it could be particularly relevant because it remains unknown whether the EWS/FLI1 translocation could result in the loss of EWSR1 function, and thus in turn contribute to the tumourigenesis process.

For all these reasons, we here aimed to gain further insight into the implication of EWSR1 in the ATR pathway and in the RS response. For that purpose, we first analysed the levels of various RS markers upon treat of immortalized EWSR1$^{-/-}$ MEFs with the RS-inducing agent HU (Li *et al.*, 2007). Remarkably, EWSR1 deletion in MEFs caused a significant increase in levels of p-RPA and p-CHK1 compared to WT MEFs upon treatment with HU, (Figure 19A), indicating increased or persistent ATR pathway activation due to RS and/or the accumulation of DNA

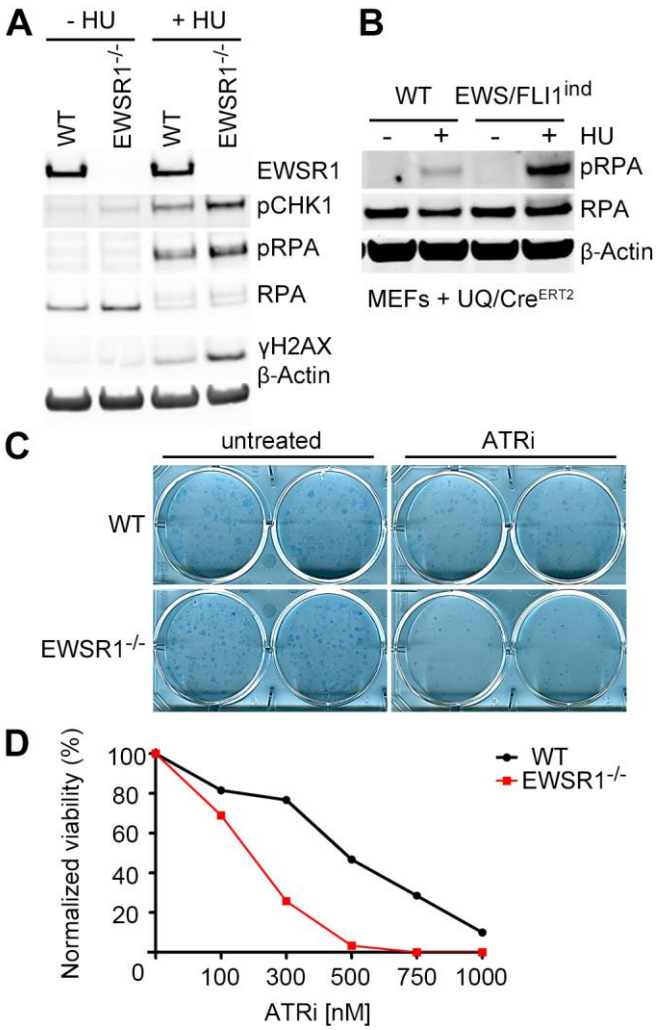


Figure 19. EWSR1$^{-/-}$ MEFs show highest levels of RS and are sensitive to RS inducing agents. (A) Western blot of analysis of the levels of RS in EWSR1$^{-/-}$ immortalized MEFs exposed to HU (2 mM, 4 hrs) using antibodies against EWSR1, pCHK1, pRPA, RPA, γH2AX. β-Actin was used as loading control. (B) Western blot of analysis of the levels of RS in primary EWS/FLI1^{ind} MEFs exposed to HU (2 mM, 3 hrs), upon OHT-induced activation of a Cre^{ERT2}, using antibodies against pRPA32 and pRPA. β-Actin was used as loading control. (C) Clonogenic assays illustrating the differential effects of ATRi (300 nM; 1 week) immortalized EWSR1$^{-/-}$ MEFs. D) Survival curves of colonies shown in (C) exposed to ATRi at indicated doses for 1 week.

breaks (Figure 19A). Similarly, expression of EWS/FLI1 in MEFs (EWS/FLI1^{ind} MEFs) also showed an increased pRPA levels upon HU treatment compared to control MEFs that do not express the EWS/FLI1 translocation (Figure 19B), suggesting that indeed the translocation could potentially lead to the loss of function of EWSR1. In addition to the biochemical analyses, colony survival assays showed that EWSR1$^{-/-}$ MEFs give rise to a reduced number of colonies compared to WT MEFs upon treatment with the ATRi (Figure 19C, D). Altogether, these results suggest that EWSR1 contributes to the response to RS in mammalian cells and that the loss of EWSR1 function can contribute to the sensitivity to ATR inhibitors observed in Ewing Sarcomas.

2. Analysis of EWSR1 interactome

To further explore why EWSR1 deficiency and EWS/FLI1 expression confers sensitivity to ATR inhibitors -and thus, why Ewing Sarcomas are sensitive to ATR inhibition- we interrogated the potential EWSR1 partners by means of proteomic analysis of EWSR1 interactions. For this purpose, we generated a doxycycline inducible streptavidin-tagged (STAG) EWSR1 expressing line in human Flp-In T-Rex 293 cells (EWSR1^{STAG}). We isolated nuclear extracts from these cells and performed STAG pull-down assays (Figure 20A) for subsequent mass spectrometry analyses. The three FET family member proteins are known co-interactors (Spahn *et al.*, 2003; Pahlich *et al.*, 2009), and as expected, our analysis identified all of them (FUL, EWSR1 and TAF15), together with SAFB2 (previously found to interact with FUS [Wang *et al.*, 2011]), among the most enriched interactors of EWSR1 (Figure 20B). Also, and in line with previous studies (Paronetto, 2013), factors involved in RNA metabolism and nucleolar proteins were also found amongst the top EWSR1 interactors (Figure 20B).

Interestingly, we observed that EWSR1 strongly interacted with the protein arginine methyltransferase 1 (PRMT1) (Figure 20C, D). PRMT1 has been shown to di-methylate the guanidine nitrogen group of arginine residues that are present in proteins such as TAF15, FUS and EWSR1 (Araya *et al.*, 2005; Jobert *et al.*, 2009; Tradewell *et al.*, 2012), and remarkably, the methylation state of EWSR1 RNA-binding domain was demonstrated to affect its subcellular localization (Belyanskaya *et al.*, 2003) EWSR1 is endowed with two main domains, the N-terminal transcriptional activation domain and the C-terminal RNA binding domain (Figure 7B). Interestingly, the RNA binding domain is lost in the EWS/FLI1 fusion protein (Figure 6B), which suggested that this might affect the interaction with PRMT1. Consistently, we found that the interaction of EWSR1 with PRMT1 is lost in the EWS/FLI1 fusion protein (Figure 20D). We next decided to investigate whether di-methylation patterns of EWSR1 are affected in the presence of DNA damage. Interestingly, we observed

increased levels of EWSR1 di-methylation at Arg^{506} upon treatment with the DSB inducing agent Neocarzinostatin (NCS) and even more with ATRi (Figure 20E), suggesting a possible implication of this post-translational modification in the role of EWSR1 in the DDR. Altogether, these experiments suggest that the loss of EWSR1 RNA-binding domain in the EWS/FLI1 fusion protein might lead to the loss of EWSR1 normal function due to the loss of relevant functional interactions such as the one with PRMT1.

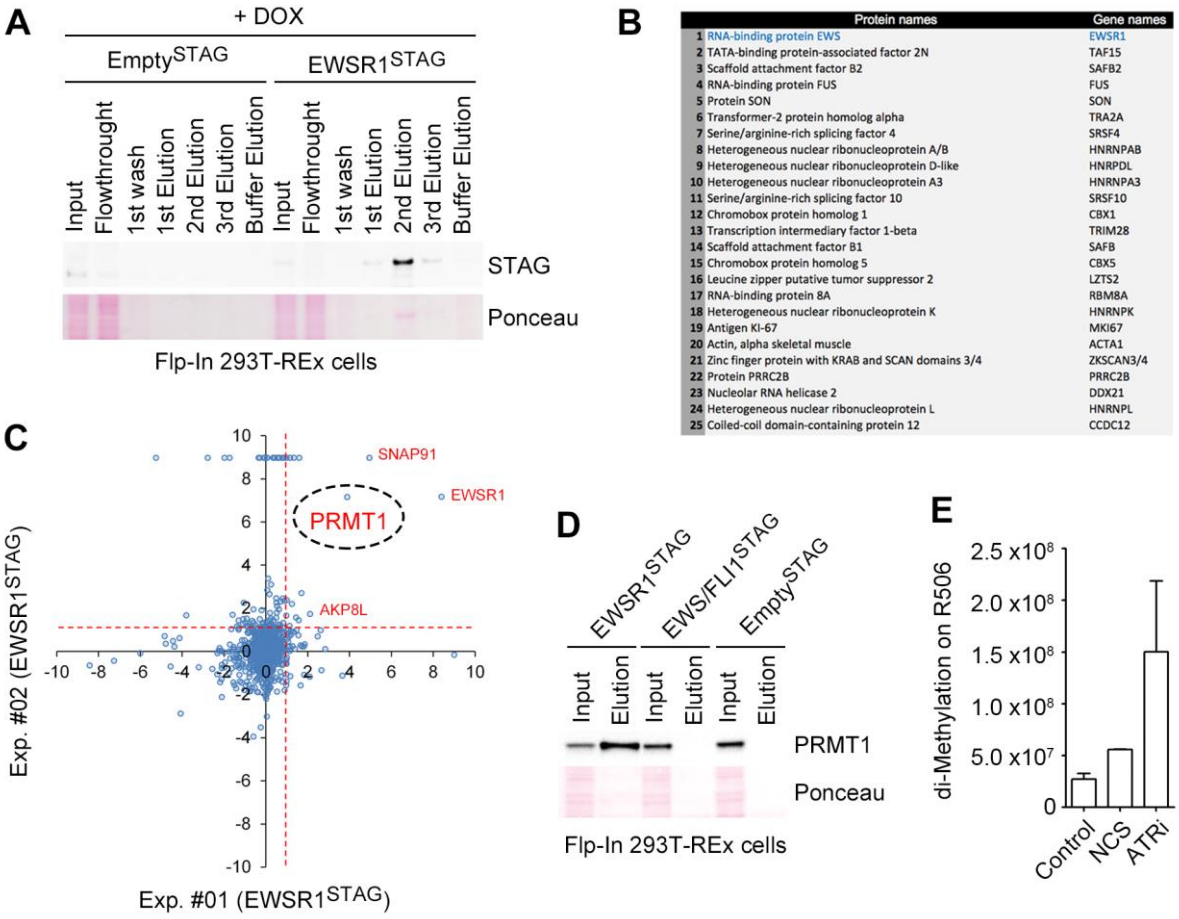


Figure 20. EWSR1 protein interacts with nucleolar proteins and PRMT1 methyltransferase. (A) Western blot illustrating the STAG pull-down assays of nuclear extracts from 293-EWSR1^{STAG} cells (EWSR1^{STAG} signal is measured with a STAG antibody). (B) The table indicates the interactors of EWSR1^{STAG} that were identified by MS, ordered by the level of enrichment when compared to proteins pulled down from cells expressing only the STAG. Most proteins identified are involved in RNA metabolism and/or nucleolar biology. (C) Graphic illustrating the PRMT1 interaction with EWSR1^{STAG} protein. (D) Western blot of PRMT1 illustrating the loss of interaction with EWS/FLI1^{STAG} fusion protein. (E) Levels of di-Methylation on EWSR1 arginine 506 (R506) after exposure to NCS (2.25 µM, 1 hr) or ATRi (30 nM, 4 hrs).

3. EWS/FLI1 expression results in a toxic effect in combination with silencing of nucleolar proteins

Due to the critical role of EWSR1 translocations in driving Ewing Sarcoma, targeting EWSR1 interactors critical for its function might provide a novel therapeutically opportunity for the treatment of these tumours. For this reason, we next addressed whether knock down of EWSR1 interactors confers synthetic lethality in cells bearing the EWS/FLI1 fusion protein. We selected 85 candidates amongst the top EWSR1 interactors and performed a screening of synthetic lethality in human inducible EWS/FLI1 expressing cells ($293^{EWS/FLI1}$) by using an endoribonuclease-prepared small interfering RNAs (esiRNA) library of the selected candidates. First, as a control, we analysed the effect of the knock down of each interactor in the viability of normal cells (non-expressing EWS/FLI1) using as a reference the condition of cells that had not been transfected (Figure 21A). In parallel, we evaluated the effect in

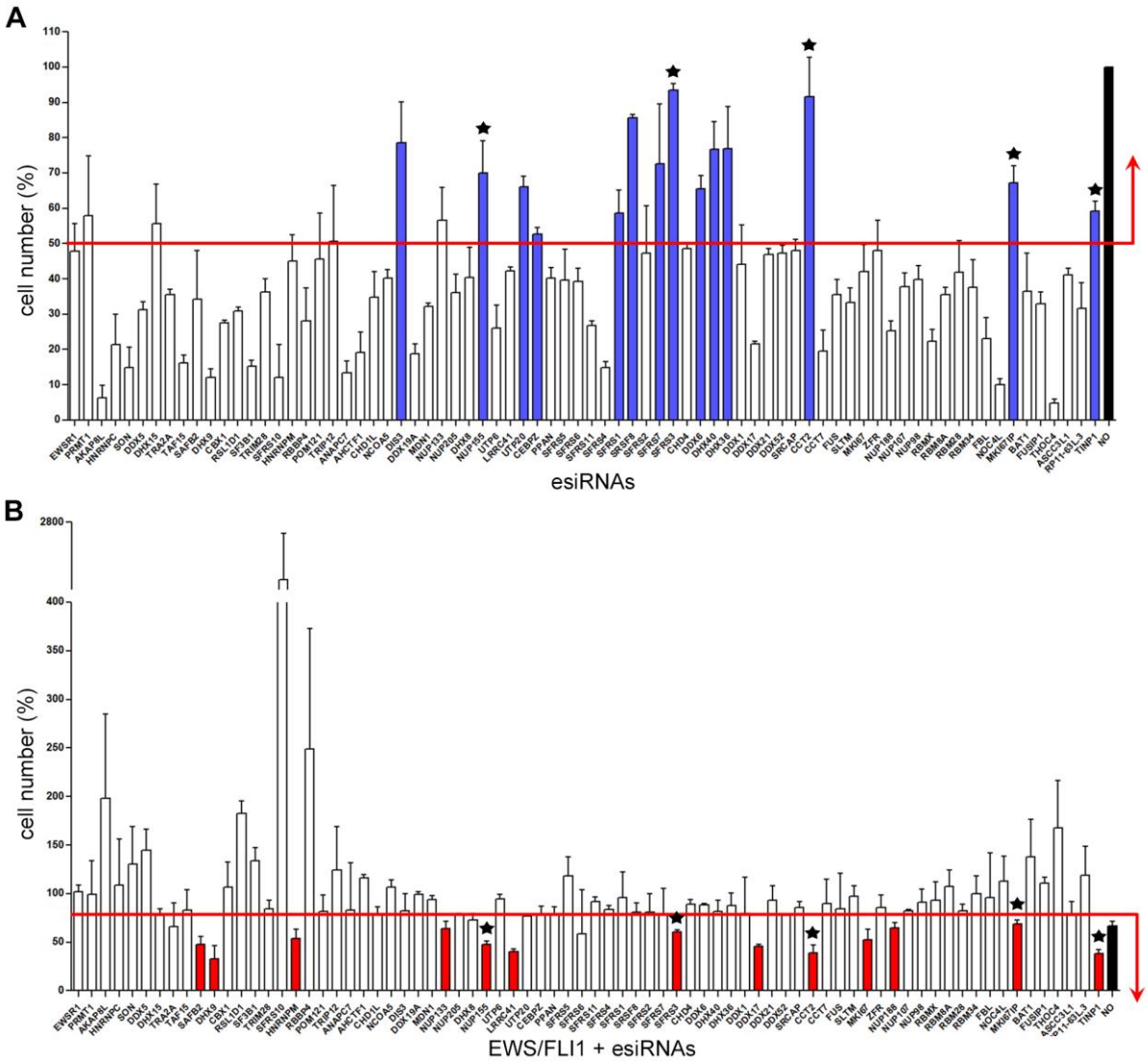


Figure 21. EWS/FLI1 expression displays synthetics lethality with knock down of nucleolar proteins. (A) Graphic illustrating the effect in the viability of knock down of esiRNA indicated. (B) Graphic illustrating the synthetic lethality of knock down of esiRNA indicated together with EWS/FLI1 expression.

viability of the knock down of the interactors together with EWS/FLI1 expression upon doxycycline administration (Figure 21B). In order to identify the most relevant outcomes, we aimed to select those interactors whose knock down did not decrease viability more than 50% in control conditions -not overexpressing EWS/FLI1- compared to the control without esiRNA (100% of viability) (Figure 21A), and from this set we selected those candidates whose knock down resulted in synergistic toxicity together with EWS/FLI1 expression (Figure 21B) for further evaluation.

Knock down of Nucleoporin 155 (NUP155), serine and arginine rich splicing factor 3 (SFRS3), chaperonin containing T-complex polypeptide 1 subunit 2β (CCT2), nucleolar protein interacting with the forkhead-associated domain of MKI67 (MKI67IP) and TGFβ-inducible nuclear protein 1 (TINP1) resulted in selective toxicity for cells expressing EWS/FLI1, but nor for healthy cells, identifying these proteins recognized as the synthetic lethality targets. Interestingly, all these EWS/FLI1 synthetic lethality targets are involved in the nucleolar biology. NUP155 and SFRS3 participate in mRNA export (Huang and Steitz, 2001; Rayala *et al.*, 2004), MKI67IP is required for ribosomal RNA maturation during cell cycle progression (Pan *et al.*, 2015) and TINP1 encodes a nucleolar protein involved in cell cycle regulation and proliferation (Zhang *et al.*, 2010). Interestingly, CCT2 is involved in the folding and activity of the oncoprotein AML^{ETO}, which is expressed in AML (Roh *et al.*, 2016).

Based on the fact that nucleolar proteins are highly enriched in $EWSR1^{STAG}$ pull-downs and that nucleolar proteins are also the predominant EWS/FLI1 synthetic lethality hits, we hypothesised that EWSR1 deficiency and/or EWS/FLI1 expression in Ewing Sarcoma (potentially resulting in EWSR1 loss of function) could disturb the nucleolar machinery of the cell. Nucleoli are structures found inside the nucleus where the transcription of ribosomal RNA (rRNA) takes place and are thus essential for ribosome biogenesis. Consequently, altered nucleolar function might lead to deficient protein synthesis, which in turn could aggravate the nucleolar defects because of poor translation of nucleolar proteins. In this context, we hypothesised that Ewing Sarcomas could potentially be highly sensitive to inhibitors of the RNA polymerase I (CX-5461, BMH-21), which is specialised in rRNA transcription. However, we did not find a preferential sensitivity of these cells to the compounds (not shown). Regardless, in spite of the fact that further analyses are required to elucidate the role of EWSR1 in the DDR, our data clearly indicate that the function of EWSR1 is intimately linked to nucleolar biology, and further studies could help to understand whether such a function might be exploited for the development of new therapies.

DISCUSSION

The backbone treatment for cancer relies on a combination of surgery, chemotherapy and radiotherapy. Remarkably, a number of treatments with chemotherapy or radiotherapy are based on the same principle; that is, to inflict DNA damage into tumour cells. Since cancer cells replicate at very high rates, they are more sensitive to the DNA replication interferences caused by these therapies and are consequently prone to enter in apoptosis and/or senescence. Following the same rationale, over the last years an arguably improved version of this strategy arose, consisting in the combination of DNA damage agents with inhibitors of the DDR thereby enhancing the effect of the damaging agents by preventing DNA damage signalling repair. In addition, since tumour cells present intrinsically higher levels of DNA damage and RS, targeting the DDR and RS response pathways has the potential to preferentially target cancer cells thus diminishing the off-target effects that are often associated to genotoxic therapies.

In fact, one of the major challenges of cancer therapy is to kill selectively tumour cells without harming healthy cells thereby avoiding hazardous and unpleasant side effects. In this regard, since genomic instability is widespread in cancer cells (Boveri, 1914), targeting genomic instability offers an opportunity to develop more selective treatments that preferentially kill cancer cells. This strategy relies on the concept of synthetic lethality, which arises when the combination of two situations causes cell death, whereas a single one does not. This concept that emerged from yeast studies, can be used to take advantage of the limitation of tumour cells for survival when fundamental processes are attacked together, as replication and DNA repair in cells harbouring cancer associated mutations (Hartwell, 1997). A recent and prominent example of the application of synthetic lethality in cancer is the toxicity of PARP inhibitors for cells deficient in HR. PARP inhibition leads to the accumulation of single-stranded breaks that become DSB during DNA replication which are effectively repair in cells proficient in HR (*e.g.* normal cells) but are toxic for HR deficient cells (*e.g.* HR deficient tumour cells). Accordingly, this synthetic lethal interaction renders PARP inhibitors as effective and selective treatments for HR deficient cancers and is currently being exploited as a therapeutic strategy for BRCA1/2 deficient tumours (Bryant *et al.*, 2005; Farmer *et al.*, 2005).

An alternative to targeting a specific mutation is to exploit the presence of high endogenous levels of DNA damage in tumours. A well-established source of genomic instability in cancer is oncogene-induced RS (Halazonetis, 2008), which, by activating the DDR, limits cancer development in its early stages (Bartkova *et al.*, 2005; Gorgoulis *et al.*, 2005). To date, much of the work in this model has been dedicated to understand how oncogenes generate DNA damage, or to what extent the enzymes from the DDR protect us

from cancer development. However, increasing evidence show that targeting RS response kinases ATR and CHK1 is preferentially toxic for tumours experiencing high levels of endogenous RS such as MYC-induced lymphomas, MLL-translocation driven leukaemias or H-RAS (Harvey sarcoma virus- RAS) driven fibrosarcomas (Murga *et al.*, 2011; Schoppy *et al.*, 2012; Santos *et al.*, 2014). While lowering the amounts of ATR or CHK1 by half could potentially lead to increased genomic instability and cancer, a severe inhibition of the RS response might be particularly deleterious for cells that replicate very fast (*e.g.* cancer cells), causing intolerable levels of DNA damage. Altogether, this indicates that ATR and CHK1 inhibitors are particularly efficient for tumours with high replication rates and, as a consequence, constitutively high amounts of RS.

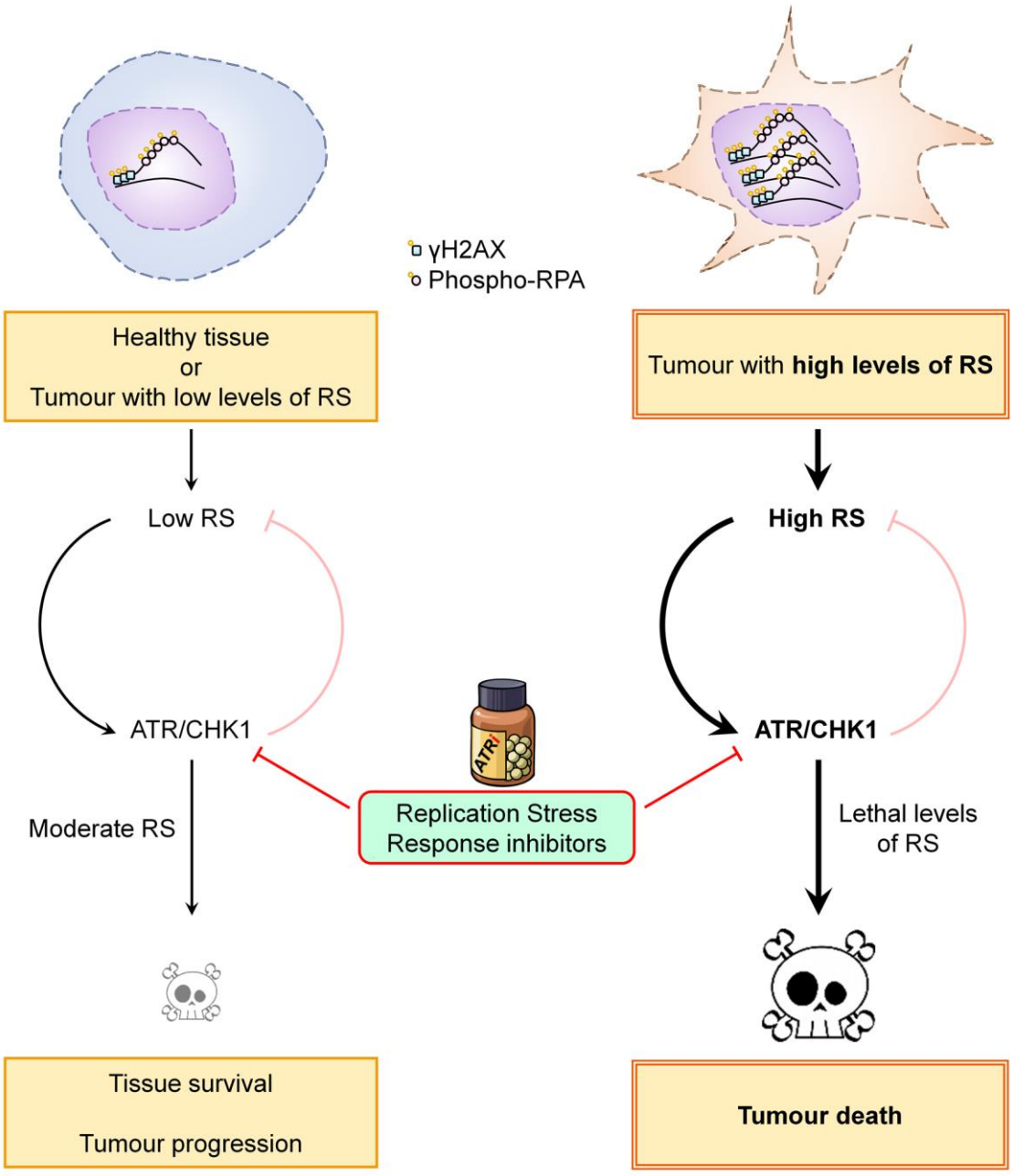


Figure 22. Synthetic lethality. Adapted from (Toledo *et al.*, 2011).

Inspired by these ideas, this Doctoral Thesis explored the idea of exploiting a synthetic lethal interaction of the RS response in cancer. If oncogenes generate RS, which is normally suppressed by the RS response, it could be possible that targeting ATR or CHK1 would be particularly toxic for cancer cells presenting considerable amounts of RS. This differs from the standard chemotherapy -which as aforementioned implies the combination of drug that generate DNA damage and DDR inhibitors- in that, in this particular case, the source of DNA damage would be intrinsic to the tumour, thereby increasing the chances to preferentially kill cancer cell. This work adds to our previous work on ATR-Seckel mice which showed that low levels of ATR are largely incompatible with tumour development and, what is more, that the loss of p53 was synthetic lethal with ATR hypomorphism (Murga *et al.*, 2009) or with ATR elimination in adult mice (Ruzankina *et al.*, 2009). This is explained by the less restrictive S-phase entry linked to p53-deficiency lead to even higher amounts of RS and cell death in the presence of a limiter ATR response (Reaper *et al.*, 2011; Toledo *et al.*, 2011).

Early work with CHK1 inhibitors showed that they failed as a general anti-cancer strategy in advanced tumours. One example of this is UCN-01, a CHK1 inhibitor that was originally discovered as an inhibitor of protein kinase C and that at some point was one of the most promising antineoplastic compounds available (Takahashi *et al.*, 1987). However, the poor efficacy in clinical trials and the off-target effects of the drug dampened the interest on it and raised doubts of the potential of the usefulness of CHK1 inhibitors in cancer. Nevertheless, and as it is the case with most chemotherapies, it is tempting to speculate that this is likely due to the fact that their efficacy could be restricted to a subset of tumours, as for example those that present high amounts of RS. Promising drugs such as Imatinib of Olaparib would have also been considered a failure if tested as generic “anti-cancer” drugs in all types of tumours. However, when these therapies are directed to tumours presenting ABL or BRCA1/2 mutations, respectively, they are very efficient, highlighting again the potential of the synthetic lethal strategy and clearly indicating the need to identify specifically which tumours types are going to benefit from these therapies. The work provided here supports this idea, as it indicated the relevance of identifying tumours with high levels of RS for the use of RS response inhibitors in cancer therapy.

Specifically, our works evidences the potential use of ATR inhibitor as anti-cancer therapy in tumours with high endogenous levels of RS. We demonstrate that inhibiting ATR in these tumours leads to p53-independent cell death due to unbearable levels of DNA damage. Following this line of thought, and by the use of preclinical models, we provide the first evidences of the efficacy ATR inhibitors as single agents in cancer therapy.

1. ATR inhibitors as a potential new therapeutic approach for MLL-driven AML

Current treatment of acute paediatric leukaemias involves the use of broad-spectrum genotoxic approaches. However, these lines of chemotherapy are largely ineffective for treatment of leukaemias that have MLL translocations. What is more, some of these therapies, such as the use of the topoisomerase II inhibitor etoposide, counterproductively promote MLL translocations and therapy-related leukaemia (Blanco, Edick and Relling, 2003; Libura *et al.*, 2009). Thus, the treatment of this subset of leukaemias would benefit from a targeted therapy that exploits a specific vulnerability in the cancer cells.

One such vulnerability is associated with the function of KMT2A, which is the protein encoded in a gene associated with MLL translocation events. KMT2A is a lysine methyltransferase that functions as an epigenetic regulator (Rao and Dou, 2015). Leukaemias carrying MLL fusion proteins require few, if any, additional mutations. Rather, fusion proteins induce leukaemia by deregulating transcription at MLL-fusion protein target genes, such as the HOXA (Homeobox protein Hox-A) gene cluster and MEIS1 (Myeloid Ecotropic viral Integration Site 1 Homolog) (Somervaille and Cleary, 2010; Bernt and Armstrong, 2011). Abnormal expression of these genes is associated with epigenetic changes, including alteration in DNA and histone methylation. For example, the H3K79 (histone 3 Lysine 79) methylase DOT1L (Disruptor of telomeric silencing 1-like) is recruited to MLL fusion protein target genes, and this subtype of leukaemia is particularly dependent on DOT1L enzymatic activity (Chen and Armstrong, 2015). Further, MLL leukaemias depend on diverse hematopoietic transcription factors, such as the bromodomain and extraterminal (BET) protein BRD4 (Bromodomain-containing protein 4), to maintain their leukaemic stem cell properties (Zuber *et al.*, 2011; Krajewska *et al.*, 2015; Roe *et al.*, 2015). Thus, one therapeutic approach for leukaemogenesis resulting from MLL translocation events is to disrupt the MLL target gene expression program with drugs that target epigenetic-modifying enzymes or the products of genes that depend on such modifications (Bernt and Armstrong, 2011). In this context, drugs that inhibit BET, such as the Bromodomain inhibitor JQ1, or DOT1L inhibitors are currently under investigation.

In addition to lineage-specific transcriptional circuits, our experiments suggested that a second point of vulnerability in MLL-driven AML is the presence of RS. Interestingly while the amount of RS markers (γH2AX) is not distinctively high in these tumours, we propose that this is because these cells have increased levels of RS response factors, such as CHK1, which help buffer the levels of RS. Accordingly, a very recent study revealed increased CHK1 levels in cells from human AML^{MLL} patients (David *et al.*, 2016). We propose that, whereas this increase in RS response factors limits the basal toxicity of RS and thus facilitates tumour

growth, it also represents a vulnerability because the tumour cells become particularly dependent on a proficient RS response. To what extent MLL translocations are responsible for the RS in AML^{MLL} cells, which have other defects such as N-RAS hyperactivity, remains to be established.

Despite the hope for new targeted therapies, mechanisms for resistance to inhibitors of BET (Fong *et al.*, 2015; Krajewska *et al.*, 2015; Rathert *et al.*, 2015) or ATR (Ruiz *et al.*, 2016) have been uncovered. Noteworthy, perturbing the chromatin-related functions of MLL fusion proteins leads to RS (Liu *et al.*, 2010; Kantidakis *et al.*, 2016). In this context, a combination of RS response inhibitors, leading to p53-independent toxicity, and epigenetic inhibitors, which may both interfere with the transcriptional properties of the MLL fusion protein and further increase the levels of RS, could help overcome the resistance of MLL-driven AML to chemotherapy.

2. Replication Stress in Ewing Sarcoma provide sensitivity to ATR inhibitors

Metastatic Ewing Sarcoma is a paediatric tumour of very poor prognosis, due to the lack of efficient therapies. Current treatments involve genotoxic agents such as temozolomide or irinotecan, whose mechanism of action involves the generation of RS. In what regards to new alternatives, Ewing Sarcoma cells were also reportedly sensitive to PARP inhibitors (Barretina *et al.*, 2012; Brenner *et al.*, 2012). However, initial clinical trials failed to see a response to these compounds in Ewing Sarcoma patients (Choy *et al.*, 2014), and thus new therapies are still needed. We here provide evidence of a distinctively high sensitivity of Ewing Sarcomas to ATR inhibitors, which correlates with high levels of endogenous RS in these tumours. Two independent ATR inhibitors were found to be significantly more toxic than the PARP inhibitor Olaparib in all Ewing Sarcoma lines tested. In addition, all Ewing Sarcoma cell lines were more sensitive to ATR inhibition than the ALT-positive cell line U2OS, recently reported as highly sensitive to ATR inhibition (Flynn *et al.*, 2015).

Our discovery of high endogenous levels of RS in Ewing Sarcoma cells also helps to explain the intrinsic sensitivity of Ewing Sarcomas to agents that perturb DNA replication. As observed in other tumours suffering from RS, such as recombination-deficient ovarian cancers (Krajewska *et al.*, 2015) or MYCN-driven neuroblastomas (Cole *et al.*, 2010), Ewing Sarcoma cells present high levels of CHK1 expression, which helps them deal with the presence of RS. As a consequence, these tumours become addicted to a proficient ATR/CHK1 pathway for their survival, explaining the high sensitivity of Ewing Sarcoma cells to ATR inhibition. At this point, we do not know why the expression of EWSR1 translocation

products drives RS in Ewing Sarcoma cells, yet since EWS/FLI1 is a transforming oncogene (May *et al.*, 1993) it could simply be another case of oncogene-induced RS. However, and in contrast to other oncogenes such as MYC or RAS, EWS/FLI1 expression does not increase DNA replication, so that a novel mechanism must be in place to explain this synthetic lethal interaction. One interesting possibility is that the expression of EWSR1 fusions could perturb the function of endogenous EWSR1, which could be the source of RS and genomic instability of these tumours. Consistently, a recent report revealed a critical role of EWSR1 in facilitating the recruitment of DNA repair factors to sites of DNA damage (Altmeyer *et al.*, 2015). In addition, previous work revealed that depletion of EWSR1 reduced levels of several DNA damage response factors, due to alterations in alternative splicing (Paronetto *et al.*, 2011). Finally, and as mentioned before, the phenotypes of EWSR1-deficient mice are reminiscent of those found in ATR mutant mice (Li *et al.*, 2007; Murga *et al.*, 2009; Cho *et al.*, 2011). Hence, it is possible that EWSR1 translocations could exert a dominant negative function over endogenous EWSR1, leading to RS and genomic instability in Ewing Sarcomas. Regardless of how EWSR1 fusions generate RS, our work provides a basis to understand the sensitivity of Ewing Sarcomas to RS-inducing agents, and identifies ATR inhibitors as a potential therapy for Ewing Sarcoma.

3. Dissecting the role of EWSR1 in genome and nucleolar maintenance

As mentioned in previous sections, it is well established that EWSR1 plays a relevant role in transcription and splicing; in fact, and quite remarkably, EWSR1 function has been linked with alternative splicing in genes involve in the DDR (Paronetto, 2013). Interestingly, in support of this relevant cellular role, *EWSR1* deficiency in mice was shown to lead to high perinatal mortality, defects in HR impairing meiosis, disrupted pre-B cell formation, hypersensitivity to ionizing radiation, premature senescence of MEFs, and an altered dynamics in the hematopoietic stem cell population. Taken together, all these evidence indicate a role for EWSR1 in preserving genomic stability (Li *et al.*, 2007), which one would speculate to be possibly indirect and not only related to its role in the splicing of some DDR genes. In line with these data, our results have identified EWSR1 as a possible new player of the ATR pathway, as they indicate that EWSR1 may be involved the RS response. Indeed, we have shown that the absence of EWSR1 causes accumulation of RS and sensitivity to the limitation of the RS response by ATR inhibitors. These results are particularly relevant, given that EWSR1 alteration has been linked to Ewing Sarcoma development, where it is commonly found as the translocation EWS/FLI1. Nonetheless, some of the physiological implications of the translocation are unclear, as it not formally demonstrated whether it implies EWSR1 loss of function. Conversely, most studies propose that EWS/FLI1 gain of

function is responsible of Ewing Sarcoma onset and progression by promoting abnormal transcription activity of FLI1 (Ohno *et al*., 1993; Wakahara *et al.*, 2008; Yang *et al.*, 2010; Niedan *et al.*, 2014), therefore providing a molecular mechanism with clinical relevance in tumorigenesis. Nonetheless, our results show that both EWSR1$^{-/-}$ cells and cells expressing heterologous EWS/FLI1 protein tend to accumulate high amounts of RS and, more importantly, the sensitivity towards ATR inhibitors found in Ewing Sarcoma cell lines and EWS/FLI1 expressing cell lines is shared by EWSR1 deficient cells. These results support the idea that EWS/FLI1 could function as a dominant negative form of EWSR1. Additionally, it is also tempting to speculate that the loss of EWSR1 function, which leads to accumulation of RS, could contribute to the tumorigenesis of Ewing Sarcoma and to the response to chemotherapy in this tumour type.

Irrespective of the high amounts of RS associated with EWSR1 ablation and EWS/FLI1 translocation, and of the possible contribution of RS to Ewing Sarcoma development, the exact role of EWSR1 in the response to RS remains unclear. Given that the genes involved in the DDR whose splicing is regulated in part by EWSR1 are not directly involved in the response to RS (Paronetto *et al.*, 2011), it is possible that EWSR1 role in the response to RS could be independent of its splicing function. But this poses the intriguing question of what is the exact role of EWSR1 in the RS response?

In an attempt to gain further insight into this function of EWSR1, we conducted proteomic studies to identify EWSR1 interactors. By doing this, we demonstrated that EWSR1 strongly interacts with the methyltransferase PRMT1, and importantly that this interactions is lost in cells expressing EWS/FLI1 fusion protein. Additionally, we also observed that EWSR1 is di-methylated on the Arg^{506} residue, and that the levels of this modification increase in the presence of DNA damage, suggesting functional relevance. In this regard, it has been suggested that the methylation of arginine residues on proteins is involved in a number of different cellular processes, such as regulation of transcription, RNA metabolism and DNA damage repair (Bedford and Richard, 2005) and that PRMT1 is arguably the predominant mammalian protein arginine methyltransferase (Tang *et al.*, 2000). Hence, it is also tempting to speculate that the patrons of methylation on EWSR1, by PRMT1 or other methyltransferases proteins (Belyanskaya, 2001; Pahlich *et al.*, 2005; Kim *et al.*, 2008; Pahlich *et al*., 2008) could regulate its function in the presence of DNA damage. Importantly, this regulation is lost in EWS/FLI1 fusion protein due to the loss of the C-terminal region of EWSR1 in this translocation.

Regardless of a role in the DDR, our proteomic analyses also demonstrated that EWSR1 strongly interacts with proteins involved in RNA metabolism and nucleolar proteins. Of note, all EWSR1 translocations in Ewing Sarcomas involve the loss of the C-terminal region, which is suggested to mediate RNA binding, and therefore, this indicates that the loss of this region would also lead to impaired RNA binding by EWSR1. Interestingly, among the all the identified interactors, the five whose silencing confers synthetic lethality in cells bearing the EWS/FLI1 fusion protein, are related with nucleolar processes such as mRNA export (Huang and Steitz, 2001; Rayala *et al.*, 2004) or ribosomal RNA maturation during cell cycle progression (Pan *et al.*, 2015). This finding might have relevant implications, as it suggests that targeting RNA metabolism pathways might be particularly detrimental for Ewing Sarcoma cells. We must note, however, that EWS/FLI1 overexpression does not

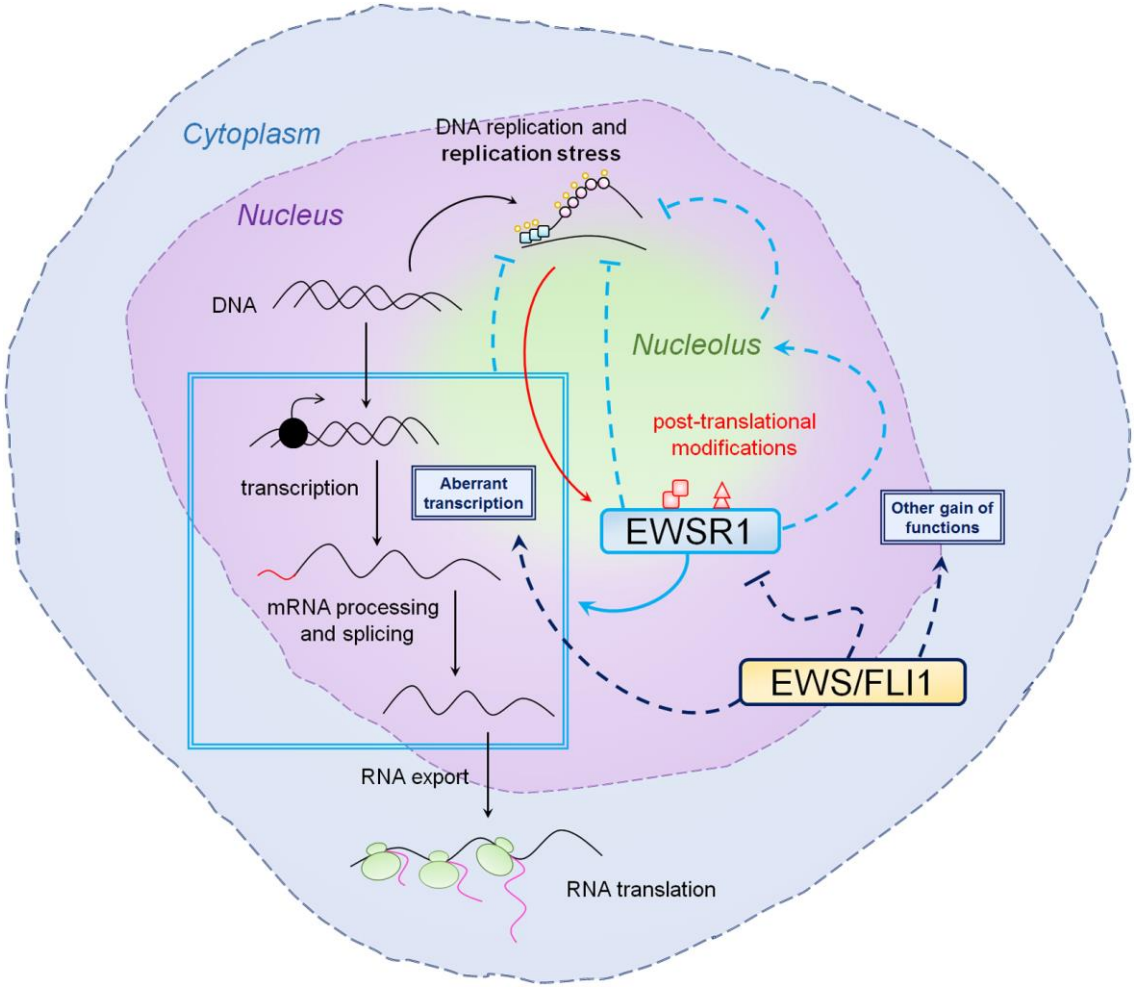


Figure 23. Possible functions of EWSR1 in the DDR and potential effects of the EWS/FLI1 translocation. In the context RS, EWSR1 could be activated through post-translational modification such as phosphorylation or di-methylation. Upon activation, EWSR1 could promote suppression of the RS via its previously described function in transcription and alternative splicing of DDR genes, possibly more specifically in the nucleolus. Remarkably, our work also suggests that EWSR1 acts operate to limits RS directly through an unknown mechanism. EWS/FLI1 expression could function as a dominant negative of EWSR1 protein and therefore, ablate EWSR1 normal functions implying an accumulation of DNA damage that could contribute to tumour onset.

particularly sensitise cells to inhibition of RNA polymerase I, which is the polymerase in charge of in rRNA synthesis (not shown). In any case, and in addition for its cancer implications, these data support an important role of EWSR1 in RNA biology, since nucleoli are structures essential for rRNA biogenesis and consequently highly enriched in RNA. Further studies are required to determine the exact role of EWSR1 in the response to replication stress, and to what extent this is linked to its functions in RNA metabolism or nucleolar integrity. However, our data supports an important role of EWSR1 for preserving genome integrity (reviewed in Kovar, 2011; Paronetto, 2013), and provide the first evidence of the potential of ATR inhibitors in the treatment of Ewing Sarcomas.

CONCLUSIONS

1. AML^{MLL} cells are highly sensitive to ATR inhibitors in vitro as independently of p53, due to the generation of high loads of DNA damage.

2. A treatment with ATR inhibitors as single agent shows remarkable efficacy in an *allograft* mouse model of MLL-translocation driven AML, as well as in *xenografts* of a human AML-MLL cell line.

3. Targeting the DSB response with ATM inhibitors as single agent also shows efficacy in a preclinical *allograft* model of AML^{MLL}.

4. Ewing Sarcomas cells present increased level of RS, as evidenced by an increase of γH2AX in replicating cells as well as intrinsically perturbed replication rates.

5. Expression of EWS/FLI1 or EWS/ERG translocations suffices to generate RS and to sensitise human or mouse cells to ATR inhibitors.

6. ATR inhibitors show efficacy as a single agents in *xenografts* of Ewing Sarcoma cells, along with a high load of treatment-induced DNA damage in tumour cells.

7. Loss of EWSR1, the gene involved in Ewing Sarcoma initiating translocation, leads to RS and sensitivity to ATR inhibition, suggesting that -in addition to the translocation- the loss of endogenous EWSR1 can contribute to the response to chemotherapy in Ewing Sarcomas.

8. EWSR1 interacts primarily with proteins involved in nucleolar biology such as NUP155, CCT2, SFRS3, MKI67IP or TINP1; and simultaneous expression of EWS/FLI1 with either knockdown of these genes leads to synthetic lethality, suggesting an important role of EWSR1 in nucleolar biology.

CONCLUSIONES

1. Las células AML^{MLL} son altamente sensibles a los inhibidores de ATR *in vitro* de forma independiente a p53, debido a la generación de una enorme cantidad de daño en el DNA.

2. El tratamiento con inhibidores de ATR como único agente muestra una remarcada eficacia en un modelo de ratón *allograft* de AML derivada de una translocación en el gen *MLL*, así como en modelos de *xenografts* de una línea celular AML-MLL humana.

3. Interferir con el mecanismo de reparación de dobles roturas del DNA usando inhibidores de ATM como agente único también muestra eficacia en un modelo *allograft* de ratón preclínico de AML^{MLL}.

4. Las células de Sarcoma de Ewing presentan un aumento en los niveles de RS, evidenciado por un incremento de γH2AX en las células que están en fase de replicación, así como un ratio de replicación intrínseco alterado.

5. La expresión de las translocaciones EWS/FLI1 o EWS/ERG son suficientes para generar RS y sensibilizar a las células, tanto humanas como de ratón, frente a los inhibidores de ATR.

6. Los inhibidores de ATR muestran eficacia como agente único en *xenografts* de células de Sarcoma de Ewing, conjuntamente con una elevada cantidad de daño en el DNA inducido por el tratamiento de las células tumorales.

7. La pérdida de EWSR1, el gen involucrado en la translocación que inicia el Sarcoma de Ewing, genera RS y sensibilidad a la inhibición de ATR, sugiriendo que -además de la translocación- la pérdida del gen EWSR1 endógeno puede contribuir a la respuesta a la quimioterapia en el Sarcoma de Ewing.

8. EWSR1 interactúa de forma primaria con proteínas involucradas en la biología nucleolar como NUP155, CCT2, SFRS3, MKI67IP o TINP1; y la expresión simultánea de EWS/FLI1 junto con el silenciamiento de estos genes lleva a una letalidad sintética, sugiriendo un papel importante de EWSR1 en la biología nucleolar.

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