Title: Effects of hypoxia on the response of the haematopoietic system to radiation-induced DNA damage

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Effects Of Hypoxia On The Response Of The Haematopoietic System To Radiation-induced DNA Damage

A thesis submitted to the National University of Ireland, Galway
in fulfilment of the requirements for the degree

Doctor of Philosophy
by
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February 2017
Abstract

Preparative regimens using DNA damaging agents such as total body irradiation (TBI) prior to bone marrow transplantation (BMT) have profound deleterious effects on the hematopoietic system including the stromal cells that support haematopoi-esis and thymopoiesis. Stromal cells are crucial for the successful regeneration of the immune system. This may be one of the main causes for the altered BM architecture and impaired HSC engraftment as well as prolonged periods of lymphopenia and T-cell deficiency common following BMT. The DNA damage response (DDR) is a complex signaling network allowing cells to respond to many different genotoxic insults. Hypoxia is known to modulate the DDR and play a role affecting the survival of different cell types. In this Thesis, the DNA damage response of thymic epithelial cells (TEC), haematopoietic stem cells (HSC), mesenchymal stromal cells (MSC) and DN2 pro-T cells, was characterized, as well as the cell-type specific effects of hypoxia on the DDR. I have shown that, while hypoxia specifically decreases the radio-resistance of mTECs by up-regulating the expression of the pro-apoptotic factor Bim, it increases the radio-resistance of HSCs by enhancing the expression of anti-apoptotic factors such as Bcl-2. In addition, in primary mouse TECs, irradiation caused impaired expression of important genes for TEC function. The molecular mechanism of Hif-1α-mediated increased radio-resistance of MSC in hypoxia has been shown to involve interaction with Arnt to constitute the Hif-1 transcription factor. Hif-1 enhances the DDR of mouse MSCs through the regulation of their transcriptional programme in hypoxia probably regulating DDR protein stability. Finally, the DDR of DN2 pro-T cells has been characterized in vitro and in vivo, and hypoxia-induced phenotypical changes have been described and investigated. These findings have important implications for improving the outcomes of BMT and promoting successful reconstitution of the haematopoietic system.
Acknowledgements

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your help and ideas, it has been great to work with you. Thanks to Janna Lusseng for her CRISPR advice, and to Alessandro Natoni for our endless discussions, your great ideas, and for lending me so many antibodies! Thanks to Tara Sugrue, from whom I “inherited” this project, for all her help when I was starting and for passing on all her knowledge to me. Many thanks to Llucia, Fran, Lilly and Patrick for making me enjoy every minute I spent in Basel.

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Thesis declaration and contributions

This PhD thesis was supported by a Marie Curie Initial Training Network (ITN) fellowship from the programme “Decision-making within cells and differentiation entity therapies (DECIDE)” which was funded by the European Union under the FP7 programme (project number 315902).

I declare that I have not obtained any previous qualification from NUI Galway based on any results contained in this thesis. I conducted all the experiments and wrote the thesis under the supervision of Professors Rhodri Ceredig and Noel Lowndes. There are a few exceptions where experiments were performed in collaboration with others. Some cell lines and reagents were also obtained from other laboratories. For clarity these are indicated clearly in the figure legends or text when appropriate and included below:

Chapter 1

The general introduction to this Thesis is an extended version of the book chapter “The radio-resistance of mesenchymal stromal cells and their potential role in the management of radiation injury” included in “The Biology and Therapeutic Application of Mesenchymal Cells”, a book edited by K. Atkinson and published by John Wiley and Sons Inc. in 2017. Authors: Tara Sugrue*, Irene Calvo-Asensio* and Rhodri Ceredig (*these authors contributed equally to this work).
Chapter 2

This chapter corresponds to the manuscript entitled “Differential response of mouse thymic epithelial cell types to ionizing radiation-induced DNA damage”, by Irene Calvo-Asensio, Thomas Barthlott, Lilly von Muenchow, Noel Lowndes* and Rhodri Ceredig*, accepted for publication in the journal *Frontiers in Immunology*.

Thymic epithelial cell lines TEC3-10 and TEC1-2 were kindly provided by Prof. Georg Holländer (Department of Biomedicine, University of Basel) and ST4.5 CD4+ CD8+ thymocyte cell line was provided by Dr. Anne Wilson (Ludwig Institute of Cancer Research, Lausanne). Isolation of primary mouse whole stroma and purification of primary mouse thymic epithelial cell subpopulations was carried out by Dr. Lilly von Muenchow and Dr. Thomas Barthlott (Department of Biomedicine, University of Basel), respectively.

Development of customized macros for microscopy image analysis was done in collaboration with Jose I. Cazalilla (Department of Automatics and Industrial Informatics in the Polytechnic University of Valencia, Spain).

Chapter 3

This chapter corresponds to the manuscript entitled “The Hif-1 transcription factor mediates hypoxia-induced increase in the radio-resistance of mouse mesenchymal stromal cells”, by Irene Calvo-Asensio, Noel F. Lowndes*, Rhodri Ceredig*, which is currently in preparation.

The MS5 mouse MSC cell line was kindly provided by Prof. Antonius Rolink (Department of Biomedicine, University of Basel). Proteomics experiments and data analysis were performed in collaboration with Dr. Eugene Dillon (Conway Institute of Biomedical and Biomolecular Sciences, University College Dublin).

Chapter 4

DN2 cells and NH-HSC cell lines, as well as the reagents necessary for their *in vitro* culture were kindly provided by Prof. Antonius Rolink (Department of Biomedicine, University of Basel). CD25+ and CD25- subpopulation sorting experiments and lineage
analyses were performed by Prof. Rhodri Ceredig. Microscopy image deconvolution was performed by Dr. Muriel Voisin.
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5'-FU  5'-fluorouracil
53BP1  p53 binding protein 1
α-SMA  alpha-smooth muscle actin
γ-H2AX  gamma-H2A.X(H2A.X Ser139 phosphorylation)
λ-Ppase  Lambda phosphatase
Apaf-1  Apoptotic protease-activating factor 1
APC  Antigen presenting cell
ARNT  Aryl Hydrocarbon Receptor Nuclear Translocator
ATM  Ataxia telangiectasia mutated
ATP  Adenosine-5'-triphosphate
ATR  Ataxia telangiectasia and Rad3 related
NH  NUP98-HOXB4
BAK  Bcl-2 antagonistic killer
BAX  Bcl-2 associated X protein
BCL-2  B cell lymphoma-2
BCL-XL  B cell lymphoma-extra long
BD  Becton Dickinson
BH3  Bcl-2 homology 3 domain
bHLH  basic helix-loop-helix domain
BIM  Bcl-2 interacting mediator of cell death
BLM  Bloom syndrome protein
BM  Bone marrow
BMT  Bone marrow transplantation
BRCA1/2  Breast cancer type 1/2
BrdU  Bromodeoxyuridine
BSA  Bovine serum albumin
BSC  Hair follicle bulge stem cell
C-TAD  C-terminal transactivation domain
CAD  Caspase-activated DNAse
CAF  Cancer-associated fibroblast
CAR  CXCL12-abundant reticular
Cas9  CRISPR-associated protein 9
CCL5  Chemokine (C-C motif) ligand 5
CCL19  Chemokine (C-C motif) ligand 19
CCL21  Chemokine (C-C motif) ligand 21
CCL25  Chemokine (C-C motif) ligand 25
CD  Cluster of differentiation
CDC  Cell division cycle
CDK  Cyclin-dependent kinase
cDNA  Complementary DNA
CFU-F/-S  Colony forming unit-fibroblast/-spleen
ChIP  Chromatin immunoprecipitation
CHK1/2  Checkpoint kinase 1/2
CIP  Calf intestinal alkaline phosphatase
CKI  CDK inhibitor
cKit  KIT Proto-oncogene receptor tyrosine kinase(CD117)
CLL  Chronic lymphocytic leukemia
CMJ  Corticomedullary junction
CML  Chronic myeloid leukemia
Ct  Cycle threshold
cTEC  Cortical Thymic Epithelial Cell
CRISPR  Clustered regularly interspaced short palindromic repeats
CtIP  CTBP-interacting protein
CXCL12  C-X-C motif chemokine ligand 12
D  Diversity gene segment / Displacement
DAPI  4',6-diamidino-2-phenylindole
dATP  Deoxyadenosine triphosphate
DC  Dendritic cell
DDR  DNA Damage Response
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<td>DL1-4</td>
<td>Delta-like ligand 1-4</td>
</tr>
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<td>DL4-Fc</td>
<td>Delta-like ligand 4 – IgG1 Fc fusion protein</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>DN</td>
<td>Double negative</td>
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<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase, catalytic subunit</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
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<td>DP</td>
<td>Double positive</td>
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<tr>
<td>DSB</td>
<td>Double Strand Break</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>Epithelial cell adhesion molecule</td>
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<td>ETP</td>
<td>Early thymus seeding progenitor</td>
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<td>Fab</td>
<td>Fragment antigen-binding</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>Fibroblast activation protein</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>gRNA</td>
<td>Guide RNA</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft-versus-host disease</td>
</tr>
<tr>
<td>FIH</td>
<td>Factor inhibiting HIF-1</td>
</tr>
<tr>
<td>H2-K</td>
<td>Histocompatibility 2, K region</td>
</tr>
<tr>
<td>H2AX</td>
<td>Histone H2A.X</td>
</tr>
<tr>
<td>H3</td>
<td>Histone H3</td>
</tr>
<tr>
<td>H3Ser10</td>
<td>Histone H3 Ser10 phosphorylation</td>
</tr>
<tr>
<td>HBO</td>
<td>Hyperbaric oxygen</td>
</tr>
<tr>
<td>HD</td>
<td>Host-derived</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HOXB4</td>
<td>Homeobox B4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stromal cell</td>
</tr>
<tr>
<td>mTEC</td>
<td>Medullary thymic epithelial cell</td>
</tr>
<tr>
<td>N-TAD</td>
<td>N-terminal transactivation domain</td>
</tr>
<tr>
<td>NBS1</td>
<td>Nimegen breakage syndrome 1</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NOXA</td>
<td>Named for “damage”</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>NUP-98</td>
<td>Nucleoporin-98</td>
</tr>
<tr>
<td>ODDDD</td>
<td>Oxygen-dependent degradation domain</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>pVHL</td>
<td>Von Hippel-Lindau tumour suppressor protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAM</td>
<td>Protospacer-adjacent motif</td>
</tr>
<tr>
<td>PAR</td>
<td>Poly-(ADP) ribose</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-(ADP) ribose polymerase</td>
</tr>
<tr>
<td>PAS</td>
<td>Per-Arnt-Sim domain</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered Saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered Saline / Tween®-20</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>pH3S10</td>
<td>phosphorylated serine 10 of histone H3</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI(3)K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 up-regulated modulator of apoptosis</td>
</tr>
<tr>
<td>RAD</td>
<td>Recombinase radiation sensitive</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>RIF-1</td>
<td>Rap1-interacting factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>RNF</td>
<td>RING finger protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Stem cell antigen-1</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immuno-deficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SF-IMDM</td>
<td>Serum-free Isocove's modified Dulbecco's medium</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SNO</td>
<td>Spindle-shaped N-cadherin+ CD45- osteoblasts</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>SSB</td>
<td>Single-strand break</td>
</tr>
<tr>
<td>ST</td>
<td>Short-term</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T-acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription activator-like effector nucleases</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline / Tween®-20</td>
</tr>
<tr>
<td>TBI</td>
<td>Total-body irradiation</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEC</td>
<td>Thymic epithelial cell</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>T-reg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>UEA-1</td>
<td>Ulex europaeus lectin agglutinin</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>V</td>
<td>Variable gene segment</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>XCL-1</td>
<td>Lymphotactin</td>
</tr>
<tr>
<td>XLF</td>
<td>XRCC4-like factor</td>
</tr>
<tr>
<td>XRCC</td>
<td>X-ray repair cross-complementing protein</td>
</tr>
<tr>
<td>ZFN</td>
<td>Zinc-finger nuclease</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1.1 Mesenchymal stromal cells (MSCs): modulators of haematopoiesis

Haematopoiesis is the process that allows the generation of specialized blood cell types, all arising from a rare population of pluripotent stem cells, known as haematopoietic stem cells (HSCs). In adults, the bone marrow (BM) is the primary organ in which haematopoiesis occurs [371]. Within the bone marrow, non-haematopoietic stromal cells and various mature immune cells form a specialized microenvironment known as the bone marrow niche. The bone marrow niche provides various developmental cues that instruct HSCs to either remain quiescent (i.e. non-cycling) or to undergo cell division and subsequently differentiate into multi-potent progenitors. These multi-potent progenitors mature into specialized cells that comprise innate (e.g. neutrophils, macrophages and dendritic cells) and adaptive (e.g. B and T cells) immunity (T cells following migration of progenitors to the thymus). Mature immune cells circulate in peripheral lymphoid organs (e.g. spleen and lymph nodes) and in the blood to exert the various functions of the immune system. Under normal conditions, the rates at which mature haematopoietic cells enter and leave the peripheral blood are balanced, a process known as haematopoietic homeostasis or haemostasis. For haemostasis to be achieved, HSC self-renewal and differentiation must be tightly regulated to ensure that mature peripheral blood cells are continuously generated without exhausting the HSC reservoir in the bone marrow. Therefore, tight regulation of HSC self-renewal and differentiation by the BM niche is essential for normal haematopoiesis to be maintained throughout adult life [123, 262, 268, 468]. The cells that comprise the BM niche are distributed between two compartments: (i) the endosteal niche located between the bone and the marrow space, which is where slowly cycling, long-term (LT) HSCs are hypothesised to be located; and (ii) the perivascular niche located within the sinusoids of the BM cavity, which is thought to be the preferential location for rapidly cycling, differentiating short-term (ST) HSCs (Figure 1.1) [63, 262, 268, 456, 484, 485].

Mesenchymal stromal cells (MSCs) are a key component of the HSC niche [123]. Traditionally, MSCs were identified as BM-derived fibroblast-like cells that adhere to plastic and generate colony-forming units (CFU-F) in vitro [142, 143]. Further investigation of the nature of these cells demonstrated their multi-potent progenitor nature, being able to differentiate along mesenchymal lineages to become adipocytes (fat cells), osteocytes
Figure 1.1: The bone marrow niche. The BM niche is segregated into two distinct compartments: (i) the endosteal niche located proximal to bone, composed of osteoblasts and osteocytes (left) and (ii) the perivascular niche located proximal to the vascular sinusoids (right). The BM niche is a hypoxic environment (2-8% $O_2$) with $O_2$ levels gradually decreasing with increasing proximity to the endosteal lining. Various MSC sub-populations of differing lineage potential, defined by specific cell surface marker expression, are distributed throughout the BM niche. These MSC sub-populations secrete various ECM components, cell adhesion molecules, growth factors and cytokines that regulate defined stages of haematopoiesis including HSC quiescence, self-renewal and mobilization, and B lymphopoiesis. Abbreviations: Fibroblast activation protein (FAP); Hematopoietic stem cell (HSC); Leptin receptor (Lepr); LT-/ST-HSC (Long-term or dormant HSC; Short-term or cycling HSC – blue arrow indicates cycling activity); Mesenchymal stromal cell (MSC); Myxovirus resistance protein 1 (Mx1); Platelet-derived growth factor-$\alpha$ (PDGF$\alpha$); Stem cell antigen-1 (Sca-1).
(bone cells) and chondrocytes (cartilage cells) [145,171,249,316,350]. By flow cytometry, MSCs are distinguished by (i) the lack of haematopoietic cell surface markers such as cluster of differentiation (CD) CD45, Ter119 and CD3 and (ii) by the presence of cell surface markers including CD44, CD90, stem cell antigen (Sca-1) and platelet-derived growth factor receptor (PDGFR)-α [241,316,442]. However, it is currently unclear whether MSCs express unique cell surface markers. As a result, the identification and isolation of purified MSC populations from the bone marrow remains technically challenging.

Unlike HSCs, the origin(s), functions and true lineage potential of MSCs in the bone marrow remain poorly understood due to the technical limitations described above. However, the general concept of a stromal cell layer that supports haematopoietic cells in vivo coincided with the discovery of the HSC in 1961. In a landmark study by Till & McCulloch, HSCs were indirectly identified by demonstrating quantitative bone marrow transplantation into lethally-irradiated mice [441]. Later, haematopoietic colonies were observed in the spleen of reconstituted mice, so-called CFU-s, which were initially thought to represent clones of HSC. However, later studies by Magli, Iscove and Odartchenko indicated that some CFU-s were transient thereby representing what we call today transient amplifying cells or proliferating precursors [281]. These findings also provided the first evidence that a stromal cell compartment capable of supporting haematopoiesis exists in vivo. Following this, a variety of MSC lines, such as ST-2, MS5 and OP9, were established in vitro from whole BM cultures, to enable the expansion of haematopoietic cells ex vivo. The ability to culture purified haematopoietic cell types on these MSC lines in vitro has greatly fuelled our understanding of haematopoietic cell differentiation, in particular, of B cell development [117,358,477].

Transgenic Cre reporter mice have become a useful tool for studying specific cell populations in vivo. These reporter mice are genetically engineered to encode a fluorescent marker, e.g. green fluorescent protein (GFP) that is only expressed upon removal of a STOP codon cassette that is flanked by loxP sites that are recognized and excised by Cre recombinase. Therefore, the use of reporter mice that express Cre recombinase under the control of tissue-specific gene promoters has enabled the identification and lineage tracing of various stem cell types in vivo. In recent years, efforts have been made to use such transgenic mice to trace MSCs in vivo. These studies have revealed that, within the BM niche, MSC sub-populations of varying differentiation potential and function co-exist. These MSC sub-populations co-ordinate various stages of haematopoiesis via
(i) direct contact with HSCs and their progenitors and (ii) the production of various cytokines, growth factors and extracellular matrix (ECM) molecules. For example, a specific Mx-1-expressing sub-population of MSC progenitors has been demonstrated to strictly differentiate into osteoblasts in vivo and to remodel the BM niche in response to injury [341].

Osteoblasts also closely interact with HSCs in the endosteal niche. Here, osteoblasts secrete extracellular matrix components [e.g. N-Cadherin and Vascular cell adhesion molecule-1 (VCAM-1)], which provide physical support for HSCs. Osteoblasts also secrete various chemokines, e.g. C-X-C motif chemokine ligand 12 (CXCL-12), which regulate HSC self-renewal in the BM [63, 430, 500]. CXCL-12 production in the BM is not confined to osteoblasts, however, as several MSC sub-populations that secrete CXCL-12 have also recently been identified. In fact, Osterix (transcription factor involved in osteogenesis)-expressing MSCs have been shown to maintain HSC progenitors and B lymphopoiesis in the endosteal niche through CXCL-12 production [168]. A subset of adipo-osteogenic progenitors known as CXCL12-abundant reticular cells (CAR) cells (also belonging to this MSC population) produce large amounts of CXCL-12 and stem cell factor (SCF) in order to promote HSC self-renewal [168, 335]. In addition, regulation of B lymphopoiesis and erythropoiesis in the BM is driven by PDGFRα+Sca1+ fibroblast activation protein-α (FAP)+ MSCs, also thanks to the production of CXCL-12 and SCF (Figure 1.1) [372, 444].

Two distinct populations of MSCs, distinguished by the expression of the leptin receptor (i.e. LepR+ MSCs) and Nestin (an intermediate filament protein) (i.e. Nestin+ MSCs) have recently been identified in the perivascular niche (Figure 1.1). These populations function (i) to maintain HSCs within the BM and (ii) to suppress HSC mobilization to the spleen in response to injury or stress, a process known as extramedullary haematopoiesis [119, 302]. Both LepR+ MSCs and Nestin+ MSCs can differentiate into osteoblasts, adipocytes and chondrocytes in vitro. However, in vivo, LepR+ MSCs mainly give rise to osteocytes and adipocytes whereas Nestin+ MSCs have only osteogenic and chondrogenic potential [119, 302]. The discrepancy between the lineage potential of these MSC populations in vitro versus in vivo suggests that the differentiation potential of MSCs may be altered by growth conditions currently used to expand these cells in vitro. Therefore, it will be important to further characterize the differentiation capacity of identified MSC populations in vivo to determine the true lineage potential of
these MSC sub-types in the physiological setting. Taken together, these findings indicate that interplay between MSC sub-populations and HSCs and their progenitors in the BM niche is likely required for the correct regulation of haematopoiesis in vivo.

Oxygen tension is emerging as an important environmental factor that modulates cell behavior, including that of stem cells. Cell lines, including MSC lines, are routinely established and expanded in vitro at the inhaled ambient oxygen concentration of 21% O₂, commonly referred to as ‘normoxia’. However, the oxygen tension in most tissues is <21% and therefore, these growth conditions are physiologically hyperoxic for a multitude of cell types including MSCs. The BM niche itself is a relatively hypoxic environment with an estimated oxygen concentration ranging between ~0.6% and 8% O₂ [124, 312, 416]. In HSCs, exposure to hypoxia has been shown to promote HSC quiescence, differentiation potential and reconstitutive capacity [124, 173, 312, 487]. Most of the studies assessing the effects of hypoxia on MSCs have focused on the effects of their in vitro culture under different oxygen tensions. These in vitro studies indicate that hypoxia has a positive impact on MSC proliferation rate, pluripotency gene expression, telomerase activity and differentiation potential while suppressing senescence and altering the cytokine and growth factor production profile [216, 266, 353, 446, 448]. Therefore, although the effects of hypoxia on MSC biology in vivo are largely unknown, oxygen tension is likely to have a similar impact on MSC proliferation, differentiation and function in vivo than it does in vitro.

1.2 Thymic epithelial cells and their support to T lymphopoiesis

1.2.1 Thymus Cellular Composition and Organization: Thymic Epithelial Cells

The thymus is the primary lymphoid organ where T lymphopoiesis takes place. Its organization and cellular composition allows the establishment of the optimal microenvironments for each stage of T cell development. Thymic epithelial cells are one of the main components of the thymic stroma, and they control the homing, proliferation, differen-
tiation and selection of the thymocyte progenitors through all the process of becoming a mature, functional and self-tolerant T cell [41].

In a mature thymus, developing thymocytes comprise more than 95% of the organ’s cellularity. This means that the thymus stroma, which comprises all the non-haematopoietic cellular components of the thymus, comprises less than 5% of the cells found in the thymus [327]. These stromal cells can be subdivided in different subsets of thymic epithelial cells (TECs), that not only provide the three-dimensional matrix in which T cells develop (together with dendritic cells, macrophages and B cells), but also control the homing, expansion, maturation and selection of these thymocytes [9]. Apart from TECs, the thymus stroma also contains mesenchymal cells (mainly of neural crest origin) and endothelial cells (that form the vasculature) [287].

The mature thymus can be anatomically subdivided in two main regions enclosed in a thin capsule: an outer cortex and an inner medulla, that are conserved throughout evolution (Figure 1.2) [155]. This allows the classification of thymic epithelial cells into cortical (cTEC) and medullary (mTEC) cells that have differential morphological, functional and antigenic properties [8]. The adult thymus contains common bipotent EpCAM$^+$ UEA1$^+$Ly51$^+$PLET1$^+$MHCII$^{hi}$ TEC progenitors that can give rise to both mTECs and cTECs [453]. However, mature TECs express distinct cortical (Cytokeratin-8$^+$ and -18$^+$, Ly51$^+$ (antibody BP1/6C3), CD205$^+$) and medullary (Cytokeratin-5$^+$ and -14$^+$, Ly51$^-$) markers that, together with their ability to bind the *Ulex europaeus* lectin agglutinin (UEA-1), allow them to be distinguished by flow cytometry [164]. Differential expression of MHCII and the accessory molecules such as CD40, CD80 or CD86 allows mTECs to be further subdivided into different subpopulations. For example, the expression of AIRE, a gene involved in regulating the expression of tissue-restricted antigens, is only found in the MHCII$^+$CD80/86$^+$UEA1$^+$ subpopulation of mTECs [7].

Each area of the thymus constitutes a functionally distinct niche [8]. While the thymic cortex is composed mainly of cTECs and macrophages, the medulla is characterized by the presence of dendritic cells (DCs), as well as mTECs and macrophages, all of which cooperate and contribute to the T cell developmental process (Figure 1.2) [240]. Thymic epithelial cells provide the cytokines, chemokines, lineage inductive ligands, selective self-antigens, cell surface molecules and extracellular matrix elements necessary for T cell development, which makes this process strictly dependent on the communication
Figure 1.2: Architecture of the thymus. The thymus can be anatomically subdivided in two main zones: the outer cortex and the inner medulla, which show differential cellular composition. While the cortex is composed mainly by cTECs and macrophages, the medulla is formed by mTECs, dendritic cells and also some macrophages. T cells migrate through these zones as they develop (Image adapted from Koch and Radtke 2011 [240]).
between TECs and developing T cells [432]. Interestingly, thymocyte/TEC interaction is not only important for T cell development, but also for the establishment of the thymic microenvironment, in which the influence of the developing thymocytes has an essential role. This reciprocal instructive process is termed as “crosstalk” [165].

1.2.2 T Cell Development and the role of thymic epithelial cells

T cell development is a sequential process in which each step takes place within a defined area of the thymus (Figure 1.3). Each of those zones constitutes the optimal microenvironment for a particular stage of T lymphopoiesis, and the developing T cells progress from one to the next one as they differentiate [240, 347]. The thymus does not contain self-renewing haematopoietic stem cells (HSC), so the production of T cells depends on its continuous seeding with T cell progenitors from the bone marrow, which (in adults) are brought to the thymus through the blood [44, 73]. These progenitors are initially found in the cortex immediately adjacent to medulla, which indicates that the homing probably takes place through the venules of the corticomedullary junction (CMJ) [265]. The exact identity of the progenitors homing to the thymus in the postnatal life remains poorly understood. However, embryonic thymopoiesis-initiating progenitors have been recently described as progenitor cells restricted to the lympho-myeloid lineage [273]. Adhesion molecules like P-selectin (expressed by thymic endothelial cells) and chemokines such as chemokine CC ligand (CCL)21, CCL25, and chemokine CXC ligand (CXCL)12 (expressed by TECs) are known to be involved in progenitor seeding of the thymus [11].

The developing T cells that populate the thymus can be classified into different subpopulations (that correspond to different developmental stages) according to their surface expression of the CD4 and CD8 co-receptors. From more un-differentiated to more mature stages, pro-T cells can be subdivided into double negative (DN) if they express neither CD4 nor CD8; double positive (DP) if they express both; or single positive (SP) if they only express either CD4 or CD8 in their surface. This classification was first described by Reinherz et al. in human thymocytes in 1980 [366] and in mouse thymocytes by Ceredig et al. in 1983 [67], using monoclonal antibodies against the CD4 and CD8 molecules from human and mice, respectively.
Figure 1.3: Developing thymocyte migration pattern through the thymus. T lymphocyte development is a sequential process. Each of its phases takes place in a discrete zone of the thymus, where the optimal microenvironment is provided. (Image adapted from Petrie and Zúñiga-Pflücker 2007 [347]). Abbreviations: Double Negative (DN), Double Positive (DP), Single Positive (SP).
The progenitors that arrive to the thymus are not committed yet to the T cell lineage, so they express neither CD4 nor CD8 co-receptors. For this reason, as previously discussed, they are known as CD4/CD8 double negative (DN) cells \[347\]. Once the T cell progenitors home to the thymus, they initiate their commitment to the T lymphocyte lineage [11, 155]. DN cells can be further subdivided in four major sub-fractions (with differential expression of CD25, CD44 and CD117), termed DN1 to DN4 depending on their degree of differentiation, DN1 being the most undifferentiated state [157].

DN1 cells (CD24\(^{-}\)CD25\(^{-}\)CD44\(^{+}\)CD117\(^{+}\)) are exposed to the Notch ligand (Dll4), interleukin-7 (IL-7), stem cell factor (SCF) and FLT-3 ligand (FLT3L) expressed by TECs, which promote their proliferation, retention, and periodic release towards the outer cortex in order to continue their differentiation process and become DN2 cells (CD24\(^{+}\)CD25\(^{-}\)CD44\(^{-}\)CD117\(^{+}\)) [11, 55, 155, 347]. The T cell receptor (TCR) rearrangements (\(\beta\), \(\gamma\) and \(\delta\), but not \(\alpha\)) start during this DN1 to DN2 transition, and are not completed until the DN3 stage (CD24\(^{+}\)CD25\(^{-}\)CD44\(^{lo}\)CD117\(^{lo}\)) is reached (Figure 1.4) [17]. DN3 is also the stage at which the \(\alpha\beta\) and \(\gamma\delta\) lineages diverge, and \(\gamma\delta\) lymphocytes are exported to the periphery [318, 418]. DN3 lymphocytes reach the subcapsular zone and start to migrate back towards the medulla (Figure 1.2) [240, 418]. DN3 cells that have successfully rearranged their TCR\(\beta\) will assemble a complete pre-TCR with a pre-TCR \(\alpha\) chain (called pT\(\alpha\)) and CD3 molecules and are selected for further differentiation [17]. The signalling through the pre-TCR leads to the rearrangement of the \(\alpha\) locus, and \(V\alpha/J\alpha\) joins can be detected at low levels in DN4 cells. However, these rearrangements are not be completed until the cell becomes a CD4/CD8 double positive (DP) cell [418].

The expression of the pre-TCR and the signalling through it also leads to differentiation into highly proliferating DN4 (CD24\(^{+}\)CD25\(^{+}\)CD44\(^{-}\)CD117\(^{+}\)) cells first and DP cells later on (Figure 1.4) [17, 318]. Some DN4 cells differentiate into DP cells through an immature CD8\(^{-}\)CD4\(^{-}\) intermediate state (immature single positive or ISP cells) [279]. As will be discussed in the next section, DP lymphocytes rearrange their \(\alpha\)-chain genes until there is signalling from a self-peptide:self-MHC complex that positively selects the receptor (Figure 1.4) [318]. Only the DP thymocytes with low to intermediate avidity for self-peptide:self-MHC complexes expressed by cTECs survive and continue to differentiate [240]. Both NOTCH and IL-7 (highly expressed by cTECs) have very important roles in these early stages of T cell development [11, 17, 347].

Positively selected lymphocytes migrate to the medulla and commit to either the CD4 or
Figure 1.4: Early T cell development. Progression of T lymphocyte development and the surface markers displayed in each cell stage. Fluorescence intensity for each marker is indicated on an arbitrary scale from negative (−) through low to very bright (+++), as measured by flow cytometry. Gene rearrangements are shown in orange boxes. (Image adapted from Ceredig and Rolink 2002 [74].) Abbreviations: Double Negative (DN), Double Positive (DP), Single Positive (SP) T cell receptor (TCR).

the CD8 lineage, becoming single positive (SP) cells (Figure 1.3 [318]). It is in the medulla where the T cells that react strongly against self-antigens are deleted, in a process called negative selection [347]. Finally, the T cells that survive the negative selection process upregulate the sphingosine-1-phosphate (S1P) receptor and are exported from the thymus into the circulation [43,240].

1.2.2.1 Positive Selection: cTECs.

Immature T cells need to undergo positive selection in order to ensure that only the thymocytes that are able to recognise MHC molecules (with a peptide bound to them) finish their development and become fully mature T lymphocytes [141]. During positive selection, the ability of the developing T cells to recognise MHC:peptide complexes is tested in a way that only those with low to intermediate affinity of the TCR-MHC:peptide interaction are induced to survive and differentiate into CD4/CD8 SP cells (only 1-5% of all DP thymocytes); whereas those with too high an affinity (potentially harmful) or too low an affinity (useless) are deleted [9,11]. Positive selection is also important because it is thought to influence which co-receptor (CD4 or CD8) each cell will finally express as a mature thymocyte, and thus the effector functions of the T cell [318].
The thymic cortex is the zone where positive selection takes place, and cTECs are the main cells in charge for this [11]. In 1994 it became clear that the molecules that provide the signals required for the T cell positive selection were present in the cTEC surface rather than being soluble factors [10]. Our understanding of this process has increased since then, although there are still some questions to address, such as the nature of MHC-associated peptides displayed by cTECs during positive selection. What has been recently shown is that cTECs express a specific form of proteasome, the thymoproteasome, which contains the cTEC-specific catalytic subunit $\beta_{5t}$, and that generates unique peptides specifically required for positive selection. In fact, $\beta_{5t}$ deficient mice show defective development of CD8$^+$ T cells [11, 317]. cTECs also express high levels of the lysosomal proteases Prss16 (also known as thymus-specific serine protease; Tssp) and cathepsin L (in contrast with the rest of peripheral tissues where cathepsin S prevails), which are required for a correct positive selection of CD4$^+$ T cells [11, 159, 193, 318]. To sum up, cTECs express a pool of characteristic peptides on their MHCI and MHCII that are essential for the correct development of T cells [11].

In addition to this, experiments performed using irradiation bone marrow (BM) chimaera systems, where donor haematopoietic cells express different MHC molecules than the host’s thymic epithelial cells, showed that positive selection also establishes MHC restriction. These studies proved that the MHC alleles expressed by the thymic epithelial cells are the ones that T cells will consider as “self” [38, 137, 336]. In an MHC-allogeneic chimera, T lymphocytes are unable to recognize antigens presented by donor-derived APCs, as they have been positively selected to recognize only the recipient’s MHC form. This showed that at least one MHC molecule in common between donor and recipient was necessary to successfully reconstitute T cell immunity [318].

1.2.2.2 Negative Selection: mTECs and DCs

Positively selected thymocytes are attracted to the medulla, where they undergo negative selection in order to establish self-tolerance, that is, to eliminate potentially self-reactive T cells and thus minimize the probability of an auto-immune response [396]. In the medulla, the two main cell types in charge of presenting self-antigens to developing thymocytes are mTECs and dendritic cells (DCs) [235]. While DCs are more efficient antigen presenting cells (APCs), mTECs play an essential role in negative selection:
they are the only cell type capable of expressing a broad range of tissue-specific antigens (known as promiscuous gene expression) \([116,236]\). AIRE (autoimmune regulator) promotes the ectopic expression of a huge repertoire of proteins that otherwise would be restricted to other organs, the so called tissue-specific self-antigens (TRAs) (although not all TRAs are expressed in an AIRE-dependent way) \([297]\). AIRE is only expressed in a subset of functionally mature CD80/86\(^{\text{high}}\) mTECs \([115,204]\), and its many functions include not only promoting promiscuous gene expression but also the regulation of the expression of many intrathymic chemokines required for the proper localization of thymocytes and dendritic cells \([250]\). mTECs attract DCs to the medulla through the expression of the chemokine XCL1 (lymphotactin), whose receptor (XCR1) is expressed by DCs \([259]\). Both mTECs and DCs cooperate in the presentation of self-antigens, establishing self-tolerance not only through the deletion of self-tolerant thymocytes but also through the production of regulatory T lymphocytes (Treg). In CXL1-deficient mice DCs are not recruited to the medulla and the production of Tregs is impaired \([11,259]\). Although mTECs are able to present self-antigens themselves, different studies have shown that they also transfer those self-antigens to neighbouring DCs for their presentation. However, the exact mechanism for this antigen transfer is still unknown \([235]\). Although negative selection and self-tolerance induction have aroused much interest for many years, there are still some outstanding questions, such as the mechanism for Treg induction, that need to be resolved \([235]\).

### 1.3 Hypoxia and HIFs

Cellular responses to hypoxia are mediated by a highly conserved family of transcription factors, known as the Hypoxia Inducible Factors (HIFs) (Figure 1.5). Each functional HIF transcription factor results from the heterodimerization of one alpha subunit (HIF-1\(\alpha\), HIF-2\(\alpha\) or HIF-3\(\alpha\)) with one of the Aryl Hydrocarbon Receptor Nuclear Translocator (Arnt, also known as HIF-1\(\beta\)) \([299]\). All HIF-alpha subunits and Arnt share a quite conserved protein domain structure comprising an N-terminal bHLH DNA binding domain and tandem PAS-A and PAS-B domains that are necessary for their heterodimerization \([114]\). In addition, HIF-1\(\alpha\) and HIF-2\(\alpha\) also possess N- and C-terminal transactivation domains (N-TAD and C-TAD) that are involved in the regulation of their function, while HIF-3\(\alpha\) contains only one TAD (N-TAD). All HIF-alpha subunits (but not Arnt)
**Figure 1.5: Domain structure comparison of the members of the HIF family.** All HIF-α and –β subunits are members of the helix–loop–helix-PER-ARNT-SIM (bHLH-PAS) family. They contain an amino-terminal bHLH domain for DNA binding, PAS domains (PAS-A and PAS-B) that allow dimerization, and transactivation domains (TADs) in order to modulate their function. In addition to these, Hif-α subunits contain an Oxygen-Dependent Degradation Domain (ODDD) which is necessary for their oxygen-mediated protein stability regulation (Figure adapted from Dengler et al. 2014 [114]). Abbreviations: Basic helix-loop-helix domain (bHLH); PER-ARNT-SIM domain (PAS); PAS-associated C-terminal domain (PAC); N-terminal transactivation domain (N-TAD), C-terminal transactivation domain (C-TAD); Oxygen-dependent degradation domain (ODDD); Leucine zipper domain (LZIP).

HIF-1α and HIF-2α also exhibit an oxygen-dependent degradation domain (ODDD) that is necessary for the control of their oxygen-regulated protein stability [172,182,201,213,214,223,357]. In consequence, ODDD-lacking Arnt protein levels are kept constant independently of the oxygen tension, while ODDD-containing HIF-α subunits are subject to oxygen-dependent proteasomal degradation as described below.

The different HIF-α subunits differ in both their functions and their expression patterns. Interestingly, despite the fact that both HIF-1 and HIF-2 recognize the same hypoxia responsive element (HRE) containing the same 5′-(A/G)CGTG-3′ consensus sequence, the target genes that each transcription factor controls differ. Studies by Hu et al. [197] and Lau et al. [256] have shown that the target-specificity of the different HIF isoforms is dictated by their N-TAD domains, maybe by mediating cooperation with specific co-activators [114]. In addition to this differential target selectivity, HIF-1 and HIF-2 also display cell-type specific regulation of target genes that has been proposed to be a result of distinct chromatin status, RNA Pol II activity, and the availability of partner transcription factors or co-activators [149,342,344,460,494]. In terms of expression pattern, while HIF-1α is ubiquitously expressed at similar levels in all human and mouse tissues, HIF-2α mRNA expression is mainly restricted to the lung, vascular endothelium, carotid body and catecholamine-producing cells [125,127,223,398,438,475].
In contrast to the HIF-1α and HIF-2α isoforms, which have been studied in much higher detail, the information available regarding HIF-3α is much more limited. Both the human and the mouse HIF3A gene encodes several different splice variants that have differential domain structures, tissue-specific expression patterns, and functions. These functions include inhibiting HIF-1 and HIF-2 activity but also acting as a transcription factor, activating gene expression of its own target genes [284, 495].

1.3.1 Oxygen-dependent regulation of HIFs

Hypoxia inducible factors (HIFs) are composed of a constitutively expressed β-subunit (HIF-1β or ARNT) and an oxygen-regulated α-subunit (HIF-1α, HIF-2α or HIF-3α), which form a heterodimer. Oxygen-dependent regulation of HIF-α proteins is essentially post-translational, their corresponding mRNAs being stably and constantly expressed independently of the oxygen tension. As a result, HIF-α proteins are produced continuously. However, in the presence of high oxygen levels (>8%–10%), a family of prolyl-4-hydroxylases (PHD), hydroxylates HIF-α at two proline residues located within ODDD that are conserved amongst the different isoforms (Pro402 and Pro564 of human HIF-1α) [59, 128, 223, 293, 294]. In mammals, at least three PHD proteins have been identified that have the ability to hydroxylate HIF: PHD1/HPH3/EGLN2, PHD2/HPH2/EGLN1, and PHD3/HPH1/EGLN3. However, not all of them have the same specificities, PHD2 (also known as EGLN1) being the most important for setting the low steady-state levels of HIF1a in normoxia [15, 35, 59, 128, 200]. Interestingly, both PHD2 (EGLN1) and PHD3 (EGLN3) are known HIF target genes, indicating the possible existence of feedback loops to tightly regulate HIF function [114, 223]. Hydroxylation of the proline residues present in the ODDD of HIF-α creates the perfect binding site for pVHL, a member of the VCB-CUL2 E3 ligase complex, resulting in polyubiquitination of HIF-α and leading to its degradation by the proteasome (Figure 1.6) [31, 207, 220, 223]. In addition to this, other post-translational modifications also contribute to HIF-α protein degradation in normoxia, such as the acetylation of the lysine residue Lys532 of HIF-1α by the ARD1 acetyltransferase, which promotes pVHL binding [212]. Another main HIF-α inhibitory mechanism is the hydroxylation introduced in the C-TAD by the Factor Inhibiting HIF (FIH) (on Asn-803 of HIF-1α or Asn-847 of HIF-2α), which impedes binding of cofactors (such as p300/CBP) to the C-TAD and therefore interferes with HIF transactivation [254, 255, 282, 348].
Figure 1.6: Oxygen-dependent regulation of HIF-1 activity. In normoxia (left panel), the presence of oxygen allows the hydroxylation of HIF-1α by prolyl hydroxylase domain-containing proteins (PHDs), allowing pVHL to target it for proteasomal degradation. In hypoxia (right panel), the low oxygen levels prevent HIF-1α hydroxylation, allowing its stabilization and translocation to the nucleus, where it dimerizes with HIF-1β and promotes the expression of oxygen-responsive genes. Abbreviations: Hypoxia Inducible Factor (HIF); prolyl hydroxylase domain-containing protein (PHD); product of the von Hippel-Lindau tumor suppressor (VHL); ubiquitin (Ub); hydroxyl groups (OH).
In contrast, in hypoxia, HIF-α subunits are no longer hydroxylated, allowing their stabilization, activation, translocation to the nucleus, dimerization with Arnt and interaction with its co-activators in order to drive the expression of its target genes (Figure 1.6) [192]. Hypoxia not only inhibits PHD-mediated HIF-α hydroxylation in the ODDD but also FIH-dependent hydroxylation of the C-TAD, allowing its interaction with its co-activators p300 and CBP [169]. Many other HIF-α post-translational modifications (acetylation, S-nitrosylation, SUMOylation, and phosphorylation) have been described to either enhance or repress transcriptional activity of HIFs, often in a cell-type specific fashion [88,303]. For example, direct or indirect HIF phosphorylation has been reported by many kinases such as mitogen-activated protein kinases (MAPK), phosphatidylinositol 3-kinase (PI3K), glycogen synthase kinase 3β (GSK3β) downstream of AKT/PI3K signalling, cyclin-dependent kinase 1 (CDK1), Polo-like kinase 3 (Plk3) or, interestingly, ataxia-telangiectasia mutated (ATM) [229].

1.3.2 The HIF-1 pathway

Once activated, HIF α/β heterodimers translocate to the nucleus and regulate a myriad of target genes that are involved in many diverse biological pathways. They do so by directly interacting with the core consensus sequence 5′-(A/G)CGTG-3′ within the hypoxia-response element (HRE), upstream of their target genes. This consensus sequence is relatively short and highly abundant in the genome. Therefore, the presence of this sequence alone is not enough to predict HIF binding [393]. In fact, recent studies investigating genome-wide HIF chromatin occupancy have shown that less than 1% of potential HIF binding sites are actually bound by HIFs in response to hypoxia, and that other features independent of the DNA sequence, such as permissive chromatin that display DNAse I hypersensitivity, RNAPII enrichment, histone modifications and basal transcriptional activity under normoxic conditions, are important for HIF binding [314,395,394,494].

Some of the best known HIF canonical targets are those important for maintaining oxygen homeostasis, such as genes involved in the metabolic switch to glycolysis: glycolytic enzymes (ALDOA, ENO1, GAPDH, HK1, HK2, PFKL, PGK1, PKM2, LDHA) and glucose transporters (GLUT1, GLUT3) [399]; or genes that increase the distribution of the available oxygen supply such as EPO and many angiogenic factors including vascular
endothelial growth factor (VEGF), stromal-derived factor 1 (SDF1), placental growth factor (PGF), platelet-derived growth factor B (PDGFB), and angiopoietin (ANGPT) 1 and 2 [367]. However, the list of HIF-1 controlled genes is continuously growing, including genes involved in many important biological processes such as cell proliferation and survival, autophagy, apoptosis, DNA repair, redox homeostasis, inflammation and immunity, stemness and self-renewal, and metastasis and invasion. Interestingly, as previously discussed, HIF-1 activity is highly influenced by the genomic landscape and the availability of its co-factor and regulating proteins. In consequence, the role of HIF-1 in the biological processes mentioned above is often tissue- or cell-type specific, adding an extra layer of complexity to the HIF-1 pathway [114,374,400,491,494].

1.4 The DNA Damage Response

The maintenance of genomic stability is essential for cell survival and for preventing malignant transformation. The genome of the cell is continuously challenged by both extrinsic and intrinsic genotoxic stresses that damage DNA such as environmental mutagens (e.g. ionizing radiation and UV light) and reactive metabolic by-products (e.g. reactive oxygen intermediates). The cellular responses to ionizing radiation (IR) are mostly due to its destructive effects on the genome [91, 222]. In general, IR-induced DNA damage can arise (i) from energy deposited directly onto DNA, or (ii) indirectly from free radicals generated by the ionization of other molecules, such as oxygen and water, which modify and/or break chemical bonds within DNA strands [471]. Cells have evolved a complex signalling network that senses DNA damage and activates biological responses that promote cell survival, or if the DNA damage present in the cell is too extensive, can activate mechanisms leading to cell death. These pathways are collectively known as the DNA Damage Response (DDR) (Figure 1.7). Conceptually, the DDR consists of (i) sensor proteins which monitor DNA for structural abnormalities (e.g. DNA breaks) (ii) transducer proteins which transmit and amplify the DNA damage signal, and (iii) effector proteins which orchestrate the desired biological response(s) following the triggering of the DDR [196,270]. These pathways can ultimately lead to either a temporary or permanent halt in cell growth and division; the activation of DNA repair mechanisms and/or to the activation of apoptosis. Therefore, the activation of specific DDR pathways ultimately determines whether a damaged cell will survive or die in
response to genotoxic stress.

The most genotoxic lesions caused by IR are DNA double-strand breaks (DNA DSBs). A DNA DSB can arise when breaks in sister DNA strands occur either directly opposite or within a few base pairs of each other, causing the chromatin to be segregated into two pieces. The lack of a DNA template at the damage site renders these lesions difficult to repair, increasing cellular susceptibility to chromosomal instability and to the induction of cell death mechanisms [471]. It is estimated that each gray (Gy) of IR introduces 33 DSBs into the mammalian genome [365]. DNA DSBs can also occur endogenously due to telomere un-capping, or during DNA replication as a result of the collapse of a stalled replication fork. Replication fork stalling can occur due to the presence of various lesions including single base lesions, cross-links and single DNA strand breaks (SSBs). It can also occur at natural replication pause sites known as fragile sites, which are frequently associated with chromosome breakage and genomic rearrangements [52,76,253]. Despite being a major threat to the genome, DNA DSBs also have defined biological purposes and are introduced deliberately, in a well-controlled manner, during meiotic recombination. In developing T and B lymphocytes, DNA DSBs are also induced during V(D)J recombination and class switch recombination, and are essential for generating the extensive repertoire of immunoglobulins and T-cell receptors expressed by B cells and T cells, respectively, which are central to the adaptive immune response [376,408].

The DDR is primarily regulated by the phosphoinositide-3-kinase related protein kinases (PI3KKs) family, which includes ataxia-telangiectasia-mutated (ATM), ATM and RAD3 related (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [92,202,407,471]. All members share a common PI3K domain that phosphorylates a Ser or Thr residue that is followed by Glu (i.e. an SQ or TQ motif) in target substrates in order to transduce DNA damage signals to various transducer and effector proteins [407]. ATM is the master regulator of DNA DSB signalling pathways whereas DNA-PKcs is primarily involved in the DNA DSB repair pathway non-homologous end-joining (NHEJ), which will be discussed later in further detail. ATR is primarily activated by single-stranded (ss) DNA structures that are generated by a range of DNA lesions, e.g. base adducts, crosslinks or single-strand breaks (SSBs). Such DNA lesions are generated naturally during DNA replication and artificially by various agents that inhibit DNA replication, such as ultra-violet (UV) light and hydroxyurea.
Figure 1.7: The DNA Damage Response. The DDR is composed of sensor, transducer and effector proteins whose activities induce biological responses to DNA damage. DNA DSBs are initially sensed by PARP and the MRN complex, resulting in the recruitment and activation of the PI(3)K kinases, ATM and DNA-PKcs (see text for details). Post-translational modifications to histones surrounding DNA DSBs, including ATM-dependent H2AX Ser139 phosphorylation (γ-H2AX), stimulate the recruitment of DDR mediators, including MDC1, 53BP1, BRCA1, CtIP and RNF8 which act to amplify the DDR signal and to modulate ATM-dependent phosphorylation of target substrates. ATM-mediated DNA DSB resection to generate ssDNA tails also results in ATR activation. Activated ATM and ATR phosphorylate and activate the DDR transducers, CHK2 and CHK1, respectively, which relay the damage signal downstream to effector proteins involved in various biological responses. These include p53 and p21 (DNA damage checkpoints, DNA damage-induced transcription; DNA repair; apoptosis and senescence); CDC25A (DNA damage checkpoints); LIG4 and RAD51 (DNA DSB repair) and Caspase-3 (Apoptosis) (see text for details). Abbreviations: Double-strand break (DSB); H2AX Ser139 phosphorylation (γ-H2AX).
Here, we will mainly focus on the branches of the DDR that mediate the cellular response to DNA double-strand breaks (DSBs) as they represent the most deleterious DNA lesions caused by IR.

1.4.1 Sensing damage: DDR initiation

The DNA damage response (DDR) is activated when DNA lesions, such as DNA DSBs, are recognized by sensor proteins. DNA DSBs are initially recognized by members of the poly-(ADP) ribose polymerase (PARP) family of proteins. Of the 17 members of the PARP family, only PARP 1-3 have so far been demonstrated to be involved in the DDR, with each member having a different specificity. PARP1 is activated by multiple types of DNA lesions, including SSBs, DNA crosslinks, stalled replication forks and DSBs; PARP2 is thought to recognize gaps and flap structures; while PARP3 responds selectively to DNA DSBs, most likely in co-operation with PARP1 [28, 42, 91]. PARPs catalyse the addition of poly-(ADP) ribose (PAR) chains (i.e. PARylation) to histone tails flanking DNA DSBs. These chains provide a platform for recruiting the MRE11-RAD50-NBS1 (MRN) complex, which forms a physical bridge between DNA DSB ends and acts as a primary sensor of DNA DSBs [28, 275]. The MRN complex is a hexameric structure consisting of dimers of each of its three subunits. The MRE11, RAD50 and NBS1 subunits collectively function to initiate the DDR, to stimulate the activation of downstream signalling pathways and also aid in the repair of DNA DSBs. MRE11 is a U-shaped nuclease that binds to short 3'OH overhangs at DNA DSB termini to form synaptic DNA complexes. RAD50 contains an ATP-binding cassette that binds and unwinds DNA DSB termini. While Mre11 facilitates short-range synapsis of the two ends at a DNA DSB, the current model suggests that RAD50 mediates long-range tethering of two different DNA molecules, for example, a broken chromosome and its sister chromatid. NBS1 acts as a flexible adaptor between MRE11 and RAD50 and mediates the assembly and nuclease activity of MRN complexes at DNA DSB sites [194, 252, 379, 478]. Recruitment of the MRN complex to DNA DSBs is essential for activating DDR signalling pathways. As previously mentioned, the PI(3)K kinases, ATM, ATR and DNA-PKcs are key regulators of DDR signalling. ATM is rapidly recruited to DNA DSBs by the MRN complex and physically interacts with NBS1. Interaction with the MRN complex stimulates ATM to be post-translationally modified on multiple residues that (i) activate (e.g. S1981 auto-phosphorylation) and (ii) modulate its function (e.g. TIP60 (HU) [52, 76, 92, 253].
acetyltransferase-dependent K3016 acetylation). The components of the MRN complex itself are subsequently phosphorylated by activated ATM which is thought to create a positive feedback loop in order to maintain ATM activity [16,194,252,258,346,379,408,427,478].

1.4.2 Sending an SOS: DDR signal transduction and amplification

Following recruitment of PARPs, the MRN complex and ATM to DNA DSBs, the surrounding chromatin is re-modelled in order to amplify the DDR signal downstream. DNA compaction in "closed" heterochromatin obstructs DNA metabolism and repair. Therefore, a more ‘open’ state of chromatin, i.e. euchromatin, is desirable in order to facilitate DNA repair processes. Consequently, immediately following DNA damage, chromatin surrounding DNA DSBs undergoes an energy-dependent local expansion, mediated by ATP-dependent chromatin remodellers (e.g. PARP1), in order to facilitate the recruitment of multiple DDR factors to damaged sites [161, 246, 414]. During the chromatin remodelling process, histones are also post-translationally modified in order to create docking sites for a family of breast cancer type 1 (BRCA1) C-terminal (BRCT)-domain containing DDR scaffold or 'mediator' proteins. DDR mediators are essential for (i) retaining sensor proteins at DNA DSB sites; (ii) modulating ATM activation and function; and (iii) facilitating DNA repair. DDR mediators are being identified on a continuing basis and include mediator of DNA damage checkpoint protein 1 (MDC1); E3 ubiquitin ligase RING finger protein (RNF) 8; p53 binding protein 1 (53BP1) and BRCA1 [91,185,248,269,355].

A well-characterized histone modification generated in response to IR-induced DNA DSBs is ATM-dependent Ser139 phosphorylation of the histone variant, H2AX, termed γ–H2AX. The γ–H2AX modification is an important signal for DDR activation in response to DNA DSBs and is generally recognized as a marker of DNA DSBs [45,60,135,373]. H2AX Ser139 phosphorylation, combined with Tyr143 dephosphorylation [via eyes absent homolog 1/3 (EYA1/3) phosphatases], facilitates the docking of MDC1 onto chromatin at DNA DSB sites where it interacts with ATM and NBS1. This stimulates the recruitment of additional ATM-MRN complexes and further ATM activation, resulting in H2AX phosphorylation over several megabases (Figure
Other histone modifications, e.g. acetylation, ubiquitination and methylation, also stimulate the recruitment of other DDR mediators such as 53BP1 and BRCA1. This complex assembly of DDR sensors and mediators at DNA DSBs results in the formation of supramolecular structures known as DNA DSB-associated foci, or IR-induced foci (IRIF), which act to amplify the DDR signal downstream and to facilitate DNA DSB repair [271, 275, 385, 425]. Crosstalk between DDR mediators and ATM at DNA DSBs enables ATM to phosphorylate numerous target substrates, including the DDR transducer, Checkpoint kinase 2 (CHK2). To facilitate DNA repair, various endonucleases are recruited to DNA DSBs to generate ssDNA tails, a process known as DNA DSB resection. As previously mentioned, ssDNA structures at damage sites are normally recognized by ATR. During DNA DSB resection, ATM mediates replication protein A (RPA) coating of ssDNA tails which subsequently triggers ATR activation and ATR-dependent phosphorylation of another DDR transducer, CHK1 [99, 202, 210, 508]. Therefore, the DDR is modulated by a crosstalk between ATM-CHK2 and ATR-CHK1 pathways, which together transmit the damage signal to multiple effector proteins that orchestrate the biological outcome(s) of DNA DSBs. These outcomes include transient delays in cell cycle progression (termed DNA damage checkpoints), DNA DSB repair, apoptosis and senescence (Figure 1.7). These processes will now be discussed in further detail.

1.4.3 DNA damage checkpoints

The cell cycle comprises the series of events that enable the replication of the genetic material and its subsequent segregation into two new cells. It is composed of four discrete phases: mitosis (M), gap 1 (G1), DNA synthesis (S), and gap 2 (G2). G0 refers to cells that are quiescent (i.e. out of cycle). (i) Gap phase 1 (G1) in which the cell is stimulated to increase in mass by extracellular mitogens and growth factors and prepares for DNA synthesis; (ii) S phase in which DNA synthesis occurs; (iii) G2 in which the cell continues to increase in mass and prepares for mitosis and (iv) M phase in which the cell segregates its newly copied chromosomes and divides [205, 325].

Cell cycle progression is driven by a series of protein kinases known as Cyclin-Dependent Kinases (CDKs). The activity of each CDK is regulated by the binding of specific cyclins whose expression levels fluctuate throughout the cell cycle, allowing dif-
different CDKs to be only active during specific cell cycle phases. Cyclin D-CDK4/6 and cyclin E-CDK2 complexes promote G₁ progression into S phase; cyclin A-CDK2 complexes are required for DNA synthesis and cyclin A- and cyclin B-CDK1 complexes regulate progression through G₂ and mitosis (Figure 1.8 A). CDK activity is also modulated by (i) phosphorylation on conserved Thr and Tyr residues by various enzymes which induce conformational changes that can either promote or inhibit cyclin binding and (ii) by CDK inhibitors (CKIs) which can bind CDKs either alone or in cyclin-CDK complexes, thereby inhibiting CDK activity [222, 292, 459, 464, 509]. Activation of the DDR signal cascade can induce transient delays at specific cell cycle transitions (G₁-to-S phase or G₂-to-M) or phases (intra-S i.e. during DNA replication), known as the DNA damage checkpoints (Figure 1.8 A) [222]. These checkpoints function as regulatory systems that ensure all processes in the cell required to replicate the genome are correctly completed before allowing subsequent division into two new daughter cells. In the presence of genotoxic or other cellular stresses, these checkpoints are activated to provide time for pathways required for the resolution of this stress to be activated.

1.4.3.1 G₁/S Checkpoint

Cells need to ensure that the genome is stable before committing to cell cycle entry. Therefore, the G₁/S checkpoint functions to prevent the initiation of DNA synthesis, i.e. entry into S phase, in the presence of DNA damage. The G₁/S checkpoint can be activated by ATM and ATR-dependent phosphorylation of the DDR signal transducers, CHK1 and CHK2. Activated CHK1 and CHK2, in turn, target two critical effectors operating in distinct branches of the G₁/S checkpoint, the CDC25A phosphatase and the transcription factor, p53. CDC25A phosphatase activates CDK2, which is involved in the loading onto chromatin of CDC45, a protein necessary for the activation of pre-replication complexes (pre-RC) during the initiation of DNA replication. In response to DNA DSBs, ATM-dependent CHK2 phosphorylation of CDC25A targets it for ubiquitin-dependent proteasomal degradation, preventing CDK2 activation, and thereby inhibiting DNA replication. This pathway is rapidly implemented, but is only capable of delaying cell cycle progression for several hours [110,111,134,150].

The other branch of the G₁/S checkpoint is regulated by the ATM-p53-p21 signalling axis (Figure 1.8 B). This pathway is activated slowly as the transcription and translation
of downstream proteins are required to induce prolonged G\textsubscript{1} arrest. p53 and p21 are two key tumour suppressors that inhibit the proliferation of genetically unstable cells by regulating the expression of genes involved in multiple DDR mechanisms including cell cycle arrest, DNA repair and apoptosis. Under normal conditions, p53 function is constitutively maintained at low levels by the mouse double minute 2 (MDM2) E3 ubiquitin ligase, which targets p53 for proteasomal degradation. However, in response to DNA DSBs, p53 and MDM2 become post-translationally modified by various DDR proteins, including ATM and CHK2, leading to p53 stabilization and accumulation, and an increase in its transcriptional activity. p21, a CDK inhibitor, is the key effector of p53-mediated G\textsubscript{1} arrest. p53 positively regulates the expression of the CKI, p21, which inhibits (i) cyclin D-CDK4/6 and cyclin E-CDK2 complexes required for the G\textsubscript{1}/S transition and (ii) proliferating cell nuclear antigen (PCNA) activity required for DNA replication, thereby blocking S phase entry \[22,23,110,111,134,150,222,283,439\].
Figure 1.8: DNA damage checkpoints.
Figure 1.8 DNA damage checkpoints (Overleaf). (A) The cell cycle is composed by four discrete phases: G₁, S, G₂ and M. The transition from one phase to the next is controlled by different Cyclins and Cyclin-dependent kinases (CDKs). These transitions can be delayed by the DDR in response to DNA damage, through the activation of specific mechanisms known as DNA damage checkpoints, which are named according to the cell cycle transition that they affect: G₁/S, intra-S or G₂/M checkpoints. (B) In response to DNA double-strand breaks, ATM and CHK2 activation leads to downstream p53 stabilization and subsequent induction of p21 transcription. p21 is a potent CDK inhibitor that prevents the activation of cyclin D-CDK4/6, cyclin E-CDK2 and cyclin B-CDK1 complexes, resulting in (i) a block in S phase entry (i.e. the G₁/S checkpoint); (ii) a reduction in the rate of DNA synthesis (i.e. the intra-S-phase checkpoint) and (iii) a block in mitotic entry (i.e. the G₂/M checkpoint). Both ATM-Chk2 and ATR-CHK1 signaling pathways lead to the phosphorylation and targeted degradation of CDC25A and CDC25C phosphatases. CDC25A and CDC25C degradation prevent the downstream activation of cyclin E-CDK2 and cyclin B-CDK1 complexes, respectively, inhibiting DNA replication (i.e. the intra-S-phase checkpoint) and blocking entry into mitosis (M) (i.e. the G₂/M checkpoint) (See text for further details). Abbreviations: Double-strand break (DSB); H2AX Ser139 phosphorylation (γ-H2AX).

1.4.3.2 Intra-S-phase Checkpoint.

The intra-S-phase checkpoint is activated in response to replication stress and DNA damage. It is characterized by a reduction in the rate of DNA synthesis. This occurs due to the inhibition of late origin firing, caused by the presence of replication inhibitors and by DNA damaging agents. This checkpoint is also initiated as a result of replication fork stalling, which occurs when DNA elongation stops due to replication complexes failing to bind to damaged template DNA. This checkpoint functions to protect stalled replication forks from aberrant recombination, promoting the relaxation of chromosomal torsional stress and the completion of replication via up-regulation of deoxyribonucleotide (dNTP) production [131]. There are likely two effector cascades that contribute to the IR-induced intra-S-phase checkpoint. The blockade of replication origin firing in response to DNA DSBs is primarily mediated by the previously described ATM-Chk2-CDC25A signalling axis (Figure 1.8B) [21,133]. The mechanism(s) underlying replication fork stalling in response to DNA DSBs, is currently poorly understood. However, Cohesin, a protein complex consisting of SMC1, SMC3, RAD21 and SCC1 that forms a ring around sister chromatids as they are being synthesized during DNA replication, has emerged as an important substrate of ATM signalling in this process. The pairing of sister chromatids, i.e. cohesion, is essential for accurate chromosome condensation and segregation in mitosis and for DNA DSB repair by homologous recombination (HR) [27,405]. NBS1/BRCA1/ATM-dependent phosphorylation of SMC1 and ATM/CHK2-dependent phosphorylation of SMC3 are known to be required for replication fork stalling in response to DNA DSBs [234,277]. The molecular mechanism(s)
linking ATM-mediated post-translational modification of Cohesin and replication fork stalling remains to be determined.

1.4.3.3 G\textsubscript{2}/M Checkpoint

The G\textsubscript{2}/M checkpoint prevents cells from initiating mitosis in the presence of DNA damage. This process is essential for preventing chromosomal instability and for ensuring that potentially harmful mutations are not inherited by daughter progeny. In response to DNA DSBs, ATM and CHK2 phosphorylate CDC25C phosphatase, targeting CDC25C for proteasomal degradation. As a result, CDC25C does not remove inhibitory threonine 14 / tyrosine 15 phosphorylations present on CDK1, preventing activation of CDK1-cyclin B complexes, thereby blocking entry into mitosis. ATR/CHK1-dependent phosphorylation and subsequent degradation of CDC25C in response to resected DNA DSBs also contributes to the activation of G\textsubscript{2}/M arrest in response to resected DSBs as described above (Figure 1.8 B) \cite{1,274}. The G\textsubscript{2}/M checkpoint is maintained via two MDC1/53BP1-dependent pathways, depending on the nature of the DNA DSB. 53BP1 and MDC1 sustain activated ATM-CHK2 signalling in response to persisting DNA DSBs whereas they promote the activation of ATR-CHK1 signalling in response to resected DNA DSBs \cite{1,406}. Overall, activation of DNA damage checkpoints is critical to provide time for persisting DNA damage to be repaired before cells undergo division.

1.4.4 DNA double-strand break repair

As previously discussed, DNA DSBs are extremely destructive for the genome. Therefore, failure to repair DNA DSBs, or their mis-repair, can result in chromosomal aberrations and subsequently cell death or cell transformation and malignancy. Cells have evolved DNA repair pathways to effectively resolve DNA lesions in order to prevent these biological outcomes. DNA DSBs are primarily repaired via two distinct pathways known as Homologous recombination (HR) and Non-Homologous End Joining (NHEJ). HR requires a complementary DNA strand within a sister chromatid as a homologous template for DNA DSB repair whereas NHEJ consists of tethering broken DNA ends together followed by their re-ligation. The requirement of a sister chromatid for repair by HR results in this pathway being restricted only to S and G\textsubscript{2} phases of the cell cycle.
whereas NHEJ can be activated throughout the cell cycle (Figure 1.9). Therefore, NHEJ is responsible for the repair of the majority of DNA DSBs while only approximately 15-20% DNA DSBs are repaired via HR [37, 81, 105, 208].

1.4.4.1 Non-Homologous end Joining (NHEJ)

NHEJ is initiated by the binding of each DNA end at the DSB by the ring-shaped KU70/KU80 heterodimeric complex. KU70/KU80 heterodimers hold the two ends in close proximity (a process known as synapsis) and recruit DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to DSB termini, together forming the DNA-PK complex in which DNA-PKcs is activated. Depending on the nature of the DNA ends and the complexity of the DSB, different processing factors are subsequently recruited to the DNA DSB. These factors include Artemis (a 5’-3’ endonuclease), polynucleotide kinase and DNA polymerase X family members that process DNA DSBs to generate 5’ and 3’ ligatable DNA ends. The processed DNA ends are subsequently re-ligated by the X4-L4 complex, consisting of X-ray repair cross-complementing protein 4 (XRCC4), XRCC4-like factor (XLF) and DNA ligase IV. XRCC4 and XLF create a scaffold for DNA Ligase IV at the DNA DSB site, resulting in the activation of DNA ligase IV and the resolution of the DNA DSB (Figure 1.9). 53BP1 has emerged as an important mediator and effector in the DDR with roles in DDR initiation, checkpoint signalling and in DNA repair [81, 187, 195, 208, 211, 264]. Interestingly, 53BP1 has been shown to interact with RAP1-interacting factor (RIF1) at DNA DSBs to block 5’ end resection, thereby promoting DNA DSB repair by NHEJ and inhibiting HR [81, 506]. 53BP1 is also involved in long-range end-joining of antigen receptor loci segments in V(D)J recombination and in class switch recombination [47, 118, 381].

1.4.4.2 Homologous Recombination (HR)

Unlike NHEJ, HR employs a complementary DNA strand within a sister chromatid as a homologous template for DNA DSB repair. Firstly, 5’-3’ DNA DSB resection is carried out to create 3’ ssDNA overhangs that are necessary for strand exchange. This process is initiated by the interaction of the MRN complex with CTBP (C-terminus binding protein of adenovirus E1A)-interacting protein (CtIP) and BRCA1, and continued by
Figure 1.9: Double-strand break repair mechanisms. In Non-Homologous End-Joining (left panel), DNA DSBs are recognized by the KU70/KU80 complex that forms a synaptic complex with DNA DSB termini and recruits DNA-PKcs to form the DNA-PK holoenzyme. DNA-PKcs is activated and the 5′-3′ endonuclease Artemis is recruited. Subsequent processing of DNA DSB ends facilitates the recruitment of the X4-L4 complex in which DNA ligase IV is activated and ligates the processed DNA DSB ends, resolving the DSB. In Homologous Recombination (right panel) DNA DSBs are resected by the 5′-3′ endonuclease activity of the MRN/CtIP/BRCA1 complex, generating ssDNA tails that are subsequently bound by replication protein A (RPA). RPA unwinds DNA secondary structure, facilitating further resection and the recruitment of HR proteins, including BRCA2, XRCC3, RAD52 and RAD54. These proteins assemble RAD51 molecules along ssDNA tails, generating a RAD51 nucleofilament that displaces RPA oligomers. The Rad51 nucleofilament invades the sister chromatid and facilitates complementary binding between the resected DNA strand (generated at the DNA DSB) and the unprocessed sister DNA strand, resulting in the formation of a displacement (D)-loop DNA intermediate. Subsequent annealing of the un-resected DNA strand at the DNA DSB to the D loop and DNA synthesis generates a four-stranded DNA structure known as a double Holliday junction. These Holliday junctions can be repaired by either (i) sister chromatid dissociation, forming a non-crossover product or (ii) cleavage and subsequent resolution of the cleaved products, generating a crossover product. While NHEJ can take place throughout the entire cell cycle, HR is restricted to S and G2 phases due to the need for a homologous template (see text for further details).
various exonucleases including EXO1 and the Bloom Syndrome (BLM) helicase. These ssDNA tails are subsequently bound and stabilized by replication protein A (RPA) oligomers, which unwind DNA secondary structures, facilitating the recruitment of HR factors. Rad51 recombinase translocates to the nucleus where various mediators, including BRCA2, XRCC3, RAD52 and RAD54 recruit and assemble RAD51 molecules along ssDNA strands, forming a nucleoprotein filament that displaces RPA. BRCA2-dependent loading of RAD51 at processed DNA DSBs results in RAD51 IRIF formation, which can be used experimentally as a marker of DNA DSB repair by HR. The RAD51 nucleoprotein filament then captures the duplex DNA of the intact homologous DNA region in the sister chromatid and invades a single DNA strand, generating a displacement (D) loop intermediate within the sister chromatid. The invasion is followed by extension along both broken DNA strands by a DNA polymerase, most likely Polκ, resulting in the formation of a four-stranded DNA structure known as a double Holliday junction. These complex DNA structures are resolved by resolvase complexes, such as Bloom syndrome protein (BLM)-topoisomerase IIIα complex, resulting in the generation of repaired crossover (reciprocal exchange of DNA sequences) or non-crossover DNA products (Figure 1.9). The type of DNA product generated by HR is tightly regulated. In mitotic cells, the generation of crossover DNA products is disfavoured when the DNA template selected fails to locate the identical sister chromatid. This is important for preventing genomic instability as loss of heterozygosity and translocations can occur due to invasion of the wrong chromosome, and copy number changes may arise as a consequence of inaccurate template selection in repetitive DNA sequence regions, such as ribosomal DNA sequences [34,130,154,187,195,261,383,429].

1.4.4.3 DNA DSB Repair Pathway Choice

The choice between NHEJ and HR pathway activation needs to be tightly regulated in order to ensure the maintenance of genomic stability. Repair pathway choice is critically influenced by the decision to initiate 5'-3' DNA DSB end resection which is now known to be regulated by the DDR mediators, 53BP1 and BRCA1. This resection process is necessary for the activation of HR and simultaneous suppression of NHEJ [81,429]. At DNA DSBs, 53BP1 collaborates with RIF1 (RAP interacting factor 1) and PTIP (PAX transactivation activation domain-interacting protein) to inhibit the association of BRCA1 with MRN complex-bound CtIP, preventing DNA DSB resection. Once the cell enters
S phase, CtIP is phosphorylated by CDKs, enabling the formation of the MRN-CtIP-BRCA1 complex. This complex displaces 53BP1, RIF1 and PTIP at DNA DSB sites, enabling DNA DSB resection to be initiated, subsequently leading to the activation of HR [34,81,130,340,383,429,497,505]. Together, NHEJ and HR enable cells to effectively repair DNA DSBs, contributing to the restoration of genomic integrity and promoting cell survival.

### 1.4.5 Apoptosis

DNA damage checkpoints and DNA repair mechanisms co-operate to restore genomic stability following genotoxic insult. However, when DNA damage is too extensive within the cell or cannot be repaired, cells can activate mechanisms to ensure that cells harbouring potentially harmful genetic mutations are eliminated or at least, lose their proliferative capacity. Apoptosis is a type of programmed cell death that eliminates damaged and stressed cells and is the most prominent type of cell death activated in the presence of DNA damage [328,428]. Apoptosis is characterized by cellular and nuclear shrinkage (pyknosis), chromatin condensation, nuclear fragmentation, blebbing and the formation of apoptotic bodies. Apoptosis is mainly executed by a family of cysteine proteases known as caspases, which co-ordinate two main apoptotic signalling pathways known as (i) the intrinsic or mitochondrial pathway and (ii) the extrinsic or death receptor-mediated pathway. Caspases are synthesized as inactive zymogens that become functionally active upon cleavage. Each pathway consists of initiator caspases (e.g. Caspase-8, -9 and -10) that cleave and activate executioner caspases (Caspase-3, -6 and -7) that act on various proteins involved in multiple biological processes. While the extrinsic apoptotic pathway is initiated by the binding of extracellular signals to Death Receptors on the cell surface, the intrinsic apoptotic pathway is activated by intracellular stresses, including DNA damage [189,333,375,504].

The intrinsic apoptotic pathway is regulated by the B cell lymphoma-2 (BCL-2) family of proteins that are characterized by the presence of 1-4 conserved Bcl-2 homology (BH) domains. The BCL-2 family consists of anti-apoptotic proteins (e.g. BCL-2, BCL-XL and MCL-1) and pro-apoptotic proteins, divided into two sub-families: (i) the BH3-only family (includes BID, BIM, NOXA, and PUMA) and (ii) the BAX family (BAX, BOK, and BAK) [2,333,420,483]. The BCL-2 family acts as a ‘life / death’ switch that is
orchestrated via interactions between the anti-apoptotic and pro-apoptotic members to ensure cell death is activated at the appropriate time and in a well-controlled manner. Under normal conditions, anti-apoptotic BCL-2 family members bind and sequester pro-apoptotic BAX family members, preventing the induction of apoptosis. In response to DNA DSBs, ATM/CHK2-dependent p53 stabilization can lead to the transcription of the p53 target genes, Puma and Noxa. Although BIM is not a direct transcriptional target of p53, it also plays a key role in activating apoptosis in response to DNA DSBs [48,129]. These activated BH3-only proteins insert their BH3 domain into the hydrophobic groove of anti-apoptotic BCL-2 proteins, releasing BAX and BAK molecules. BAX, BAK and other activated BH3-only proteins subsequently oligomerize at the outer mitochondrial membrane, forming pores that facilitate the release of pro-apoptotic factors from the mitochondrial intermembrane space into the cytosol [2,189,333,420,483].

Cytochrome C is one of the most important pro-apoptotic factors released in this process that interacts with apoptotic protease-activating factor (APAF-1) and deoxyadenosine triphosphate (dATP) in the cytosol, collectively forming the apoptosome. Formation of the apoptosome initiates a signalling cascade in which a sequential series of initiator caspases (e.g. caspase-9) and downstream executioner caspases (e.g. caspase-3) cleave and inactivate target proteins. For example, executioner caspase-3 can cleave PARP (normally involved in DNA SSB repair) in the presence of extensive DNA damage, preventing PARP-dependent recruitment of other DNA repair proteins to DNA damage sites, ultimately resulting in DNA degradation. Caspase-3 also cleaves and activates the negative regulatory subunit of the caspase-activated DNase (CAD), resulting in DNA cleavage. Cleavage of nuclear lamina results in nuclear shrinking and blebbing and cleavage of cytoskeletal proteins and cell adhesion molecules contributes to the loss of cell shape. These processes altogether culminate in cellular disassembly and death [189,334,420,483].

1.4.6 Cellular senescence

Cellular senescence is defined as an irreversible cell cycle arrest that is restricted to proliferating cells. The causes of cellular senescence are still under investigation and include (i) telomere dysfunction, (ii) unrepaired DNA damage, (iii) oxidative stress and (iv) excessive mitogenic signals, such as oncogene over-expression [65,101,364,382]. Se-
nescent cells remain alive and metabolically active, but are characterized by the stable loss of proliferative capacity and altered gene expression that results in multiple cellular changes including enlarged and defective mitochondria; increased β-galactosidase production in lysosomes; protein aggregation in the endoplasmic reticulum and an enlarged, flattened morphology \[364\]. In response to DNA DSBs, cellular senescence can be activated by the ATM / CHK2 signalling axis via the phosphorylation and stabilization of p53 \[101\]. As previously described, in response to DNA damage, p53 can induce the expression of the tumour suppressor, p21. p21, in turn, inhibits cyclin D-CDK4/6; cyclin E-CDK2 and PCNA activity, halting G\(_1\) progression into S phase. The exact mechanisms that underlie the decision to either transiently arrest (i.e. checkpoint) or permanently arrest (cellular senescence) cell cycle progression at the G\(_1\)/S transition are poorly understood. However, it is hypothesized that the presence of un-repaired DNA damage within cells may cause p53-p21 signalling to be sustained long-term, ultimately leading to permanent cell cycle arrest, i.e. senescence \[65\].

Cellular senescence can also be activated via the p16\(^{INK4A}\)-pRB (Retinoblastoma protein) pathway in response to DNA damage. This pathway controls the G\(_1\)-S phase transition via the regulation of the transcription factor, E2F1, whose target genes regulate passage through the restriction point in late G\(_1\) and stimulate entry into S phase. p16\(^{INK4A}\) is a CKI encoded by the INK4A/ARF locus. In response to DNA damage, p16\(^{INK4A}\) binds to the CyclinD-CDK4/6 complex, preventing it from phosphorylating and inactivating RB. As a result, RB remains active and suppresses the activity of E2F1, preventing it from activating target genes required to transit from G\(_1\) to S phase, thereby halting cell cycle progression in G\(_1\) \[101, 292, 502\]. Overall, apoptosis and senescence are key tumour-suppressive mechanisms that prevent the survival and/or growth of cells harbouring persistent DNA damage.

### 1.5 Cell-type specific responses to DNA damage

For multicellular organisms, preservation of genomic integrity from extrinsic and intrinsic DNA damage that constantly challenges the cellular environment is vital in order to avoid premature ageing and/or malignant transformations \[502\]. However, there is high variability in the way different cell types respond to insults in their DNA. While
some cell types opt for promoting repair of their DNA lesions, others quickly undergo apoptosis in response to DNA damage. Stem cells are a clear example of this phenomenon [369]. Preservation of the genomic integrity of stem cells is essential for tissue development and maintenance throughout adult life. Multiple studies have focused on the deleterious effects of DNA damaging agents, such as IR, on tissue viability and regeneration, demonstrating that the level of radio-sensitivity varies widely between different stem cell types, and resulting in an increasing interest in the DDR of stem cells and their progenitors to explain those differences. For example, HSCs, embryonic stem cells (ESCs), and intestinal stem cells display high radio-sensitivity, undergoing extensive apoptosis in response to IR [136,184,301]. In contrast, other stem cell types such as hair follicle bulge stem cells (BSCs) or keratinocyte stem cells (KSCs) show high radio-resistance and less prone to IR-induced cell death [415,440]. Interestingly, DNA DSB repair capacity is highly correlated with the radio-resistance of these stem cells, with radio-resistant stem cells (e.g. BSCs and KSCs) being able to efficiently repair IR-induced DNA DSBs whereas radio-sensitive stem cells fail to do so [184,306,415]. These studies collectively indicate that the response of stem cells to DNA damaging agents is cell type – dependent, and that the individual response of each cell type is strongly linked to distinct DNA repair efficiency and kinetics, repair pathway choice (non-homologous end joining vs. homologous recombination), checkpoint activation or sensitivity to apoptosis or senescence [492].

1.5.1 The response of the haematopoietic system to ionizing radiation

Exposure to ionizing radiation (IR), e.g. γ-rays and X-rays, has a multitude of adverse dose-dependent effects on human tissues, including skin burns, hair loss and shedding of the gut-lining epithelium. The haematopoietic system is one of the first cellular systems to fail following acute exposure to IR. Lymphocytes, in general, are highly radio-sensitive and die within 24 hrs following exposure to even low IR doses whereas granulocytes, erythrocytes and platelets are more radio-resistant [104,138]. HSCs and their progenitors are also radio-sensitive ($D_0$ of 0.6-1.6 Gy, i.e. the dose required to reduce the proportion of surviving cells by 37%), preventing continuous renewal of functional blood and immune cell types in the periphery [138,176]. Allogeneic bone marrow trans-
plantation (BMT) remains the only practical treatment available for haematopoietic failure following IR exposure. In this process, patients are transplanted with genetically mis-matched bone marrow cells that have been isolated from a healthy donor. Since radiosensitive HSCs and their progenitors are depleted in transplant recipients, healthy donor HSCs can engraft into the bone marrow of the transplant patient and reconstitute the haematopoietic system. Without adequate BM transplantation (BMT), exposed patients will eventually die due to haematopoietic failure associated with anemia, thrombocytopenia, agranulocytosis and lymphopenia [104,138].

However, unlike haematopoietic cells, MSCs are relatively radio-resistant. Studies performed in mice and in man have demonstrated that MSCs isolated from BM transplant recipients originate from the irradiated host, rather than the engrafted donor bone marrow [4,24,144,257,370,411]. In addition, MSCs isolated from these recipients can be subsequently expanded in vitro to generate cell lines, such as MS5, which (i) retain mesenchymal differentiation capacity; (ii) continue to support haematopoiesis in vitro and (iii) do not give rise to tumours when injected into mice [4,24,144,257,370,411]. Therefore, these findings demonstrate that MSCs within the bone marrow can survive doses of irradiation that are detrimental to the haematopoietic system.

Similar to MSCs, thymic epithelial cells (TECs) are also able to survive irradiation and retain functionality, as demonstrated by the fact that in MHC incompatible radiation bone marrow chimeras, the functional MHC-restricted T cell repertoire of peripheral T cells derived from donor HSCs is determined by the MHC of the irradiated host and not by that of the original bone marrow donor, as discussed in Section 2.2.1 [38,137].

1.5.2 Radio-resistance of DN2 thymocytes: the exception to the rule

As previously discussed, haematopoietic cells are highly sensitive to ionizing radiation and as such, B-lymphocytes quickly succumb shortly after irradiation [324,395]. T-lymphocytes, although showing slightly higher radio-resistance than B cells and a slower death kinetics, also extensively die in response to IR doses even lower than 1Gy [12,356]. Consequently, acute radiation exposure results in rapid loss of thymic cellularity, due to extensive T cell death [431]. However, many authors have reported
a single wave of cellularity increase occurring shortly after radiation exposure (~5-7 days), which is independent of reconstitution with donor cells, and that is known as auto-reconstitution. This effect was found to be the result of the proliferation and differentiation of host-derived intrathymic radio-resistant T cell precursors that are able to survive irradiation and resume their normal development in the thymus. Thanks to a series of elegant experiments comparing T cell pools produced in lethally irradiated thymectomized or nonthymectomized C57BL/6 host mice that had been reconstituted with syngeneic bone marrow genetically incapable of generating T cells, Bosco et al. recently identified these radio-resistant early T cell precursors as DN2 thymocytes. However, the DN2-specific features that allow this subpopulation—and not others—to survive lethal irradiation remain unclear.

### 1.5.3 The DNA Damage Response of Mesenchymal Stromal Cells

Studies to date clearly indicate that MSCs are radio-resistant. However the mechanisms that underlie MSC radio-resistance are poorly understood. Long-term proliferation of primary human MSCs following exposure to high dose irradiation is comparable to that of known radio-resistant cell types, such as the A549 lung cancer cell line. Irradiated human MSCs phosphorylate and activate ATM and induce γ-H2AX formation which is abrogated in the presence of inhibitors that target ATM (KU55933 and caffeine) and DNA-PKcs (NU7441 and wortmannin). Therefore, the DDR is activated by irradiated human MSCs in vitro. The radio-resistance of mouse MSCs is comparable to that of human MSCs. However, primary mouse MSC cultures exhibit variable induction of γ-H2AX expression and foci formation following irradiation. This suggests that primary mouse MSC cultures exhibit a heterogeneous response to IR-induced DNA damage, probably due to the fact that they are a very heterogeneous mixture. In fact, primary MSC cultures are currently established from whole BM aspirates previously depleted of haematopoietic cells. However, as previously described, different stromal cell populations of varying function and differentiation potential are present within the BM. As a result, these ‘bulk’ uncloned MSC cultures are highly heterogeneous, with phenotypic characteristics varying between passage number and also between independent isolations. The inherent heterogeneity of these MSC cultures makes it difficult to extensively characterize the mechanisms that contribute to MSC radio-resistance.
To overcome the difficulties presented by using bulk MSC cultures, recent studies within our group have been performed with clonal mouse MSC lines in order to investigate MSC radiobiology in greater detail [421, 424]. These studies indicate that the radio-resistance of the MSC lines MS5 and ST-2 is comparable to that of primary mouse MSCs [421]. Detailed investigation into the response of these MSC lines to irradiation has demonstrated that several aspects of DDR signalling are likely to contribute to MSC radio-resistance. The kinetics of γ-H2AX formation following treatment with IR were compared between irradiated MSC lines and the radiosensitive immature (CD4^+ CD8^+) thymocyte line ST4.5. These experiments demonstrated that irradiated MSCs induced maximal H2AX phosphorylation and γ-H2AX IRIF formation at a faster rate than radiosensitive ST4.5 thymocytes. ATM and DNA-PKcs function redundantly to phosphorylate H2AX and chemical inhibition of their kinase activity delays H2AX phosphorylation [419, 466]. Interestingly, MSCs were found to endogenously express higher levels of the key DNA DSB response proteins, ATM and DNA-PKcs, than ST4.5 thymocytes. Taken together, these findings may indicate that the abundance of important DDR initiator proteins may promote rapid γ-H2AX formation at DNA DSB sites in irradiated MSCs, enabling robust activation of DDR signalling pathways that promote MSC survival.

The resolution over time of H2AX Ser139 phosphorylation and/or γ-H2AX foci in irradiated cells is an indirect indicator of DNA DSB repair. Following low-dose irradiation (1 Gy), γ-H2AX was resolved at a faster rate in irradiated MSCs compared with ST4.5 thymocytes. Furthermore, while γ-H2AX was resolved in MSCs following high-dose irradiation (10 Gy), γ-H2AX persisted long-term in irradiated ST4.5 thymocytes, probably because these cells quickly undergo apoptosis in response to IR. These findings indicate that MSCs have an enhanced ability to repair IR-induced DNA DSBs compared to immature thymocytes [421]. Similar to human MSCs, mouse MSC lines can repair IR-induced DNA DSBs using both NHEJ and HR pathways [83, 354, 421, 424]. It has recently been shown that the majority of IR-induced DNA DSBs are repaired at early time-points post IR, primarily via NHEJ [37]. Similarly, DNA DSBs are largely repaired in MSCs by 4-8h post high-dose (10 Gy) irradiation [424]. Interestingly, the MSC line, MS5, exhibited an increased capacity to repair DNA DSBs via NHEJ compared to radiosensitive ST4.5 thymocytes [421]. In addition, mouse MSCs expressed higher levels of key DNA DSB repair proteins, e.g. Atm, DNA-PKcs, DNA ligase IV and Rad51, than...
ST4.5 thymocytes. Overall, these findings indicate that MSCs have a robust capacity to repair IR-induced DNA DSBs that is likely promoted by expressing high endogenous levels of key DNA DSB repair proteins.

The presence of DNA damage, including DNA DSBs, can lead to the activation of signaling pathways that result in either delayed cell cycle progression, or transient arrest at transitions between cell cycle phases, known as DNA damage checkpoints. In response to DNA DSBs, activation of the $G_1$/S DNA damage checkpoint is primarily mediated via p53-dependent induction of p21 as previously described. Irradiated human MSCs maintain p53 stabilization over time and strongly induce p21 expression post IR, indicating robust activation of the $G_1$/S checkpoint [46, 106, 354]. However, both primary and clonal mouse MSCs only transiently activate the $G_1$/S checkpoint, indicated by (i) transient p53 stabilization and (ii) progression of irradiated $G_1$ cells into S phase post high dose irradiation [421, 424]. The lack of a definitive $G_1$/S checkpoint response in mouse MSCs may be correlated with p53 dysfunction in these cultured cells. However in the presence of persisting DNA DSBs, which can be induced in cells using specific chemotherapeutic drugs (e.g. etoposide), persistent p53 stabilization and downstream induction of p53 target proteins, p21 and Puma, are readily detectable in mouse MSC lines. Interestingly, transient activation of the $G_1$/S checkpoint has also been reported to occur in irradiated hair follicle bulge stem cells, embryonic stem cells and human induced pluripotent stem (iPS) cells [106, 315, 415]. Like MSCs, BSCs also have a high capacity for repairing IR-induced DNA DSBs via NHEJ and repair DSBs at a similar rate to MSCs [415]. Taken together, these findings suggest that the transient activation of the $G_1$/S checkpoint in irradiated mouse MSCs may be correlated with their ability to rapidly resolve DNA DSBs, thereby permitting $G_1$ cells to progress through the cell cycle post irradiation.

Induction of DNA DSBs in S phase cells can activate the intra-S-phase checkpoint that is characterized by a reduction in the rate of DNA replication, delaying S phase progression. Cells that accumulate DNA damage during $G_2$ or carry remaining DNA damage from S phase can activate the $G_2$/M checkpoint, which inhibits mitosis in the presence of DNA DSBs. Activation of the intra-S and $G_2$/M checkpoints can be assessed by monitoring the cell cycle progression of 5'-bromodeoxyuridine (BrdU) labelled cells. BrdU is a thymidine analogue that is incorporated into cells' DNA during DNA replication. Flow cytometric analysis of BrdU labelled cells with an anti-BrdU antibody and a DNA mar-
ker (e.g. propidium iodide) enables one to distinguish BrdU labelled S phase cells from BrdU unlabelled G₁, G₂ and mitotic cells. Analysis of BrdU labelled MSCs demonstrated that both primary and cloned mouse MSCs delay S phase progression and subsequently accumulate in G₂ for at least 12 hours post high-dose irradiation in vitro [421]. These observations indicate that although irradiated mouse MSCs only transiently activate the G₁/S checkpoint, the intra-S-phase and G₂/M checkpoints are robustly activated by this cell type. The reason(s) why irradiated mouse MSCs preferentially activate intra-S and G₂/M checkpoints whereas irradiated human MSCs induce a robust G₁/S checkpoint are unclear. It is evident that both mouse and human MSCs can repair DNA DSBs via NHEJ and HR [83, 354, 421, 424]. However, whether the capacity of MSCs to repair DNA DSBs by these two pathways is comparable between mouse and man is unknown and may account for the preferential activation of specific DNA damage checkpoints in these cell types.

Importantly, irradiated MSCs can subsequently recover from G₂/M arrest and re-enter cell cycle. In addition, irradiated MSCs retain the ability to differentiate along mesenchymal-derived lineages [321, 421]. These findings indicate that the proliferative capacity and differentiation potential of MSCs remain intact long-term post irradiation. However, radiosensitive ST4.5 thymocytes undergo rapid cell death post irradiation, independent of cell cycle phase status. This is due to the rapid activation of apoptosis, indicated by strong p53 stabilization and extensive cleavage of caspase-3 and of PARP in this cell type post irradiation [421]. This phenomenon, previously coined as ‘rapid interphase death’ commonly occurs in irradiated lymphocytes. However, unlike ST4.5 cells, despite harbouring an intact apoptotic machinery, apoptosis is weakly activated by irradiated mouse MSCs and cell death mainly only occurs when these cells attempt to re-enter cell cycle following G₂/M arrest [421]. In addition, MSCs express high levels of the anti-apoptotic proteins, Bcl-2 and Bcl-XL, and low levels of the potent pro-apoptotic proteins, Bim and Puma [421]. The intrinsic expression levels of various apoptotic proteins are known to influence the therapeutic sensitivity of various cell types, including HSCs, BSCs, KSCs and lymphocytes [121, 129, 330, 415]. Therefore, abundant expression of anti-apoptotic proteins in MSCs may also enhance their ability to activate pro-survival mechanisms by suppressing the activation of cell death.

Overall, these findings demonstrate that in response to irradiation, MSCs preferentially activate DNA damage signalling pathways that promote cell survival. Rapid induction
of γ-H2AX in irradiated MSC nuclei, presumably promoted by high expression levels of key DDR proteins, is likely to aid MSCs in rapidly initiating protective DNA damage checkpoints and DNA DSB repair. DNA damage checkpoints enable cells to extend the time frame in which DNA damage can be repaired before entering the next phase of the cell cycle. Therefore, coordinated activation of DNA damage checkpoints and DNA repair mechanisms is likely to be important for efficient resolution of genomic instability in MSCs.

Why MSCs preferentially activate pro-survival pathways in response to DNA damage is unknown. However, mounting evidence indicates that the DDR plays fundamental roles in stem cell biology. The DDR functions in stem cells to monitor endogenous DNA damage caused by environmental factors, e.g. ROS production, and by telomere shortening, preventing stem cell dysfunction and exhaustion in vivo [79, 206, 226, 417]. In addition, several key DDR proteins (e.g. Atm, p53 and p21) and DNA DSB repair proteins are also required in stem cells for (i) maintaining self-renewal; (ii) inhibiting senescence and (iii) regulating their differentiation potential [79, 85, 203, 206, 226, 276, 306, 323, 417, 467]. Interestingly, proteins involved in genome stability and cell cycle control have also been directly implicated in MSC biology. For example, loss of (i) the cell cycle regulator, Rb; (ii) the microRNA processor, Dicer, or (iii) telomerase (required for telomere maintenance and stability); impairs the ability of MSCs to regulate haematopoiesis, resulting in myelo-proliferation and leukemia development [217, 359, 463]. In addition, deletion or loss-of-function mutations in several DDR genes, including Atm, Atr and DNA ligase IV, can result in growth delay, skeletal abnormalities and osteoporosis [191, 329, 380]. Overall, these findings suggest that MSC radio-resistance may perhaps be a consequence of the importance of (i) the maintenance of genomic stability for stem cell homeostasis and (ii) DDR components in regulating MSC self-renewal, differentiation and function in the BM.

1.5.4 Effects of hypoxia on MSC radio-resistance

MSCs are radio-resistant progenitors that reside in hypoxic niches in vivo, particularly in the BM [268, 313, 416, 424, 485]. In cancer, hypoxia within tumours and HIF-1α over-expression in tumour cells are associated with increased malignancy and metastatic potential, and with poor patient outcome [454, 457]. This prognosis is highly correlated
with the increased resistance of hypoxic cancer cells to cancer therapies, including radiotherapy [36,457]. Mounting evidence indicates that exposure to hypoxia can alter the DDR of cancer cells, contributing to their increased resistance to DNA damaging agents including IR [179,374]. In addition, cellular exposure to severe hypoxia (<1% O_2) can activate the DDR independently of DNA damage, indicating that DDR signalling may contribute to adaptation to low oxygen environments [50,152,153,179,490]. Hyperbaric oxygen (HBO) treatment involves the administration of 100% oxygen above normal atmospheric pressure to increase the amount of dissolved oxygen in plasma [310]. This treatment has been proposed as a means to increase oxygen levels within tumours in the hope of augmenting the efficacy of anti-cancer treatments. However, a recent review indicated that various tumour types respond differentially to HBO treatment [310]. Further studies in this area are clearly necessary. Whether exposure to hypoxia affects the resistance of MSCs to irradiation is largely unknown.

In our group, the effects of γ-irradiation on mouse MSCs cultured in normoxia (21% O_2) and hypoxia (5% O_2) have been directly compared. This study demonstrated that both primary mouse MSCs and MSC lines display increased resistance to γ-irradiation in vitro when cultured in hypoxia [424]. DDR activation (indicated by γ-H2AX formation) was comparable between normoxic and hypoxic MSCs. In addition, MSCs also activated potent intra-S-phase and G_2/M checkpoints in hypoxia. However, irradiated MSCs were found to recover from cell cycle arrest at a faster rate in hypoxia than in normoxia [424]. Furthermore, H2AX Ser139 phosphorylation and γ-H2AX IRIF were resolved at a faster rate in MSC nuclei in hypoxia, compared with normoxia. Interestingly, hypoxic MSCs showed increased levels of key NHEJ repair proteins, including DNA-PKcs and DNA ligase IV in hypoxia. The increase in the protein levels of these repair factors coincided with HIF-1α stabilization in MSCs upon exposure to hypoxia. Rad51 IRIF formation, a key step in HR activation, was also accelerated in hypoxic MSCs. siRNA-mediated depletion of HIF-1α in MSCs prevented the increase in the levels of these DNA repair proteins in hypoxic MSCs [424]. These results indicate that HIF-1α may function as a transcriptional modulator of DNA DSB repair in mouse MSCs. Importantly, HIF-1α knockdown rendered MSC lines more radiosensitive. Overall, this study demonstrated that enhanced MSC radio-resistance in hypoxia is likely due to alterations in the DDR of MSCs to IR-induced DNA DSBs. These alterations include an enhanced DNA DSB repair capacity which is mediated, at least in part, by the activation of HIF-1α signalling.
Figure 1.10: The DNA Damage Response of mouse MSCs. In normoxia (left panel) and hypoxia (right panel), irradiated MSCs activate DNA damage checkpoints and DNA DSB repair (NHEJ and HR) to resolve genotoxic DNA DSBs. Resolution of DNA DSBs likely enables irradiated MSCs to recover from cell cycle arrest and promotes MSC survival. In hypoxia (right panel), HIF-1α stabilization resulted in (i) increased endogenous expression of the two key NHEJ enzymes, DNA-PKcs and DNA ligase IV, and (ii) accelerated recruitment of Rad51 to DNA DSBs, an essential component of repair by HR. Consequently, the DNA DSB repair capacity of MSCs is increased in hypoxia, accelerating recovery from cell cycle arrest and improving long-term survival. Abbreviations: DSB (Double-strand break); HIF-1α (Hypoxia inducible factor-1α); HR (Homologous recombination); IRIF (IR-induced foci); NHEJ (Non-homologous end-joining).

in hypoxic MSCs (Figure 1.10) [424].

1.6 Genome-editing approaches to study gene function.

Gene knockout and mutagenesis approaches have proven to be a very useful tool for investigation of gene function. For many years, traditional forward and reverse genetic strategies have allowed the elucidation of the function of thousands of genes.
However, the emergence of new generation tools for DNA manipulation has revolutionized these classical approaches thanks to their target site specificity, flexible design, and ease of operation \[278\]. In recent years, the development of new, highly versatile genome-editing technologies has made it possible to rapidly, easily and economically introduce sequence-specific modifications into the genomes of many different cell types and organisms \[148\]. The most widely used genome-editing tools are zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and most recently, clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) \[174\]. The main features of these technologies are summarized in Table 1.1 \[148,174,278\].

<table>
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<th>Table 1.1: Comparison of ZFN, TALEN and CRISPR/Cas9 main features</th>
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<td><strong>Mechanism</strong></td>
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All three systems are based in the same principle: they induce the cleavage of DNA at the desired genomic location and they take advantage of the subsequent repair process by either NHEJ or HR in order to promote knock-out or knock-in events \[361\]. However, in contrast to TALENs and ZFNs, the design of which involves costly and laborious protein engineering, CRISPR/Cas9 supposes a much more affordable, flexible and easily engineered system, requiring only basic molecular biology and cell culture techniques \[493\]. This has allowed CRISPR/Cas9 to quickly become the system of choice in many laboratories.
1.6.1 The CRISPR/Cas9 System.

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system is a form of adaptive immune system developed by bacteria and archaea in order to detect and degrade foreign nucleic acids [120]. The original Strep-tococcus pyogenes SF370 type II CRISPR locus consists of four genes (the Cas9 nuclease, a trans-activating CRISPR RNA (crRNA) known as tracrRNA, a precursor crRNA (pre-crRNA) array containing nuclease guide sequences (spacers) interspaced by identical direct repeats (DRs), and the SpRNase III necessary to process the crRNAs) [93].

However, optimization of the CRISPR system has allowed it to be reduced to two essential components: the Cas9 nuclease and a gRNA, which results from the expression of a crRNA-tracrRNA chimeric transcript containing all essential crRNA and tracrRNA components [285].

The gRNA consists of a 20 nt protospacer sequence at the 5´-end that matches the DNA sequence of the target site and a conserved 3´-end scaffold with a special stem-loop structure that allows Cas9 binding, thereby directing it to the specific genomic locus to which the protospacer sequence is complementary (Figure 1.11). In order for the Cas9 to cut, the target DNA sequence must be directly followed by a protospacer-adjacent motif (known as PAM), which in the case of the S. pyrogenes Cas9 must be a 5´-NGG-3´ trinucleotide downstream of the targeted DNA [120]. Interestingly, other Cas9 orthologs from different bacteria have different PAM requirements [361]. The requirement of the presence of the PAM sequence immediately adjacent to the target site is a limiting factor for the use of the CRISPR/Cas9 system in some applications. For this reason, alternative Cas9 versions with different PAM specificities have been designed, allowing the targeting of sequences previously impossible to mutate using the CRISPR/Cas9 system [238].

Once the Cas9 has recognized the gRNA and the PAM sequence, it will make a blunt cut between the 17th and 18th bases of the target sequence complementary to the protospacer (3 bp 5´ of the PAM) [89]. These double-strand breaks will be then recognized by the cellular DDR machinery and repaired using either error-prone non-homologous end joining or more accurate homologous recombination (Figure 1.11). NHEJ-mediated DNA repair can generate small insertion and deletion mutations at target sites that often disturb or abolish the function of target genes by disrupting the reading frame.
**Figure 1.11: CRISPR/Cas9 mechanism of action.** The protospacer sequence (shown in green) adjacent to the 5’-NGG-3’ PAM sequence of the gRNA directs the Cas9 to its target site, where it introduces a DSB 3 nucleotides upstream of the PAM. This double strand break can be repaired by either error-prone NHEJ, which is likely to introduce insertions or deletions, eventually resulting in a premature stop codon; or by HR, which allows precise gene editing when an exogenous HDR template is provided. (Image adapted from Wyvekens et al. 2016 [493] and D’Agostino and D’Aniello 2017 [102]). Abbreviations: CRISPR-associated protein 9 (Cas9); protoscaler-adjacent motif (PAM); guide RNA (gRNA); double strand break (DSB) non homologous end joining (NHEJ), homologous recombination (HR); homology-directed repair (HDR).
of the resulting mRNA [499]. DSBs can also be repaired by homologous recombination (HR), which requires a homologous template in order to carry out accurate repair, and this allows specific sequence substitutions or insertions to be easily introduced in the target sequence by providing an exogenous HR template (Figure 1.11) [499].

However, one major concern regarding CRISPR/Cas9-mediated genome modifications is the potential introduction of off-target mutations in sites that resemble the on-target sequence [493]. For this reason, efforts are being made in order to minimize the CRISPR/Cas9 potential for off-target effects, such as engineering of the Cas9 nucleases to generate high-fidelity variants that would reduce non-specific DNA contacts [237]. In summary, CRISPR/Cas9 is a very useful, novel and highly efficient genome-editing tool that is continuously being improved and that has opened a broad range of possibilities for rapid, robust, and multiplexable genome engineering applications.

1.7 Introduction to the present project. Hypotheses and Aims.

Total body irradiation treatments (TBI) used prior to bone marrow transplantation (BMT) not only affect the radiosensitive endogenous HSCs and resident host immune cells, but also have deleterious effects on the radio-resistant supportive cell types that orchestrate the haematopoietic process, such as mesenchymal stromal cells (MSCs) in the bone marrow and thymic epithelial cells in the thymus (TECs). For this reason, investigation of the mechanisms underlying the radio-resistance of mesenchymal stromal cells and thymic epithelial cells can provide the basis for the development of improved pre-conditioning regimens that allow adequate depletion of host hematopoietic cells while maintaining supportive BM and thymic niches. The DNA damage response (DDR) is the signalling network that allows cells to respond to the deleterious effects that DNA damaging agents, such as ionizing radiation, cause in their genomes. Therefore, understanding how the DDR of mesenchymal stromal cells and thymic epithelial cells contributes to their radio-resistance is crucial for improving the outcomes of BMT. In addition, the mechanisms that mediate the particularly high radio-resistance of DN2 pro-thymocytes, opposite to their radiosensitive immediate progenitors (DN1 cells) and progeny (DN3 cells), remain poorly characterized. Hypoxia has been long known to
impact the DDR of hypoxic cancer cells, mediating their increased resistance to DNA
damaging agents such as IR. Furthermore, in our group, hypoxia has been shown to
specifically enhance the radio-resistance of mouse MSCs in a Hif-1α-dependent manner.

For these reasons, the main aims of my Ph.D. studies were:

1. To investigate the role of the DDR in mediating the radio-resistance of mouse
thymic epithelial cells (TECs) in vitro, as well as to determine the effects of hypoxia
on their DDR. In addition, the effects of radiation exposure on primary mouse TEC
functionality were assessed (Chapter 2).

2. To determine the specific mechanism that allows Hif-1α to modulate the DDR of
mouse MSCs and enhance their radio-resistance (Chapter 3).

3. To characterize the particularities of the DDR of DN2 pro-T cells in comparison to
other radiosensitive cell types such as HSCs, as well as to define the effects that
hypoxia may exert on their DDR (Chapter 4).

The experimental work performed to achieve these goals will now be discussed in detail.
Chapter 2

Differential response of mouse thymic epithelial cell types to ionizing radiation-induced DNA damage
2.1 Introduction

The thymus is the main organ for T lymphocyte development, for which its structure and its composition are specialized, providing the necessary microenvironments for each step of T cell differentiation and selection \[7, 432\]. In a mature thymus, developing thymocytes account for around 95% of thymic cellularity \[287\], meaning that the thymic stroma, comprising all the non-hematopoietic cellular components, accounts for less than 5% of the cells found in the thymus \[155, 166\]. The majority of stromal cells consist of thymic epithelial cells (TECs), which not only provide the three-dimensional matrix in which T cells develop but also control the homing, expansion, maturation and selection of these thymocytes \[8, 11, 155\].

The mature thymus, which is encased within a thin fibrous capsule can be anatomically subdivided in two main regions: the peripheral cortex and the inner medulla \[7, 8, 11\], which are conserved throughout evolution \[155\]. This allows the classification of the thymic epithelial cells into cortical (cTECs) and medullary (mTECs), which have differential morphological, functional and antigenic properties \[8, 155\]. Both mTECs and cTECs can derive from a common bipotent EpCAM$^+$UEA1$^+$Ly51$^+$PLET1$^+$MHCII$^+$TEC progenitor \[453\]. However, they express distinct cortical (Cytokeratin-8$^+$and -18$^+$, Ly51$^+$) and medullary (Cytokeratin-5$^+$and -14$^+$, Ly51$^-$) markers that, together with the mTEC-specific ability to bind the *Ulex europaeus* lectin agglutinin (UEA-1), allow them to be distinguished \[7, 155, 164\]. mTECs can be further subdivided in different subpopulations by the expression of MHCII and accessory molecules such as CD40 and CD80/86, with AIRE expression being found specifically in a subpopulation of MHCII$^+$CD80/86$^+$UEA1$^+$ mTECs \[5, 479\]. All these subsets of thymic epithelial cells are highly specialized to provide the cytokines, chemokines, lineage inductive ligands, selective self-antigens, cell surface molecules and extracellular matrix elements necessary for T cell development, a process that is strictly dependent on the communication between TECs and developing T cells \[9, 347\].

Allogeneic bone marrow transplantation (BMT) is currently the most effective treatment for lymphoid and myeloid cancers as well as for the treatment of certain genetic immune-deficiency or autoimmune disorders \[94\]. Prior to transplantation, a patient must undergo a combination of conditioning or preparative regimes, normally consisting of radiotherapy (frequently in combination with chemotherapeutic drugs), in order
to eliminate endogenous HSC and resident host immune cells. Ionizing radiation (IR) causes many deleterious and dose-dependent effects on the hematopoietic system, which is highly radio-sensitive and is one of the first cellular systems to be affected by exposure to IR. However, other cell types such as thymic epithelial cells are also vulnerable to damage inflicted during the BMT process by agents such as radiation or chemotherapy. In order for a bone marrow transplantation to be successful, not only is the presence of viable progenitors necessary but the maintenance of a functional microenvironment to support differentiation of these cells is also crucial. This deleterious effect on the thymus functionality is one of the main causes that has been hypothesized to explain the prolonged periods of T-cell deficiency that BMT patients often suffer, and that render them highly susceptible to common and opportunistic infections, as well as occurrence and relapse of cancers. For this reason, investigation of the effects that ionizing radiation causes on thymic epithelial cells and their ability to perform their normal function is crucial for improving the outcomes of BMT.

Ionizing radiation causes extensive damage to the genome of the cells, either by direct energy transfer to the DNA or most frequently through the generation of free radicals by ionization of molecules, primarily water. Of all lesions induced, DNA double strand breaks (DSBs) are the most genotoxic due to their difficulty to be repaired. This destructive impact on genomic integrity triggers the activation of the DNA damage response (DDR), which is a complex signalling network that allows the cells to mount an orchestrated response to their damaged DNA. The DDR is composed of sensors that monitor DNA for structural abnormalities (damaged DNA), transducers, that transmit and amplify the damage signal and effectors in charge of triggering and coordinating biological processes. Such processes include transient cell cycle arrest (checkpoints), DNA repair, alteration of transcriptional programs, apoptosis or senescence.

We have previously shown how the execution of the DNA damage response can deeply impact on the cells’ sensitivity to IR. Here we characterized the DNA damage response of thymic epithelial cell lines and compared the specific responses of cortical and medullary TECs in order to identify the main mechanisms underlying TEC survival after IR. Since we previously demonstrated a role of hypoxia in enhancing the DDR of mesenchymal stromal cells, we also analysed whether hypoxia plays a role in regulating TEC responses to IR. We show that by markedly decreasing their expression...
of factors that are essential for their functions, exposure to IR has a profound effect on primary mouse TEC functionality. To the best of our knowledge, this is the first time that the DDR of thymic epithelial cells has been studied in detail.

2.2 Materials and methods

2.2.1 Cell culture and treatment

The cortical thymic epithelial cell line cTEC 1-2 and the medullary thymic epithelial cell line mTEC 3-10 were kindly provided by Prof. Georg Holländer (Department of Biomedicine, University of Basel) and ST4.5 CD4+CD8+ thymocyte cell line was provided by Dr. Anne Wilson (Ludwig Institute of Cancer Research, Lausanne). These cells are cytoplasmic TCRβ and CD3ε positive (not shown). All cell lines were cultured in Dulbecco's modified Eagle's medium high glucose (Gibco) supplemented with 10% foetal bovine serum (FBS) (Sigma Aldrich) and 1% penicillin/streptomycin sulphate solution (Gibco).

All cell types were continuously cultured in humidified incubators at 37°C containing 21% O₂ (normoxia) or 5% O₂ (hypoxia) for at least one week prior to experimentation.

Cells were γ-irradiated at the indicated doses using a Mainance Millennium Sample Irradiator containing a ¹³⁷Cs source at a dose rate of approximately 102 cGy/min. Cells were treated with 1μM Staurosporine solution (Cell Signaling Technologies) or 25 μM 2-bromodeoxyuridine (BrdU) and harvested at the indicated time points post-treatment.

2.2.2 Mice

C57BL/6 mice were bred under pathogen-free conditions at the Centre for Biomedicine at the University of Basel. All animal experiments were carried out within institutional guidelines (authorization numbers 1886 and 1888 from Kantonaes Veterinäramt, Basel).
2.2.3 Isolation and Sorting of Mouse Thymic Epithelial Cell Sub-populations

Two groups of 20 C57BL/6 mice were used in this experiment. One of the groups was irradiated with 9 Gy, while the other was left untreated as control. 24h after irradiation, thymic stromal cells were isolated from the 20 control and 20 irradiated thymi and sorted according to their cell surface phenotypes. For TEC isolation, thymic lobes were separated, cleaned from fat and connective tissue, pierced with watchmaker forceps and placed in PBS containing Liberase TM research grade (1 Wünsch unit/ml; Roche) at 37°C. After 10 min, lobes were gently resuspended using a 1 ml pipet tip cut off to provide a 3-4 mm opening. Lobes were further incubated for 5 min intervals at 37°C and resuspended using increasingly smaller tip openings until a homogeneous cell suspension was achieved. Cells were washed with IMDM containing 10% FCS and 5 mM EDTA, resuspended in PBS/2% FCS at a concentration of 100x10⁶/ml and stained with biotinylated EpCAM for 15min on ice. Washed cells were resuspended at 250 x10⁶/ml and 60μl/ml Streptavidin beads (Miltenyi) were added for 15 min at 4°C. Washed cell preparations were then separated on the AutoMACSpro (Miltenyi) with the POSSEL program. The enriched TEC fraction was re-stained with EpCAM PE-Cy7 and Streptavidin PE-Cy7, CD45 Alexa700, Ly51 PE, UEA-1 FITC, MHCII APC-Cy7, CD80 PerCP-Cy5.5 and CD86 APC. Dead cells were discriminated by DAPI and TEC subsets were sorted on a FACSARia (BD Biosciences). Sorted cells were pelleted, resuspended in 500 μl Trizol (Life Technologies) and stored at -20°C for further processing.

2.2.4 Growth curve analysis

Cells were seeded into 6 well plates (Nunc) at a concentration of 5x10⁴ cells / well. Individual cultures were harvested daily for 7 days and cell counts were performed in duplicate in a haemocytometer using Trypan blue exclusion of dead cells.

2.2.5 Clonogenic Survival Assay

Adherent TEC cell lines were irradiated at 1-8Gy and seeded into six-well plates (Nunc) at a concentration of 200 cells per well. Cells were incubated for 8 days until colonies
were clearly visible. Colonies were stained with Coomassie Blue (Sigma-Aldrich) and counted. All colony images are representative of one of four independent experiments. Non-adherent ST4.5 cells were irradiated with 1-8 Gy, seeded into 6-well plates at a concentration of 3x10^4 cells per well, harvested 5 days post irradiation, and cell numbers were counted in duplicate using a haemocytometer and Trypan blue exclusion of dead cells was performed. The percentage survival of each cell type was determined by normalizing the number of colonies / cells generated by irradiated cultures to the number of colonies / cells generated by control un-irradiated cultures.

2.2.6 Flow Cytometry

Cells were trypsinized to obtain a single cell suspension, filtered through a 30 μm filter (Cell Trics) and counted prior to staining following the different protocols described below. Cells were then analysed using BD FACS Canto® or BD Accuri™ C6 flow cytometers (BD Biosciences) and FlowJo® software (TreeStar Inc.). All information regarding solutions and antibodies used can be found in Supplementary Tables S.1 and S.2, respectively.

2.2.6.1 Surface marker analysis by surface staining

Cells were trypsinized to obtain a single cell suspension and counted. 5x10^5 cells per sample were resuspended in FACS buffer (2% FCS, 0.05% Sodium Azide, PBS) and stained with the appropriate primary antibodies or isotype controls. If applicable, cells were then washed and stained with fluorescently-labelled streptavidin prior to analysis using a BD Accuri™ C6 flow cytometer and FlowJo® software.

2.2.6.2 Cell cycle analysis using BrdU-Propidium Iodide Staining

Cells were labelled for 45 min with 25 μM 5'-bromo-deoxyuridine (BrdU) (Sigma Aldrich), washed in PBS and re-suspended in growth medium. Cells were harvested at the indicated time points post irradiation (3 Gy), fixed in ice-cold 70% ethanol and stained with anti-BrdU and FITC-conjugated anti-mouse IgG antibodies and Propidium Iodide
(PI) / RNase staining buffer (BD Biosciences) as previously described \[421\]. The progression of cells through the cell cycle was analysed by measuring the percentage of BrdU-positive cells in G\textsubscript{1} phase until 24h post IR using a BD FACS Canto® flow cytometer and FlowJo® software.

2.2.6.3 \textbf{G\textsubscript{2}/M checkpoint analysis using phospho-Histone H3 Ser10 - Propidium Iodide Staining}

Cells were harvested at the indicated time points post irradiation (3 Gy), washed in PBS and fixed in ice-cold 70% ethanol. Following permeabilization in PBS / 0.25% Triton X-100, cells were sequentially stained with anti-phospho-histone H3 (Ser10) and FITC-conjugated anti-rabbit IgG antibodies for 2h and 30min at room temperature, respectively, separated by two washing steps in PBS / 1% BSA. Cells were then re-suspended in PI / RNase staining buffer (BD Biosciences) and mitotic index analysed up to 24h post irradiation using a BD FACS Canto® flow cytometer and FlowJo® software.

2.2.6.4 \textbf{Analysis of Apoptosis using cleaved Caspase-3 Staining}

TEC cell lines were harvested at the indicated time points post irradiation (3 Gy), washed in PBS and fixed with 2% PFA. Cells were then permeabilized using PBS / 0.5% Saponin and sequentially stained with anti-Caspase-3 and FITC-conjugated anti-rabbit IgG antibodies diluted in PBS / 1% BSA for 2h and 1h respectively at 37°C, separated by 2 washes with PBS / 1% BSA. Apoptotic cells were quantified by flow cytometry as Caspase-3 positive cells up to 96h post IR using a BD Accuri™ C6 flow cytometer and FlowJo® software. Staurosporine-treated cells were used as positive control for this assay.

2.2.7 \textbf{RNA methods}

2.2.7.1 \textbf{RNA isolation and retro-transcription}

Total RNA was isolated from cells by TRIzol® Reagent (Life Technologies) -chloroform extraction. In short, \(10^6\) cells per condition were dissolved in 1ml of TRIzol reagent and
incubated for 5 min at room temperature. 200 μl of chloroform (Sigma) were added and the tubes were thoroughly vortexed to maximize phase exchange. Phases were then allowed to separate by incubating for 5 min at room temperature before spinning down at 12,000 g for 30 min at 4°C. Aqueous layer was recovered and RNA was precipitated by addition of 2 volumes of 100% ethanol (Sigma). RNA was pelleted by centrifugation at 12,000 g for 30 min at 4°C. Three washes with 70% ethanol were performed, separated by 5 min spins at 8,000 g. Remaining ethanol was allowed to dry and RNA was resuspended in nuclease-free H₂O (Qiagen). RNA quantification and purity assessment was performed by measuring the absorbance at 260, 230 and 280 nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific). RNA integrity was assessed by visualization on MOPS/agarose gels. cDNA was generated using Applied Biosystems’ High-Capacity cDNA Reverse Transcription Kit according to the manufacturer’s instructions. All information regarding solutions used can be found in Supplementary Table S.1.

2.2.7.2 RNA agarose gel electrophoresis

1% agarose (Sigma) gels were prepared in 1x MAE buffer containing formaldehyde at a final concentration of 2.2%. RNA Loading Buffer at a final 1x dilution was used to load the samples onto the gel, after incubation at 56°C for 5 min, and gels were run in 1x MAE containing 1.1% formaldehyde in Owl mini (50 ml gel) or wide mini (100 ml gel) cells at 80-100 V until the required separation was achieved. The analysis of the gel was carried out using a trans-illuminator with UV light and photographs were taken with a digital camera (ChemiImager 5500, Alpha Innotech). All information regarding solutions used can be found in Supplementary Table S.1.

2.2.7.3 Real-time PCR

10-80 ng of cDNA was used as template in semi-quantitative real-time PCR reactions with specific primers on a Step One Plus Real-Time PCR System (Applied Biosystems). The reactions were prepared with SYBR Select reaction mix from Applied Biosystems. Predesigned KiCqStart® primer pairs for mouse Prkdc, Lig4, Rad51, Aire, Dll4, Flt3l, Il7, Kitl, β3t, Ctsd, Ccl17, Ccl19, Ccl21, Ccl22, Ccl25, Cxcl1, Cxcl12, Bim, β-Actin (Actb) and Gapdh were purchased from Sigma Aldrich. PCR conditions were optimized for
every primer pair and standard curves were performed to determine the efficiency of each of them. Gene expression changes were determined by the $\Delta \Delta \text{Ct}$ method, using $\beta$-Actin as housekeeping gene for normalization and adjusting according to the primer efficiencies previously calculated.

2.2.7.4 Whole Thymic Stroma Gene Expression Analysis

C57BL/6 mice were irradiated with 9Gy and thymic stroma was subsequently obtained from control and irradiated thymi. T cells were depleted by gently pressing thymuses through a 70$\mu$m pore size cell strainer followed by several washes with ice-cold PBS. The remaining stroma was then fragmented and disaggregated in TRIzol® Reagent (Life Technologies) for RNA isolation using the TRIzol-chloroform method. Resulting RNA was used as template for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems according to the manufacturer’s instructions and qPCR reactions were performed as described above. Gene expression analysis was carried out separately for each technical replicate using the $2^{-\Delta \Delta \text{Ct}}$ method, prior to averaging, as described in [267], and standard error of the mean of both control and irradiated samples are reported. Gapdh was used as endogenous control gene and untreated samples were used as reference for normalization.

2.2.7.5 Primary Mouse Thymic Epithelial Cell Subpopulations Gene Expression Analysis

RNA was isolated from irradiated or control sorted thymic epithelial cell subpopulations using the TRIzol-chloroform method. cDNA was then synthetized using either the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems or the QuantiTect Whole Transcriptome Amplification Kit from Quiagen, according to the manufacturer’s instructions. qPCR reactions were performed as described above.

2.2.7.6 DNA Damage Response qPCR Arrays

RNA was isolated from TEC cell lines cultured in normoxia (21% O$_2$) or hypoxia (5% O$_2$) using the TRIzol-chloroform method. 500ng per sample of the resulting total RNA were
used as a template for cDNA synthesis using Quiagen’s RT2 First Strand Kit according to the manufacturer’s protocol. qPCR reactions were prepared using the RT2 SYBR Green ROX qPCR Mastermix from Quiagen and loaded into the commercial customized Mouse DNA Damage Response RT2 Profiler PCR Arrays which include primers for DNA Ligase IV (Lig4), Bcl2, Bcl-XL (Bcl2l1) and Puma (Bbc3) in addition to the 84 DDR genes present in the standard PCR arrays.

2.2.8 Western blotting

Whole cell extracts were prepared from control or irradiated cells at the indicated time-points post irradiation by direct addition of 1x Laemmli buffer to the cells still adhered to the culture plates following one wash with ice-cold PBS. Cells were disaggregated into the Laemmli buffer using a cell scraper, heated at 95°C for 5 min and sonicated prior to separation using SDS-PAGE gels and transferred to nitrocellulose membranes. Chemiluminescence was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and medical x-ray film (Konica Minolta Medical & Graphic Imaging Inc.). In assays in which protein quantification was necessary, this was performed using a LiCor Odyssey infrared imaging system according to manufacturer’s instructions. Information regarding all solutions and antibodies used for western blotting can be found in Supplementary Tables S.1 and S.3 respectively.

2.2.9 Immunofluorescence microscopy

2.2.9.1 Immunofluorescence Staining

Cells were cultured on glass coverslips in 21% or 5% O2 for 48 hours prior to irradiation. All cultures were fixed in 4% paraformaldehyde (Sigma Aldrich), permeabilized in 0.1% Triton®-X 100 solution and nuclei stained for γH2AX and Rad51 IRIF as previously described [421]. All images were captured using 40X or 60X magnification on a Delta Vision integrated microscope system (Applied Precision) controlled by SoftWoRx software mounted on an IX71 Olympus microscope. Images were deconvolved using the ratio method and maximal intensity projections obtained using SoftWoRx. All images shown are representative of one of five independent experiments. The number of γH2AX
and Rad51 IR-induced foci (IRIF) per nucleus was quantified blindly using customized macros for Fiji [388], as described below, in a minimum of 50 cells per time-point in each experiment. The use of this semi-automated system for image processing and analysis is advantageous since it is much faster, ensures equal treatment of all images and allows blind quantifications to be easily performed. In addition, the macros described below were designed in a generic and reusable way, so that they can be readily applied to other similar applications. Information regarding all solutions and antibodies used can be found in Supplementary Tables S.1 and S.4 respectively.

2.2.9.2 Image processing using Fiji

Images acquired using the Delta Vision integrated microscope system and the SoftWoRx software were automatically saved in .dv format. This Fiji macro can be divided into 2 main parts. The first one consists of converting the original .dv files into .tif files (separated by channel) with no image processing at all. In a second step, the macro separates the single images corresponding to each channel acquired in each multi-channel image. The display range of each channel is then adjusted (not affecting the real underlying pixel values) in order to enhance the particular features of interest in each image (for example, the IRIFs) [19]. The best minimum and maximum values to be applied to each channel must be previously determined by the user and specified within the text body of the macro. Once these have been defined, they will be applied to every image so that all of them are treated in the exact same way, allowing subsequent comparisons. Finally, all channel images corresponding to the same original file are merged into a single multi-channel adjusted image, which is saved in a different folder to the raw data, therefore avoiding over-writing of the original images. In addition, the name of the file is modified by the addition of “Adj” at the end, standing for “adjusted image”. This is an iterative process which analyses each file present in a folder (defined by the user when the macro is launched, and empty at the start of the process) one by one. At the end, a “Log” text file specifying the specific details of the image processing is created. The code of this macro can be found in Annex 1.
2.2.9.3 IRIF quantification using *Fiji*

This macro allows accurate and automated quantification of all IRIFs present per cell nucleus, whose intensity is above a certain threshold previously defined by the user. In order to do this, the user must manually determine the best detection threshold and the approximate particle size, which will then be specified within the text body of the macro to be equally applied to all images. Using the DAPI channel the macro permits the detection of the cell nuclei and then uses this information to quantify the number of IRIF present within the area of each nucleus. This is a semi-automated process which allows the iterative analysis of the images one by one, and in which the user is required to review each step of the analysis, having the option to improve the threshold for the detection of the nuclei (which is otherwise automatically determined by the software) and also eliminate “wrong” particles that must be excluded from the study (for example, two or more nuclei located too close to each other that might be detected as one single particle), therefore increasing the accuracy of the analysis. The input for the macro must be a folder containing only the multi-channel .tif images to analyse (such as the ones obtained using the macro described in Section 2.2.9.2), and the output is a folder (defined by the used when the macro is launched, and empty at the start of the process) containing one .xls file per image, which details the IRIF analysis performed for each cell nucleus detected in each of the images. The code of this macro can be found in Annex 2.

2.3 Results

2.3.1 Characterization Of Thymic Epithelial Cell Lines

To study the responses of TEC lines to ionizing irradiation, we used the cell lines TEC 3-10 (medullary TEC) and TEC 1-2 (cortical TEC). These cell lines were originally established by T. Mizuochi et al. from C56BL/6 mice, who characterized their medullary and cortical nature by immunostaining with the Th-3 and Th-4 antibodies [309]. Prior to our experiments, we verified the phenotype of these cells by morphological (Figure 2.1A) and flow cytometric analysis of CD45, EpCAM, Ly51 and MHCII surface marker expression and binding of the UEA-1 lectin. Thus, we were able to confirm the identity
of the mTEC 3-10 cell line as CD4<sup>−</sup>EpCAM<sup>+</sup>Ly5<sup>1</sup>−UEA1<sup>+</sup>MHCI<sup>+</sup> and the cTEC 1-2 cell line as CD4<sup>−</sup>EpCAM<sup>+</sup>Ly5<sup>1</sup>−UEA1<sup>−</sup>MHCI<sup>+</sup> (Figure 2.1 B, C).

### 2.3.2 TEC lines are resistant to ionizing radiation, and hypoxia reduces mTEC radio-resistance in vitro.

To determine how hypoxia influenced cell growth, TEC lines were cultured in either normoxia (21% O<sub>2</sub>) or hypoxia (5% O<sub>2</sub>) and growth curves plotted. A tendency for enhanced proliferation of mTEC 3-10 cells was observed under hypoxic conditions (with an average doubling time of 18.3h in normoxia and 14.6h in hypoxia), whereas cTEC 1-2 cells grew at a similar rate in both hypoxia and normoxia (17.4h doubling normoxia and 17.3h in hypoxia) (Figure 2.2 A, B). Interestingly, cTEC 1-2 cells grew to higher cell number (~2 fold) under normoxic conditions, whereas mTEC 3-10 cells, despite faster growth in hypoxia, reached the same plateau cell concentration in both conditions. To study the effects of irradiation on cell lines, clonogenic survival assays were carried out. Images of colonies obtained are shown for mTEC and cTEC in Figures 2.2 E and F respectively. Confirming their relative enhanced growth in hypoxia, colony sizes of mTEC 3-10 cells were detectably larger in hypoxia, although there were fewer colonies. For cTEC 1-2 cells, there was no observable change in colony size in hypoxia and colony numbers seemed unchanged. Results of a series of such experiments are shown for mTEC 3-10 and cTEC 1-2 cells in Figures 2.2 C and D respectively. In these experiments, the ST4.5 CD4/CD8 double positive (DP) T cell line was included as a radio-sensitive control [421]. Both mTECs and cTECs showed a much higher radio-resistance than the DP cell line ST4.5. Both mTEC 3-10 and cTEC 1-2 lines showed a very similar survival to low IR doses, however, mTEC 3-10 cells show an increased radio-resistance to the highest doses of IR (particularly noticeable at 6-8Gy). Taken together, these results indicate that mTEC 3-10 cells are more resistant to high doses of IR, and that hypoxia specifically reduces the radio-resistance of this cell line. Our clonogenic survival assays also showed that both cTEC 1-2 and mTEC 3-10 cell lines retained approximately 50% of their colony formation capacity after treatment with 3Gy compared to the untreated condition (Figure 2.2 C-D), therefore, this dose was chosen for most subsequent experiments.
Figure 2.1: Thymic epithelial cell line characterization. (A) Morphological characteristics of mTEC 3-10 (left) and cTEC 1-2 cell lines. Scale bar corresponds to 125 μm. (B) Flow cytometry analysis of surface marker expression of mTEC 3-10 and cTEC 1-2 cell lines. Isotype control is indicated in light blue while surface markers are indicated in dark blue. (C) MFI of isotype control and surface marker staining of mTEC 3-10 and cTEC 1-2 cell lines. Average of 3 replicates is shown, with error bars indicating the standard deviation (SD). * p<0.05, multiple t-tests with Holm-Sidak post correction.
Figure 2.2: TEC sensitivity to ionizing radiation. Growth curves of (A) mTEC 3-10 and (B) cTEC 1-2 cell lines cultured in 21%/5% O<sub>2</sub>. Clonogenic survival assays of (C) mTEC 3-10 and ST4.5 cells and (D) cTEC 1-2 and ST4.5 cell lines in 21% or 5% O<sub>2</sub>. * p<0.05, ** p<0.01 compared with normoxic samples, two-way ANOVA. (E) Representative images of TEC colonies generated in clonogenic survival assays following treatment with 0, 2, 4, 6 and 8Gy.
2.3.3 Oxygen Level Does Not Affect The Cell Cycle Regulation Of Thymic Epithelial Cells.

In response to genotoxic lesions such as those introduced by ionizing radiation (IR), cells activate the DNA Damage Response (DDR), a complex signalling network that orchestrates the cellular response to such lesions. One of the cell's earliest responses to DNA damage is to induce a cell cycle arrest \[186\]. To study the cell cycle checkpoints activated by thymic epithelial cells in response to IR, cell cycle progression of BrdU pulse labelled mTECs and cTECs was analyzed by flow cytometry. Combined BrdU and PI staining allows to distinguish cells in G\(_1\), S and G\(_2\)/M phases of the cell cycle as well as progression of BrdU-labelled (S phase) cells through the cell cycle and their return to G\(_1\) phase (Figure 2.3 A, B). After receiving a 3Gy IR treatment, both mTEC 3-10 and cTEC 1-2 cells accumulated in G\(_2\)/M phase until about 8h, which indicates a strong prevalence of the G\(_2\)/M checkpoint in these cells, with very little or no activation of the G\(_1\) or intra-S checkpoints. As the cell cycle progresses, a subpopulation of newly formed BrdU-labelled G\(_1\) cells appears and increases in size. Quantification of this new subpopulation was used as readout for the kinetics with which cells resumed the cell cycle after the genomic insult and left the G\(_2\)/M arrest. The delay in cell cycle progression induced by IR can be clearly observed in comparison with the untreated cells (Figure 2.3 C, D), although no differences were detected between normoxic and hypoxic conditions for both mTEC 3-10 and cTEC 1-2 cell lines (Figure 2.3 A-D). However, comparison between mTEC 3-10 and cTEC 1-2 cells indicated a faster recovery from the cell cycle arrest in cTEC 1-2 cells than in mTEC 3-10, as indicated by the higher proportion of BrdU-positive G\(_1\) cells present 8h and 12h after IR (Figure 2.3 C, D).

Since both TEC cell lines seem to mainly rely on the G\(_2\)/M checkpoint, and because of the fact that the BrdU/PI assay does not allow the discrimination between G\(_2\) and M phases of the cell cycle, a G\(_2\)/M checkpoint assay was used. To do so, a mitotic index analysis was performed flow cytometrically using combined intracellular staining for phosphorylated histone H3 Serine10 (pH3S10) and PI. The pH3S10 phosphorylation is a mark of chromosomal condensation and is broadly used to identify mitotic cells. In response to IR, the activation of the G\(_2\)/M checkpoint results in the arrest of cells in G\(_2\) and the consequent loss of the mitotic cell population (Figure 2.4 A, B; 2h time point). Only after several hours (4h for cTEC 1-2 and 8h for mTEC 3-10) do cells begin
Figure 2.3: TEC cell cycle regulation and checkpoint analysis. Cytograms of (A) mTEC 3-10 and (B) cTEC 1-2 cells stained for BrdU incorporation and DNA content (propidium iodide) in 21% or 5% O₂ at different time points following BrdU pulse, with or without treatment with 3Gy of IR. Representative gating strategy for the identification of BrdU⁺G₁ cells is shown in black, while gates for the identification of cells in different phases of the cell cycle are shown in green (G₁ cells), blue (S-phase cells) and red (G₂/M cells). Quantification of average percentage of BrdU-labeled G₁ phase cells in (C) mTEC 3-10 and (D) cTEC 1-2 cells cultured in either 21% or 5% O₂, 0-24h post BrdU pulse, with or without treatment with 3Gy of IR. n=4.
to resume the cell cycle and mitotic cells begin to be detectable again. This difference between the timing with which mTEC 3-10 and cTEC 1-2 cells resume mitosis indicates a distinct cell cycle regulation between the two cell types, with cTEC 1-2 cells releasing from the G₂/M arrest faster than mTEC 3-10 cells. However, the quantification of the mitotic index did not show any significant difference between normoxia and hypoxia (neither for cTEC 1-2 nor for mTEC 3-10 cells) (Figure 2.4C, D), indicating that oxygen levels do not affect cell cycle regulation in these cells.

2.3.4 Hypoxia does not influence the double strand break (DSB) repair capacity of TEC lines.

In light of the decreased radio-resistance of mTEC 3-10 cells in hypoxia we wondered whether the DNA repair capacity might be altered in this condition. The phosphorylation of Ser139 of the histone variant H2AX (γH2AX) was used as a marker of unrepaired DSBs by both western blotting and immunofluorescence analysis (Figure 2.5) in order to determine the kinetics of DSB repair. In both mTEC 3-10 and cTEC 1-2, the highest levels of γH2AX phosphorylation were observed 30 min after IR, with a progressive decrease consistent with DSB repair (Figure 2.5A). The quantification of the number of IR-induced γH2AX Foci (IRIF) showed no significant difference between normoxic and hypoxic mTEC 3-10 or cTEC 1-2 cells (Figure 2.5C, D), indicating that hypoxia does not have significant effects in the DSB repair capacity of the cells. Consistent with this observation, quantification of Rad51 IRIF, a direct mark of DNA DSB repair by homologous recombination, also did not show any significant difference between normoxic and hypoxia (Figure 2.6). In line with this observation, western blot analysis of the levels of expression of different DNA damage response factors (DNA-PKcs, DNA Ligase IV, Rad51, Chk1 and Chk2) showed only cell type-related differences (higher expression of the NHEJ factors DNA-PKcs and DNA Ligase IV and the effector kinase Chk1 by mTEC 3-10 than cTEC 1-2) but no effect caused by the hypoxic treatment of the cells (Figure 2.7D).
Figure 2.4: G2/M checkpoint analysis. Cytograms of (A) mTEC 3-10 and (B) cTEC 1-2 cells stained for histone H3 Ser10 phosphorylation (pH3S10) and DNA content (propidium iodide) in 21% or 5% O2 at different time points following treatment with 3Gy of IR. Representative gating strategy for the identification of pH3S10+ cells is shown in black. Quantification of average mitotic index (% of pH3S10 positive cells) in (C) mTEC 3-10 and (D) cTEC 1-2 cells, 0-24h post irradiation. n=4.
Figure 2.5: Double strand break repair kinetics in TECs. (A) Representative western blots showing γH2AX and β-Actin levels in mTEC 3-10 and cTEC 1-2 cells cultured in 21% or 5% O₂, 0-24h after irradiation with 3Gy. (B) Representative images of mTEC 3-10 and cTEC 1-2 nuclei stained for γH2AX IRIF, in 21% or 5% O₂, 0-24h post 3Gy irradiation. Scale bars correspond to approximately 20 μm. Average number of γH2AX IRIF per nucleus in (C) mTEC 3-10 cells and (D) cTEC 1-2 cells, 0-24h post IR, n=5.
Figure 2.6: Double strand break repair kinetics in TECs (II). (A) Representative images of mTEC 3-10 and cTEC 1-2 nuclei stained for Rad51 IRIF, in 21% or 5% O₂, 0-24h post 3Gy irradiation. Scale bars correspond to approximately 20 µm. (B) Average number of Rad51 IRIF per nucleus 0-24h post-IR.
Figure 2.7: TEC DNA Damage Response gene expression analysis. Volcano plots of qPCR array data comparing (A) normoxic mTEC 3-10 vs. cTEC 1-2; (B) normoxic vs. hypoxic mTEC 3-10; and (C) normoxic vs. hypoxic cTEC 1-2 gene expression. Green and black vertical lines represent 0 and 2-fold expression changes, respectively. Blue horizontal lines represent a p value of 0.05, with significantly regulated genes being shown above them. All genes up-regulated more than 2-fold are shown in red, while all genes down-regulated more than 2-fold are shown in green (independently of their statistical significance). (D) Representative western blots showing mTEC 3-10 and cTEC 1-2 expression levels of DNA-PKcs, DNA Ligase IV, Rad51, Chk1, Chk2 and β-Actin in normoxia (21% O_2) and hypoxia (5% O_2). n=3.
2.3.5 mTECs express higher levels of DDR factors and exposure to hypoxia results in their down-regulation.

Our results so far indicate some differences in the DNA damage response of medullary and cortical thymic epithelial cell lines, as well as normoxic and hypoxic mTEC 3-10 cells. To further characterize the DDR components of each cell type, a comprehensive analysis of the gene expression of an array of 87 genes belonging to the DDR signalling network was performed using commercial qPCR arrays. Comparison of the gene expression of mTEC 3-10 and cTEC 1-2 cells showed a marked trend towards higher levels of expression of DDR genes in mTEC 3-10 cells compared to cTEC 1-2. Although ~60% of the genes showed greater than 2-fold increase in mRNA expression in mTEC 3-10 cells (Figure 2.7A, shown in red and Supplementary Table S.5), only ~35% of all the genes analysed were significantly more expressed (p value > 0.05) in mTEC 3-10 cells (Figure 2.7A and Supplementary Table S.5). This finding may indicate the presence of a more robust DNA damage response in mTEC 3-10 cells than in their cortical counterparts.

Amongst the significantly differentially regulated genes, mTEC 3-10 cells showed enrichment in DNA DSB repair factors involved in HR (Rad51b, Rad51c, Rad52, Fancd2, Blm, Brca1 and Brca2) and NHEJ, such as Prkdc (DNA-PKcs) and Lig4, (confirming the western blot results), as well as key players involved in excision repair pathways such as Parp2, Ddb2, Xpa, Xpc, Ercc1 and Gadd45a). mTEC 3-10 cells also showed higher levels of genes involved in sensing and coordinating the DDR such as Nbs1, Rad50, Chk1 and Atr, and also cell cycle regulation such as Cdkn1a (p21) and Cdc25c, which may explain the differential checkpoint regulation observed between the two cell lines (Figures 2.3 and 2.4). Western blot analyses of DDR factors showed that this regulation is also maintained at the level of protein for at least some of the transcripts analysed (Figure 2.7D). Consistent with the previous qPCR data, mTEC 3-10 cells express higher protein levels of DNA-PKcs, DNA Ligase IV and Chk1 than cTEC 1-2 cells, but no difference was observed for the HR factor Rad51 or the other main effector kinase Chk2.

When comparing the effects of hypoxia on each cell line, mTEC 3-10 cells seem to be more responsive to the hypoxia treatment, showing a marked trend towards a down-regulation of most of the genes when exposed to low oxygen levels (Figure 2.7B). However, only six genes show a greater than two-fold up-regulation in normoxia compared to hypoxia (Figure 2.7B, shown in red, and Supplementary Table S.5) and only two genes
(Lig1 and Rad18) showed a modest but significant up-regulation (Figure 2.7 B and Supplementary Table S.5). In contrast, culturing cTEC 1-2 cells in hypoxia did not induce many changes of expression of genes involved in the DDR pathway, with only one gene up-regulated (Ogg1) over 2-fold but showing no statistical significance (Figure 2.7 C, shown in green, and Supplementary Table S.5).

2.3.6 Hypoxia promotes mTEC apoptosis upon irradiation through the up-regulation of Bim.

Since the decreased radio-resistance of mTEC 3-10 cells in hypoxia does not seem to be related to differences in repair capacity of DNA lesions or differential regulation of cell cycle checkpoints, we wondered whether this could be due to an enhanced susceptibility to undergo apoptosis in response to IR. Apoptosis was measured by cleaved Caspase-3 staining and flow cytometric analysis at different times up to 96h following irradiation with 10Gy and using staurosporine treatment as positive control. In contrast with previous experiments, a higher IR dose of 10Gy was chosen in this case in order to efficiently study cell death rather than repair of the DNA lesions. cTEC 1-2 cells showed higher sensitivity to IR as evidenced by the faster increase in Caspase-3 positive cells, reaching 30% after 72h, when only 10% of mTEC 3-10 cells had activated the apoptotic pathway (Figure 2.8 A-D). Hypoxic mTEC 3-10 cells showed a faster accumulation of Caspase-3 positive apoptotic cells over time, with significant differences being observed at 72h and 96h after IR (Figure 2.8 A, B). Consistent with this, significantly higher apoptotic rates were also observed in hypoxic mTEC 3-10 cells upon treatment with staurosporine. In contrast, cTEC 1-2 cells only showed higher apoptosis in hypoxia when treated with staurosporine, but not following IR at any of the time points analysed (Figure 2.8 C, D). This result correlates with those from the clonogenic survival assays previously described.

In order to investigate the mechanism underlying the increased propensity of hypoxic mTEC 3-10 cells to undergo apoptosis, the level of different pro- and anti-apoptotic proteins was analysed by western blotting. mTEC 3-10 and cTEC 1-2 cells showed differential responses to IR in terms of their regulation of apoptotic factors. Whereas in mTECs, there is a higher induction in expression of the pro-apoptotic proteins Bim, Bax, Bak, Noxa and Puma upon irradiation, this is also accompanied by an increase in the levels of
Figure 2.8: IR-induced apoptosis analysis in TECs. (A) Representative cytograms of mTEC 3-10 cells stained for cleaved Caspase-3, and (B) quantification of average percentage of Caspase-3 positive mTEC 3-10 cells, 0-96h post 10Gy of IR. Staurosporin treatment was used as a positive control for the activation of the apoptosis pathway. Representative gating strategy for the identification of Caspase3+ cells is shown in black. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 compared to normoxic samples, two way ANOVA, n=4. (C) Representative cytograms of cTEC 1-2 cells stained for cleaved Caspase-3, and (D) quantification of average percentage of Caspase-3 positive cTEC 1-2 cells 0-96h following treatment with 10Gy of IR. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 compared to normoxic samples, two way ANOVA, n=4. (E) Representative western blots and quantification of (F) mTEC 3-10 and (G) cTEC 1-2 protein expression level of pro- and anti-apoptotic proteins. β-Actin was used as reference gene for the quantification and all values were normalized against the untreated normoxic samples. *p<0.05, multiple t tests with Holm-Sidak post-test correction, n=4.
anti-apoptotic proteins such as Bcl-XL or Bcl-2 (Figure 2.8 E, F). This induction of anti-apoptotic proteins may counteract the effect of the increase of pro-apoptotic factors. In contrast, cTEC 1-2 cells show a less pronounced IR-induced increase in the levels of pro-apoptotic factors, and a very mild induction of anti-apoptotic proteins (Figure 2.8 E, G). This differential response may explain the previously observed higher sensitivity of cTEC 1-2 cells to IR-induced apoptosis.

Interestingly, while most of the apoptotic factors studied followed the same pattern of expression in normoxia and hypoxia for both TEC cell lines, hypoxic mTEC 3-10 cells showed a significantly higher induction of the expression of Bim after IR (both Bim-EL and Bim-L isoforms) (Figures 2.8 E, F). This specific increase in hypoxia is not accompanied by an increase in the levels of any anti-apoptotic protein that could counteract the effect of Bim, and this may be the key to the greater propensity of mTEC 3-10 cells to undergo apoptosis under hypoxic conditions.

### 2.3.7 Ionizing radiation profoundly affects expression of functional factors in primary mouse thymic epithelial cells

Finally, we investigated the effects of IR treatment on the functional properties of primary mouse thymic epithelial cells. To do so, mRNA expression of a number of genes known to have an important role in TEC function in vivo was analysed with or without IR treatment. Initial experiments were carried out with mRNA isolated from lymphocyte-depleted total thymic stroma. These preparations showed a marked and consistent decrease in expression of most of the genes analysed, including KitL, Dll4, Il-7, Flt3L, Ccl17, Ccl21, Ccl22 and Ccl25 (Figure 2.9 A), suggesting that the function of the thymic stroma may be compromised following exposure to IR.

In light of these preliminary results, we investigated the expression of these genes in sorted subpopulations of thymic stromal cells from control and irradiated mice (Figure 2.9 B-C, Supplementary Figures S.1 and S.2). In addition, given the important role of Bim up-regulation in response to IR for survival of mTEC 3-10 cells previously described, mRNA expression of Bim was also included in the analysis. Gene expression in sorted cTEC, mTEC MHCIILow mTEC MHCIIfishCD86 and mTEC MHCIIfishCD86+ sorted cells (untreated or irradiated) was analysed by real-time PCR. Due to the low
Figure 2.9: Effects of ionizing radiation on the functional properties of primary mouse TEC subpopulations. (A) mRNA expression levels of TEC functional factors in total primary mouse thymic stroma. All values were normalized against Gapdh and expressed relative to the untreated sample. Graphs show the average of four biological replicates. * p<0.05, multiple t-tests with Holm-Sidak post-test correction. (B) Gating strategy for the sorting of mouse primary cTEC, mTEC MHCII low, mTEC MHCII high CD86− and mTEC MHCII high CD86+ subpopulations. (C) mRNA expression levels of TEC functional factors in mouse primary sorted TEC subpopulations. Data was normalized against Gapdh and expressed as fold change relative to the untreated sample. All values correspond to the average of three technical replicates and one biological sample corresponding to twenty thymi per group pooled together prior to the analysis. * p<0.05, multiple t-tests with Holm-Sidak post-test correction.
number of mTEC MHCII\textsuperscript{high}CD86\textsuperscript{+} obtained from irradiated mice, a cDNA amplification step was necessary in order to obtain enough material for complete analysis. First of all, gene expression of the different TEC functional factors was compared amongst the different cell types in order to confirm cell identity and establish the relative contribution of each of the cell types to the overall gene expression in the thymus. The different genes analysed were classified according to the information available from the Immunological Genome Project (Immgen) database into genes that are highly expressed in cTECs and progressively lower in the different mTEC subtypes (such as β5t, Il-7, Dll4, KitL, Cxcl12, Ccl21 or Ccl25); and genes that are expressed at low levels in cTECs and increase progressively in mTECs (such as Aire, Ctss, Xcl1, Ccl17, Ccl19 or Ccl22). We detected expression of genes traditionally described as mTEC-specific (such as Aire) in cTECs and vice versa. However, comparison amongst cell types confirmed that our gene expression data nicely correlated with the information found in the Immgen database and that the expression of mTEC-specific genes in cTECs and cTEC-specific genes in mTECs was extremely low in comparison (Figure 2.10).

Next, IR-induced variation in the expression of TEC functional factors was analysed. cTECs seemed to be the stromal cell subpopulation most affected by irradiation, showing the most pronounced decrease in all of the studied genes (Figure 2.9 C). mTEC MHCII\textsuperscript{low} cells also showed a significant decrease in all genes, although to a lesser extent than cTECs. In contrast, both mTEC MHCII\textsuperscript{high} (CD86 positive and negative) subpopulations had a quite similar response to IR treatment, showing down-regulation of some genes but also up-regulation of others. Amongst the genes down-regulated in mTEC MHCII\textsuperscript{high}CD86\textsuperscript{+} cells were Aire, Xcl-1, Ccl-17, Ccl-19, and Ccl-22, whereas they showed up-regulation of Il-7, KitL, Ccl21 and Ccl25. In contrast, mTEC MHCII\textsuperscript{high}CD86\textsuperscript{−} cells showed up-regulation in Il-7, Ccl17, Ccl21 and Ccl25 with no change in Aire, Flt3L or Xcl-1 expression (Figure 2.9 C). Interestingly, all genes up-regulated in mTEC MHCII\textsuperscript{high} cells were very weakly expressed in these cells compared to mTEC MHCII\textsuperscript{low} cells and most especially cTECs. This response pattern probably explains why expression of these genes was down-regulated in the total thymic stromal extract analysed previously (Figure 2.9 A, Figure 2.10 A). In line with this, no significant changes were detected in total thymus expression of Aire and Xcl-1, corresponding with the results found for mTEC MHCII\textsuperscript{high}CD86\textsuperscript{−} cells, which are the primary contributors to the expression of these genes (Figure 2.9 A, Figure 2.10). Interestingly, in the case of Bim,
MHCII$^{\text{high}}$CD86$^{-}$ cells showed a significant induction in Bim mRNA expression in response to IR, while mTEC MHCII$^{\text{low}}$ cells showed a mild but significant downregulation, and cTECs showed no changes. Overall, our data suggest that ionizing radiation causes profound changes in expression of many genes encoding factors critical for thymic epithelial function and thymocyte differentiation.

2.4 Discussion

Thymic epithelial cells are one of the main components of the thymic stroma, and they control the homing, proliferation, differentiation and selection of thymocyte progenitors throughout the process of becoming a mature, functional and self-tolerant T cell [7][11]. Following TBI and BMT, reconstitution of the T cell compartment takes several weeks and requires a fully functional thymus [443]. During this period when de novo T cell production is impaired and the T cell compartment is incapable of mounting specific immune responses, patients are highly susceptible to infectious diseases, disease relapse, and graft-versus-host disease [481]. For this reason, investigating the main causes of poor thymic functionality following BMT is critical to improve the outcomes of this therapy. Surprisingly, there is very little published information available on the functional outcomes of irradiation or other modalities of cyto-reductive regimens on thymic stromal cell function. Historically, demonstration that host thymic stroma retained functionality following irradiation came from the seminal papers of Bevan demonstrating the phenomenon of positive selection. Thus, in MHC incompatible radiation bone marrow chimeras, the functional MHC-restricted T cell repertoire of peripheral T cells derived from donor HSC became that of the MHC of the irradiated host and not that of the original bone marrow donor [38][137]. It is now known that cTECs mediate positive selection. Far less attention has been paid to the ability of the post-irradiated thymic stroma, in chimeras, in this case of mTECs, to orchestrate negative selection of the T cell repertoire. This involves the re-expression, including the appropriate mRNA splicing of tissue-specific genes in thymic epithelial cells [224].

Clinical studies have shown that reduced-intensity cyto-reductive regimens result in enhanced T lymphopoiesis (including higher numbers of CD4$^{+}$ T cells, greater T-cell receptor diversity and higher peripheral T-cell receptor excision circle frequency) [80].
Figure 2.10: Mouse primary sorted TEC subpopulation gene expression comparison. mRNA expression of TEC functional factors in untreated TEC subpopulations, expressed as $2^{-\Delta Ct}$ normalized to Gapdh expression. Genes are classified in two groups according to their expression pattern across TEC subpopulations: (A) genes expressed highly in cTECs and with decreasing expression across the mTEC subpopulations and (B) genes expressed at low levels in cTECs and with increasing expression across the mTEC subpopulations. All values correspond to the average of three technical replicates and one biological sample corresponding to twenty thymi pooled together prior to the analysis. Error bars represent the standard error of the mean. * $p<0.05$, multiple t-tests with Holm-Sidak post-test correction.
[84][215], suggesting that deleterious effects on the thymic stroma are directly linked with the efficiency of the recovery of the T cell compartment. Therefore, development of new strategies to improve T cell production in the thymus requires finding ways to protect the thymic stroma from the insults derived from the BMT process, including DNA damage caused by TBI and chemotherapeutic drugs. To try and understand in some detail the DDR of TEC, we have begun by using continuously cultured cell lines representative of cTEC and mTEC respectively. Some of the results obtained with these cell lines have then been applied to semi-purified preparations of fresh thymic stromal cells. Finally, preliminary experiments are reported on FACS-purified subpopulations of TEC.

We therefore began by studying in detail the DDR of two different thymic epithelial cell lines (one cortical and one medullary): cTEC 1-2 and mTEC 3-10. The DNA damage response is the signalling network that allows cells to detect and respond to lesions in their DNA [91] that in physiological conditions follow endogenous damage mediated by free radicals and replicative stress. However, development of this DDR allows cells to respond to damage mediated by external sources such as that caused by ionizing radiation. Although this signalling pathway is present in every cell and is conserved throughout evolution, there is a high variability in the way different cell types respond to insults in their DNA, with different cell types showing distinct DNA repair efficiency and kinetics, repair pathway choice (non-homologous end joining vs. homologous recombination), checkpoint activation or sensitivity to apoptosis or senescence [492]. Comparison between the radio-sensitivity of thymic epithelial cell lines (mTEC 3-10 and cTEC 1-2) and the ST4.5 CD4/CD8 double positive (DP) T cell line by clonogenic survival assays demonstrated a much higher radio-resistance of the TEC lines than the DP T cells used as a radio-sensitive control (Figure 2.2 C, D). When comparing the TEC lines to each other, cell type-specific differences were also observed. While the survival curves were similar for both cell lines at low IR doses (up to 4Gy), cTEC 1-2 cells showed higher radio-sensitivity at higher doses (Figure 2.2 C, D). In line with this, cleaved Caspase-3 analysis showed a higher propensity of cTEC 1-2 cells to undergo apoptosis in response to both IR and staurosporine treatment (Figure 2.8) than mTEC 3-10 cells. Cell cycle checkpoint regulation in response to IR also showed a faster recovery of cTEC 1-2 cells from the IR-induced cell cycle arrest, which may be partially explained by their lower expression of checkpoint regulators such as Cdkn1a (p21) and Cdc25c. Commercial DDR
qPCR array analysis also revealed significantly higher expression of approximately 30% of all genes analysed in mTEC 3-10 cells. These genes mainly encoded DNA repair factors such as \textit{Rad51b}, \textit{Rad51c}, \textit{Rad52}, \textit{Fancd2}, \textit{Brca1}, \textit{Brca2}, \textit{Lig4} or \textit{Prkdc} (DNA-PKcs), probably indicating a more robust DNA damage response in these cells (Figure 2.7). The higher level of DNA-PKcs in mTECs is of special importance since it plays a very important role in their function in T-cell negative selection, acting as a co-factor for Aire-mediated de-repression of tissue-restricted antigen expression [297,511].

Our group has previously identified hypoxia as an enhancer of the DNA damage response of mesenchymal stromal cells [424]. For this reason, we also studied the effects of hypoxia on the radio-resistance of our TEC cell lines. Interestingly, only mTECs showed a cell-type specific responsiveness to hypoxia, which increased their sensitivity to IR. Although growth curves and colony formation assays demonstrated a faster growth rate of mTEC 3-10 cells in hypoxia, clonogenic survival was significantly lower in this condition (Figure 2.2). No difference was observed in checkpoint regulation or DNA repair capacity of mTEC 3-10 cells cultured at different oxygen tensions (Figures 2.3-2.6). However, cleaved Caspase-3 analysis showed higher apoptosis rates in hypoxic mTEC 3-10 cells in response to treatment with both IR and staurosporin (Figure 2.8 A,B). In order to study the mechanism behind this phenotype, a detailed analysis of pro- and anti-apoptotic protein levels was performed, providing evidence for a stronger induction of Bim expression in hypoxia compared to normoxia. BIM is a very strong apoptosis inducer thanks to its ability to bind to many anti-apoptotic proteins (MCL-1, BCL-2, BCL-XL, BCL-W, BFL-1 and Epstein-Barr virus BHRF-1) as well as directly binding to the pro-apoptotic proteins BAX and BAK and directing them to the mitochondrial membrane and inducing its permeabilization [413]. Since this pro-apoptotic protein increase was not accompanied by any specific anti-apoptotic protein induction that could counteract the effects of Bim, it is likely that this is one of the main drivers increasing the susceptibility of hypoxic mTEC 3-10 cells to undergo apoptosis. In light of these results, \textit{Bim} mRNA expression changes in response to IR were subsequently studied in primary sorted TEC subpopulations (Figure 2.9 C), demonstrating that mTEC 3-10 cells behave similarly to mTEC MHCII\textsuperscript{high}CD86\textsuperscript{−} cells, which show an induction in \textit{Bim} expression in response to IR. In contrast, primary cTECs do not show any significant induction of \textit{Bim} mRNA expression, in line with the very modest Bim protein upregulation observed in cTEC 1-2 cells.
Our preliminary data with whole thymic stromal preparations showed a marked decrease in the mRNA levels of most of the transcripts analysed (Figure 2.9 A). Previous studies have shown depletion of mTEC and cTEC populations and enrichment of fibroblastic components in the thymi of irradiated mice [482]. Other authors have described similar decreases of specific transcripts such as II-7 or Ccl25, although these changes have been mainly attributed to changes in thymic cellularity [90, 501]. For this reason, we performed a more detailed analysis of the gene expression of purified sorted thymic epithelial cell types, in order to exclude the possibility that the decrease in mRNA levels was due to a decrease in total TEC numbers and not to a specific down-regulation of gene expression. In contrast to the reports mentioned above [90, 501], our experiments did not show differences in the number of sorted cells between irradiated and un-irradiated groups (data not shown), although this is probably due to the fact that our sorts were performed 24h after irradiation whereas other groups have studied changes in TEC numbers at later time points after IR [90, 482]. Our analysis of different purified TEC subpopulations individually confirmed the overall functional factor down-regulation and revealed cTECs as the subpopulation most affected by ionizing radiation (Figure 2.9 D). These molecules have important roles in attraction, commitment, survival, proliferation, migration and selection of thymocytes throughout their development [7, 11]. Previous work by different groups has shown the important implications of this decrease of TEC functional factors in T-cell reconstitution following BMT. Observations by Zlotoff et al. and Zhang et al. revealed a marked decrease in thymic seeding by progenitors in irradiated thymi compared with un-irradiated controls [501, 507], which could be rescued by supplementation with Ccl21 and Ccl25 [501]. Other studies have also shown enhanced post-transplantation thymic recovery by exogenous administration of II-7 [6, 280] or Flt3l [146, 225]. Thus, elucidation of the mechanisms behind damage-induced loss of thymic function may be useful for the design of promising strategies to improve T-lineage recovery following BMT.

In conclusion, to the best of our knowledge, we have for the first time studied in detail the DNA damage response of thymic epithelial cells and the short-term effects of ionizing radiation on their expression of many genes that are essential for T cell development. We have shown that TECs exhibit a relatively high radio-resistance, although IR has detrimental effects in their survival and functionality, inducing a profound down-regulation of functional factors in primary murine thymic epithelial cells. We have also
shown how cTECs and mTECs respond differently to DNA damage, by displaying differential checkpoint recovery and sensitivity to undergo apoptosis in response to IR, as well as differential expression of DDR genes such as DNA repair factors or proteins involved in cell cycle regulation. Finally, we have demonstrated that hypoxia reduces the radio resistance of our mTEC 3-10 cell line through the up-regulation of the pro-apoptotic protein Bim. These findings constitute a first step towards understanding TEC response to IR and the mechanisms behind their radio-resistance, which is crucial for improving the outcomes of BMT and promoting successful T cell reconstitution.
Chapter 3

The role of Hif-1 in enhancing the radio-resistance of mouse MSCs
3.1 Introduction

Haematopoiesis is the process leading to the formation of all types of blood cells, originating from haematopoietic stem cells, through a complex series of commitment and differentiation events [58, 247]. In adults, the bone marrow is the primary organ where haematopoiesis takes place, and constitutes a highly specialized environment in which both non-hematopoietic stromal cells and haematopoietic cells in various developmental stages form the bone marrow niche [262, 268]. Mesenchymal stromal cells (MSCs) are key components of the bone marrow microenvironment, providing support for hematopoietic stem cells (HSCs) and regulating their maintenance and the production and maturation of haematopoietic progenitors (Figure 1.1) [319]. Apart from providing microenvironmental support for HSCs, MSCs are themselves also multi-potent progenitors capable of differentiating along various mesenchymal lineages to become adipocytes (fat cells), osteocytes (bone cells) and chondrocytes (cartilage cells) [350]. Apart from the bone marrow, MSCs are also known to localize to solid tumors to which they are attracted following signals from the tumor mass and therein develop into cancer-associated fibroblasts (CAFs), becoming an integral part of the tumor microenvironment [308]. Once in the tumors, CAFs promote tumorigenesis in multiple ways, such as i) cancer cell growth stimulation and inhibition of tumor cell apoptosis, ii) angiogenesis promotion and contribution to the tumor vasculature, iii) suppression of the activity of anti-tumor immune cells and iv) promotion of tissue invasion and metastasis [20, 100, 180, 181, 228, 244, 351]. A feature shared by the bone marrow niche and tumors is that they are relatively hypoxic environments [124, 313, 377, 416, 457]. Hypoxia has been shown to play a key role in both HSC and MSC biology, modulating their proliferation rates, quiescence, differentiation potential, and re-constitutive capacity [124, 173, 216, 266, 313, 353, 447, 448, 487]. In the tumor microenvironment, hypoxia is involved in angiogenesis, proliferation, metabolism, metastasis, differentiation, and response to radiation therapy, therefore being an important adverse prognostic factor in cancer [57, 176, 348, 362].

Cellular adaptation to hypoxia is mediated by a family of transcription factors known as the hypoxia inducible factors, or HIFs. These consist of a constitutively expressed HIF-1β-subunit (also known as Arnt) and an oxygen-responsive HIF-α subunit (HIF-1α, HIF-2α or HIF-3α) [299]. Amongst these, HIF-1α is considered as the main contributor
to the general acute cellular response to hypoxia, mainly due to the fact that it is ubiquitously expressed in all tissues, whereas its HIF-2α and HIF-3α counterparts are expressed in a tissue-specific fashion and are thought to contribute to more specific processes \[125,127,223,284,398,438,475,495\]. All members of the HIF family share their protein domain structure, which is composed mainly of a DNA-binding bHLH domain and a tandem of two PAS domains (PAS-A and PAS-B) for heterodimerization \[114\]. Apart from these, the alpha subunits also contain an oxygen-dependent degradation domain (ODDD) involved in the regulation of protein stability, and transactivation domains (TAD) for the regulation of protein function (Figure 1.5) \[114\].

In normoxic conditions, proline residues in the HIF-α degradation domain (ODDD) are hydroxylated by prolyl hydroxylase domain-containing proteins (PHDs), enabling the interaction with pVHL (product of the von Hippel-Lindau tumor suppressor). pVHL is a subunit of the VCB-CUL2 E3 ligase complex that polyubiquitinates HIF-1α, which ultimately results in its proteasomal degradation \[31,59,128,207,220,223,293,294\]. Additionally, factor inhibiting HIF (FIH) hydroxylates an asparagine residue in the C-terminal transactivation domain, impeding its interaction with important co-factors such as p300/CBP and therefore rendering HIF-1 transcriptionally inactive \[254,255,282,348\]. In hypoxic conditions, however, these enzymes no longer post-translationally modify HIF-1α, allowing HIF-1α stabilization and accumulation within the cells. Stabilized HIF-1α is subsequently activated and translocated to the nucleus where it dimerizes with ARNT, composing the fully-active HIF-1 transcription factor that goes on to regulate the expression of hypoxia-responsive genes (Figure 1.6) \[114,223,260,398\].

HIF-1-dependent regulation has been attributed to a myriad of genes involving many important cellular processes such as the metabolic switch to glycolysis, angiogenesis, growth factor and cytokine production, cell growth and migration, extracellular matrix production, autophagy, apoptosis, redox homeostasis, inflammation and immunity, and the DNA damage response, all of them crucial for cells to functionally adapt to low oxygen environments \[114,223,374,400,491,494\]. However, HIF-1 activity in these biological processes is often tissue- or cell-type specific due to the impact of the particular chromatin status, RNA POL II activity, and the availability of partner transcription factors or co-activators found in each cell type \[149,342,344,460,494\].

Previous studies in our group directly comparing MSC response to ionizing radiation in normoxia (21% O₂) and hypoxia (5-2% O₂) have shown that both primary mouse MSCs
and MSC lines display a hypoxia-dependent increase in resistance to γ-irradiation in vitro [424]. Although DDR activation (indicated by γ-H2AX phosphorylation and IRIF formation) was comparable between normoxic and hypoxic MSCs, these γ-H2AX IRIF were resolved at a faster rate in MSC nuclei in hypoxia, and Rad51 IRIF formation, a key step in DNA repair by HR, was also accelerated in hypoxic MSCs. In addition, monitoring of the cell cycle progression of 5′-bromodeoxyuridine (BrdU) labeled cells indicated that irradiated MSCs recovered from cell cycle arrest at a faster rate in hypoxia than in normoxia, while in vitro and in vivo measurement of the DNA repair capacity of the cells by both NHEJ and HR showed increased repair efficiency by both mechanisms in hypoxia. Interestingly, upon exposure to hypoxia, MSCs up-regulated the protein levels of the key NHEJ repair proteins DNA-PKcs and DNA ligase IV (but not the HR factor Rad51), which coincided with Hif-1α stabilization and accumulation in the cells. siRNA-mediated depletion of Hif-1α in MSCs prevented the increase in the protein levels of these DNA repair factors in hypoxic MSCs and significantly reduced the radio-resistance of hypoxic MSCs, indicating that Hif-1α is likely to be involved in enhancing the DDR of MSCs in hypoxia (Figure 1.10) [424].

In this chapter I describe the progress made in deciphering the exact molecular mechanism underlying the role of Hif-1α in the DDR of hypoxic MSCs. This was achieved by generating and characterizing Hif-1α knockout clones of the mouse MSC line MS5. The Hif-1α knockout MS5 cells were also reconstituted to express mutant Hif-1α proteins. My results indicate that both subunits of the Hif-1 transcription factor, and their interaction, are essential for mediating the hypoxia-dependent increase in MSC radio-resistance. In addition, the ability of Hif-1α to directly interact with DNA is also crucial for this effect, indicating that it might be doing so thanks to its transcription factor function. However, the fact that hypoxia did not cause changes in the mRNA expression of almost any of the many DDR genes analyzed (especially Prkdc and Lig4, whose protein levels are increased by exposure to hypoxia in a Hif-1α-dependent manner) is an important indicator that Hif-1 may be indirectly affecting DDR protein stability through the transcriptional regulation of an (or several) intermediary factor(s), whose identity remains unknown.
3.2 Materials and Methods

3.2.1 Cell culture and treatments

The MS5 mouse MSC cell line was provided by Prof. Antonius Rolink (Department of Biomedicine, University of Basel) and was cultured in Dulbecco's modified Eagle's medium high glucose (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich) and 1% penicillin/streptomycin sulfate solution (Gibco). MS5 cells were continuously cultured in humidified incubators at 37ºC containing 21% O\(_2\) (normoxia) or 2% O\(_2\) (hypoxia) for at least one week prior to experimentation. Experiments that required hypoxia treatment were performed into a hypoxic chamber (Coy Lab Products) in order to maintain O\(_2\) levels stable.

HEK293T cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium high glucose (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma Aldrich) and 1% penicillin/streptomycin sulfate solution (Gibco) using humidified incubators at 37ºC and 5% CO\(_2\).

MS5 Cells were γ-irradiated at the indicated doses using a Mainance Millennium Sample Irradiator containing a \(^{137}\)Cs source at a dose rate of approximately 102 eGy/min. Cells were treated with 500\(\mu\)M DMOG (Dimethyloxalylglycine) (Sigma Aldrich) and harvested at the indicated time points post-treatment.

3.2.1.1 siRNA Transfection

Hif-1\(\alpha\) (s67532 Mouse Hif1\(\alpha\) and n414564 Mouse Hif1\(\alpha\) Silencer Select Predesigned siRNA) and Arnt (s62616 Mouse Arnt Silencer Select Predesigned siRNA) siRNAs, as well as the Control siRNA (Silencer® Select Negative Control No. 1 siRNA - 4390843) were obtained from Ambion. 1.5x10\(^5\) MS5 cells were seeded per 60mm tissue culture plate. 24h later, cells were transfected with 50nM of siRNA using 6\(\mu\)l of Oligofectamine (Invitrogen) in 1ml of Opti- MEM I (Invitrogen) per 60mm dish. 3h post transfection, 0.5ml of DMEM (without penicillin and streptomycin) supplemented with 30% FBS and 4mM L-glutamine was added. After 24h, medium was substituted with the usual DMEM with 10% FBS and 1% penicillin-streptomycin. 48h after transfection,
knockdown efficiency was assessed by western blotting and cells were used for the required experiments.

### 3.2.2 Clonogenic survival assay

Adherent MS5 cells were irradiated at 2-10 Gy and seeded into six-well plates (Nunc) at a concentration of 300 cells per well. Cells were incubated for 8 days until colonies were clearly visible. Colonies were stained with Coomassie Blue (Supplementary Table S.1) and counted. The percentage survival of each cell type was determined by normalizing the number of colonies / cells generated by irradiated cultures to the number of colonies / cells generated by control un-irradiated cultures.

### 3.2.3 DNA Methods

#### 3.2.3.1 Genomic DNA extraction

5x10^5 cells were collected and lysed in 500μl TAIL buffer. After an overnight incubation at 37°C, the genomic DNA was precipitated with saturated NaCl (6M) and isopropanol. The DNA pellet was washed twice in 70% ethanol and allowed to air-dry before resuspension in 50μl of TE buffer. Samples were left overnight at 4°C to allow complete resuspension of the DNA pellet and were stored at -20°C until used for downstream applications. Information regarding all solutions used can be found in Supplementary Table S.1.

#### 3.2.3.2 Polymerase Chain Reaction (PCR)

KOD polymerase (Novagen) was used for isolation of Hif1a cDNA from total MS5 cDNA and for amplification of the genomic regions harboring CRISPR/Cas9-induced mutations. Reactions were set up following manufacturer’s instructions as shown in Table 3.1 and amplification was carried out using an Eppendorf Mastercycler ep Gradient S thermocycler. Programs used are shown in Tables 3.2 and 3.3 while primer sequences are shown in Supplementary Table S.6. Annealing temperatures depended on melting temperatures of primers and were determined by previous optimization.
### Table 3.1: KOD standard reaction setup.

<table>
<thead>
<tr>
<th></th>
<th>Initial Concentration</th>
<th>Final Concentration</th>
<th>Volume (for 50 μl reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOD Buffer</td>
<td>10x</td>
<td>1x</td>
<td>5μl</td>
</tr>
<tr>
<td>MG₂SO₄</td>
<td>25mM</td>
<td>1.5mM</td>
<td>3μl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>2mM each</td>
<td>0.2mM each</td>
<td>5μl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>10μM</td>
<td>0.4μM</td>
<td>2μL</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>10μM</td>
<td>0.4μM</td>
<td>2μL</td>
</tr>
<tr>
<td>KOD Polymerase</td>
<td>1 U/μl</td>
<td>0.02 U/μl</td>
<td>1μL</td>
</tr>
<tr>
<td>Template</td>
<td>100-200 ng/μL</td>
<td>10-20 ng/μL</td>
<td>5μl</td>
</tr>
<tr>
<td>DMSO (optional)</td>
<td>100%</td>
<td>5%</td>
<td>2.5μl</td>
</tr>
<tr>
<td>milliQ H₂O</td>
<td>-</td>
<td>-</td>
<td>Up to 50μL</td>
</tr>
</tbody>
</table>

### Table 3.2: KOD PCR conditions used for amplification of specific genomic regions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>2min</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Denaturation</td>
<td>95°C</td>
<td>20s</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Annealing</td>
<td>55-60°C</td>
<td>10s</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>70°C</td>
<td>5s/kbp</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Final Extension</td>
<td>70°C</td>
<td>2min</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Final Hold</td>
<td>4°C</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>
### Table 3.3: Gradient PCR conditions for isolation of MS5 Hif-1α cDNA.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>2min</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Denaturation</td>
<td>95°C</td>
<td>20s</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Annealing</td>
<td>70 to 60°C (-1°C per cycle)</td>
<td>10s</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>70°C</td>
<td>5s/kbp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Denaturation</td>
<td>95°C</td>
<td>20s</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>60°C</td>
<td>10s</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>70°C</td>
<td>5s/kbp</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Final Extension</td>
<td>70°C</td>
<td>2min</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>Final Hold</td>
<td>4°C</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

#### 3.2.3.3 Site-directed mutagenesis

In order to introduce specific mutations in the Hif-1α cDNA, complementary pairs of primers containing the desired nucleotide changes were designed (Supplementary Table S.6). The mutagenesis reaction was carried out using KOD polymerase as previously described (Table 3.1), using an Eppendorf Mastercycler ep Gradient S thermocycler. Cycling conditions are shown in Table 3.4. The products obtained were checked on a 1% Agarose gel before addition of 1.5μl of DpnI restriction enzyme directly to the mutagenesis reactions in order to eliminate the original plasmid template molecules that (unlike the newly generated mutant plasmids) are methylated and thus prone to digestion with the enzyme. Digestion reactions were incubated for 12-16h at 37°C and then transformed into competent Top10 *E. coli*. Single colonies were picked and plasmids were checked for the presence of the desired mutations by sequencing.

#### 3.2.3.4 DNA agarose gel electrophoresis and gel extraction

1% agarose (Sigma) gels were prepared in 1xTAE buffer. Ethidium bromide was added at a final concentration of 0.5μg/ml before gel setting. 6x Orange G Loading Buffer was used to load the samples at a final 1x dilution. 1kb Plus DNA Ladder from Fermentas
Table 3.4: Site-directed mutagenesis cycling conditions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>2min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>95°C</td>
<td>30s</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Annealing</td>
<td>56°C</td>
<td>20s</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>68°C</td>
<td>30s/kbp</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Final Extension</td>
<td>72°C</td>
<td>5min</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Final Hold</td>
<td>4°C</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

was used to determine the sizes of the DNA molecules. Gels were run in 1x TAE in Owl mini (50ml gel) or wide mini (100ml gel) cells at 80-100V until the required separation was achieved. The analysis of the gel was carried out using a transilluminator with UV light and photographs were taken with a digital camera (ChemiImager 5500, Alpha Innotech). In the cases when gel extraction of DNA molecules was required, appropriate DNA bands were excised from gel and purified using Nucleospin Gel and PCR Clean-up kit from Machery-Nagel, according to the manufacturer’s instructions. Information regarding all solutions used can be found in Supplementary Table S.1.

3.2.3.5 Plasmid preparation and cloning

All cloning steps and preparation of plasmid DNA required before the transfection and transduction of mouse cells were carried out using TOP10 competent *E. coli* cells. Thermo Scientific plasmid purification kits were used for DNA mini-preps and Machery-Nagel kits for midi- and maxi-preps, respectively, and performed according to the manufacturer’s recommendations. Restriction digests and ligation reactions were performed with restriction enzymes and T4 DNA ligase purchased from NEB, using the buffers and protocols supplied with the enzymes. Gateway cloning was performed using BP and LR clonases from Thermo Fisher Scientific using the buffers and solutions provided, according to the manufacturer’s instructions.
3.2.3.6 DNA sequencing

DNA was sent to Eurofins MWG for sequencing. Sequencing of 50-100ng of plasmid DNA or 5-20ng of purified PCR products was carried out using either a T7 forward primer provided by the sequencing company or specific primers that were mixed and sent together with the DNA (Supplementary Table S.6).

3.2.4 Protein Methods

3.2.4.1 Protein Extraction

Whole cell extracts were prepared from control or irradiated cells at the indicated time-points post irradiation by direct addition of 1x Laemmli buffer to the cells that still adhered to the culture plates following one wash with ice-cold PBS. Cells were disaggregated into the Laemmli buffer using a cell scraper, heated at 95°C for 5 min, sonicated and spun down at 14,000g for 2 min at 4°C prior to quantification by the Bradford method. Information regarding all solutions used can be found in Supplementary Table S.1.

3.2.4.2 Western Blotting

30-50μg of total cell extracts were separated using SDS-PAGE gels and transferred to nitrocellulose membranes as previously described. Chemiluminescence was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and medical x-ray film (Konica Minolta Medical & Graphic Imaging Inc.). In assays in which protein quantification was necessary, this was performed using a LiCor Odissey infrared imaging system according to manufacturer’s instructions. Information about all solutions and antibodies used for western blotting can be found in Supplementary Tables S.1 and S.3 respectively.

3.2.4.3 Label-free proteomics analysis

Protein isolation: Protein was isolated with the addition of trichloroacetic acid (20%). After centrifugation at 14,000g for 10mins and aspiration, cell pellets were twice
washed in ice-cold acetone with centrifugation repeated. Protein pellets were resuspended in buffer of 8M Urea in 50mM Ammonium Bicarbonate (NH₄HCO₃). Protein concentration was determined using the Bradford Assay. Information regarding all solutions used for proteomic analyses can be found in Supplementary Table S.1.

**In-solution digestion:** Cysteine of plasma protein samples were reduced using dithiothreitol followed by alkylation with iodoacetamide. Dithiothreitol, iodoacetamide and urea concentrations were diluted using 50mM NH₄HCO₃ before Trypsin SingleT™ proteomic grade (Sigma) was added, ensuring a urea concentration lower than 2M. Digestion was carried out overnight at 37°C. After drying in vacuum centrifuge, peptides were acidified by trifluoroacetic acid (TFA), desalted with c18 STAGE tips [363], and resuspended in 0.1% TFA.

**Mass Spectrometry:** Peptide fractions were analyzed on a quadrupole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer equipped with a reversed-phase NanoLC UltiMate 3000 HPLC system (Dionex LC Packings, now Thermo Scientific). Peptide samples were loaded onto C18 reversed phase columns (5 cm length, 75 µm inner diameter) and eluted with a linear gradient from 8 to 40% acetonitrile containing 0.5% TFA in 60 min at a flow rate of 3 µL/min. The injection volume was 5 µl. The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS2 acquisition. Survey full scan MS spectra (m/z 350 – 1600) were acquired in the Orbitrap with a resolution of 70,000. MS2 spectra had a resolution of 17,500. The twelve most intense ions were sequentially isolated and fragmented by higher-energy C-trap dissociation.

**Protein identification:** Raw data from the Orbitrap Q-Exactive was processed using MaxQuant version 1.5.1.0 [96][450], incorporating the Andromeda search engine [97]. To identify peptides and proteins, MS/MS spectra were matched to the Uniprot mus musculus database (2016_10) containing 50,306 entries. All searches were performed with tryptic specificity allowing two missed cleavages. The database searches were performed with carbamidomethyl (C) as fixed modification and acetylation (protein N terminus) and oxidation (M) as variable modifications. Mass spectra were searched using the default setting of MaxQuant, namely a false discovery rate of 1% on the peptide
and protein level. For the generation of label-free quantitative (LFQ) ion intensities for protein profiles, signals of corresponding peptides in different nano-HPLC MS/MS runs were matched by MaxQuant applying a mass accuracy of at least 20 ppm and a maximum time window of 1 min [95].

**Proteomic data analysis:** The Perseus statistical software (version 1.4.1.3) was used to analysis the LFQ intensities. Protein identifications were filtered to eliminate the identifications from the reverse database and common contaminants and the total number of proteins remaining was defined as the number of proteins identified. Data was log transformed and t-test comparison of fractions carried out. For tests derived from volcano plots, missing values were imputed with values from a normal distribution and a t-test comparison was performed. For visualization using heat maps, the dataset was normalized by z-score [112][451]. The Metascape online tool (http://metascape.org) was used for GO term enrichment analysis [445].

### 3.2.5 Immunofluorescence microscopy

#### 3.2.5.1 Immunofluorescence staining

Cells were cultured on glass coverslips in 21% or 5% O₂ for 48 hours prior to irradiation. All cultures were fixed in 4% paraformaldehyde (Sigma-Aldrich) and permeabilized in 0.1% Triton®-X 100 solution. Unspecific antibody binding was prevented by blocking with 5% FCS/2% Goat Serum/PBS for 1h at 37°C. Cells were then stained with anti-Hif-1α primary antibody (Abcam) (1:50 dilution in blocking buffer) for 1h at 37°C and washed 3x with PBS before staining with FITC-conjugated goat anti-rabbit secondary antibody (1:200 dilution in blocking buffer) for 1h at 37°C. Cultures were washed 3x with PBS and nuclei were counterstained with DAPI-containing Vectashield antifade mounting medium (Vector Labs). All images were captured using 40X magnification on a Delta Vision integrated microscope system (Applied Precision) controlled by Soft-WoRx software mounted on an IX71 Olympus microscope. Images were adjusted using customized macros for Fiji [388], as previously described (Section 2.2.9.2). Information regarding all solutions and antibodies used for immunofluorescence staining can be found in Supplementary Tables S.1 and S.4 respectively.
3.2.6 RNA methods

3.2.6.1 RNA isolation and retrotranscription

Total RNA was isolated from cells by TRIzol® Reagent (Life Technologies) - chloroform extraction and ethanol precipitation. In short, $10^6$ cells per condition were dissolved in 1ml of TRIzol reagent and incubated for 5min at room temperature. 200μl of chloroform (Sigma) were added and the tubes were thoroughly vortexed to maximize phase exchange. Phases were then allowed to separate by incubating for 5min at room temperature before spinning down at 12,000g for 30min at 4°C. Aqueous layer was recovered and RNA was precipitated by addition of 2 volumes of 100% ethanol (Sigma). RNA was pelleted by centrifugation at 12,000g for 30min at 4°C. Three washes with 70% ethanol were performed, separated by 5min spins at 8,000g. Remaining ethanol was allowed to dry and RNA was resuspended in nuclease-free $H_2O$ (Qiagen). RNA quantification and purity assessment were performed by measuring the absorbance at 260, 230 and 280nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific). RNA integrity was assessed by visualization on MOPS/agarose gels as described below. cDNA was generated using Applied Biosystems’ High-Capacity cDNA Reverse Transcription Kit according to the manufacturer’s instructions. Information regarding all solutions used can be found in Supplementary Table S.1.

3.2.6.2 RNA agarose gel electrophoresis

1% agarose (Sigma) gels were prepared in 1xMAE buffer containing formaldehyde at a final concentration of 2.2%. RNA Loading Buffer at a final 1x dilution was used to load the samples onto the gel, after incubation at 56°C for 5min, and gels were run in 1x MAE containing 1.1% formaldehyde in Owl mini (50ml gel) or wide mini (100ml gel) cells at 80-100V until the required separation was achieved. The analysis of the gel was carried out using a trans-illuminator with UV light and photographs were taken with a digital camera (ChemiImager 5500, Alpha Innotech). Information regarding all solutions used can be found in Supplementary Table S.1.
3.2.6.3 Real-time PCR

10-80ng of cDNA were used as template in semiquantitative real-time PCR reactions with specific primers on a Step One Plus Real-Time PCR System (Applied Biosystems). The reactions were prepared with SYBR Select reaction mix from Applied Biosystems. Predesigned KiQqStart® primer pairs for mouse Prkdc, Lig4, Rad51, 53BP1, Mcl-1, Brca1, AldoA, Bnip3, Egln1, and β-Actin (Actb) were purchased from Sigma Aldrich. PCR conditions were optimized for every primer pair and standard curves were performed to determine the efficiency of each of them. Gene expression changes were determined by the ΔΔCt method, using β-Actin as housekeeping gene for normalization and adjusting according to the primer efficiencies previously calculated.

3.2.6.4 DNA Damage Response qPCR Arrays

RNA was isolated from MS5 cells cultured in normoxia (21% O₂) or hypoxia (2% O₂) using the TRIzol-chloroform method. 500ng per sample of the resulting total RNA were used as a template for cDNA synthesis using Quiagen's RT2 First Strand Kit according to the manufacturer’s protocol. qPCR reactions were prepared using the RT2 SYBR Green ROX qPCR Mastermix from Quiagen and loaded into the commercial customized Mouse DNA Damage Response RT2 Profiler PCR Arrays which include primers for Lig4, Bcl2, Bcl-xl (Bcl2l1) and Puma (Bbc3) in addition to the 84 DDR genes present in the standard PCR arrays.

3.2.7 CRISPR/Cas9 Methods

CRISPR/Cas9 methodology was used to disrupt Hif-1α from the MS5 mouse MSC cell line. In order to do this, the pX330 WT Cas9 plasmid (obtained from Addgene, catalogue number #42230) was used. Guide RNA sequences are shown in Table 3.5.

3.2.7.1 Oligonucleotide cloning into pX330

Guide RNA sequences targeting the first and third exon of mouse Hif1α were designed manually and blasted against the mouse genome to verify their specificity. Correspond-
Table 3.5: Guide RNA sequences designed for Hif-1α knock out. Overhang sequences for cloning are shown in blue.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Targeted Exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guide RNA 1 Forward</td>
<td>CACCGGGCACCCGATCGCCATGG</td>
<td>Exon 1.2</td>
</tr>
<tr>
<td>Guide RNA 1 Reverse</td>
<td>AAACCTCCATGGCGAATCGGTGCC</td>
<td>Exon 1.2</td>
</tr>
<tr>
<td>Guide RNA 2 Forward</td>
<td>CACCGCTCGCTCGGGCCTAACGC</td>
<td>Exon 1.2</td>
</tr>
<tr>
<td>Guide RNA 2 Reverse</td>
<td>AAACGCGTTTAGGCCCGAGCGAGC</td>
<td>Exon 1.2</td>
</tr>
<tr>
<td>Guide RNA 3 Forward</td>
<td>CACCGCTAACAGATGACGGCGACA</td>
<td>Exon 3</td>
</tr>
<tr>
<td>Guide RNA 3 Reverse</td>
<td>AAACCTGTCGCCGTCATCTGGTAGC</td>
<td>Exon 3</td>
</tr>
</tbody>
</table>

Designing oligonucleotides (forward and reverse) including overhangs for cloning were purchased from Sigma and reconstituted at a concentration of 100 μM in nuclease-free H₂O. 25 μl of each oligonucleotide were incubated at 95°C for 5 min and annealed by allowing them to cool down slowly until room temperature for approximately 2 hours. The oligonucleotides were then treated with T4 polynucleotide kinase (NEB) and re-annealed as previously described.

4 μg of pX330 plasmid were linearized by digestion with BbsI restriction enzyme (Thermo Scientific) and treated with Calf Intestinal Alkaline Phosphatase (CIP, from NEB). The linearized plasmid was then purified using a PCR Clean Up kit from Thermo Scientific.

1 μl of a 500nM solution of the annealed oligonucleotides was used for ligation into 50 ng of the linearized pX330 vector using T4 Ligase (NEB). Ligation products were then transformed into competent Top10 E. coli, which were grown under Ampicillin selection. Clones corresponding to each guide RNA were picked and screened by BbsI digestion. Positive clones were sequenced to confirm the presence of the oligonucleotides and the absence of mutations using the pX330_seq primer (Supplementary Table S.6).

Correct clones were purified from large-scale E. coli cultures using the NucleoBond® Xtra Midi / Maxi-prep kit from Macherey-Nagel and re-sequenced prior to transfection.

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3.2.7.2 CRISPR plasmids transfection into MS5 cells

$10^6$ MS5 cells were seeded in a 10 cm petri dish with 10 ml of DMEM supplemented with 10% FBS and without Penicillin/Streptomycin. 24h later, ~75% confluent cells were transfected using Lipofectamine reagent (Thermo Fisher Scientific) with 3μg of each gRNA/Cas9 plasmid, 1μg of pLOX Puromycin resistance plasmid for selection of transfected cells and 0.5μg of eGFP expression plasmid to test for transfection efficiency. After incubation for 24h with the transfection mixture, cells were trypsinized and serial dilutions were performed in complete medium containing 10μM of Puromycin. Spare cells not used for the serial dilutions were used to assess transfection efficiency by flow cytometry. Cells were grown under selection for 48 hours before medium with puromycin was replaced with fresh medium and cells were cultured for 14 days until colonies were clearly visible.

3.2.7.3 Clone selection and screening

Single colonies were picked using cloning discs (Sigma) and transferred into 12 well plates. Cultures were scaled up until three wells of a 6-well plate were obtained per clone. One well was harvested for cryopreservation, the second one for protein extraction and screening and the third one for genomic DNA extraction and characterization of the mutations introduced.

Clones were screened for the presence or absence of the Hif-1α protein by western blotting, using treatment with 500μM of DMOG to prevent the degradation of the protein and ensure its detection. 15 clones were also randomly selected for PCR and sequencing of the genomic region where the Cas9 was directed by the guide RNAs and determining the targeting efficiency. Positive clones missing the Hif-1α band were also further characterized by PCR and sequencing to determine the exact mutations introduced in each allele, using the primers shown in Supplementary Table S.6.
3.2.8 Lentiviral Transduction Methods

3.2.8.1 Hif-1α cDNA cloning into pLENTI PGK Blast plasmid

Hif-1α cDNA was isolated from whole MS5 cDNA by PCR, using specific primers complementary to the start of the coding sequence in Exon 1.2 (F primer) and to the end of the coding sequence in Exon 15 (R primer), finishing in the last nucleotide before the transcriptional stop codon. In addition, primers contained overhangs corresponding to attB sites to allow cloning by Gateway cloning through a BP reaction (Supplementary Table S.6). Amplified Hif-1α cDNA was run on an agarose gel in order to assess specificity of the reaction. The band corresponding to the Hif-1α cDNA predicted size (~2.5kbp) was extracted from the gel, sequenced and cloned into pDONR201 plasmid (Invitrogen) by gateway cloning.

Different clones were screened by digestion and sequencing to confirm the absence of mutations caused during the PCR amplification or the cloning process. Correct clones were purified from large-scale E. coli cultures using the NucleoBond® Xtra Midi / Maxi-prep kit from Macherey-Nagel and re-sequenced.

bHLH- and PAS-A-mutated Hif-1α cDNA versions were generated by site-directed mutagenesis (Section 3.2.3.3), using the primers found in Supplementary Table S.6. Resulting plasmids were screened by sequencing and correct clones were again purified from large-scale E. coli cultures as previously described, and re-sequenced.

WT and mutated versions of the Hif-1α cDNA contained in pDONR201 plasmids were cloned into the pLENTI PGK Blast lentiviral mammalian expression vector (Adgene) by Gateway cloning through an LR reaction. The clones obtained were screened by enzymatic restriction and sequencing, and correct clones were purified from large-scale E. coli cultures using the NucleoBond® Xtra Maxi-prep kit (Macherey-Nagel) to achieve higher plasmid yield and concentration, and sequenced.

3.2.8.2 Lentiviral particle generation

5x10⁵ HEK293T cells were seeded per 10cm petri dish and cultured until they reached a ~70% confluency level. Each of the pLENTI PGK Blast vectors containing the different
versions of the Hif-1α cDNA was mixed with the three packaging plasmids psPAX2, pMS2.g and Rev plasmids as indicated in Table 3.6 and diluted in a 150mM NaCl solution up to a 250μl volume. 40μl JET-PEI transfection reagent (Polyplus) were mixed with 210μl of a 150mM NaCl solution and incubated at room temperature for 5min prior to mixing it with the plasmid mix previously prepared and incubating this new mixture for 20min at room temperature.

Table 3.6: Plasmids used for lentiviral particle generation.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLENTI Expression Vector</td>
<td>7μg</td>
</tr>
<tr>
<td>psPAX2 Packaging Plasmid</td>
<td>7μg</td>
</tr>
<tr>
<td>pMD2.g Envelope Plasmid</td>
<td>3μg</td>
</tr>
<tr>
<td>pRSV-Rev Plasmid</td>
<td>3μg</td>
</tr>
</tbody>
</table>

HEK293T cell growth medium was replaced with 10ml fresh DMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin solution. After incubation, pDNA/JET-PEI mix was added gently to the tissue culture plates, and mixed well. Cells were then cultured for 24-48 hours at 37°C and 5% CO₂ in humidified incubators in order for the virus particles to be produced.

24h and 48h after transfection, cell culture supernatant (now containing viral particles) was harvested, and replaced with fresh medium. Supernatants were filtered through a 45nm filter and stored at 4°C for up to a week or at -80°C long term.

3.2.8.3 Viral transduction of MS5 cells

_Hif1α⁻/⁻_ MS5 cells were seeded in 6-well tissue culture plates at low density (10⁵ cells per well) to be ~30% confluent the next day. After 24h, tissue culture medium was removed and substituted with 2ml of undiluted viral supernatant. Plates were then spun down at 400g for 90min, at room temperature, to increase viral transduction efficiency, and then transferred to the cell culture incubator. 24h after, viral supernatant was removed and fresh medium was added. Cells were then allowed to recover for 24h before adding blasticidin at a concentration of 50μg/ml (this optimal concentration had been previously determined by performing a kill curve with the _Hif1α⁻/

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for selection of cells that had successfully integrated the transgenes. Cells were kept under selection for one week prior to performing any experiments with them.

### 3.3 Results

#### 3.3.1 Hif-1α-mediated increase of the radio-resistance of hypoxic mouse MS5 cells is not caused by an effect on the mRNA expression of DDR factors.

Previous findings in our group [424] demonstrated that hypoxia enhances the radio-resistance of both primary and immortalized mouse mesenchymal stromal cells by affecting the efficiency of their DNA Damage Response. Hypoxic MSCs displayed a Hif-1α-dependent increase in the protein levels of the DNA repair factors DNA-PKcs and Ligase IV (involved in NHEJ) while the levels of the HR factor Rad51 remained unchanged. In line with these results, an increased efficiency of DNA repair by non-homologous end joining was also detected. It was also shown that the immortalized mouse MSC cell lines MS5 and ST2 behave very similarly to primary mouse MSCs, which is why the MS5 cell line was chosen for a more detailed molecular investigation of the mechanism behind the effects of hypoxia on the DDR of MSCs. Hif-1α is the oxygen-regulated subunit of the hypoxia inducible factor Hif-1, which also contains the constitutively expressed subunit Hif-1β (also known as Arnt) [223]. Hif-1 is a transcription factor that controls the expression of many hypoxia-inducible genes in order to allow cellular adaptation to hypoxia [170]. Since the canonical function of Hif-1 is to control the expression of a myriad of hypoxia-responsive genes, and hypoxia resulted in an increased DNA-PKcs and Ligase IV protein level (that correlated with Hif-1α accumulation in the cells), we hypothesized that Hif-1α might be involved in mediating an increase in the mRNA expression level of DNA repair genes involved in NHEJ such as DNA-PKcs (Prkdc) and DNA Ligase IV (Lig4), but not in HR, since the protein levels of Rad51 remained unchanged. In order to test this hypothesis, the effect of hypoxia on the mRNA expression levels of these genes was investigated. Real-time PCR was used to measure the mRNA levels of Hif1α, Prkdc, Lig4 and Rad51 in normoxic and hypoxic MSCs at different time-points after treatment with 10Gy of IR (Figure 3.1 A). Surprisingly, hypoxia treatment did not result
in the increase of the mRNA levels of any of the genes analyzed, but instead it caused a mild but statistically significant decrease in the mRNA levels of Rad51. Significant up-regulation in the mRNA expression of all genes studied only occurred in response to IR, 24h after irradiation in normoxic conditions. In hypoxia, a similar but less pronounced trend was observed, with only DNA-PKcs showing a statistically significant up-regulation. Despite the fact that hypoxia did not up-regulate the mRNA expression of DNA-PKcs and DNA Ligase IV, the previously described hypoxia-mediated increase in MS5 radio-resistance was reproduced (Figure 3.1 B), indicating that this effect is not correlated with an increase of DNA-PKcs and DNA Ligase IV mRNA expression as had been previously hypothesized. In light of these results, it was decided to investigate whether hypoxia influences the mRNA expression of other genes implicated in the DNA damage response.

Customized commercial qPCR arrays were used in order to compare the mRNA expression of 87 genes belonging to the DDR signalling network in hypoxia (2% O_2) relative to normoxia (21% O_2). This array allows the analysis of genes involved in the ATM and ATR signalling cascade, different DNA repair pathways (such as nucleotide excision repair, base excision repair, mismatch repair and double strand break repair), as well as cell cycle control and apoptosis. In line with the previous results, hypoxia did not significantly affect the expression of the vast majority of the genes, with only one gene (Mgmt) up-regulated over two fold (although this difference did not show statistical significance) and only one gene (Ogg1) showing a significant but mild down-regulation (Figure 3.1 C, D). Neither DNA-PKcs nor DNA Ligase IV (the proteins up-regulated as a result of the hypoxia treatment) showed significant differences at the level of mRNA, suggesting that the regulation of the NHEJ factors occurs at a post-translational level rather than at an mRNA expression level. In order to confirm the qPCR array results, individual real-time PCRs for 53BP1, Brca1, DNA-PKcs, DNA Ligase IV, Mdc1 and Rad51 were performed, showing the same results (Figure 3.1 E). Rad51 was the only gene displaying a statistically significant (although mild) down-regulation in hypoxia compared to normoxia, similarly to what had been observed in previous experiments (Figure 3.1 A). In order to confirm the transcriptional responsiveness of the cells to hypoxia, mRNA expression of three well-known Hif-1α target genes: AldoA, Bnip3 and Egln1 [170] was analyzed. As expected, the hypoxia treatment resulted in all three genes showing a significant up-regulation (Figure 3.1 F).
Figure 3.1: Hypoxia treatment does not affect mRNA expression of DNA Damage Response genes. (A) mRNA expression levels of Hif1a and the DDR factors DNA-PKcs, DNA Ligase IV and Rad51, measured 0-24h after treatment with 10Gy IR. β-actin was used as housekeeping gene and normoxic samples as reference for mRNA relative expression quantification using the ΔΔCt method. * p < 0.05, paired t tests, n=9. (B) Clonogenic survival assay of MS5 cells in 21% or 2% O₂. * p<0.05, ** p<0.01 compared with normoxic samples, two-way ANOVA with Bonferroni post-test correction, n=9. (C) Scatter plot and (D) volcano plot of qPCR array data comparing normoxic (21% O₂) and hypoxic (2% O₂) MS5 cells. In the scatter plot, diagonal lines represent -2, 0 and +2 fold changes, from left to right. In the volcano plot, green and black vertical lines represent 0 and 2-fold expression changes, respectively. Blue horizontal lines represent a p value of 0.05 (n=3), with significantly regulated genes being shown above them. All genes up-regulated more than 2-fold are shown in red (independently of their statistical significance). mRNA expression levels of (E) DDR factors S3BP1, Brc1, DNA-PKcs, DNA Ligase IV, Mdc1 and Rad51 and (F) Hif-1α target genes AldoA, Bnip3 and Egln1 in hypoxia (2% O₂) compared to normoxia (21% O₂). β-actin was used as housekeeping gene and normoxic samples as reference for mRNA relative expression quantification using the ΔΔCt method. * p < 0.05, paired t tests, n=4.
3.3.2 Generation of a Hif-1α knockout MS5 cell line using CRISPR/Cas9 technology.

3.3.2.1 CRISPR/Cas9 Strategy

To investigate the role of Hif-1α in the DNA Damage Response of MS5 cells, a Hif1a knockout MS5 cell line was generated using CRISPR/Cas9 technology. The Hif1a gene locus, which is located on mouse chromosome 12, contains 15 exons separated by 14 introns, dispersed over 45 kb [397,473]. In 1997, Wenger et al. described two Hif1a mRNA isoforms in mice, resulting from two alternative first exons (exon 1.1 and exon 1.2) and differing mainly in their 5’ UTR (Figure 3.2 A) [474]. When comparing the sequence information found in the NCBI database (https://www.ncbi.nlm.nih.gov), the exon 1.2-containing transcript (Transcript Variant 1) encodes a Hif-1α protein that is 12 amino acids longer in the N-terminal region than the exon 1.1-containing isoform (Transcript Variant 3). Transcript Variant 2, also described in the NCBI database, is nearly identical to Transcript Variant 1 except for the lack of three nucleotides in the N-terminal region and a single nucleotide substitution. According to this information, three different guide RNA sequences were designed, and the wild-type endonuclease version of the Cas9 enzyme was used in order to introduce double-strand breaks at the desired sites [93]. As depicted in Figure 3.2 B, two of the gRNAs were designed in exon 1.2, in order to target Hif1a transcript variants 1 and 2 at both sides of the translational start codon (ATG), in an attempt to generate a deletion that would impede transcription initiation at this site. Transcript variant 3 (whose expression is driven by a tissue-specific promoter [476]) has its translational start codon in exon 2. However, this exon is relatively short (~190 bp) and no potential high quality gRNA sequences were found in it. For this reason, the third gRNA, which would target all mRNA isoforms, was designed in exon 3. Guide RNA specificity was tested by blasting the sequence against the mouse genome in order to avoid off-target effects, and its quality and predicted efficiency was assessed using two online available tools: MIT CRISPR design tool (http://crispr.mit.edu/) and the DESKGEN genome-editing tool (https://www.deskgen.com).
Figure 3.2: *Hif1α* knock-out CRISPR/Cas9 strategy. (A) Mouse *Hif-1α* genomic locus and cDNA structure. Untranslated regions are shown in white while translated regions are shown in gray. Two alternative ATG sites are indicated with green arrowheads, while stop codon (TGA) is indicated in red. (B) *Hif-1α* CRISPR/Cas9 knockout strategy. Guide RNAs designed according to the two 1st alternative exons described by Wenger et al. in 1998 [476]. Cas9 cut sites are indicated by red arrows. ATG in first exon is indicated in bold.
3.3.2.2 Generation and characterization of Hif1α knockout MS5 cells. CRISPR/Cas9 results.

Mouse MS5 cells were transfected simultaneously with a pool of the three pX330 plasmids containing the three gRNA sequences that had been designed, in order to increase targeting efficiency. Resulting clones were screened for the presence or absence of the Hif-1α protein by western blot. Prior to this, different antibodies against Hif-1α were tested in order to select the best one for these purposes, using two different commercial siRNAs against murine Hif-1α, either alone or in combination. In addition, the PHD inhibitor DMOG, a cell permeable, competitive inhibitor of prolyl hydroxylase domain-containing proteins (such as the PHDs that hydroxylate HIF-1α in the presence of oxygen), which prevents Hif-1α protein degradation even in normoxic conditions and allows its accumulation in the cells (Figure 1.6), was used. As shown in Supplementary Figure S.3, different antibodies recognize the Hif-1α protein with different levels of specificity, ranging from the NB100-105 antibody from Novus Biologicals which only recognizes an unspecific band (which is slightly smaller than Hif-1α) to the Abcam antibody which is the most specific antibody amongst the ones tested and does not give rise to any unspecific bands. The NB100-449 antibody from Novus Biologicals recognizes an unspecific band that co-migrates very closely to Hif-1α and impedes the proper interpretation of the results. The Bethyl antibody shows the same problem although the unspecific band is less strong compared to Hif-1α. Finally, the antibody purchased from R&D also recognizes an unspecific band, although in this case it is smaller than the Hif-1α band and does not co-migrate with it. For all these reasons, the chosen antibody to perform the screening of the CRISPR/Cas9 clones obtained was the one distributed by Abcam. Interestingly, this experiment also allowed us to test the quality of the two different commercial siRNAs against Hif-1α obtained from Dharnacon. Hif-1α knockdown was detected only when the cells were transfected with siRNA1 (alone or in combination) but not with siRNA2.

Hif-1α protein levels are very tightly regulated and its degradation is induced within seconds in the presence of oxygen. In order to avoid Hif-1α degradation during sample preparation that would result in false positives for the protein deletion, the 95 CRISPR clones selected were treated with 500μM DMOG for 8h prior to protein extraction for screening by western blot. Apart from this, genomic DNA from all these clones was also
harvested in order to characterize the mutations introduced in their Hif1a loci. In order to characterize the targeting efficiency of the CRISPR/Cas9 strategy, 15 clones were randomly picked and gDNA regions to which the Cas9 was directed by the gRNAs were amplified by PCR and sequenced. Out of the 15 clones analyzed, 13 showed mutations in at least one exon, and only two were wild type, resulting in a targeting efficiency of ~87% (Table 3.7). Most of these mutations had occurred at the exact sites where the Cas9 endonuclease was predicted to introduce double-strand breaks. Interestingly, when the forward primer complementary to exon 1 and the reverse primer complementary to exon 3 were used together, the gDNA from some clones gave rise to a ~400bp PCR product which was absent in the WT MS5 gDNA used as control. Sequencing of this DNA fragment revealed that it was the result of a ~20kb deletion between the Cas9 cut sites in exons 1.2 and 3 (data not shown). However, no mutations were detected at the site where the first gRNA would direct the Cas9 to cut, indicating that this particular gRNA had not worked properly. In order to investigate the reasons for this, the MS5 Hif1a cDNA was isolated and most of it (except for the 3'UTR and the beginning of the 5'UTR) was sequenced and compared to the reference sequence used to design the gRNAs. Supplementary Figure S.4 shows the alignment between the MS5 Hif1a cDNA sequence and the reference sequence found in the NCBI database. A total of 10 single nucleotide changes, a 1 nucleotide deletion (in the 5'UTR, so not affecting reading frame) and a 42 nucleotide deletion were detected (Supplementary Figure S.4), which result in 4 amino acid substitutions and the deletion of a fragment of 14 amino acids in the protein sequence (Supplementary Figure S.5). This 14 amino acid deletion falls in the ODD domain and had been previously described by Wenger et al. in 1996 [475]. Unfortunately, two of the single nucleotide changes were found in the sequence of the first gRNA, explaining why it did not work.

Figure 3.3A shows an example of three of the Hif1a knockout clones obtained: clones 41, 55 and 76. Sequencing of their gDNA (Figure 3.3B) allowed characterizing the specific mutations present in each of their two alleles (Figure 3.3 C, D). Exon 3 of clone 41 presented a single-nucleotide and a two-nucleotide deletion in each allele, respectively, resulting in the disruption of the reading frame of the protein and, eventually, the introduction of a premature stop codon. Similarly, clone 55 showed two single-nucleotide insertions in exon 1 and a 40-nucleotide deletion and a 4-nucleotide insertion in each allele in exon 3. Finally, clone 76 presented a single-nucleotide deletion and a single-
Table 3.7: Targeting efficiency of the Hif1α knockout CRISPR/Cas9 strategy

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Exons Mutated</th>
<th>Overall Targeting Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Exon 3</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Exons 1 and 3</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Exons 1 and 3</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>Exon 3</td>
<td>87%</td>
</tr>
<tr>
<td>55</td>
<td>Exons 1 and 3</td>
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<tr>
<td>73</td>
<td>Exons 1 and 3</td>
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</tr>
<tr>
<td>76</td>
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A nucleotide insertion in exon 1 and a two-nucleotide and a four-nucleotide deletions in the two alleles in exon 3. Two of these Hif1α knockout clones (41 and 76) were selected as two independent clones for subsequent experiments.

3.3.3 Both subunits of the transcription factor Hif-1 are required for the hypoxia-induced increase in MS5 radio-resistance.

The newly generated Hif-1α−/− MS5 cell lines were used to study the effect of Hif-1α loss in MS5 radio-resistance. Two independent clones (41 and 76) were used in parallel in order to control for possible off-target effects resulting from the CRISPR/Cas9 Hif1α knockout protocol. First of all, disruption of the Hif-1 pathway by Hif-1α deletion was confirmed by analyzing mRNA expression of known Hif-1α target genes (Figure 3.4A). AldoA, Bnip3 and Egln1 are significantly up-regulated in hypoxic WT MS5 cells. However, Hif1α knockout not only completely abolished this up-regulation but also caused a significant defect in the mRNA expression of these genes, irrespective of the oxygen levels. We then proceeded to analyze the effect of Hif-1α depletion on the radio-resistance of MS5 cells. As previously shown (Figure 3.1B), WT MS5 survival to increasing doses of ionizing radiation is enhanced in hypoxic conditions. In contrast, Hif1α knockout prevented hypoxia-mediated increase in MS5 radio-resistance, indicating that this effect is
Figure 3.3: Generation of Hif1α knockout MS5 cell line using CRISPR/Cas9 technology. (A) Representative western blot showing three of the Hif-1α−/− MS5 cell lines obtained (Clones 41, 55 and 76). (B) Sequencing raw data corresponding to WT and mutated Hif-1α Exon 3. Specific mutations present in each allele of (C) exon 1 and (D) exon 3 of the Hif1a genomic locus of Hif-1α−/− MS5 clones 41, 55 and 76. Cas9 cut sites are indicated with red arrows.
Hif-1α-dependent (Figure 3.4 B, C). In line with this result, treatment of normoxic WT MS5 cells with DMOG, a PHD inhibitor that prevents Hif-1α degradation in the presence of oxygen, resulted in an increase of their radio-resistance in comparison with the untreated cells, although not to the same level as the hypoxic untreated cells (Figure 3.4 D, E).

Hif-1α is the oxygen-regulated subunit of the Hif-1 transcription factor, forming a heterodimer with the constitutively expressed subunit Arnt (aryl hydrocarbon receptor nuclear translocator, also called Hif-1β) [401]. In order to investigate the mechanism behind Hif-1α-mediated effect of hypoxia on MS5 response to IR, the requirement of its partner Arnt was also investigated. An Arnt-specific siRNA was used to deplete this protein from WT MS5 cells, which resulted in a reduction in the radio-resistance specifically in hypoxia (Figure 3.4 F, G), becoming comparable to the survival levels displayed by the normoxic cells. Cells transfected with control siRNA showed no significant difference compared to the un-transfected cells, indicating the siRNA transfection process had no effect on the cellular response to IR. This result suggests that not only Hif-1α, but also Arnt, are required for the hypoxia-mediated increase in MS5 radio-resistance.

### 3.3.4 Generation of Hif-1α mutant MS5 cell lines.

I have so far demonstrated that, although the hypoxia-induced increase in MS5 radio-resistance is dependent on both Hif-1α and Arnt (the two subunits that compose the Hif-1 transcription factor), there is no effect of hypoxia on mRNA expression of a great number of the main genes that function in the DDR. This is particularly surprising given that several of these factors (DNA-PKcs and Ligase IV) show a Hif-1α-dependent increase of their protein levels in hypoxia. In light of this information, two hypotheses were formulated that could explain the previous results: either Hif-1α and Arnt have a different role in the DDR than their canonical transcription factor function (maybe controlling protein stability), or they are indeed acting as a transcription factor, but controlling the expression of an unknown intermediary protein that in turn affects the components of the DDR. In order to investigate which of these hypotheses is true, two different Hif-1α mutant cDNA constructs were designed and stably expressed in Hif-1α−/− MS5 cells, and their ability to increase MS5 radio-resistance tested. The first mutant protein would lack the ability to interact with the DNA double helix (which would prevent
Figure 3.4: Both subunits of the transcription factor Hif-1 are required for the hypoxia-induced increase in MS5 radio-resistance. (A) Relative mRNA expression of the Hif-1α target genes AldoA, Bnip3 and Egln3, normalized against β-Actin and WT MS5 cultured in normoxia for mRNA relative expression quantification using the ΔΔCt method. * p < 0.05, paired t tests, n=3. Clonogenic survival assay of (B) WT and Hif-1α−/− MS5 clones 41 and 76, (D) untransfected, control and Arnt knock down WT MS5 cells and (F) WT MS5 cells untreated or treated with 500 µM DMOG for 48h cultured in normoxia (21% O₂) and hypoxia (2% O₂). n=6. Representative Hif-1α western blot showing (C) WT MS5 cells and Hif-1α−/− MS5 clones 41 and 76 and (E) WT MS5 cells untreated or treated with 500 µM DMOG for 48h. (G) Representative Arnt western blot of WT MS5 cells transfected with Control or siArnt siRNA.
Figure 3.5: Three-dimensional structure of the Hif-1 transcription factor. Representation of the interaction between the bHLH and PAS domains of the two subunits of the Hif-1 transcription factor (Hif-1α in green and Arnt in orange) and the DNA double helix. This model has been obtained according to the information from the crystal structure published by Wu et al. in 2015 [491], which is publicly available in the Protein Data Bank under accession number 4ZPR.

3.3.4.1 Design of Hif-1α mutant constructs.

In December 2015, Wu et al. [491] published a partial crystal structure of the Hif-1 transcription factor interacting with the DNA double helix (Figure 3.5). This crystal structure is composed of the N-terminal segments of both Hif-1α and Arnt, thereby allowing the mapping of the main specific residues that mediate both the interaction between Hif-1α and Arnt and also their interaction with DNA. Both Hif-1α and Arnt are members of the helix–loop–helix-PER-ARNT-SIM (bHLH-PAS) family, and as such they are composed of an amino-terminal bHLH domain for DNA binding, PAS domains (PAS-A and PAS-B) that allow dimerization, and transactivation domains (TADs) in order to modulate their function. In addition to these, Hif-1α contains an Oxygen-Dependent Degradation Domain (ODDD) which is critical for its regulation and function (Figure 3.6A) [227]. In order to prevent Hif-1α interaction with DNA, two point mutations were...
introduced in its bHLH domain that would result the following two amino acid substitutions: K19Q and R30Q (Figure 3.6 B). These are the two positively charged amino acids that directly interact with the negatively charged DNA molecule. According to the COSMIC Database, these two specific substitutions have been found in cancer patients in which Hif-1α function is altered [139, 491]. Wu et al. 2015 [491] also demonstrated that interaction between Hif-1α and Arnt could be abolished by mutating two single residues found in the PAS-A domain of Hif-1α: R170A and V191D [491], resulting from two double-nucleotide mutations in the Hif-1α cDNA (Figure 3.6 C).

### 3.3.4.2 Generation and characterization of Hif-1α mutant MS5 cell lines.

Once the desirable mutations had been introduced, three different versions of the Hif-1α cDNA (WT cDNA, bHLH-mutated cDNA and PAS-A-mutated cDNA) were cloned into a lentiviral mammalian expression vector driven by the hPGK promoter and containing a Blasticidin resistance cassette. Lentiviral particles were generated and Hif-1α−/− MS5 cells were transduced with the different cDNA expression constructs and also an empty vector to be used as control for the subsequent experiments. After selection, expression of the Hif-1α proteins was assessed by western blot. As shown in Figure 3.7 A, lentiviral transduction with the three Hif-1α constructs resulted in an over-expression of the Hif-1α proteins when compared with WT MS5 cells, in hypoxia. Interestingly, the recombinant proteins were regulated in the same fashion as the endogenous Hif-1α, showing high protein levels in hypoxia (2% O₂) but very low levels in normoxia (21% O₂). The WT cDNA and PAS-A-mutated cDNA transduced cell lines showed the highest Hif-1α expression, while the levels of the bHLH-mutated Hif-1α protein were slightly lower, maybe due to an effect of the mutations on protein stability, although this was not confirmed.

Immunofluorescence staining was used to assess subcellular localization of the Hif-1α recombinant proteins in order to confirm their correct translocation to the nucleus, where Hif-1α performs its functions. In line with the previous western blot results, in hypoxia, the recombinant Hif-1α proteins were over-expressed when comparison with the WT MS5 cells, while their levels were extremely low in normoxia (Figure 3.7 B). In addition, proper translocation to the nucleus in hypoxia was confirmed by co-localization of the Hif-1α signal with the DAPI used to stain the nuclei. Hif-1α staining revealed a
Figure 3.6: Design strategy of MS5 cell lines containing WT and mutant Hif1α cDNA. Schematic representation of (A) Hif-1α wild type (WT) and mutant proteins with (B) defective interaction with DNA (Hif-1αK19Q/R30Q (bHLH-mutated) or (C) defective interaction with Arnt (Hif-1αR170A/K191D) (PAS-A-mutated). Mutated nucleotides and amino acids are shown in red, while the original codons and amino acids are indicated in blue.
Figure 3.7: Generation of MS5 cell lines containing WT and mutant Hif-1α cDNA. Representative (A) Hif-1α western blot and (B) immunofluorescence images showing the levels of expression and subcellular localization of the Hif-1α protein in WT, Hif-1α−/−, and Hif-1α−/− MS5 transduced with the different versions of the Hif-1α cDNA previously described (Figure 3.6) in normoxia (21% O₂) and hypoxia (2% O₂). Hif-1α is shown in green, while DAPI is shown in blue. Scale bars correspond to approximately 20 μm.
Once the proper regulation of the recombinant Hif-1α proteins was confirmed, real-time PCR was used to assess their functionality. As previously shown, mRNA expression of the Hif-1α target genes AldoA, Bnip3 and Egln1 is significantly up-regulated in hypoxic WT MS5 cells under hypoxic conditions, and this up-regulation is completely abolished when Hif-1α is knocked out (Figure 3.8 and Figure 3.4 A). Transduction of Hif-1α−/− MS5 cells with WT Hif1α cDNA rescued the mRNA expression of all three Hif-1α target genes (although this rescue was only partial in the case of Bnip3) (Figure 3.8). On the contrary, transduction with wither bHLH-mutated or PAS-A-mutated Hif1α cDNA did not affect Hif-1α target gene mRNA expression (Figure 3.8), indicating that their inability to interact with either DNA or Arnt renders them unable to perform their transcription factor function.
3.3.5 Hif-1α ability to interact with both Arnt and DNA is required for its function in the DDR of mouse MS5 cells.

I have now confirmed that, although expression of the mutant Hif-1α proteins is correctly regulated in the cells, the two single amino acid changes present in each of them impede their normal function as part of the Hif-1 transcription factor. This is quite a conservative approach in terms of protein structure, since no domain has been deleted and all mutations consist of discrete amino acid substitutions. For these reasons, the capability of these mutant proteins to function in the DDR of mouse MS5 cells (and that of the WT version to rescue the hypoxia-mediated increase in radio-resistance) was tested by clonogenic survival assays. First of all, WT and Hif-1α−/− MS5 cells, used as positive and negative controls, respectively, confirmed the previously shown increase in WT MS5 radio-resistance in hypoxia, and the loss of this effect caused by knocking out Hif1a (Figure 3.9 A, B). Interestingly, only the transduction of the Hif-1α−/− MS5 cells with WT Hif1a cDNA rescued the WT phenotype (Figure 3.9 C), while none of the mutant proteins was able to mediate an increase in MS5 radio-resistance (Figure 3.9 D, E), showing the same phenotype as the empty vector control (Figure 3.9 F). These observations indicate that interaction of Hif-1α and Arnt to form the Hif-1 transcription factor, as well as its interaction with the DNA are required for their function in the DDR of MS5 cells, which is most likely to involve regulation of gene expression by Hif-1.

Sugrue et al. also demonstrated in 2014 that hypoxic MS5 cells are more efficient in DSB repair following treatment with IR than normoxic cells [424]. In order to investigate whether Hif-1's ability to function as a transcription factor is also required for a more efficient DSB repair in hypoxia, and confirm the previous results obtained by clonogenic survival assay, DSB repair kinetics were analyzed by γH2AX western blot (as described in Chapter 2). Protein samples were harvested at different time points after treatment with 10Gy of IR, both in hypoxia (2% O₂) and normoxia (21% O₂). γH2AX signal (measured by infrared florescence of the bands obtained by western blotting) peaked 30min after irradiation, and the signal progressively decreased over time indicating DSB repair (Figure 3.10). In hypoxic WT MS5 cells, decrease in γH2AX signal occurred faster than in their normoxic counterparts, denoting a significantly faster repair of the DNA lesions. In contrast, Hif-1α−/− MS5 cells show the same DNA repair kinetics in hypoxia than in normoxia. The fact that γH2AX phosphorylation is not cleared faster
Figure 3.9: Effect of Hif-1α cDNA mutants on MS5 radio-resistance. Clonogenic survival assays of (A) WT MS5 cell line, (B) Hif-1α−/− MS5 Clone 41 cell line, (C) Hif-1α−/− MS5 Clone 41 cell line transduced with a WT Hif-1α cDNA lentiviral construct, (D) Hif-1α−/− MS5 Clone 41 cell line transduced with a bHLH-mutated Hif-1α cDNA lentiviral construct, and (E) Hif-1α−/− MS5 Clone 41 cell line transduced with a PAS-A-mutated Hif-1α cDNA lentiviral construct, and (F) Hif-1α−/− MS5 Clone 41 cell line transduced with an empty lentiviral construct, cultured in 21% or 2% O₂. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 compared with normoxic samples, two-way ANOVA with Bonferroni post-test correction, n=9.
in hypoxia in the absence of Hif-1α confirms that this protein is involved in enhancing the DNA repair efficiency of MS5 cells. In line with the previous results, reconstitution using the WT Hif-1α cDNA rescued the faster DSB repair observed in WT MS5 cells in hypoxia, while the two mutated cDNA versions showed the same phenotype as the Hif-1α−/− MS5 cells (Figure 3.10). Taken together, evidence obtained so far supports the hypothesis that, although Hif-1α may not be affecting the mRNA expression of the DDR factors studied so far, its ability to interact with Arnt to form the Hif-1 transcription factor and their interaction with DNA are critical for its role in enhancing the DDR of mouse MS5 cells.

### 3.3.6 WT and Hif1α knockout MS5 proteome comparison.

It seems now clear that Hif-1α modulates the DDR of mouse MS5 cells through its canonical function as a transcription factor in collaboration with Arnt. However, the exact mechanism allowing it to do so remains unclear. In order to investigate the global changes in protein abundance caused by Hif-1α in hypoxic MS5 cells, proteomic analyses were performed comparing WT MS5 cells and Hif-1α−/− cells in normoxia and hypoxia. Traditionally, isotopic labelling-based techniques have represented the gold standard for quantitative proteomics due to their accuracy and robustness. However, advances in the resolution that can be achieved from mass spectrometers, combined with advances in proteomic data analysis algorithms have allowed label-free proteomic studies to become increasingly useful for quantitative proteomic studies. In label-free quantification (LFQ) applications, fragment-ion intensities that are unique to a specific peptide are used for quantification. The fact that no expensive and time-consuming isotopic labelling is needed makes LFQ experiments much easier and economical. In addition, in contrast to isotopic labelling–based experiments, where the number of available isotopic tags limits the number of samples that can be analysed, no such limitation exists in the case of LFQ experiments [3,95,450,451].

Here, a total of 12 samples (WT MS5 and Hif-1α−/− MS5 grown in either 21% or 2% O₂, three biological replicates each) were analysed following the protocol described in Section 3.2.4.3. A total of ~2200 different proteins could be identified in the WT samples, while only ~1800 proteins were identified in the case of the Hif-1α−/− MS5 cells. Comparison of normoxic and hypoxic WT samples led to the detection of 173 proteins
Figure 3.10: Effect of Hif-1\(\alpha\) cDNA mutants on MS5 DSB repair kinetics. Left panel: Representative western blots showing \(\gamma\)H2AX, H2AX and \(\beta\)-Actin levels in WT MS5 cell line, Hif-1\(\alpha^{-/-}\) MS5 Clone 41 cell line, and Hif-1\(\alpha^{-/-}\) MS5 Clone 41 cell line transduced with and empty lentiviral vector, WT Hif1a cDNA, bHLH-mutated Hif1a cDNA, and PAS-A-mutated Hif1a cDNA lentiviral constructs, cultured in 21% or 2% \(O_2\), 0-24h after irradiation with 10Gy. Right panel: quantification of the relative \(\gamma\)H2AX signal in the corresponding western blots, normalizing the signal against the 0.5h timepoint (peak of the \(\gamma\)H2AX signal) and using both H2AX and \(\beta\)-Actin as loading controls. * \(p<0.5\), **\(p<0.01\), ***\(p<0.001\), ****\(p<0.0001\), two-way ANOVA with Bonferroni post-test correction, \(n=4\).
whose expression changes were statistically significant when a cut-off p value of 0.05 was applied. Of these, only 16 had LoG_{2}(Fold Change) values higher than 1.5 (which corresponds to fold changes higher than ~3-fold), and are indicated in Figure 3.11 A. In the case of Hif-1α−/− samples, 175 proteins were found to be significantly up- or down-regulated in normoxia compared to hypoxia, while only 14 had a LoG_{2}(Fold Change) higher than 1.5 (Figure 3.11 B). Despite the number of proteins significantly changed between the two sets of samples, only 17 are common between the two, highlighting the importance of Hif-1α in mediating protein expression changes in response to hypoxia.

Reproducibility amongst the sample replicates can be observed in the heat maps depicted in Figure 3.12. In them, ion current intensity of proteins significantly changed using a cut-off p value of 0.05 are shown for both the WT and the Hif-1α−/− MS5 groups of samples.

GO term enrichment using the list of proteins differentially regulated in WT cells in hypoxia (2% O_{2}) compared to hypoxia (21% O_{2}) gave as a result many processes related with cellular adaptation to hypoxia such as glycolysis, oxidation-reduction processes or hemostasis, many of which were not found in the Hif-1α−/− samples (Figure 3.13), indicating that Hif-1α plays an important role in the regulation of the proteins belonging to these groups, as expected. Interestingly, GO term enrichment analysis with Hif-1α−/− MS5 samples showed enrichment in proteins belonging to the mitotic checkpoint and apoptosis pathways, which were mainly down-regulated and up-regulated, respectively, in a Hif-1α-independent fashion. However, the previously observed metabolic processes observed to be enriched in hypoxic WT MS5 cells (glycolysis, oxidation-reduction processes, etc.) were not found, indicating that the lack of Hif-1α prevents the metabolic switch that allows cells to adapt to low oxygen conditions.

I have previously shown how, despite the effect of Hif-1α on the DDR being dependent on its ability to act as a transcription factor, no changes in mRNA expression of DDR proteins were detected in hypoxia compared to normoxia. However, Sugrue et al. [424] had previously shown a Hif-1α-mediated increase in DNA-PKcs and DNA Ligase IV protein levels in response to hypoxia. A possible explanation for this could be that Hif-1α may transcriptionally regulate the expression of proteins involved in regulating protein stability and/or degradation, which would in turn mediate an increase in the half-life of these (and maybe also other) DDR factors. For this reason, the presence of proteins involved in regulation of protein stability and degradation amongst the significantly
Figure 3.11: Volcano plots to compare effect of hypoxia on the proteome of WT and Hif1a knockout MS5 cells. Gene expression fold changes of normoxic vs. hypoxic (A) WT MS5 and (B) Hif-1α−/− MS5 cells are plotted in the x axis versus Log_{10} of the p-values derived from a t-test (y axis). Red horizontal dashed lines represents a p value of 0.05 (-Log (0.05) = ~1.3), while green vertical lines indicate Log_2 fold changes of -1.5 (left) and 1.5 (right). Names of proteins with p<0.05 and Log_2 fold changes >1.5 are indicated.
Figure 3.12: Heat maps comparing effect of hypoxia on the proteome of WT and 
Hif1α knockout MS5 cells. Heat maps with hierarchical clustering comparing the 
significant proteins (p<0.05) obtained through a Log transformed intensity t-test in (A) 
WT MS5 and (B) Hif-1α−/− MS5 samples. Intensity color code represents ion current 
intensity (LoG₂ transformed, z-score normalized).
regulated protein lists obtained by analysis of the proteomics data was investigated. Interestingly, all protein ubiquitinases (Cul2, Uba5 and Ube2n) differentially regulated in WT MS5 samples showed down-regulation in hypoxia compared to normoxia, while the only deubiquitinase, Uchl5, was up-regulated. In addition, all members of the proteasome found in this list (Psma3, Psmc5 and Psmc6) were also expressed at lower levels in hypoxic WT MS5 cells. None of these proteins, however, was found to be differentially regulated in \( \text{Hif-1}^\alpha-/- \) MS5 cells, indicating that their regulation might be Hif-1\( \alpha \)-dependent. In turn, protein ubiquitinases (Ube3a, Otub1, Nedd4) found to be expressed differently in \( \text{Hif-1}^\alpha-/- \) MS5 samples were all up-regulated in hypoxia, opposite to the observations made regarding WT MS5 cells. Proteasome members showed a mixed response to hypoxia in these samples, although there was a tendency towards up-regulation of the majority of them (Psmd1, Psmb3, Psmd13). Although these are interesting observations, these results must be confirmed and investigated further before any firm conclusions can be drawn from them.

### 3.4 Discussion

Mesenchymal stromal cells are an essential component of the bone marrow haematopoietic stem cell niche, where they contribute to the regulation of HSC maintenance and self-renewal, and co-ordinate various stages of the haematopoietic process [123, 352]. The bone marrow is a hypoxic environment, and hypoxia is thought to be an important factor in regulating the biology and function of both MSCs and HSCs [124, 173, 216, 266, 313, 353, 447, 448, 487]. In addition, MSCs are also known to migrate to solid tumours, another hypoxic environment, where by developing into cancer-associated fibroblasts (CAFs), they become an integral part of the tumour microenvironment contributing to tumour growth, survival and invasiveness [20, 100, 180, 181, 228, 244, 308, 351]. In cancer cells, hypoxia, by impairing DNA repair mechanisms such as mismatch repair (MMR) and homologous recombination (HR) [40, 57, 377, 386] has been broadly related to poor prognosis. Hypoxia results in increased genetic instability and resistance to radio- and chemotherapy. In contrast, I and others have shown that mouse MSCs cultured under hypoxic conditions display increased DNA repair efficiency as well as radio-resistance (Figures 3.1 B, 3.10) [424], and that this effect is dependent on Hif-1\( \alpha \) (Figures 3.4 and 3.10).
Figure 3.13: Main GO terms enriched in hypoxic WT and Hif1a knockout MS5 samples. GO terms enriched in hypoxic WT MS5 samples when considering (A) the 173 proteins that change significantly according to the Log transformed intensity t-test between WT normoxic and hypoxic samples (p<0.05), and (B) the 175 proteins that change significantly according to the Log transformed intensity t-test between Hif-1α−/− normoxic and hypoxic samples (p<0.05). GO terms are shown in histogram plots in which the length of the bars corresponds to the statistical significance of the enrichment (-log p value). (C) Heat map of GO terms enriched in hypoxic WT MS5 samples compared to hypoxic Hif-1α−/− MS5 samples when considering all proteins that change significantly according to the Log transformed intensity t-test with a p value lower than 0.05. Graphs obtained using the Metascape online analysis tool.
Cellular adaptation to hypoxia is primarily mediated by the Hypoxia Inducible Factors (HIFs) [125, 127, 223, 284, 299, 398, 438, 475, 495]. Given the transcription factor nature of hypoxia inducible factors, it was first hypothesized that the increased levels of the NHEJ factors DNA-PKcs and DNA Ligase IV, and the subsequent enhanced DNA repair capacity of hypoxic mouse MS5 cells described by Sugrue et al. [424] might be due to a Hif-1-mediated transcriptional up-regulation of DNA repair genes. Hif-1α mRNA is constitutively expressed independently of oxygen levels, and the control of protein levels is achieved post-translationally through the regulation of protein stability and proteasomal degradation [223]. Therefore, the fact that we did not find any differences in Hif1a mRNA expression between the normoxic and hypoxic samples is in line with previous results. However, the fact that exposure of MS5 cells to hypoxia did not result in increased mRNA levels of either DNA-PKcs or DNA ligase IV indicates that the higher levels of these proteins in hypoxic MS5 cells described by Sugrue et al. [424] are not the result of differential mRNA expression. In contrast, significant up-regulation in the expression of all four genes analysed (Hif1a, DNA-PKcs or DNA ligase IV and Rad51) occurred 24h after irradiation with 3Gy of IR in normoxia, whereas in hypoxia this up-regulation was less pronounced and only reached statistical significance in the case of DNA-PKcs, whose IR-dependent up-regulation had been previously reported in cancer cell lines at the level of protein [410]. In addition, using commercial qPCR arrays, analysis of the mRNA expression of an array of 87 genes belonging to the DDR pathway indicated that oxygen level did not influence the expression of the vast majority of these genes, while the up-regulation of well known Hif-1α target genes AldoA, Bnip3 and Egln1 [170] demonstrates that the results obtained for the DDR genes are not due to a lack of responsiveness of MS5 cells to the decreased oxygen tension.

In order to further investigate the mechanism underlying this Hif-1α-mediated increase in radio-resistance of mouse MSCs, Hif-1α was knocked out from the MS5 mouse MSC cell line using a CRISPR/Cas9 strategy. Clonogenic survival assays performed on Hif-1α−/− MS5 cells confirmed that the effect of hypoxia in enhancing the radio-resistance of MS5 cells was indeed dependent on the presence of Hif-1α [424]. In addition, this conclusion was also reinforced by the fact that treatment of normoxic WT MS5 cells with DMOG, a PHD inhibitor that prevents Hif-1α degradation in the presence of oxygen, resulted in an increase in radio-resistance, although not to the same levels as in hypoxic cells. As described in Section 1.3.1, the regulation of HIFs in hypoxia is not only
based on the prevention of their proteasomal degradation but also involves many regulatory post-translational modifications such as hydroxylation or phosphorylation, which cannot be mimicked by simply treating the cells with DMOG. Apart from that, it must be noted that the increase in the levels of Hif-1α achieved through DMOG treatment did not reach the levels found in hypoxic cells. Therefore, these two facts alone or in combination may explain why the treatment with DMOG could not completely reproduce the levels of radio-resistance achieved in hypoxia.

Hif-1α exerts its functions as a transcription factor through the heterodimerization with Arnt. We therefore wondered whether the presence of Arnt would be necessary for the hypoxia-dependent increased survival of MS5 cells in response to IR. To test this, Arnt was knocked down in the cells using a specific siRNA, and clonogenic survival assays were performed under both normoxic and hypoxic conditions. Arnt knock-down prevented the increase in MS5 radio-resistance, therefore indicating that this effect is not only dependent on Hif-1α but also on Arnt, and that it is probably the whole Hif-1 transcription factor that is necessary to enhance the radio-resistance of MS5 cells.

However, I have previously shown that the Hif-1α-dependent increase in protein levels of the NHEJ factors DNA-PKcs and DNA Ligase IV reported by Sugrue et al. [424] was not linked to increased levels of mRNA expression in hypoxic cells. To investigate whether Hif-1α was acting through its canonical transcription factor function with regards to influencing the DDR of MS5 cells, three cell lines were generated containing different versions of the Hif1a cDNA (WT, bHLH-mutated and PAS-A-mutated) were generated. Wu et al. recently published their work describing the crystal structure of the Hif-1α-Arnt heterodimer and their interaction with the DNA double helix [491]. Thanks to this, they were able to map the specific amino acids responsible for the interaction of Hif-1α with the DNA double helix and with Arnt. By introducing the same mutations that we have introduced in the PAS-A domain (R170A and V191D), Wu et al. [491] showed that the interaction between recombinant N-terminal portions of the Hif-1α and Arnt proteins was abolished in vitro. We have now been able to confirm this result in vivo, demonstrating that these two single amino acid substitutions disrupt Hif-1α function within the cells. In addition, we have also confirmed that the two amino acids identified by Wu et al. as the main interactors with the DNA double helix (K19 and R30) are essential for Hif-1α function in vivo and their substitution (K19Q and R30Q) also abolishes the ability of Hif-1α to regulate the expression of its target genes.
Once characterized, these $Hif-1\alpha^{-/-}$ cells transduced with WT, bHLH-mutated or PAS-A-mutated Hif-1z cDNA were used to investigate the mechanism allowing Hif-1 to enhance the DDR of hypoxic MS5 cells. Clonogenic survival assays and post-IR gH2AX time-course experiments demonstrated that, while transduction of MS5 cells with WT Hif-1z cDNA could rescue the hypoxia-induced increase in radio-resistance and the increased DNA repair efficiency, the inability of Hif-1z to interact with either DNA or Arnt prevented the rescue of the WT phenotype (Figure 3.9). Therefore, both these interactions are required to enhance MS5 survival in response to IR.

In summary, my results so far demonstrate that it is the Hif-1 transcription factor (and not only Hif-1z on its own) that enhances MS5 radio-resistance in hypoxia. Given the dependence of this effect on the direct interaction with DNA, it is likely that Hif-1 is influencing the DDR of MS5 cells through transcriptional regulation. However, the fact that the vast majority of DDR factors analysed are not transcriptionally regulated in response to hypoxia indicates that this is probably an indirect effect. Therefore, we propose an update to the previous model (Figure 1.10) in which upon hypoxia, Hif-1z is stabilized and accumulates in the cells, being able to dimerize with Arnt to compose the Hif-1 transcription factor, which will then drive the expression of one (or several) unknown intermediary factor(s) that will in turn contribute to enhancing the DDR of MS5 cells and their survival post irradiation (Figure 3.14). Unfortunately, attempts to determine the identity of this intermediary factor(s) have not yet been successful. Sugrue et al. [424] showed that protein levels of the NHEJ factors DNA-PKcs and DNA Ligase IV were increased in hypoxia in a Hif-1z-dependent manner, and that DNA repair by NHEJ was enhanced in hypoxia. However, we could not detect up-regulation of the mRNA expression of these genes in hypoxia compared to normoxia. For this reason we hypothesized that the increased protein levels of DNA-PKcs and DNA Ligase IV might be due to a differential regulation of the protein turnover, and that perhaps Hif-1 coordinates the expression of proteins involved in controlling protein stability. Yet, attempts to measure DNA-PKcs and DNA Ligase IV protein half-life in normoxia and hypoxia and in the presence or absence of Hif-1z through cycloheximide treatment (an inhibitor of protein synthesis) were not successful, and this hypothesis could not be confirmed. However, preliminary proteomic analyses of the protein expression changes induced by hypoxia in both WT and $Hif-1\alpha^{-/-}$ MS5 cells indicated that proteins involved in induction of protein degradation by the proteasome (such as ubiquitinases and
Figure 3.14: Updated model for the influence of hypoxia on the DNA Damage Response of mouse MSCs. In normoxia (left panel) and hypoxia (right panel), irradiated MSCs activate DNA damage checkpoints and DNA DSB repair to resolve genotoxic DNA DSBs. Resolution of DNA DSBs likely enables irradiated MSCs to recover from cell cycle arrest and promotes MSC survival. In hypoxia (right panel), HIF-1α stabilization results in its accumulation in the cells and allows its interaction with Arnt to conform the Hif-1 transcription factor, which translocates to the nucleus and regulates the transcription of hypoxia-responsive genes. Activation of the Hif-1 transcriptional programme in MSCs results in increased DNA DSB repair capacity, accelerating recovery from cell cycle arrest and improving long-term survival. Abbreviations: DSB (Double-strand break); HIF-1α (Hypoxia inducible factor-1α); Arnt (aryl hydrocarbon receptor nuclear translocator, Hif-1β).
members of the proteasome itself) were downregulated in hypoxic WT MS5 samples, while an opposite effect was observed in the case of Hif-1α−/− cells. Although these are encouraging results, further confirmation and investigation of these results is necessary in order to establish the real impact of these changes in the Hif-1α-mediated enhancement of the DDR in hypoxic MSCs. Unfortunately, this preliminary proteomic analysis was not powerful enough to fully characterise the entire extent of proteomic changes induced by hypoxia in these cells, as demonstrated by the fact that known hypoxia-regulated proteins, such as Hif-1α itself, were not found to be significantly up-regulated in WT hypoxic samples, while western blot analysis of the same samples confirmed its differential regulation (data not shown). Therefore, more powerful proteomic analyses would be necessary in order to get the full picture of the proteomic changes induced by Hif-1.

Since its discovery in 1992 by Wang and Semenza [402], the mechanisms of HIF-1 regulation of gene expression and the identity of the whole array of genes under its transcriptional control have been under continuous investigation. Many studies have attempted to identify the complete list of genes regulated by HIFs in different cellular types and species. This has been done using different approaches ranging from genome-wide transcript profiling using expression micro-arrays to more recent chromatin immunoprecipitation coupled to high-throughput sequencing (ChIP-Seq) techniques [126, 170, 198, 307, 314, 392, 394, 433, 435, 472, 494]. One important lesson learnt from these studies is that HIF-1 dependent transcriptional control shows a high complexity and cell-type specificity, being greatly influenced by chromatin status, epigenetic marks and direct or indirect interaction with other transcription factors [114, 393]. In line with this, hypoxia and HIFs have been shown to regulate and be regulated by many members of the DNA damage response in many ways in different cell types (mainly cancer cells). To cite some examples, ATM activation in response to DNA damage has been shown to be reduced in Hif1a knock-out murine fibroblasts [488], while loss of ATM prevented the stabilization and activity of HIF-1α under hypoxic conditions [64]. Also, in human colon cancer cells, HIF-1α was shown to inhibit BRCA1 activity indirectly by counteracting C-MYC under hypoxic and normoxic conditions [243], while BRCA1 was found to enhance hypoxia-induced stabilization of HIF-1 [221]. Similarly, Parp-1 directly interacts with the HIF-1α protein and contributes to its activation in several human cancer cell lines as well as murine embryonic fibroblasts [374]. In conclusion,
these examples indicate that HIF-1α is notably interwoven with molecules centrally involved in the DDR (apart from many other cellular processes), which leads us to think that there is probably not a single mechanism for Hif-1-mediated reinforcement of the DDR and the survival capacity of MSCs, but rather that this is most likely a complex process involving the regulation of a broad transcriptional programme that will require further research in order to be fully understood.
Chapter 4

Effects of Hypoxia on the Radio-resistance of Mouse DN2 pro-T cells and Haematopoietic Stem Cells
4.1 Introduction

In adults, the bone marrow is the main organ in which haematopoiesis takes place, giving rise to all types of haematopoietic cell types originating from self-renewing, multipotent haematopoietic stem cells (HSCs) that reside in the bone marrow niche (Section 1.1) [86, 209, 371, 503]. However, unlike all other haematopoietic cells, T lymphocytes are not produced in the bone marrow but rather in the thymus [304, 305], which constitutes the optimal microenvironment for all stages of T cell development and selection (Section 1.2) [8, 287]. Under normal physiological conditions, the thymus does not contain self-renewing haematopoietic stem cells (HSC). Instead, it is continuously seeded with T cell progenitors originating in the bone marrow and (in adults) transported to the thymus through the blood [44, 74]. Once within the thymus, these progenitors receive signals from the thymic stroma that induce them to proliferate, differentiate and commit to the T cell lineage in order to become mature, functional and self-tolerant T cells [7, 11].

Developing T cells at different developmental stages can be distinguished according to their pattern of expression of the CD4 and CD8 co-receptors on their surface. From the more undifferentiated to the more mature states, ~5% of normal mouse thymocytes express neither CD4 nor CD8 (double-negative, DN cells); ~80% express both CD4 and CD8 (double-positive, DP cells); ~10% are CD4 single-positive, (CD4SP) and ~5% CD8 single-positive (CD8SP) [67]. T cell progenitors homing to the thymus express neither CD4 nor CD8 and therefore, upon entrance to the thymus, they are contained within the DN cell population [347]. DN cells can be further subdivided according to their surface expression of CD25, CD44 and CD117 into four major cellular subsets, known as DN1 to DN4. As depicted in Figure 1.4, DN1 cells, which are the most undifferentiated, can be identified as CD25<sup>-</sup>CD44<sup>+</sup>CD117<sup>+</sup>, DN2 cells are characterized as CD25<sup>+</sup>CD44<sup>+</sup>CD117<sup>+</sup>, DN3 cells are defined as CD25<sup>+</sup>CD44<sub>low</sub>CD117<sub>low</sub>, and finally DN4 cells, the most differentiated DN sub-population, are negative for all three markers (CD25<sup>-</sup>CD44<sup>-</sup>CD117<sup>-</sup>) [74, 158].

Upon irradiation, thymic cellularity is dramatically reduced as a consequence of the high radio-sensitivity of thymocytes [431]. However, a single wave of auto-reconstitution with donor cells takes place via a CD25<sup>+</sup> intermediate [512] shortly after exposure to IR as a result of the proliferation and differentiation of host-derived intrathymic radio-
resistant T cell precursors \[70, 218, 512\]. The latter were recently identified as DN2 thymocytes (Section 1.5.2) \[46\]. However, further investigation is required in order to identify the particular features allowing DN2 cells (and not other pro-T cells) to survive irradiation and resume their normal development in the thymus to generate functional mature T lymphocytes capable of re-constituting \(\sim 35\%\) of the normal T cell compartment, and displaying a polyclonal TCR repertoire, as described by Bosco et al. in 2010 \[46\].

Historically, different systems have been developed to study T cell development, including foetal thymus organ culture (FTOC), reaggregated FTOC, bone marrow chimae-ras, transgenesis, etc. \[26, 183\]. More recently, in vitro expansion and development of thymocytes using stromal cell lines ectopically expressing the Notch ligands have been used to dissect the signalling events required for T cell development \[311, 390, 391, 465\]. However, in these in vitro systems, the exact combination of signals delivered by the stromal cells as well as their intensity, which might be variable, are difficult to control. The presence of stromal cells in these cultures makes detailed genetic analysis of uniquely T cell-specific events difficult to dissect. The recent development of a stromal-free pro-T cell culture system in the laboratory of Prof. Antonius Rolink is already proving to be a very useful tool in order to study the minimal requirements necessary for T-cell commitment and differentiation \[449\]. Importantly, the pro-T cells generated and expanded in vitro using this methodology retain their normal functionality and are able to reconstitute T cell compartments of irradiated mice \[151\]. This stromal-free culture system commonly known as “The Plastic Thymus” is based on the immobilization of DL4-human IgG1-Fc (DL4-Fc) fusion protein to the surface of tissue culture plates previously pre-coated with anti-human IgG1-Fc antibody (Figure 4.1 A, Section 4.2.1.1 \[449\]). In addition, the culture medium is supplemented with IL-7 and SCF, allowing the maintenance and expansion of purified DN2 thymocytes long term in vitro \[151\]. For the purposes of the present study, the “Plastic Thymus” culture system was used to overcome the technical challenges derived from the low numbers of DN pro-T cells (especially DN1 and DN2) present in the mouse thymus, in order to generate enough DN2 pro-T cell numbers to allow characterization of the molecular pathways contributing to their high radio-resistance.

Similarly to pro-T cells, the number of HSCs that can be found in a mouse bone marrow is very low, which, as previously discussed, increases greatly the difficulty of their study
at the molecular level [78]. However, the work performed by Humphries, Sauvageau, Antonchuk et al. over the past twenty years [13,14,384,437] demonstrated that overexpression of the homeobox HOXB4 transcription factor in primary murine HSCs greatly enhances their self-renewal capacity both in vivo and in vitro, without affecting their capacity to give rise to all haematopoietic lineages. Importantly, this increased self-renewal capacity in vitro did not result in an over-expansion of the HSC compartment in transplanted mice. In contrast, the HSC pool numbers were re-established to normal pre-transplantation levels, suggesting that in vivo control mechanisms are able to regulate the expansion of HOXB4-transduced HSCs in these mice, and no anomalies in the peripheral blood were detected. HOXB4 is a member of the Hox family of transcription factors involved in the regulation of embryonic development and haematopoiesis. In line with this, HOXB4 has been shown not only to promote HSC self-renewal and expansion, but also to promote the differentiation of embryonic stem cells towards the haematopoietic lineage [140,239,332].

This system was subsequently optimized by Pineault et al. and Ruedl et al. (Figure 4.1B) [349,378], who showed that viral transduction with constructs encoding Nucleoporin 98 (NUP)–HOXB4 fusion protein resulted in even higher self-renewal capacity of primary murine HSCs, which also maintained their ability to repopulate all the haematopoietic lineages in vivo even after long periods of in vitro expansion. Furthermore, unlike fusions of nucleoporins with other members of the Hox family, ectopic expression of the NUP98-HOXB4 fusion protein did not give rise to haematological malignancies in re-constituted mice [349,378]. The exact mechanism by which fusion of the HOXB4 transcription factor to NUP98 results in this more robust expansion and survival of HSCs is not well understood [378]. However, recent findings demonstrating that nucleoporins not only control nucleo-cytoplasmic trafficking in the nuclear pore complex (NPC) but also have important functions in gene expression regulation might provide some insight as to their mechanism of action. In fact, NUP98 has been recently shown to localise to promoters of developmentally regulated genes and modulate the expression of genes that are active during differentiation of human embryonic stem cells, stimulating developmental and cell-cycle gene expression [132,219,263].

Through the Marie Curie Initial Training Network (ITN) programme of which I was part, I had the opportunity to do a two-months placement in the laboratory of Prof. Rolink, where I used the “Plastic Thymus” DN2 culture system to study the radio-biology
of DN2 pro-T cells in vitro, as well as being able to perform preliminary studies to investigate the response of DN pro-T cell subsets irradiated in vivo. In addition, NUP-HOX (NH) HSC cell lines generated in the Rolink laboratory were also used to study the DNA Damage response of murine HSCs in vitro.

4.2 Materials and Methods

4.2.1 Cell culture and treatments

4.2.1.1 In vitro expansion of DN2 thymocytes using the “plastic thymus” culture system

DN2 thymocytes were isolated from the thymi of C57BL/6 mice in the Rolink laboratory (Department of Biomedicine, University of Basel), being identified as CD4−CD8−CD44+CD25+cKit+CD127+, and sorted using a BD FACS Aria® cell sorter. As previously discussed, the “plastic thymus” culture system allows long-term expansion of DN2 pro-T cells in vitro in the absence of support from stromal cells. To do this, the surface of 6 well culture plates was coated with 2 ml of 10 μg/ml mouse monoclonal anti-human IgG1 Fc antibody (HUF5.4) diluted in sterile PBS (Sigma-Aldrich) by overnight incubation at 4°C. The PBS containing HUF5.4 was then removed and wells washed twice with 5 ml of IMDM growth medium prior to the addition of DL4-Fc at a final concentration of 2 μg/ml diluted in growth medium. 2ml of this DL4-Fc solution were added per well and incubated overnight at 4°C. The wells were then washed twice with growth medium in order to eliminate any free DL4-Fc molecules not bound to the surface of the culture plate. DN2 thymocytes were cultured in 2 ml Serum Free-Iscove’s Modified Dulbecco’s Medium (SF-IMDM) (Gibco) supplemented with 5% HyClone FCS (Thermo Scientific), 10% IL-7 supernatant, 0.1 μg/ml SCF and 0.2% Ciproxin® (Bayer Pharmaceutical) per well at 37°C in 21% or 5% O2 (Figure 4.1).

4.2.1.2 In vitro culture of NUP98-HOXB4 (NH) HSC and HSC-Bcl-2 cell lines

NH HSCs (HSC) and HSCs over-expressing Bcl-2 (HSC-Bcl-2) were previously generated from C57BL/6 CD3e−/− and H-2K.BCL-2 transgenic (Tg) mice, respectively, in the
Figure 4.1: DN2 and HSC in vitro culture systems. (A) “The plastic thymus” culture system: Tissue culture wells used for DN2 thymocyte culture were coated with DL4-Fc fusion protein bound to the well surface using anti-human IgG1-Fc antibody (HUF5.4). In addition, culture medium was supplemented with Interleukin-7 (IL-7) and Stem Cell Factor (SCF) [151]. (B) NH-HSC generation and culture system: retroviral transduction of the NUP98-HOXB4 fusion construct allows long-term expansion of HSCs in vitro. Culture medium is supplemented with Interleukin-6 (IL-6) and Stem Cell Factor (SCF) [461].
Rolink laboratory (Department of Biomedicine, University of Basel) [461], following the protocol established by Sauvageau et al. in 1995 [384] and subsequently optimized by Ruedl et al. in 2008 [378]. In brief, mice were intra-peritoneally injected with 300 µl of 10 mg/ml 5’-fluorouracil (5'-FU) prepared in PBS in order to enrich for primitive quiescent HSCs by selectively inducing death of cycling HSCs. Four days later, the bone marrow was flushed from mouse femurs; and the cell suspension obtained after erythrocyte lysis using ACK buffer was cultured for 4 days. Resulting HSCs were then transduced with a pMYc-Nup98-HoxB4-IP retroviral vector produced in Phoenix-Eco cells and expanded under selective pressure using 1 µg/ml Puromycin (Sigma-Aldrich). NH-HSC and Bcl-2-NH-HSC lines obtained following this protocol were confirmed to display the typical surface phenotype CD45+Lin−cKit+Sca-1+CD11c−CD19−B220−CD8− and were capable of successfully re-constituting all haematopoietic lineages in sub-lethally irradiated mice [461]. For the purposes of this study, NH-HSC and Bcl-2-NH-HSC lines were maintained in SF-IMDM (Gibco) supplemented with 5% FCS (Gibco), 3% v/v IL-6 supernatant, 0.1 µg/ml SCF and 0.2% v/v Ciproxin® (Bayer Pharmaceutical) at 37°C (as described in [461]), in either normoxia (21% O₂) or hypoxia (5% O₂).

4.2.1.3 Cell treatments

γ-irradiation at the indicated doses was performed using a Gammacell 40 irradiator containing a 137Cs source at a dose rate of approximately 80 cGy/minute. BrdU was added to cells at a 25 µM concentration during 1 hour. Etoposide and cycloheximide were added at final concentrations of 50 µM and 50 µg/ml, respectively.

4.2.2 Mice

C57BL/6 mice were bred under pathogen-free conditions at the Centre for Biomedicine at the University of Basel. All animal experiments were carried out within institutional guidelines (authorization numbers 1886 and 1888 from Kantonaes Veterinäramt, Basel).
4.2.3 Isolation and Sorting of mouse CD4/CD8 double negative (DN) 1-3 Subpopulations

DN cells were isolated from 5 thymi per time point after irradiation (9 Gy) and sorted according to their cell surface phenotypes. For the DN cell isolation, thymi were crushed through a 70μm pore size cell strainer in sterile IMEM (Gibco) to generate a thymocyte suspension.

CD4+ and CD8+ T lymphocytes depletion was achieved by incubating the thymocyte suspension with culture supernatants of rat IgM anti-mouse-CD4 (RL172.4) and rat IgM anti-mouse-CD8 monoclonal antibodies (3.168.8.1) [68, 75] and lysing them using Low-Tox®-M Rabbit Complement (Cedarlane®). Viable DN thymocytes were then separated from the cell debris using Ficoll-Hypaque density media (Sigma-Aldrich) and stained with anti-CD25, anti-CD44 and anti-CD117 antibodies at 4°C for 30 minutes in IMDM culture medium. DN thymocytes were then re-suspended in 500 μl of IMDM following a washing step and filtered prior to sorting using a BD FACS Aria II® (BD Biosciences). Dead cells were excluded using propidium iodide exclusion. DN1 were sorted as CD117highCD25−CD44high, DN2 as CD117highCD25+CD44high, and DN3 as CD117lowCD25+CD44low (Figure 4.11 A, B; Figure 1.4) [74]. Sorted cells were pelleted, resuspended in 100 μl IMDM and cytopun onto poly-L-lysine-coated microscope slides for immunofluorescence staining as described below (Section 4.2.9.1).

4.2.4 Growth curve analysis

DN2 cells were seeded into 6 well plates at a concentration of 10^6 cells/well and cultured in either normoxia (21% O2) or hypoxia (5% O2). Cell counts were performed every 4-5 days and an aliquot of the culture was used for surface staining analysis by flow cytometry. Relative contribution of each subpopulation (CD25+ and CD25− cells) to the total cell numbers was calculated according to the surface marker expression data obtained by flow cytometry, which allowed analysis of the growth of each subpopulation individually.
4.2.5 Clonogenic Survival Assay

NH-HSC, Bcl-2-overexpressing NH-HSC and DN2 cells were irradiated with 1-6Gy, seeded into 6-well plates at a concentration of $5 \times 10^4$ cells per well, harvested 3 or 5 days post irradiation (for NH-HSCs and DN2 cells, respectively), and cell numbers were counted in duplicate using a haemocytometer using Trypan blue for exclusion of dead cells. The percentage survival of each cell type was determined by normalizing the number of cells generated in irradiated cultures to the number of cells generated in control un-irradiated cultures.

4.2.6 Flow Cytometry Methods

Cells were harvested and counted prior to staining following the different protocols described below. Cells were then analysed using BD FACS Canto® or BD FACS Calibur flow cytometers and FlowJo® software.

4.2.6.1 Surface marker analysis by surface staining

Cells were spun at 1200rpm for 10 min and resuspended in a known volume of FACS buffer (2% FCS, 0.05% Sodium Azide, PBS) for quantification. $5 \times 10^5$ cells per sample were stained with the appropriate primary antibodies or isotype controls. If applicable, cells were then washed and stained with fluorescently-labelled streptavidin prior to analysis using a BD FACS Calibur flow cytometer (BD Biosciences) and FlowJo® software.

4.2.6.2 Cell cycle analysis using BrdU-Propidium Iodide Staining

Cells were labelled for 1h with 25μM 5'-bromo-deoxyuridine (BrdU) (Sigma Aldrich), washed in PBS and re-suspended in growth medium. Cells were harvested at the indicated time points post irradiation (4 Gy), fixed in ice-cold 70% ethanol and stained with anti-BrdU and FITC-conjugated anti-mouse IgG antibodies and Propidium Iodide (PI) / RNase staining buffer (BD Biosciences) as previously described [421]. The progression of cells through the cell cycle was analysed by measuring the percentage of
BrdU-positive cells in each G\textsubscript{1} phase until 24 h post IR using a BD FACS Canto® flow cytometer (BD Biosciences) and FlowJo® software (TreeStar Inc., OR, USA).

### 4.2.7 RNA Methods

#### 4.2.7.1 RNA isolation and retrotranscription

Total RNA was isolated from cells by TRIzol® Reagent -chloroform extraction, as described in Section [2.2.7.1](#) and cDNA was generated using Applied Biosystems’ High-Capacity cDNA Reverse Transcription Kit according to the manufacturer’s instructions.

#### 4.2.7.2 Real-time PCR

10-20ng of cDNA was used as template in semi-quantitative real-time PCR reactions with specific primers on a Step One Plus Real-Time PCR System (Applied Biosystems). The reactions were prepared with TaqMan® gene expression master mix (Thermo Fisher Scientific) using predesigned TaqMan® gene expression assays for amplification of mouse **Lig4** (DNA Ligase 4), **Prkdc** (DNA-PKcs), **Rad51** and **β-Actin** (Thermo Fisher Scientific). Gene expression changes were determined by the ΔΔCt method, using **β-Actin** as housekeeping gene for normalization and adjusting according to the primer efficiencies previously calculated.

#### 4.2.8 Western blotting

Whole cell extracts were prepared from control or irradiated cells at the indicated time points post irradiation by direct addition of 5μl of 4x Laemmli buffer per 100,000 cells, previously harvested by centrifugation, washed with ice-cold PBS and counted. Cells were disaggregated into the Laemmli buffer, heated at 95°C for 5 min and sonicated prior to separation using SDS-PAGE gels and transferred to nitrocellulose membranes. Chemiluminescence was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and medical x-ray film (Konica Minolta Medical & Graphic Imaging Inc.). Information regarding all solutions and antibodies used for western blotting can be found in Supplementary Figures [S.1](#) and [S.3](#) respectively.
4.2.9 Immunofluorescence microscopy

4.2.9.1 Immunofluorescence staining

DN cells were spun onto poly-L-lysine-coated microscope slides using a Cytospin® centrifuge (Shandon), fixed in 4% paraformaldehyde (Sigma Aldrich), and permeabilized in 0.1% Triton®-X 100 solution. Nuclei were then stained for γH2AX IRIF as previously described [421] (Section 2.2.9.1). Image Z-stacks were captured using 60X magnification on a Leica SP5 integrated microscope system (Leica Microsystems).

4.2.9.2 Image processing and IRIF quantification.

Images were deconvoluted using the Huygens Software by Scientific Volume Imaging. Image Z-stacks were projected using the maximal intensity method using Fiji [388]. Customized macros for Fiji described in sections 2.2.9.2 and 2.2.9.3 were used to adjust the projected images and blindly quantify the number of γH2AX IR-induced foci (IRIF) per nucleus.

4.3 Results

4.3.1 Hypoxia enhances HSC and DN2 radio-resistance in vitro.

As I have previously shown with thymic epithelial cells (Chapter 2) and mesenchymal stromal cells (Chapter 3) in vitro, hypoxia has cell-type dependent effects on the radio-resistance of these cells. Given these results, the influence of hypoxia on the response of HSCs and DN2 cells was also investigated. To do so, NH HSC, Bcl-2 transgenic NH HSC and DN2 cells, preconditioned in normoxia (21% O₂) or hypoxia (5% O₂) for at least a week prior to the experiments, were irradiated with increasing doses of IR and cultured again for 3 days (HSC cells) or 5 days (DN2 cells) before surviving cells were quantified. Results obtained from these survival assays (Figure 4.2) indicated that hypoxia significantly increased the survival of the three cell types investigated. Bcl-2 overexpression in Bcl-2 transgenic NH-HSC cells resulted in an increased radio-resistance in comparison with the NH-HSCs expressing WT levels of Bcl-2, as well as DN2 cells, in
both normoxia and hypoxia. When comparing NH-HSCs to DN2 cells, although at lower doses, DN2 cells show similar levels of radio-resistance to NH HSC cells (or even slightly lower), at doses higher than 2Gy, HSC radio-resistance drops dramatically, while DN2 survival is maintained at higher levels, in both normoxia and hypoxia (Figure 4.2). However, as will be later discussed (Section 4.3.6), hypoxic DN2 cultures were found to be enriched in cells not matching the typical DN2 surface marker phenotype, and therefore this must be taken into consideration when drawing conclusions regarding these experiments.

Figure 4.2: Hypoxia enhances the radio-resistance of mouse HSCs and DN2 cells in vitro. Clonogenic survival assays of (A) NH-HSC and Bcl-2 transgenic NH-HSC cell lines and (B) DN2 pro-thymocytes cultured in normoxia (21% O₂) and hypoxia (5% O₂). , #, * p<0.05; ##, ** p<0.01; ###, *** p<0.001; ####, **** p<0.0001; two-way ANOVA with Bonferroni post-test correction, n = 4 for HSC cell lines; n = 7 for DN2 cells.
Figure 4.3: Cell cycle checkpoint activation of normoxic and hypoxic DN2 cells. Representative cytograms of (A) N-HSC, (B) Bcl-2 transgenic NH-HSC, and (C) DN2 cells stained for BrdU incorporation and DNA content (propidium iodide) cultured in normoxia (21% O\(_2\)) and hypoxia (5% O\(_2\)) at different time points following BrdU pulse, with or without treatment with 4Gy of IR. Representative gating strategy for the identification of G\(_1\) phase (blue), S phase (red), G\(_2\)/M phase (green) and BrdU+ G\(_1\) cells are shown. (D) Comparison of the percentage of cells in each phase of the cell cycle in NH-HSC, Bcl-2 NH-HSC and DN2 cells cultured in 21% or 5% O\(_2\). * p<0.05, paired t tests with Holm-Sidak post-test correction, n = 3. Quantification of average percentage of BrdU-labeled G\(_1\) phase (E) NH-HSC, (F) Bcl-2-transgenic NH-HSC and (G) DN2 cells cultured in either 21% or 5% O\(_2\), 0-36h post BrdU pulse, with or without treatment with 4Gy of IR.
4.3.2 Checkpoint activation in normoxic and hypoxic HSC and DN2 cells in response to IR.

To investigate the checkpoints activated by NH-HSC and DN2 cells in response to IR, the BrdU pulse-chase method previously described (Section 4.2.6.2) was used. Figure 4.3 A-C depicts the results obtained for NH-HSC, Bcl-2-overexpressing NH-HSC and DN2 cells cultured in normoxia (21% O\textsubscript{2}) or hypoxia (5% O\textsubscript{2}) during undisturbed growth or in response to IR. Despite the fact that some technical issues arose during the generation of this data, which resulted in artifactual 2n and 4n DNA content peaks, meaningful information could still be obtained from these experiments. Quantification of the percentages of cells in each phase of the cell cycle (in normal conditions) in the different cell lines revealed marked differences in cell cycle distribution (Figure 4.3 D). While ~70% of DN2 cells were in G\textsubscript{1} phase of the cell cycle in normal conditions, only ~40% of NH-HSC and Bcl-2 NH-HSC cells were in this phase. In line with this, only ~25% of DN2 cells were actively replicating their DNA (S phase), while this proportion went up to ~50% for both HSC cell lines. Similarly, a smaller proportion of DN2 cells (~2%) than HSC cells (~6%) were in G\textsubscript{2}/M phases of the cell cycle. These results correlate with the observation of a faster cycling of both NH-HSC cell lines than DN2 cells (data not shown).

Shortly after irradiation with 4Gy, NH-HSC cells underwent extensive cell death, as evidenced by the sub-G\textsubscript{1} population (Figure 4.3 A, indicated with red arrow heads), although the number of cells that survived irradiation and resumed the cell cycle was clearly higher in hypoxia (Figure 4.3 A, 24h time point). This was also evidenced by the results of the quantification of BrdU+ G\textsubscript{1} cells (Figure 4.3 E). In line with the previous clonogenic survival results, Bcl-2-transgenic NH-HSC showed higher levels of survival following the genotoxic insult, although a large proportion of cells still underwent apoptosis quickly after IR (Figure 4.3 B, red arrow heads). Again, similar to results obtained by clonogenic survival assays, a slightly higher proportion of cells survived irradiation in hypoxia (Figure 4.3 B, F), although this effect was very subtle. This higher survival allowed a more accurate study of the checkpoints activated in response to IR. A population of late-S/G\textsubscript{2} cells accumulated 8h to 12h after irradiation, maybe indicating the activation of intra-S-phase or G\textsubscript{2}/M checkpoints (Figure 4.3 B, black arrow heads). This accumulation could also be detected in NH-HSC expressing WT levels of Bcl-2, alt-
hough to a lesser extent than in their Bcl-2-overexpressing counterparts, especially in hypoxia (Figure 4.3A, black arrow heads). In the case of the DN2 cells, lower levels of IR-induced cell death were detected, in comparison with the NH-HSC cell lines (Figure 4.3C). However, the main difference amongst the two cell types was related to the type of checkpoint activated in response to IR. Regardless of the oxygen tension, DN2 cells equally accumulated in $G_1$ in response to IR (Figure 4.3C, black arrow heads), and in fact, all the cells that were able to survive the IR treatment were those that were in $G_1$ at the time of irradiation (since BrdU-positive cells are practically non-existent in the later time points after IR) (Figure 4.3G). Taken together these results indicate dramatic differences in the cell cycle regulation between DN2 and HSC cells. However, differences observed between normoxic and hypoxic culture conditions were most likely only due to the distinct survival capacities of the cells, and not to a specific effect of hypoxia on cell cycle checkpoint regulation.

### 4.3.3 Hypoxia accelerates the resolution of DSBs in HSC cell lines, while delaying it in DN2 cells.

I have previously shown how the DNA repair capacity of mouse MSCs is enhanced in hypoxia, correlating with an increase of their radio-resistance in this condition (Chapter 3). Since hypoxia also increases HSC and DN2 radio-resistance, the kinetics of DNA DSB resolution in normoxia and hypoxia was compared. To do so, phosphorylation of H2AX in serine 139 ($\gamma$H2AX) was analysed by western blotting at different time points after irradiation with 4Gy (Figure 4.4). Both HSC cell lines behaved very similarly in terms of the kinetics of $\gamma$H2AX phosphorylation. For both of them, the $\gamma$H2AX peak occurs two hours after IR, independently of the oxygen tension. However, in hypoxia, both HSC cell lines show a faster decrease in $\gamma$H2AX phosphorylation, which can be interpreted as a faster repair of the DNA lesions. In contrast, DN2 cells show an earlier peak of the $\gamma$H2AX levels, occurring 1h after IR. However, in this case culture in hypoxic conditions results in slower kinetics of DSB repair, opposite to the results obtained with both HSC cell lines. Interestingly, DNA repair kinetics of DN2 cells seems to contradict results obtained by clonogenic survival assays. However, as previously mentioned, hypoxic DN2 cultures were subsequently found to be a heterogeneous mixture (further discussed in Section 4.3.6), which complicates the interpretation of these results.
4.3.4 Hypoxia enhances anti-apoptotic protein expression in HSC cell lines.

In light of the previous results, expression of DNA repair and apoptotic factors was analysed at the level of mRNA and/or protein (Figure 4.5). mRNA expression of the DNA repair factors DNA-PKcs (Prkdc), DNA Ligase IV (Lig4) and Rad51 in normoxia and hypoxia was analysed by real-time PCR. Culture under different oxygen levels did not cause significant changes in mRNA expression of any of the DNA repair factors analysed, in any of the three cell lines studied (Figure 4.5 A-C). However, comparison between the different cell lines indicated important cell-type specific differences. DN2 cells expressed much higher levels of the NHEJ factor DNA Ligase IV, while showing a modest but statistically significant lower expression of the HR factor Rad51. Amongst the two HSC cell lines, the only significant difference was the slightly lower expression of Rad51 in Bcl-2-transgenic NH-HSC cells compared with WT NH-HSC cells (Figure 4.5 D). Interestingly, the cell-type specific differences in DNA ligase IV expression were also detected at the level of protein (Figure 4.5 E), with DN2 cells expressing much higher levels of this protein, as well as 53BP1, another important factor for DNA repair.
via NHEJ. Hypoxic DN2 cells, however, showed decreased levels of both proteins in comparison to their normoxic counterparts, which may explain the lower DSB repair efficiency shown by these cells in hypoxia (Figure 4.4). Since no differences were found at the level of mRNA between normoxic and hypoxic DN2 cells, this difference at the level of protein is likely to be regulated in a post-translational level.

In addition, protein levels of the anti-apoptotic factors Bcl-2, Mcl-1 and Bcl-XL, and the pro-apoptotic factors Bim and Puma were analysed by western blotting. Interestingly, culture of NH-HSC under hypoxic conditions resulted in a large increase in the Bcl-2 protein levels compared to normoxia, being comparable to those seen in the Bcl-2-transgenic NH HSC cells (Figure 4.5 F), which may at least partially explain the increased radio-resistance of hypoxic NH HSC cells. DN2 cells also showed high Bcl-2 protein levels, similar to those of Bcl-2-transgenic NH HSC cells, which were not affected by oxygen tension. In contrast, HSC and DN2 cells showed different patterns of expression of the two Mcl-1 bands detected by western blotting. While DN2 cells express higher levels of the upper band regardless of the oxygen levels, both NH-HSC cell lines express equal levels of both bands in normoxia, but show preferential expression of the bottom one in hypoxia (Figure 4.5 F). The expression of the third anti-apoptotic protein analysed, Bcl-XL, showed no changes related to either cell type or level of oxygen.

With regards to the pro-apoptotic proteins, while the levels of Puma were similar between the three cell types analysed, DN2 showed much higher levels of both Bim isoforms detected, Bim-EL and Bim-L, in comparison with the NH-HSC cell lines (Figure 4.5).

**4.3.5 NH-HSC cells preferentially express a longer-lived Mcl-1 isoform in response to hypoxia.**

In the previous section (4.3.4) I described the differential expression of two Mcl-1 forms by NH-HSC cell lines and DN2 cells, as well as the hypoxia-related differences in the relative expression of the two Mcl-1 forms in both NH-HSC cell lines. Since Mcl-1 is very important for HSC survival [337], it was decided to study the nature of the two differentially expressed Mcl-1 forms. In mice, two Mcl-1 isoforms have been reported, both with pro-survival functions but different protein stability [242]. The smaller isoform, named Mcl-1V, lacks 46 amino acids in the N-terminus, a region important for the induction of
Figure 4.5: DNA repair and apoptotic proteins expression in normoxic and hypoxic HSC and DN2 cells. mRNA expression levels of Hif-1α and DNA repair factors DNA-PKcs, DNA Lig IV and Rad51 in (A) NH-HSC, (B) Bcl-2 transgenic HN-HSC and (C) DN2 cells in normoxia (21% O₂) and hypoxia (5% O₂). All values were normalized against β-Actin and expressed relative to the normoxic sample. (D) mRNA expression comparison between cell lines, normalized using β-Actin as housekeeping control and NH-HSC as the reference sample. All graphs show the average of three biological replicates. * p<0.05, multiple t-tests with Holm-Sidak post-test correction. Representative western blots of (E) DDR factors DNA Ligase IV and 53BP1; and (F) pro- and anti-apoptotic proteins in NH-HSC, Bcl-2 transgenic HN-HSC and DN2 cells in normoxia (21% O₂) and hypoxia (5% O₂).
protein degradation, and thus has a longer half-life than the bigger Mcl-1 isoform. Another possible explanation for this doublet band could be that the upper band is actually a phosphorylation product with reduced electrophoretic migration potential. In order to investigate the real nature of the two Mcl-1 versions detected, a lambda-phosphatase treatment experiment and a cyclohexamide pulse experiment were performed. In order to perform the de-phosphorylation test using lambda-phosphatase, protein extracts were treated with the phosphatase and with its inhibitor Na$_3$VO$_4$ either alone or in combination (Figure 4.6 A). Treatment with lambda-phosphatase alone resulted in the loss of the $\gamma$H2AX phosphorylation used as positive control, whereas treatment with both the phosphatase and its inhibitor Na$_3$VO$_4$ prevented this effect. In contrast, none of these treatments affected the two forms of Mcl-1, suggesting that the top band is not a phosphorylation product.

Cells were then treated with cyclohexamide, an inhibitor of protein synthesis, and the kinetics of the decay of the two Mcl-1 bands was analysed (Figure 4.6 B). In line with the information found in the literature, the smaller Mcl-1 isoform showed an increased half-life compared to the larger form, which quickly disappeared as a consequence of the cyclohexamide treatment.

### 4.3.6 Hypoxia induces phenotypic changes in DN2 cells cultured *in vitro*.

As previously mentioned, DN2 cells cultured in hypoxic conditions resulted in important phenotypic changes. Surface staining using antibodies specific for CD25, CD44 and CD117 (cKit) showed that, whereas in normoxia the typical CD25$^+$cKit$^+$CD44$^+$ DN2 phenotype is stably maintained over time, hypoxia resulted in the emergence of a greater proportion of CD25$^+$cKit$^+$CD44$^+$ cells in hypoxic DN2 cultures, and this became more prominent with time (Figure 4.7 A, B). We first hypothesized that this expanded cell population might result from the outgrowth of contaminant cell types already present in the cultures (given that a small number of CD25$^-$ cells can be also observed in normoxia), which might find hypoxic conditions more advantageous for their more rapid proliferation. In order to address this possibility, hypoxic DN2 cultures were pulsed with BrdU for 1h and subsequently stained for both CD25 surface expression and BrdU.
Figure 4.6: Identification of Mcl-1 variants. (A) Representative western blots showing γH2AX, total H2AX, Mcl-1 and β-Actin in protein extracts treated with different combinations of Lambda phosphatase (λ-Ppase) and its inhibitor Na3VO4. Protein extracts were obtained from cells treated with 50μM Etoposide for 3h in order to induce γH2AX phosphorylation. (B) Representative western blots showing Mcl-1 levels at different timepoints ranging from 0h to 4h after treatment with 5μg/ml cycloheximide.
Figure 4.7: Phenotypic changes induced by hypoxic culture in DN2 cells. Representative cytograms of DN2 cells stained for their surface expression of (A) CD25 and CD117 (cKit) and (B) CD25 and CD44, after 5 and 12 days of either normoxic (21% O\(_2\)) or hypoxic culture (5% O\(_2\)). (C) BrdU incorporation of CD25\textsuperscript{high} and CD25\textsuperscript{low} cells, after 1h treatment with 25\(\mu\)M BrdU in hypoxia (5% O\(_2\)). (D) Growth curves of CD25\textsuperscript{+} and CD25\textsuperscript{−} cells cultured in normoxia (21% O\(_2\)) and hypoxia (5% O\(_2\)).

incorporation into their DNA. Comparison of the relative amounts of BrdU incorporated by CD25\textsuperscript{+} and CD25\textsuperscript{−} cells indicated that CD25\textsuperscript{+} cells incorporated much more BrdU than their CD25\textsuperscript{−} counterparts. These results show that CD25\textsuperscript{+} cells were cycling faster than their CD25\textsuperscript{−} partners, making it unlikely that the outgrowth of CD25\textsuperscript{−} cells was due to their enhanced proliferation in hypoxia. In addition, growth analysis of normoxic and hypoxic CD25\textsuperscript{+} and CD25\textsuperscript{−} cells indicated that in general, both cell populations showed a lower proliferation rate in hypoxia. Therefore, the emergence of this CD25\textsuperscript{−} cell population in hypoxia must most likely the result of a phenotypic change of the original CD25\textsuperscript{+} “DN2-like” cells.
4.3.7 The CD25\(^{-}\) cell population arising in hypoxic DN2 cultures is a mixture of cell types from different lineages.

In order to investigate the nature of this new CD25\(^{-}\) population, the surface expression of different lineage markers by CD25\(^{-}\) cells present in normoxic and hypoxic cultures was analysed by multi-colour flow cytometry (Figures 4.8 and 4.9 respectively). Within the small CD25\(^{-}\) population, a proportion of cells were found to positively stain for B220, TCR\(\gamma\delta\), NK1.1 and CD11c, while all of them stained positively for CD117 (cKit) (Figure 4.8), as had previously been observed (Figure 4.7 A). When the same analysis was performed on CD25\(^{-}\) cells present in hypoxic cultures, the same staining pattern as in the normoxic CD25\(^{-}\) population was observed, with the only difference that in this case, the cell numbers were much greater (Figure 4.9 A). However, gating out all cells that stained positively for lineage markers showed the presence of a lineage negative population that did not express any of the lineage markers analysed in this experiment (Figure 4.9 B). In addition, this same lineage negative subpopulation was also detected to be present in the normoxic cultures, although in much smaller numbers (data not shown).

4.3.8 Hypoxia abolishes the potential of CD25\(^{-}\) cells to regenerate the CD25\(^{+}\) “DN2-like” cell population.

It was then decided to investigate whether the CD25\(^{+}\) and CD25\(^{-}\) cell populations from normoxic and hypoxic cultures had the potential to give rise to each other. Therefore, CD25\(^{+}\) and CD25\(^{-}\) cells were purified by cell sorting and then cultured under either normoxic or hypoxic conditions for three days before their phenotype was analysed again by flow cytometry (Figure 4.10). Interestingly, both CD25\(^{+}\) and CD25\(^{-}\) cells originally isolated from normoxic cultures were able to regenerate both CD25\(^{+}\) and CD25\(^{-}\) populations to some extent (Figure 4.10 A), whereas CD25\(^{-}\) cells originally isolated from hypoxic cultures failed to regenerate the CD25\(^{+}\) population (unlike their normoxic counterparts) (Figure 4.10 B). In addition, CD25\(^{+}\) cells isolated from hypoxic cultures were able to regenerate the CD25\(^{-}\) population faster than the ones isolated from normoxic cultures.
Figure 4.8: Surface marker analysis of the normoxic CD25^- cell subpopulation. Representative cytograms of CD25^- cells present in normoxic cultures stained with the lineage markers B220, TCRγδ, NK1.1, CD11c, CD11b and CD117. n=3.
Figure 4.9: Surface marker analysis of the hypoxic CD25<sup>−</sup> cell subpopulation. Representative cytograms of (A) CD25<sup>−</sup> cells present in hypoxic cultures stained with the lineage markers B220, TCRγδ, NK1.1, CD11c, CD11b and CD117; and (B) Lineage negative population of CD25<sup>−</sup> cells present in hypoxia that did not stain with any of the lineage markers used. n=3.
Figure 4.10: Analysis of the subpopulations generated from CD25⁺ and CD25⁻ cells sorted from normoxic and hypoxic cultures. Representative cytograms showing CD25 and CD44 staining of cells found in (A) normoxic and (B) hypoxic DN2 cultures, and the staining pattern of the populations arising from sorted CD25⁺ and CD25⁻ subpopulations cultured in either normoxia (21% O₂) or hypoxia (5% O₂) for 13 days after sorting.
4.3.9 \textit{In vivo} response of DN pro-T cell subpopulations to IR.

Finally, the response of the radio-resistant DN2 cell population to ionizing radiation \textit{in vivo} was studied and compared with that of their radiosensitive progenitors (DN1 cells) and progeny (DN3 cells). To do so, DN pro-T cells were isolated from either control or irradiated mice at different time points after \textit{in vivo} treatment with 9Gy, following the protocol described in Section 4.2.3 (Figure 4.11A). DN cells were then stained with specific antibodies against CD25, CD44, cKit and CD3, and DN1, DN2 and DN3 subpopulations sorted according to their surface phenotype (Figure 4.11B). The numbers of both DN1 and DN3 pro-T cells recovered dramatically dropped after the IR treatment, whereas the number of DN2 pro-T cells remained higher in proportion at all time points post-IR (Figure 4.11C-E). Interestingly, CD117 (cKit) surface expression by DN cells decreased over time following the IR treatment, an effect that was not correlated with changes in cell size (measured through the cells' FCS).

Sorted DN subpopulations were used to perform a preliminary study of the DDR of these cells \textit{in vivo}, by indirectly analysing the induction and repair of DSBs through the appearance and resolution of $\gamma$H2AX IRIF following treatment with IR (Figure 4.12). DN2 cells irradiated \textit{in vivo} show a very quick activation of the DDR pathway, as evidenced by the peak in $\gamma$H2AX IRIF formation happening after only 30min, correlating with the results obtained by western blot for \textit{in vitro} cultured DN2 cells, which also displayed a fast accumulation of $\gamma$H2AX in response to IR. Resolution of the DNA DSBs then occurs rapidly, with most of the $\gamma$H2AX IRIF having disappeared by 4h after IR. DN3 cells, however, show a delay in the formation of $\gamma$H2AX IRIF in comparison with DN2 cells, with the maximum being reached 1h after irradiation. Surprisingly, resolution of the DNA DSBs in DN3 cells happens rapidly thereafter, although the numbers of $\gamma$H2AX IRIF remain higher than in DN2 cells at all time points studied (Figure 4.12B). In addition, it must be taken into account that all times referred to in this experiment correspond to the time after IR when the thymi were isolated, and therefore the time elapsed during sample preparation and sorting until cells were fixed (~2h) is likely to have an effect on the DNA repair kinetics of the cells, which is difficult to assess in these conditions. Therefore, these times should be simply considered as a relative reference for the interpretation of these results.
Figure 4.11: Characterization of DN pro-T cells in vivo response to IR. (A) Protocol followed (from Ceredig and Rolink 2002) and (B) sorting strategy used for the purification of mouse DN pro-T cells from control and irradiated mice. (C) Number of DN1, DN2 and DN3 pro-T cells and (D) number of DN1 and DN2 pro-T cells only and (E) percentage of DN1, DN2 and DN3 pro-T cells recovered at different time points 0 to 12h after irradiation with 9Gy. (F) cKit staining and (G) MFI of DN thymocytes 0 to 12h after irradiation with 9Gy.
Figure 4.12: DNA DSB repair kinetics of sorted DN subpopulations. (A) Representative images of DN2 and DN3 nuclei stained for γH2AX IRIF and DAPI, corresponding to cells irradiated in vivo and isolated 0-12h post-IR. Scale bars correspond to approximately 10μm. (B) Average number of γH2AX IRIF per nucleus in DN2 and DN3 cells isolated 0-12h post-IR.
4.4 Discussion

Following BMT, patients undergo a period of lymphopenia until their immune system is successfully regenerated. This lymphopenia renders them susceptible to life-threatening opportunistic infections and reactivation of endogenous viruses [103, 486]. Although thymic cellularity drops drastically following irradiation due to the high radiosensitivity of thymocytes [431], many authors have reported a single wave of thymic auto-reconstitution occurring shortly after radiation exposure [70, 218, 512]. Bosco et al. recently characterized this phenomenon and determined that it was due to the survival of relatively radio-resistant CD25+, CD44+, CD117high conventional DN2 pro-thymocytes capable of recapitulating normal thymic differentiation and export to the periphery, and that they speculated that their mature peripheral T cell progeny may act as a first barrier against infections during the lymphopenic periods that follow BMT [46]. In fact, it has been shown that in some cases, host-derived anti-CMV T cells are able to protect the patients against viral infection during the lymphopenic period that follows BMT [77]. However, the mechanisms that mediate the particularly high radio-resistance of DN2 pro-thymocytes, unlike that of their radiosensitive immediate progenitors (DN1 cells) and progeny (DN3 cells), are so far poorly characterized. One of the main difficulties to be overcome in studying the molecular pathways responsible for this high radio-resistance of DN cells is their low number (especially DN1 and DN2) in the normal mouse thymus. Here, I have used “the plastic thymus” culture system to characterize the DNA damage response of in vitro-cultured DN2 pro-T cells. I have shown that DN2 cells display high radio-resistance (Figure 4.2 B), quickly activate their DDR in response to IR (Figure 4.4), preferentially induce the G1 checkpoint following irradiation (Figure 4.3) and express high levels of proteins important for DNA repair by NHEJ such as DNA Ligase IV and 53BP1 (Figure 4.5).

As previously described (Section 1.2.2), developing T cells undergo a process of rearrangement of their T cell receptor (TCR) through the mechanism of V(D)J recombination, which begins during the DN1 to DN2 transition [17]. V(D)J recombination is initiated by the introduction of DNA DSBs by the RAG1 and RAG2 proteins [147], triggering the activation of the DNA damage response and the subsequent repair of the lesions by NHEJ [29, 30, 188]. The DDR pathway is crucial for lymphocyte development as demonstrated by the fact that deficiency or loss-of-function mutations in
important DDR genes such as Atm, Nbs1, 53BP1, TopBP1 and DNA ligase IV cause multiple defects in lymphocyte development and function, resulting in aberrant V(D)J rearrangements, lymphopenia, and increased susceptibility to haematological malignancies [51,54,82,199,232,322]. The necessity for an efficient DDR pathway in place at the time of TCR gene rearrangements is crucial for lymphocyte development and may explain the particularly high levels of 53BP1 and DNA ligase IV observed in DN2 cells compared to HSCs (Figure 4.5 D, E), as well as the fast DDR activation and high efficiency of DNA DSB repair displayed by DN pro-T cells. Also, in lymphocytes, ATM and p53 have been directly implicated in limiting DSBs caused by V(D)J recombination exclusively to the G1 phase of the cell cycle [122], through the repression of Cyclin D3 protein levels in response to DNA DSBs [113]. This mechanism, which is specific for developing lymphocytes and not observed in mature T cells, may explain the preferential activation of the G1 checkpoint in DN2 pro-T cells in response to DNA damage. However, the V(D)J recombination process not only occurs in DN2 cells, but also in more advanced stages of T cell development, as well as in pro-B cells [74], which are highly radio-sensitive. Therefore, this fact alone is not sufficient to explain the particularly high radio-resistance of DN2 cells. In this regard, it would be interesting to compare the DDR of DN2 pro-T cells with pro-B cells in a similar differentiation stage. The current availability of stromal-free culture systems for the expansion of these cells [462] would greatly facilitate these studies.

In addition, in comparison with HSCs, DN2 cells also expressed particularly high levels of the pro-apoptotic factor Bim (Figure 4.5 F). Bim is an important apoptotic mediator in thymocyte biology. It has been shown to be crucial in many stages of T cell development, such as the regulation of lymphocyte progenitor survival and negative selection [49,167,190,345]. Furthermore, Bim activity is crucial for thymocyte cell death induction in response to IR [129], and it has been shown to be highly expressed in thymocytes prior to pre-TCR expression, that is, at the DN3 stage, and is down-regulated upon signalling through the pre-TCR [286]. Therefore, our observation of high levels of Bim being expressed in DN2 pro-T cells is in line with literature in the field. These high levels of Bim would be lethal for the cells if they were not counteracted by high expression of anti-apoptotic proteins such as Bcl-2 (also in line with our results), which is also highly expressed in DN pro-T cells and is down-regulated at the DP stage [162,163]. Interestingly, this high Bcl-2 expression is dependent on IL-7, an indispensable cytokine.
for T lymphopoiesis, whose receptor IL7Rα (CD127) is under the control of Notch1 signalling \[^{72,160}\], two of the main components to “The Plastic Thymus” culture system.

However, although these previous observations support the notion that DN2 pro-T cells cultured in vitro retain many of their intrinsic characteristics, which makes it a very useful tool for the study of DN2 cells at the molecular level, this culture system might have limitations, mainly derived from the lack of interaction and signalling from thymic epithelial cells. For this reason, as an indicator of DDR induction and DNA repair capacity displayed by these cells in vivo (Figures 4.11 and 4.12), the kinetics of induction and resolution of γH2AX IRIF in ex-vivo sorted DN2 and DN3 populations in response to IR was used. Cell numbers recovered at different time-points after IR indicated a faster drop in DN1 and DN3 than in DN2 cell numbers (Figure 4.11 C-E), with a higher proportion of DN2 cells retaining viability, probably due to the higher radio-resistance of this latter cell type described by Bosco et al. in 2010 \[^{46}\]. Surprisingly, cKit surface expression by DN cells was observed to decrease over time after IR. Previous studies \[^{291}\] have indicated that ckit is a target gene for Notch signalling. Therefore, the decrease in CD117 expression may be the result of decreased Notch ligand expression by thymic epithelial cells following irradiation (also discussed in Chapter 2), although further research would be necessary to confirm this hypothesis.

Interestingly, both DN2 and DN3 pro-T cells displayed discrete, single, γH2AX IRIF in the absence of IR treatment, which according to the observations from Chen et al. \[^{82}\], might correspond to sites of V(D)J recombination-induced DSBs (Figure 4.12). Similar to the results obtained for the cells in vitro, DN2 thymocytes ex-vivo displayed a fast activation of their DDR following IR, with γH2AX IRIF numbers peaking at 30min after irradiation, whereas DN3 cells show a delayed accumulation of γH2AX IRIF, reaching their maximum 1h after IR. Although the number of unrepaired DSBs was higher in the case of DN3 cells at all late time points, both cell types display high efficiency in DSB repair thereafter, as indicated by the quick disappearance of γH2AX IRIF, which was practically complete by 4h after IR. This was surprising in the case of DN3 cells, which show a higher radio-sensitivity than DN2 cells, and were expected to display a poorer DNA repair capacity. However, these results might actually indicate that the difference in survival between DN2 cells and other DN subpopulation might not be so much related to a higher DNA repair capacity but more to a distinct propensity to undergo apoptosis in response to DNA damage. In line with this, given more time, it would be interesting...
to investigate the pro- and anti-apoptotic protein expression profile of the different DN subtypes. Another important factor to take into account when interpreting this data is the fact that a viability dye was used to exclude dying cells during the sorting process. Consequently, especially at the later time points, samples might have been enriched with the few cells that were able to retain viability and repair their DNA, therefore giving the impression of a more efficient DNA repair capacity.

In contrast to DN2 pro-T cells, HSCs are highly radio-sensitive [184, 301]. Here, I have characterized the DDR of NH-HSCs cultured in vitro and compared it to that of DN2 cells, showing that NH-HSCs display higher radio-sensitivity than DN2 cells particularly at high IR doses (closer to those used as cytoreductive regimens prior to BMT), and that Bcl-2 overexpression in Bcl-2 transgenic NH-HSCs increased their radio-resistance (Figure 4.2 A). Also, cell cycle checkpoint regulation was investigated in these cells, demonstrating that, contrary to DN2 cells, which preferentially activate the G\textsubscript{1} checkpoint in response to IR, NH-HSCs rely mainly on the G\textsubscript{2}/M checkpoint (Figure 4.3). The maximum level of phosphorylation of γH2AX in response to IR is reached 2h after IR in both NH-HSC cell lines, while it occurs much faster in DN2 pro-T cells (peaking 1h after IR), indicative of a delay in the recruitment of DDR factors to DNA DSBs, which may help explain the higher radio-sensitivity of these cells (Figure 4.4). This correlates with the fact that NH-HSCs express lower levels of important DDR factors such as DNA ligase IV and 53BP1 (Figure 4.5), as well as Atm, DNA-PKcs and Nbs1 (Tara Sugrue, unpublished data). Interestingly, culturing both NH-HSC cell lines under hypoxic conditions (5% O\textsubscript{2}) increased their radio-resistance (Figure 4.2 A) and the efficiency of DNA repair (Figure 4.4) in these cells. This increased survival capacity was partially explained by the important up-regulation of Bcl-2 protein levels detected in hypoxic NH-HSCs (Figure 4.5 F), which reached similar levels to that in their Bcl-2 transgenic counterparts. However, hypoxia did not seem to further up-regulate Bcl-2 expression in Bcl-2-overexpressing NH-HSCs, which also show increased survival in this condition, therefore indicating that this is not the only mechanism accountable for the increased radio-resistance of hypoxic NH-HSCs. In addition, a differential pattern of expression of two Mcl-1 isoforms was detected depending on the levels of oxygen. While in normoxia (21% O\textsubscript{2}), the two Mcl-1 bands are equally expressed, in hypoxia there is a preferential expression of the lower form in both NH-HSC cell lines (Figure 4.5 F). According to the literature, the human MCL-1 gene encodes 3 splicing variants named MCL-1L, MCL-1S
and MCL-1ES. Of the three, only the bigger isoform (MCL-1L) has anti-apoptotic activity, the other two having pro-apoptotic functions [233]. However, in the mouse, only one smaller splicing isoform has been identified, named Mcl-1V, which lacks 46 amino acids in the N-terminus, has a longer half life than the bigger Mcl-1 isoform, and retains a comparable anti-apoptotic activity [242]. A similar MCL-1 form has also been identified in humans, however, in this case it was described as the product of a post-translational N-terminal truncation of the longer protein [109]. Apart from this, the human N-terminal truncated form of Mcl-1 also showed a longer half-life (similarly to the mouse Mcl-1V isoform), and a higher anti-apoptotic capacity than its bigger MCL-1L counterpart. Both the human and the mouse Mcl-1 versions lacking the N-terminal regions have been found to be overexpressed in a range of cancer cell types, and therefore have been proposed to be involved in enhancing cell survival [109,242]. Here I have confirmed that the lower band preferentially expressed by NH-HSC cells in hypoxia does in fact show a longer half-life (Figure 4.6), and is hence likely to correspond to the Mcl-1V isoform previously described. However, attempts to investigate whether this shorter isoform was the product of a differently spliced mRNA or a protein truncation were unfortunately not successful. Mcl-1 is very important for HSC survival [337], therefore, the preferred expression of Mcl-1V by hypoxic NH-HSCs might also be contributing to their higher radio-resistance in this condition.

Unfortunately, the number of pro- and anti-apoptotic factors that could be analysed in these cells was limited, and therefore it would be interesting to further characterize the changes in the levels of these proteins induced by hypoxia. However, although this differential pattern of expression of apoptotic factors might at least partially explain the differential radio-resistance of NH-HSCs, it does not account for the faster DNA repair observed in hypoxic cells. I have previously shown how the transcription factor HIF-1 is involved in enhancing MSC survival and DNA repair in hypoxic conditions (Chapter 3). Therefore, given more time, it would be interesting to investigate whether HIF-1 has the same role in haematopoietic stem cells.

Initially, it was thought that hypoxia also increased the radio-resistance of DN2 cells cultured in vitro (Figure 4.2A), although this observation contrasted with the fact that a less efficient DNA repair (Figure 4.4) and decreased levels of DNA ligase IV and 53BP1 (Figure 4.5) were detected in this condition. However, surface marker analysis of normoxic and hypoxic in vitro DN2 cultures demonstrated that, while DN2 cells cultured
in normoxia maintain a stable phenotype, culturing the same cells in hypoxia results in the accumulation of a CD25− subpopulation that increases in number over time (Figure 4.7). This fact makes it impossible to compare the DDR of DN2 cells in normoxia and in hypoxia, since in this last condition, a mixed population of CD25+ DN2-like cells and CD25− cells of unknown nature are analysed jointly and it is impossible to distinguish the specific contributions of each one to the overall result.

It was then decided to characterize the nature of the CD25− population arising in hypoxic DN2 cultures. Since normoxic cultures also show a small but constant proportion of CD25− cells, I first investigated whether the increase in the CD25− population was due to the overgrowth of contaminating cell types whose proliferation rates were favoured in hypoxia much more than that of DN2 cells. BrdU incorporation experiments coupled with CD25 surface marker staining demonstrated that CD25− cells incorporated less BrdU over time than CD25+ cells, therefore ruling out the possibility of an overgrowth. It was then clear that the CD25− population had to be arising from the original CD25+ cells, and since the phenotype of the CD25− coincides with that of DN1 cells, this raised the question whether hypoxia was causing DN2 cells to dedifferentiate. Since DN2 cells are not still fully committed to the T-cell lineage and still harbour other lineage potentials [18,272,295,404,409], another possible option was that the CD25+ DN2 cells were actually differentiating into other lineages instead. It was then decided to investigate the nature of the CD25− cells present in both normoxic and hypoxic cultures by analysing the expression of different lineage markers such as B220, TCRγδ, NK1.1 or CD11c (Figures 4.8 and 4.9). Our results indicate that the CD25− cell population present in both normoxic and hypoxic cultures contain fractions of cells that stain positively for all lineage markers analysed, in a similar proportion, differing only in the total number of cells which was much higher in hypoxic cultures (Figures 4.8 and 4.9), while CD25+ cells were negative for those markers in both conditions (data not shown). However, gating on the cells negative for all the different lineage markers demonstrated the existence of a lineage negative cell fraction within the CD25− cell population (Figure 4.9 B), which was present both in normoxic and hypoxic cultures, and whose nature remained unclear.

Finally, the ability of the different cell populations to give rise to each other was investigated by sorting CD25+ and CD25− populations from normoxic and hypoxic cultures and growing them either under normoxic or hypoxic conditions (Figure 4.10). Interestingly,
these experiments demonstrated that, while CD25⁻ cells originating from normoxic cultures are able to give rise to CD25⁺ cells, the same was not true for CD25⁻ cells originally sorted from hypoxic cultures, indicating that long-term culture under low oxygen levels abolishes the ability of the cells to regenerate the CD25⁺ population, even in normoxia. Surprisingly, CD25⁺ cells originally isolated from both normoxia and hypoxia and then subsequently cultured in normoxia gave rise to at least a limited number of CD25⁻ cells, providing evidence for the existence of some sort of “equilibrium” between the two populations in normoxia that is somehow disturbed by hypoxia, resulting in the accumulation of CD25⁻ cells, which then eventually lose the ability to become CD25⁺ cells again.

These results may be highlighting an important role of hypoxia in thymocyte survival and differentiation. In fact, the thymus is itself a hypoxic environment [53,175], and Hif-1α activity has been shown to regulate thymocyte survival [39]. Classical experiments using FTOCs showed that liquid-air interphase cultures gave rise predominantly to CD8⁺ T cells, while submerged cultures resulted in the preferential generation of γδ T cells [71,389], which may indicate an important role for oxygen tension in regulating T cell fate. Also, Notch1 signalling, that is essential for thymocyte proliferation and differentiation [17,360,496], is modulated by Hif-1α in hypoxic conditions [260]. However, despite all these interesting indications of the potential roles of hypoxia and HIFs in thymocyte survival, proliferation and differentiation, to the best of our knowledge, no studies have been undertaken so far that address this question, and efforts have been directed almost exclusively to the investigation of the role of hypoxia and HIFs in the modulation of the already mature T cell functions [62,300,338,434].
Chapter 5

Conclusions and Future Perspectives
F
or decades, bone marrow transplantation (BMT) has remained a well-established therapy to treat patients with a variety of lymphoid and myeloid cancers, genetic immune disorders (e.g. severe combined immunodeficiency, sickle cell anemia or thalassemia major) and various autoimmune disorders (e.g. rheumatoid arthritis and multiple sclerosis), even being the only treatment currently available for some of them \[231, 458\]. However, prior to transplantation, the patient must undergo a series of highly aggressive preparative regimens that consist of total body irradiation (TBI), usually in combination with chemotherapeutic drugs. TBI is frequently used prior to allogeneic BMT because the bone marrow (BM) is distributed throughout various regions of the body, with ~60% of total body BM located in the pelvis and the vertebrae. It also reaches sites that are unaffected by chemotherapy and represents a means for targeting cancer cells that are resistant to chemotherapeutic agents [94, 298]. Since the haematopoietic system is highly sensitive to ionizing radiation, TBI treatments result in the elimination of all endogenous HSCs and resident host immune cells, in order to allow engraftment of donor HSCs [94, 298]. The fact that the development of a donor-derived immune system after BMT is possible is a clear indication that the stromal cells that support and regulate haematopoiesis (mesenchymal stromal cells in the bone marrow and thymic stromal cells in the thymus, primarily) are, to some extent, relatively radio-resistant. However, TBI and other preparative regimens not only have important destructive effects in the host immune system, but also negatively affect all the supportive cell types that orchestrate the haematopoietic process [66, 480]. In fact, reduction of the intensity of the preparative regimens has been shown to improve HSC engraftment in the bone marrow as well as the subsequent reconstitution of the immune system, including the T cell compartment, probably due to the better preservation of the bone marrow and thymic microenvironments [25, 215]. In addition, until the immune system is successfully regenerated, the period of lymphopenia that follows BMT renders patients susceptible to life-threatening opportunistic infections and re-activation of endogenous viruses [103, 486]. In this context, the phenomenon of thymic auto-reconstitution from radio-resistant DN2 pro-T cells and migration of their polyclonal mature T cell progeny to peripheral lymphoid tissues might help provide a first line of defence [46, 70, 77, 218, 512]. Therefore, investigation of the mechanisms underlying the radio-resistance of mesenchymal stromal cells, thymic epithelial cells and DN2 pro-T cells, and the radio-sensitivity of haematopoietic stem cells can provide the basis for the development of improved pre-conditioning regimens that allow adequate depletion
of host hematopoietic cells while sparing supportive BM and thymic niches.

Nowadays, although it results in more cures and remissions than other alternative treatments, a high mortality and morbidity are still associated with allogeneic BMT and its preparative regimens, with approximately 40% of patients with advanced cancer dying following transplantation [94, 156, 231, 296, 368, 510], indicating that there is still a significant need for new strategies that improve the clinical outcomes of BMT. The DNA damage response (DDR) is the signalling network that allows cells to respond to the deleterious effects that DNA damaging agents, such as ionizing radiation, generate in their genomes [91]. For this reason, in this Thesis, I have focused on providing a deeper understanding of how the DDR contributes to the different levels of radio-resistance of MSCs, TECs, HSCs and DN2 pro-T cells, while characterizing how hypoxia, an important environmental factor in both the bone marrow and the thymus [53, 124, 175, 313, 416], influences the DDR of these cell types.

In Chapter 2, I have shown that different subsets of primary mouse thymic epithelial cells irradiated in vivo undergo an important impairment in mRNA encoding important functional factors crucial for the control and support of T cell development. Other authors have reported reduced thymic epithelial cell numbers following irradiation [339, 482], whereas we found no changes in the numbers of cells recovered from control and irradiated groups in the immediate post-irradiated thymus. In line with our results, similar observations regarding lower expression of TEC factors such as Il-7 or Ccl25 have been reported by other authors, however, they were attributed to the previously mentioned reduction in thymic cellularity [90, 501]. Since these differences are most likely due to the different time points after IR used by each group, it would be interesting to investigate more fully the kinetics of TEC depletion (if any) following irradiation, as well as that of the expression of functional factors, in order to characterize whether TECs ever fully recover functionality up to control levels with time. Foxn1, a key transcription factor for TEC biology, controlling TEC differentiation, function, and maintenance [455], has been shown to drive the expression of many TEC functional factors such as Ccl25, Cxcl12, Dll4, Scf, Aire or β5t [61, 326], most of which we have shown to be down-regulated in response to IR. Therefore, it would be interesting to investigate whether the expression of Foxn1 is also impaired in irradiated TEC subsets, correlating with the lower expression of some of its target genes. Pan et al. [339] showed recently that Foxn1 expression was increased in an IR dose-dependent manner.
when analysed 5 days after irradiation, and that thymocyte depletion caused by IR had an important role in the induction of Foxn1 expression. This is not surprising given the importance of the crosstalk between TECs and developing T cells for the biology of both cell types \([32, 41, 165]\). For this reason, it would also be interesting to investigate how the phenomenon of thymic auto-reconstitution (as well as the re-seeding of the thymus with donor-derived progenitors following BMT) might contribute to a faster recovery of thymic function.

The post-natal thymus has been shown to contain both bi-potent and mTEC-restricted progenitors, which are able to self-renew and give rise to cortical and medullary or uniquely medullary thymic epithelial cells, respectively. However, the relationship between these different types of progenitors, the possible existence of a cTEC-restricted progenitor, the exact mechanisms of TEC maintenance and regeneration in the adult thymus, their contribution to thymic involution, as well as the possible existence of a TEC stem cell niche remain poorly characterized \([177, 178, 331, 452, 489]\). Therefore, it would be interesting to investigate the effects of irradiation specifically on TEC progenitors and their ability to promote TEC regeneration. In addition, overexpression of Foxn1 has been recently shown to be sufficient to mediate the regeneration of the involuted thymi, enhancing the production of differentiated TECs from progenitor cells and up-regulating the expression of TEC functional factors, resulting in increased T cell production \([56]\). Hence, it would be interesting to investigate whether Foxn1 overexpression in irradiated TECs results in enhanced regeneration of TEC numbers and function, similar to what has been observed in the case of thymic involution.

In addition, in this chapter, thymic epithelial cell lines cTEC 1-2 and mTEC 3-10 have been used to study in detail the DDR of thymic epithelial cell lines, which has allowed the description of the role of hypoxia in decreasing the survival capacity of mTEC cells through the up-regulation of Bim protein levels. Cell lines are a very useful tool for the molecular characterization of cell types whose low numbers in the mouse pose difficulties for their study \textit{in vivo}, as it is the case for TECs. However, the use of cell lines poses certain limitations, including the effects of their immortalization and \textit{in vitro} culture, which must be taken into account before extrapolating the results obtained to the primary cells in their normal environment. Finding out which specific mechanisms are able to modulate TEC survival in response to IR can have important implications for the development of drugs capable of enhancing TEC survival following DNA damage indu-
ced by agents used as preparative regimens for BMT. Here, we have shown that, while primary mTEC MHCI$^{\text{high}}$CD86$^{-}$ cells up-regulate Bim mRNA expression in response to IR, their cortical counterparts do not show significant differences in Bim expression after irradiation, similarly to what was observed at the level of protein in the cell lines. Although this result is encouraging, it would be interesting to investigate whether this is also the case in terms of Bim protein levels in primary cells, and most importantly, whether hypoxia-mimicking treatments or HIF chemical inhibitors are able to affect these levels to match the observations made in the cell lines, therefore providing a target for the design of strategies to enhance TEC survival after IR. Similarly, investigation of the mechanisms behind the transcriptional repression of TEC functional factors and whether this is translated into protein levels could provide the means to improve TEC functional recovery following irradiation.

In Chapter 3, I investigated the molecular mechanism involved in the previously described Hif-1$\alpha$-mediated increase in MSC radio-resistance and DNA repair capacity in hypoxia [424]. The mesenchymal stromal cell compartment is highly heterogeneous, being composed of multiple sub-populations of varying function, proliferative capacity and differentiation potential that coexist and contribute to the BM niche in vivo (Section 1.1) [119, 168, 302, 335, 341, 372, 444]. As previously discussed (Section 1.5.3), this heterogeneity complicates the detailed characterization of the mechanisms that contribute to MSC radio-resistance. To overcome this difficulty, different mouse MSC cell lines have been previously used to study their DDR [421, 424], demonstrating that the response of MSC cell lines MS5 and ST2 were highly comparable to that of primary mouse MSCs, and validating them as useful tools for further studies. Here, I have used the MS5 mouse MSC cell line in order to introduce the genetic modifications that would allow dissecting the function of Hif-1$\alpha$ in the DNA damage response of Mouse MSCs. My results indicated that Hif-1$\alpha$ acts in conjunction with Arnt to form the Hif-1 transcription factor, which requires its DNA-binding capacity to induce transcriptional changes in the cells that ultimately result in an increased radio-resistance and a faster DNA DSB repair. Previous experiments using primary bulk MSCs have demonstrated that the hypoxia-induced increase in MS5 radio-resistance is also observed in primary cells cultured in vitro [424], which is an important indication that the Hif-1-dependent enhancement of the DDR of MS5 cells probably also occurs in primary mouse MSCs. However, discrepancies between self-renewal capacity, differentiation potential, surface
marker expression and propensity to become senescent between MSCs studied *in vitro* or *in vivo* \[423\] indicates that these culture conditions may alter the characteristics of the cells in some ways. Therefore, *in vivo* validation of these observations would help clarify the impact of important factors such as MSC heterogeneity and the contribution of microenvironmental factors in MSC radiobiology. In fact, studies attempting to investigate whether MSCs retain their functionality following irradiation have obtained contradictory results. While some reported no effect or even an enhancement of the differentiation potential or immunomodulatory properties of MSCs after IR, others reported an impairment of these functions \[98,107,288,412,421,470\]. These differences might reflect the effect of different IR doses, time points after IR, or origin of the MSCs. Interestingly, Singh et al. \[412\] described that, while MSCs cultured *in vitro* showed decreased osteogenic differentiation following high IR doses (18Gy), the same cells irradiated *in vivo* did not experience any decrease in differentiation potential in response to IR, therefore demonstrating that culture conditions and microenvironmental factors have a crucial role in MSC radiobiology.

The radio-resistance of MSCs can be therapeutically beneficial or unfavorable, depending on the clinical setting. While MSCs are likely to have beneficial effects in improving allogeneic BMT procedures and in treating GVHD \[422\], MSC are also known to be recruited into the tumor microenvironment \[33,180,228,289,387\] where they are transformed into cancer-associated fibroblasts (CAFs) and promote tumorigenesis through various mechanisms \[20,100,180,181,228,244\]. Here, Hif-1 has been confirmed to be an important mediator of MS5 radio-resistance, implicating the potential use of Hif-1 as a therapeutic target to modulate MSC survival in response to IR, for which verification of these results in primary mouse cells, as well as its translation to humans would be crucial. The growing interest in hypoxia and HIFs in the context of cancer therapy has resulted in the development of many drugs that allow either HIF-1 chemical inhibition or activation \[290,320\], which could be very useful for the development of these studies. Further investigation into MSC radio-resistance and our understanding of how MSCs contribute to restore hematopoiesis, modulate the immune system and sustain cancer development in response to DNA damaging agents such as IR, will likely facilitate the development of more effective therapies for BMT and cancer.

Finally, in Chapter 4, I characterised the DNA damage response of mouse HSCs and DN2 pro-T cells *in vitro*, as well as the influence of hypoxia on their radio-resistance.
Here I have shown that DN2 pro-T cells display higher radio-resistance to high IR doses, faster activation of the DDR pathway in response to IR, higher levels of expression of DDR factors that are important for NHEJ and differential checkpoint activation than NH-HSCs. In addition, hypoxia has been shown to enhance the radio-resistance and DNA repair kinetics of NH-HSCs through a mechanism that involves the anti-apoptotic proteins Bcl-2 and Mcl-1, and that most likely involves other factors. Since the Hif-1 transcription factor has been shown to enhance mouse MSC radio-resistance, it would be interesting to investigate whether it is also involved in improving HSC survival after IR, constituting again an important therapeutic target. The effect of hypoxia on the expression of pro- and anti-apoptotic factors has been described to be highly cell-type specific [403]. In fact, Bcl-2 has been reported to be both up-regulated or down-regulated in response to hypoxia in different cell types, both in Hif-1α dependent and independent ways [87, 230, 426, 469, 498]. Therefore, further research will be necessary in order to investigate the mechanism behind this strong hypoxia-mediated up-regulation of Bcl-2 protein levels. In addition, similarly to what has been discussed before, it would be important to validate these results using untransformed cells in vivo.

The characterization of the DDR of DN2 pro-T cells in vitro indicated that several aspects of their biology might be contributing to their high levels of radio-resistance. For example, the fact that they are actively re-arranging their TCR locus through the process of V(D)J recombination is probably the reason for the presence of a more active DDR and NHEJ mechanism of DNA repair, which is likely to contribute to a more efficient repair of DNA DSB. However, V(D)J recombination also occurs at other stages of T cell development, such as the DN3 stage. Comparison of the kinetics of γH2AX IRIF formation and resolution in DN2 and DN3 cells irradiated in vivo demonstrated that, although the peak in γH2AX IRIF generation is delayed in DN3 cells, both DN2 and DN3 thymocytes are able to quickly repair the DNA DSBs in their genome. Therefore, other mechanisms might account for the particularly high radio-resistance of DN2 cells, which remain to be investigated. In addition, B cells also undergo the process of V(D)J recombination during their development, but no radio-resistant pro-B cell subpopulation has been described to date in normal mice. However, B-cell auto-reconstitution has been reported in Bcl-2-overexpressing transgenic mice [121], which may indicate an important role of apoptotic pathways in this phenomenon. Therefore, the investigation of the mechanisms of DN2 radio-resistance could greatly benefit from the comparison...
of the DDR of DN2 pro-T cells and pro-B cells at an equivalent stage in their development along the B cell lineage, which would be highly facilitated by the fact that stromal cell-free cultures are currently available for the \textit{in vitro} expansion of pro-B cells [462].

Finally, I have shown that culturing DN2 pro-T cells under hypoxic conditions using “The Plastic Thymus” culture system results in the accumulation of a heterogeneous CD25− cell population expressing different lineage markers (B220, NK1.1, TCRγδ or CD11c) as well as lineage negative cells (that may or may not correspond to true DN1-like cells). The fact that hypoxia abolishes the ability of these cells to give rise to DN2-like cells upon transfer of sorted cells into normoxic conditions indicates that the level of oxygen might be influencing the developmental potential of these cells. Therefore, this might constitute an interesting system for the investigation of the signals involved in the determination of pro-T cell lineage commitment and differentiation. Further investigation will be required to fully characterize the nature of the CD25− lineage− cells arising in hypoxia, as well as whether the CD25+ cells regenerated from normoxic CD25− population are real DN2 pro-T cells, which would have important implications for the study of T cell development. Under certain conditions \textit{in vivo}, the mouse thymus has been shown to become autonomous [108] and the results obtained with hypoxia offer the possibility that a similar situation is being observed \textit{in vitro}.

In conclusion, I have characterized the multiple facets of the DNA damage response that contribute to the different levels of radio-resistance displayed by crucial cell types involved in haematopoiesis, as well as the mechanisms behind the cell-type specific effects that hypoxia exerts on the DDR of these cells. This information, together with further research regarding the radiobiology of the haematopoietic system, will likely contribute to the development of advanced therapeutic regimens for allogeneic BMT, with important implications for the improvement of its outcomes.
Supplementary material
**Table S.1: General use buffer and solutions.** In general, all solutions were made using ddH₂O and reagents supplied by Sigma (unless otherwise indicated).

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
<th>Preparation</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coomassie Blue Staining Solution</strong></td>
<td>10% Acetic Acid 40% Methanol 0.05% w/v Coomassie Blue powder</td>
<td>100 ml Acetic Acid 400 ml Methanol 0.5 g Coomassie Blue powder H₂O up to 1L</td>
<td>Clonogenic survival assay</td>
</tr>
<tr>
<td><strong>Coomassie Blue De-Staining Solution</strong></td>
<td>10% Acetic Acid 45% Methanol</td>
<td>100 ml Acetic Acid 450 ml Methanol H₂O up to 1L</td>
<td>Clonogenic survival assay</td>
</tr>
<tr>
<td><strong>DNA Loading Buffer</strong></td>
<td>0.25% Orange G 30% Glycerol</td>
<td>0.025g Orange G 3ml 100% Glycerol H₂O up to 10ml</td>
<td>DNA agarose gel electrophoresis</td>
</tr>
<tr>
<td><strong>EDTA Stock Solution</strong></td>
<td>0.5M EDTA pH8</td>
<td>95.05g Na₂•EDTA•2H₂O 9g Solid NaOH H₂O up to 500ml Adjust pH to 8</td>
<td>Various B buffers preparation</td>
</tr>
<tr>
<td><strong>FACS Buffer</strong></td>
<td>2% FBS 0.05% Sodium Azide PBS</td>
<td>20ml FBS 0.5g Sodium Azide PBS up to 1L</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td><strong>LB</strong></td>
<td>1% w/v bacto-tryptone 0.5% w/v yeast extract 1% w/v NaCl dH₂O</td>
<td>10g bacto-tryptone 5g bacto-yeast extract 10g NaCl dH₂O up to 1L Adjust pH to 7 Sterilize by autoclaving</td>
<td><em>E.Coli</em> culture</td>
</tr>
<tr>
<td><strong>LB/Agar</strong></td>
<td>1% w/v bacto-tryptone 0.5% w/v yeast extract 1% w/v NaCl 1.5% w/v Agar dH₂O</td>
<td>10g bacto-tryptone 5g yeast extract 10g NaCl 15g Agar dH₂O up to 1L Adjust pH to 7 Sterilize by autoclaving</td>
<td><em>E.Coli</em> culture</td>
</tr>
<tr>
<td><strong>1X MAE Buffer</strong></td>
<td>20mM MOPS pH7 5mM NaOAc 1mM EDTA</td>
<td>100ml 10x MAE H₂O up to 1L</td>
<td>RNA agarose gel electrophoresis</td>
</tr>
<tr>
<td><strong>10X MAE Buffer</strong></td>
<td>0.2M MOPS, pH7 50 mM NaOAc 10 mM EDTA</td>
<td>200ml 5X 1M MOPS pH7</td>
<td>RNA agarose gel electrophoresis</td>
</tr>
<tr>
<td>Buffer Type</td>
<td>Composition</td>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------------------------------------</td>
<td>--------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>5X MOPS Buffer</td>
<td>1M MOPS, pH 7</td>
<td>Adjust pH to 7 dH₂O up to 1L</td>
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</tr>
<tr>
<td>PBST</td>
<td>0.1% Tween-20® PBS</td>
<td>Western blotting</td>
<td></td>
</tr>
<tr>
<td>Ponceau-S</td>
<td>0.5% w/v Ponceau-S 5% w/v acetic acid H₂O</td>
<td>Western blotting</td>
<td></td>
</tr>
<tr>
<td>1x SDS Protein Loading Buffer</td>
<td>65mM Tris-HCl pH6.8 2% SDS 12.5 mM EDTA 10% Glycerol 2% β-mercaptoethanol 0.02% Bromophenol Blue</td>
<td>Protein extraction and SDS-PAGE electrophoresis</td>
<td></td>
</tr>
<tr>
<td>RNA Loading Buffer</td>
<td>6% Formaldehyde 1x MAE 0.04% Bromophenol Blue 6.4% Glycerol 44% Deionized Formamide</td>
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</tr>
<tr>
<td>1x Running Buffer</td>
<td>1x TG Buffer 0.1% SDS</td>
<td>SDS-PAGE electrophoresis</td>
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<tr>
<td>1X TAE</td>
<td>40mM Tris-Acetate pH8 2mM EDTA H₂O</td>
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<td>50X TAE</td>
<td>2M Tris 5.71% Glacial Acetic Acid 100mM EDTA pH8 H₂O</td>
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<td>TAIL Buffer</td>
<td>50mM Tris-HCl pH 8.8 100mM EDTA (pH 8) 100mM NaCl 1% SDS</td>
<td>Genomic DNA extraction</td>
<td></td>
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<tr>
<td>10x TBS</td>
<td>0.2M Tris-HCl pH 7.5 0.15M NaCl 16mM KCl H₂O</td>
<td>Western blotting</td>
<td></td>
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RAW TEXT: 5X MOPS Buffer
1M MOPS, pH 7
209.26g MOPS
Adjust pH to 7 dH₂O up to 1L

RAW TEXT: PBST
0.1% Tween-20® PBS
1ml Tween-20® PBS up to 1L

RAW TEXT: Ponceau-S
0.5% w/v Ponceau-S 5% w/v acetic acid H₂O
650μl 1M Tris-HCl pH6.8

RAW TEXT: 1x SDS Protein Loading Buffer (Laemmli Buffer)
65mM Tris-HCl pH6.8 2% SDS 12.5 mM EDTA 10% Glycerol 2% β-mercaptoethanol 0.02% Bromophenol Blue
β-mercaptoethanol H₂O up to 10ml
(Add 20x Bromophenol Blue to a 1x concentration after protein quantification)

RAW TEXT: RNA Loading Buffer
6% Formaldehyde 1x MAE 0.04% Bromophenol Blue 6.4% Glycerol 44% Deionized Formamide

RAW TEXT: 1x Running Buffer
1x TG Buffer 0.1% SDS
100ml 10X TG Buffer 10ml 10% SDS H₂O up to 1L

RAW TEXT: 1X TAE
40mM Tris-Acetate pH8 2mM EDTA H₂O

RAW TEXT: 50X TAE
2M Tris 5.71% Glacial Acetic Acid 100mM EDTA pH8 H₂O

RAW TEXT: TAIL Buffer
50mM Tris-HCl pH 8.8 100mM EDTA (pH 8) 100mM NaCl 1% SDS

RAW TEXT: 10x TBS
0.2M Tris-HCl pH 7.5 0.15M NaCl 16mM KCl H₂O
24.2g Tris Base 8.8g NaCl 1.2g KCl H₂O up to 1L

Adjust pH to 7.5
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<thead>
<tr>
<th>Buffer Type</th>
<th>Ingredients</th>
<th>Volume/Concentration</th>
<th>Use</th>
</tr>
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<tr>
<td>1x TBST</td>
<td>1xTBS 0.1% Tween-20®</td>
<td>100ml 10X TBS 1ml Tween-20® H₂O up to 1L</td>
<td>Western blotting</td>
</tr>
<tr>
<td>1x TE Buffer</td>
<td>10mM Tris-HCl pH 7.5-8 1mM EDTA pH8</td>
<td>100ml 10X TBS 1ml Tris-HCl pH 7.5-8 0.2ml 0.5M EDTA pH8 H₂O up to 100ml</td>
<td>DNA extraction</td>
</tr>
<tr>
<td>10X TG (Tris-Glycine) Buffer</td>
<td>250mM Tris 1.92M Glycine H₂O</td>
<td>100ml 10X TG Buffer 300ml Methanol 600ml H₂O</td>
<td>Western blotting</td>
</tr>
<tr>
<td>1x Transfer Buffer</td>
<td>1X TG Buffer 30% Methanol H₂O</td>
<td>100ml 10X TG Buffer 300ml Methanol 600ml H₂O</td>
<td>Western blotting</td>
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<tr>
<td>1.5M Tris-HCl pH8</td>
<td>1.5M Tris-HCl pH8</td>
<td>181.71g Tris Base H₂O up to 1L</td>
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<tr>
<td>1M Tris-HCl pH6.8</td>
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<td>121.14g Tris Base H₂O up to 1L</td>
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<tr>
<td>Urea Protein Extraction Buffer</td>
<td>8M Urea</td>
<td>48.48g Urea H₂O up to 100ml</td>
<td>Label-Free proteomics analysis</td>
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Table S.2: Antibodies used for flow cytometry.

<table>
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<tr>
<th>Antibody</th>
<th>Source</th>
<th>Usage Conditions</th>
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<tr>
<td>Anti-BrdU mouse monoclonal antibody</td>
<td>BD Biosciences</td>
<td>1:20 5% BSA/0.1% TrytonX100/PBS</td>
</tr>
<tr>
<td>Anti-Caspase 3 rabbit polyclonal antibody</td>
<td>Abcam</td>
<td>1:50 5% BSA/PBS</td>
</tr>
<tr>
<td>Anti-phospho-Histone H3 (Ser10) rabbit polyclonal antibody</td>
<td>Millipore</td>
<td>1:50 5% BSA/0.1% TrytonX100/PBS</td>
</tr>
<tr>
<td>FITC-conjugated anti-mouse IgG (whole molecule) antibody</td>
<td>Sigma-Aldrich</td>
<td>1:50 5% BSA/0.1% TrytonX100/PBS</td>
</tr>
<tr>
<td>FITC-conjugated anti-rabbit IgG (whole molecule) antibody</td>
<td>Bethyl</td>
<td>1:50 5% BSA/0.1% TrytonX100/PBS</td>
</tr>
<tr>
<td>FITC-anti-CD45 (30-F11)</td>
<td>BD Biosciences</td>
<td>1:100/ FACS Buffer</td>
</tr>
<tr>
<td>PECy7-anti-CD45 (30-F11)</td>
<td>BD Biosciences</td>
<td>1:100/ FACS Buffer</td>
</tr>
<tr>
<td>FITC Rat IgG2b, κ Isotype Control (A95-1)</td>
<td>BD Biosciences</td>
<td>1:100/ FACS Buffer</td>
</tr>
<tr>
<td>FITC-anti-EPCAM (BD Biosciences)</td>
<td>BD Biosciences</td>
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<td>DSHB, University of Iowa</td>
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<td>PE-anti-Ly51 (6C3)</td>
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<td>BioLegend</td>
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<td>Cy5-UEA-01 and biotinylated UEA-01 lectin</td>
<td>Vector Laboratories</td>
<td>1:100/ FACS Buffer</td>
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Table S.3: List and usage conditions of antibodies used for western blotting.

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<th>Antibody</th>
<th>Host Species</th>
<th>Source</th>
<th>Usage Conditions</th>
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<td>Anti-ATM</td>
<td>Goat polyclonal</td>
<td>Abcam</td>
<td>1:1000 5% Milk/PBST</td>
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<tr>
<td>Anti-ATR (N-19)</td>
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<td>Rabbit polyclonal</td>
<td>Sigma-Aldrich</td>
<td>1:10,000 5% Milk/PBST</td>
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<td>Cell Signaling Technology</td>
<td>1:500 5% BSA/PBST</td>
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<td>Anti-Bad</td>
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<td>Cell Signaling Technology</td>
<td>1:500 5% BSA/PBST</td>
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<td>Cell Signaling Technology</td>
<td>1:1000 5% BSA/PBST</td>
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<td>Anti-Bax</td>
<td>Rabbit polyclonal</td>
<td>Cell Signaling Technology</td>
<td>1:1000 5% BSA/PBST</td>
</tr>
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<td>Cell Signaling Technology</td>
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<td>Abcam</td>
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<td>Millipore</td>
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<td>Anti-Histone H2AX</td>
<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>1:10,000 5% Milk/PBST</td>
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<tr>
<td>Anti-Hif-1α (EPR16897)</td>
<td>Rabbit monoclonal</td>
<td>Abcam</td>
<td>1:2000 5% Milk/PBST</td>
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<td>Host Species</td>
<td>Source</td>
<td>Usage Conditions</td>
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<tr>
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<td>Millipore</td>
<td>1:200 5% FBS/PBS</td>
</tr>
<tr>
<td>Anti-Rad51</td>
<td>Rabbit polyclonal</td>
<td>Abcam</td>
<td>1:200 5% FBS/PBS</td>
</tr>
<tr>
<td>Anti-Hif-1α (EPR16897)</td>
<td>Rabbit monoclonal</td>
<td>Abcam</td>
<td>1:200 5% FBS/ 2% Goat Serum /PBS</td>
</tr>
<tr>
<td>Texas Red-conjugated AffiniPure $F(ab′)_2$ Fragment goat anti-rabbit IgG antibody</td>
<td>Jackson ImmunoResearch Laboratories</td>
<td>Jackson ImmunoResearch Laboratories</td>
<td>1:200 5% FBS/PBS</td>
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<tr>
<td>Texas Red-conjugated AffiniPure $F(ab′)_2$ Fragment goat anti-rabbit IgG antibody</td>
<td>Jackson ImmunoResearch Laboratories</td>
<td>Jackson ImmunoResearch Laboratories</td>
<td>1:200 5% FBS/ 2% Goat Serum /PBS</td>
</tr>
</tbody>
</table>

Table S.4: List and usage conditions of antibodies used for immunofluorescent staining.
Annexe 1: Fiji macro for image processing

// This macro converts all files in a directory to TIFF using the Bio-Formats macro extensions. Then it adjusts the brightness/contrast according to the parameters specified by the user.

// The user must provide with a value for minimum and maximum values to display in each channel.

// The input directory must contain only the .dv files to process.

// The output directory must be empty.

varminBlue = 100;
varminGreen = 70;
varminRed = 230;
varmaxBlue = 500;
varmaxGreen = 100;
varmaxRed = 200;
directory = getDirectory("Chose input files");
fileList = getFileList(directory);
outputDirectory = getDirectory("Choose output directory");
run("Bio-Formats Macro Extensions"); setBatchMode(true);
for (i=0; i<fileList.length; i++) {
    file = directory + fileList[i];
    Ext.setId(file);
    Ext.getImageCount(imageCount);
    for (image=0; image<imageCount; image++) {
        Ext.openImage("", image);
        outFile = outputDirectory + fileList[i] + "+" + image + ".tif"
        saveFile(outFile);
    }
    Ext.close();
    Ext.openImagePlus(file);
    outFile = outputDirectory + fileList[i] + ".tif";
    saveFile(outFile);
}

// At this point, we have all the .tif (3 for each .dv, as they are split by channel) in the output directory

// We have to get a filelist for the output, where the .tif are.
fileListOut = getFileList(outputDirectory);
outDirAdj = outputDirectory;
for (i=0; i<fileListOut.length - 1; i = i + 3) {
    file = outputDirectory + fileListOut[i];
    // Opening each channel and adjusting display range open (fileListOut[i]);
    setMinAndMax(minBlue, maxBlue);
    open (fileListOut[i+1]); setMinAndMax(minGreen, maxGreen);
    open (fileListOut[i+2]); setMinAndMax(minRed, maxRed);
    // Merging the three channels run("Merge Channels...", "c1="+ fileListOut[i+2] +" c2 ="+ fileListOut[i+1] +" c3="+ fileListOut[i] +" create");
    out1 = substring(fileListOut[i], 0, lengthOf(fileListOut[i]) - 10);
    // Saving into an "adjusted" image saveAs("Tiff", outDirAdj + out1 + "_ADJ.tif");
    close();
// Deleting old images
for (int i = 0; i < fileListOut.length; i++) {
    tmp1 = File.delete(outputDirectory + fileListOut[i]);
    tmp2 = File.delete(outputDirectory + fileListOut[i+1]);
    tmp3 = File.delete(outputDirectory + fileListOut[i+2]);
}

// Creating a log after processing
getDateAndTime(year, month, dayOfWeek, dayOfMonth, hour, minute, second, msec);
outLog = outDirAdj + "log.txt";
outTxt = File.open(outLog);
print(outTxt, dayOfMonth + "-" + month + "-" + year + "	" + hour + ":" + minute + ":" + second);
print(outTxt, " ");
print(outTxt, "------------------------------------------\n")
print(outTxt, "Parameters used:\n")
print(outTxt, " ");
print(outTxt, "minBlue = \t " + minBlue + "\tmaxBlue = \t " + maxBlue);
print(outTxt, "minGreen = \t " + minGreen + "\tmaxGreen = \t " + maxGreen);
print(outTxt, "minRed = \t " + minRed + "\tmaxRed = \t " + maxRed);
print(outTxt, "------------------------------------------\n")
File.close(outTxt)

// Showing the confirmation message
showMessage("Info", "<html>" +"<font size=+1>Process finished successfully!! Congrats!<br>" +"<br>");
showStatus("Finished."); setBatchMode(false);
Annexe 2: Fiji macro for IRIF quantification

// This macro automatically counts the foci present in two channels of the input
// multichannel TIFF images.
// The user must provide with a value for the noise tolerance and the particle size.
// The input directory must contain only the .tif files to process.
run("Set Measurements...", "area mean min integrated redirect=None decimal=3");
var noise_tolerance_C2 = 500;
var noise_tolerance_C1 = 500;
var particle_size = 3000;
directory = getDirectory("Chose input files");
fileList = getFileList(directory);
outputDirectory = getDirectory("Choose output directory");
// In this function, the user will be asked to select a threshold and verify
// that the particles detected are correct.
// If not, the user will be able to add/remove appropriate particles.
function process_image(inputDir, currFile) {
auxFile = inputDir + currFile;
auxwinC1 = "C1-" + fileList[i];
auxwinC2 = "C2-" + fileList[i];
auxwinC3 = "C3-" + fileList[i];
outPathC1 = outputDirectory + substring(auxwinC1, 0, lengthOf(auxwinC1) - 3) + "xls"
outPathC2 = outputDirectory + substring(auxwinC2, 0, lengthOf(auxwinC2) - 3) + "xls"
open (auxFile);
// Channels are split
run("Split Channels");
selectWindow(auxwinC3);
run("Threshold...");
// The user can specify the threshold
title = "WaitForUser";
msg = "Set the threshold, then click "OK".";
waitForUser(title, msg);
// The user can add/remove appropriate particles if necessary
run("Analyze Particles...", "size=particle_size Infinity pixel show=[Overlay

Outlines]
display exclude clear include add");
run("Clear Results");
title = "WaitForUser";
msg = "If necessary, delete the wrong particles, then click "OK".";
waitForUser(title, msg);

// Finding the points with intensity greater than the specified threshold for the second channel
selectWindow(auxwinC2);
run("Find Maxima...", "noise=noise_tolerance_C2 output=[Single Points]");
roiManager("Show None");
roiManager("Show All");
roiManager("Measure");

// Saving the results into an .xls file
saveAs("Results", outPathC2);

// Finding the points with intensity greater than the specified threshold for the first channel
selectWindow(auxwinC1);
run("Find Maxima...", "noise=noise_tolerance_C1 output=[Single Points]");
run("Clear Results");
roiManager("Show None");
roiManager("Show All");
roiManager("Measure");

// Saving the results into an .xls file
saveAs("Results", outPathC1);
close("*");
run("Clear Results");
title = "Do you wish to continue?";
msg = "Results successfully saved for " + currFile + ". To continue, press \"OK \".";
waitForUser(title, msg);

for (i =0; i<fileList.length; i++) {
    file = directory + fileList[i];
    process_image(directory, fileList[i]);
}

title = "Finished";
msg = "A total of " + fileList.length + " images have been analyzed";
waitForUser(title, msg);
Table S.5: TEC DNA Damage Response gene expression analysis. List of genes showing greater than 2-fold up- or down-regulation and/or p value lower than 0.05.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold Change</th>
<th>p Value</th>
<th>Gene Symbol</th>
<th>Fold Change</th>
<th>p Value</th>
<th>Gene Symbol</th>
<th>Fold Change</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdkn1a</td>
<td>9.7867</td>
<td>0.049561</td>
<td>Rpa1</td>
<td>6.6947</td>
<td>0.4525</td>
<td>Ogg1</td>
<td>-2.2717</td>
<td>0.068328</td>
</tr>
<tr>
<td>Ogg1</td>
<td>6.69</td>
<td>0.027358</td>
<td>Mbd4</td>
<td>2.6039</td>
<td>0.086309</td>
<td>Mbd4</td>
<td>5.112</td>
<td>0.018754</td>
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<tr>
<td>Gadd45a</td>
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<td>Fancc</td>
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<td>0.306775</td>
<td>Mbd4</td>
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<tr>
<td>Mbd4</td>
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<td>0.018754</td>
<td>Mlh1</td>
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<td>Rad18</td>
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Figure S.1: Cell sorting strategy of untreated mouse primary TEC subpopulations. Gating strategy and post-sort purity data of untreated TEC subpopulations.
Figure S.2: Cell sorting strategy of irradiated mouse primary TEC subpopulations. Gating strategy and post-sort purity data of irradiated TEC subpopulations.
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**Figure S.3: Hif-1α antibodies and siRNA validation.** Representative western blots of MS5 cells untreated or treated with control siRNA (Scr.), two different commercial Hif-1α siRNAs alone or in combination, or 500μM of DMOG, in normoxia (N) (21% O₂) or hypoxia (H) (2% O₂), probed with different antibodies against Hif-1α. Commercial normoxic and hypoxic HeLa protein extracts were used as control for one of the antibodies.
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Figure S.4: Reference vs. MS5 Hif-1α cDNA sequence comparison. Alignment of the Hif-1α cDNA sequence isolated from MS5 cells against the mouse Hif-1α cDNA reference sequence found in the NCBI database used to design the CRISPR/Cas9 Knock-out strategy. The initial part of the 5'UTR and the 3' UTR are not shown. Coding sequence is shown in blue, the two alternative start codons are indicated in green, the stop codon is indicated in orange and the differences between the two sequences are highlighted in red. gRNA sequences are indicated with purple boxes, and Cas9 cut sites are indicated with red arrows.
**Figure S.5: Reference vs. MS5 Hif-1α protein sequence comparison.** Alignment of the Hif-1α protein sequence isolated from MS5 cells against the mouse Hif-1α protein reference sequence found in the NCBI database. Protein domains are described according to the information found in the UniProt Database: Red = bHLH domain; Green = PAS Domains; Blue = ODD Domain; Turquoise = N-TAD Domain; Orange = C-TAD Domain. Differences between the two sequences are highlighted in bold and underlined.
References


mal cells display variable anatomic site-dependent response and recovery from irradiation.

DNA double-strand breaks.

City of human bone marrow-derived mesenchymal stromal cells

1193.

lineage-specific and developmental stage specific mechanisms suppress cyclin D3 expression in response

Classification of Diffuse Large B-cell Lymphoma Subtypes by Their Protein Expression Profiles

Intrathymic progenitor cell transplantation across histocompatibility barriers results in the persistence of early thymic progenitors and T-cell differentiation.


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inhibited by IL-7 signaling dependent DN3 to DP transition requires Notch signaling, is improved by CXCL12 signaling and is bone marrow multipotent stromal cells. (2012), pp. 169–182.


G. J. Williams, S. P. Lees-Miller, and J. A. Tainer, Mre11-Rad50-Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks, DNA Repair (Amst.), 9 (2010), pp. 1299–1306.


