Vrije Universiteit Brussel

Katholieke Universiteit Leuven

Universiteit Antwerpen

Interuniversity Program Molecular Biology (IPMB)

The role of p53 in radiation-induced microcephaly in mice

Thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science in Molecular Biology

Haris Bin Fida

Promoter: Dr. Roel Quintens
Supervisor: Andre Claude Mbouombouo Mfossa
Radiobiology Unit, SCK•CEN

Co-promoter: Prof. Dr. Luc Leyns
Laboratory of Developmental and Stem Cell Biology,
Vrije University Brussel

Academic year 2017-2018

DISCLAIMER

This MSc thesis with title "The role of p53 in radiation-induced microcephaly in mice" contains confidential and proprietary information belonging to the Studiecentrum voor Kernenergie/Centre d'Etude de l'Energie Nucléaire [also known as the Belgian Nuclear Research Centre], Foundation of Public Utility, SCK•CEN or "Party", with its Registered Office in Belgium, Avenue Herrmann-Debroux 40, BE-1160 BRUSSELS and its Operational Office also in Belgium, Boeretang 200, BE-2400 MOL, with enterprise number 0406.568.867 and VAT number BE406.568.867. Any communication or reproduction of this thesis, and any communication or use of its content without explicit authorization is prohibited. Any infringement to this rule is illegal, and entitles to claim damages from the infringer, without prejudice to any other right, e.g. in case of granting a patent or registration in the field of intellectual property. Vis-à-vis the Vrije Universiteit Brussel, reference is made to the applicable "Raamovereenkomst wetenschappelijke samenwerking VUB - SCK•CEN" dated 28/10/2008, and the resulting "Projectovereenkomsten". Vis-à-vis the author, reference is made to the applicable general internship conditions and declaration of secrecy both dated 07/09/2017. This thesis is distributed only to a limited list of involved people under confidentiality agreement. The content does not jeopardize the novelty of underlying inventions.

Acknowledgments

Foremost, I would like to express my sincere gratitude to my promoter Dr. Roel Quintens for his immense support and guidance. I am highly thankful to Andre Claude Mbouombouo Mfossa, you have been a tremendous mentor and support for me. I would like to thank you for your critical suggestions which really helped me out in getting the right direction during my experiments and thesis writing. I would also like to thank rest of the members of the Radiobiology Unit, for their support, and particularly Prof. Sarah Baatout who always inquired about my progress and motivated me. My sincere thanks to Dr. Mieke Verslegers who helped me out in image analysis and always gave me effective feedbacks.

I am highly thankful to the IPMB coordinator Dr. Vincent Delespaux, Rudi Willems and Greta Verhasselt. All of you have been there to support me throughout my academic period. A special thanks to my co-promoter Prof. Dr. Luc Leyns, Dr. Lieve Moons, and Dr. Mohamed Ahmed for their time, and critical evaluation of my work.

A special gratitude to my parents and family members. Words cannot describe for their immense support, motivation, and encouragement throughout my years of study. Especially I am thankful to my dearest brother, Talha Bin Fida who always stood by to me in difficult moments, and always been a source of motivation to me.

Summary

Objective: A previous study suggested that radiation-induced p53-mediated gene activation leads to the development of microcephaly. We, therefore, investigated the potential role of p53 in the development of radiation-induced microcephaly in mice.

Methods: *Trp53* conditional knock-out (cKO) mice were X-irradiated at E11 and E14, and brains were harvested and compared with *Trp53* wild-type (WT) mice. We used immunohistochemistry to assess the underlying cellular and molecular mechanisms in relation to p53.

Results: After radiation exposure, WT mice showed a significant decrease in their brain size in comparison to non-irradiated WT mice. In contrast, irradiated cKO mice showed the partial restoration of their brain size. Further immunohistochemical analysis revealed the massive apoptosis in the irradiated WT brains, whereas the irradiated CKO brains showed significantly less apoptosis. Besides, radial glial cells depletion was observed in irradiated WT brains, followed by an ectopic immature neurons emergence, and decrease expression of an epithelial marker Qki5, indicating the premature neuronal differentiation. In contrast, premature neuronal differentiation was prevented in the irradiated cKO brains.

Conclusion: Our results show that p53 plays a significant role in the development of radiation-induced microcephaly, which is partly on account of its pro-apoptotic activity. However, we also demonstrated the potential involvement of p53 in the premature neuronal differentiation which may involve an EMT like mechanism, hence provides an interesting reference for future research.

Keywords: central nervous system, ionizing radiation, p53, apoptosis, microcephaly, neuronal differentiation.

List of Abbreviations

aIPCs	Apical intermediate progenitor cells
aRGCs	Apical radial glial cells
ASPM	Abnormal spindle-like microcephaly-associated protein
ATM	Ataxia Telangiectasia Mutated
Bax	Bcl2-associated X protein
bIPCs	Basal intermediate progenitor cells
Bq	Becquerel
bRGCs	Basal radial glial cells
CDKS	Cyclin-dependent Kinases
CHK2	Checkpoint Kinase2
cKO	Conditional knock-out
CNS	Central nervous system
CP	Cortical plate
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSBs	Double strand breaks
E	Embryonic day
EMT	Epithelial to mesenchymal transition
GW	Gestational week
Gy	Gray
hNPCS	Human neuronal progenitor cells
HRR	Homologous recombination repair
IKNM	Interkinetic nuclear migration
IPCs	Intermediate progenitor cells
IQ	Intelligence quotient
IR	Ionizing radiation
ISVZ	Inner subventricular zone
L	Layer

LET	Linear energy transfer
MCPH	Autosomal recessive primary microcephaly
MDM2	Mouse double minute 2 homolog
MRN	Mre11, Rad50, Nbs1
MZ	Marginal zone
NECs	Neuro-epithelial cells
NHEJ	Non-homologous end joining
NPCs	Neuronal progenitor cells
OSVZ	Outer subventricular zone
PP	Preplate
RF	Radio frequency
RGCs	Radial glial cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Sievert	Sv
SP	Subplate
SVZ	Subventricular zone
VZ	Ventricular zone
WT	Wild-type
ZIKV	Zika virus

Table of Contents

1 Chapter 1: Introduction

1.1 Introduction

The central nervous system (CNS) development is an extensive process which initiates from the embryonic period and continues until adolescence. Among all the body systems, the CNS is the most susceptible to developmental injury (1, 2). In humans, the early fetal period (8 to 15 week) is being described to be the most vulnerable to environmental insults like ionizing radiation (IR) (3). During this period neurons are extensively generated and migrate towards their respective position in the neocortex where they will ultimately form networks necessary for cognitive functions (4–6). Any perturbation of this process by IR might result in altered brain structure and function (4).

The impacts of prenatal radiation exposure on the developing brain had been evidenced by many epidemiological studies (4, 5, 7, 8). For instance, epidemiological studies on the atomic bomb and fallout survivors of Hiroshima and Nagasaki (1945) indicated the occurrence of severe mental retardation which was often associated with microcephaly (small head size). These deleterious effects were found to be more pronounced in survivors who were exposed *in utero* between a gestational week (GW) 8 to 15, and to a lesser extent between GW16 to 25 (5, 9).

A previous study at the Radiobiology Unit of the Belgian Nuclear Research Centre (SCK•CEN) has demonstrated that radiation exposure at embryonic day 11 (E11), which corresponds to the onset of neurogenesis in mouse, results in the development of microcephaly (10). Furthermore, they showed that shortly after IR exposure gene expression changes in the embryonic brain are mostly regulated by the tumor suppressor p53 (11). Interestingly these genes were found to highly overlap with those induced in $Magoh^{+/-}$ mouse which is a genetic mouse model of microcephaly (12), and more relevantly to those induced in Zika Virus (ZIKV) infected human neuronal progenitor cells (hNPCs) (12), as ZIKV infection during pregnancy is linked to the development of congenital microcephaly (13, 14). Based on these findings, we hypothesize that early p53-dependent gene activation after radiation exposure might be responsible for the microcephaly later in life.

To verify the validity of this hypothesis, another study was conducted at SCK•CEN. For this study *Trp53* conditional knock-out (cKO) mice were bred. These cKO mice were irradiated and their brains were compared with p53 wild-type mice (WT mice). The results indicated that after radiation exposure, WT mice showed a reduction in the brain size. In contrast, the brain size of cKO mice was significantly larger than those of WT mice (unpublished results). These results clearly indicate that p53 has a key role in the development of radiation-induced microcephaly. However, the molecular and cellular mechanisms behind the radiation-induced microcephaly are not yet fully dissected.

Therefore, the aim of this study is to illuminate the possible cellular and molecular mechanisms behind the etiology of this neurodevelopmental disorder in relation to p53. Understanding these mechanisms will help especially pregnant women who suffer from cancer. The probability of having cancer during pregnancy is 1 in 1000, which is quite low

but not negligible (15). During pregnancy, women can encounter several types of cancers, out of which cervical and breast cancer are the most common ones (16). Cancer during pregnancy is challenging as it is difficult to maintain the balance between the benefits and risks associated with radiotherapy for the mother and the fetus (17). Radiation exposure during pregnancy, particularly in the early fetal period (GW8 to 15 after conception) is teratogenic and results in growth and mental retardation, including microcephaly (3). On account of these side effects most often it is advisable to defer the radiotherapy until the delivery of the child, and or in some cases, termination of pregnancy is suggested (18).

In the following chapter, a detail literature review is given. This review covers mammalian central nervous system development with particular emphasis on the cerebral cortex. Furthermore, a detail description of different types of IR, and their biological impacts is given, which will help out in understanding the deleterious effects of IR at the cellular level. Finally, an overview of the epidemiological and experimental data, with the aim of providing a deep understanding of the topic of our study.

2 Chapter 2: Literature Review

2.1 Mammalian Central Nervous System

The mammalian CNS comprises the brain and the spinal cord. The present form of mammalian CNS is the result of million years of evolution, enabling them in higher behavioral and cognitive capacities. Among mammalian brains, the human brain is more complex, and it can be exemplified on account of the higher intellect and cognitive abilities of humans (19).

The development of the human brain is an extensive process which initiates from the third gestational week (GW3) and remains in progress throughout the childhood till adolescence. The third week of gestation is marked by profound changes like gastrulation during which the embryo transforms into the three-layered structure. The uppermost layer comprises of epiblast cells which differentiate into three primary stem cell lines, and give rise to the various structures in the developing embryo. Among these stem cell lines, neural stem cells give rise to different cells that form the brain and central nervous system (20).

After gastrulation, rudimentary structures of the brain begin to develop and it starts with the assembly of neural progenitor cells in the vicinity of the rostral-caudal midline of the developing embryo. This zone of neural progenitor cells is termed as the neural plate and surrounding this neural plate the neural tube starts to develop. Neural tube development (neurulation) initiates with the appearance of ridges which subsequently fold and fuse together to form a hollow tube-like structure. Right before the closure of the neural tube its anterior region expands and differentiates into three primary brain vesicles: the Prosencephalon, Mesencephalon, and Rhombencephalon (Figure 2-1). The prosencephalon gives rise to the forebrain, whereas mesencephalon and rhombencephalon give rise to the midbrain and hindbrain respectively (20, 21).

The forebrain (prosencephalon) is further subdivided into the telencephalon and diencephalon. The telencephalon develops into the cerebral cortex, whereas the diencephalon develops into the thalamus and hypothalamus (22).

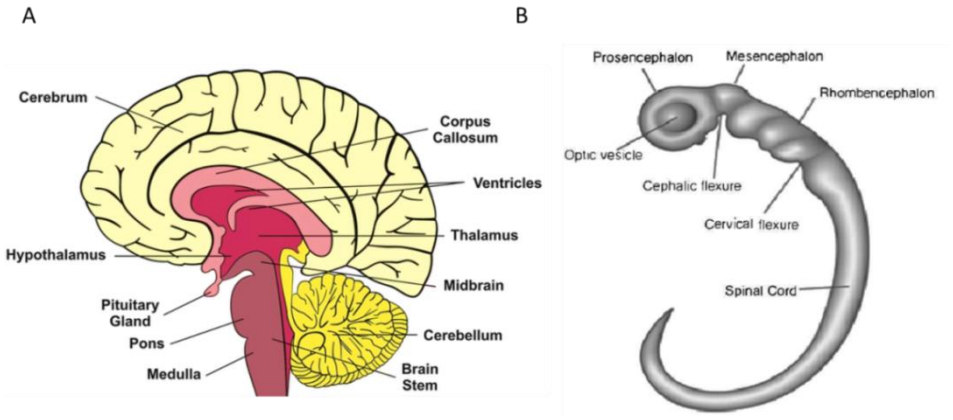

Figure 2-1 Human brain anatomy.
Anatomical division of brain (A). Subdivision of the anterior portion of the brain into three primary vesicles: the Prosencephalon, Mesencephalon, and Rhombencephalon (B) (20, 23).

2.1.1 Human Cortex Development

The cerebral cortex is the most external part of the brain, and comprises two bilaterally symmetric structures known as hemispheres (24). In the evolutionary context, it is the most recent addition to the mammalian brain that helps out in the development of cognitive abilities such as sensory perception, motor skills as well as language, emotions, and conscious thoughts (22). Another attribute of evolution is the addition of folds (sulci) and ridges (gyri) in the cortex due to which the large volume of the brain can be confined in the small cranial vault (22, 25). The cerebral cortex is composed of 6 layers of cells, which are generally classified into neurons and glia. Neurons can be categorized broadly into glutamatergic projection neurons and GABAergic inhibitory neurons, and together they constitute the functional part of the cortex, whereas glia can be categorized into astrocytes, oligodendrocytes, and microglia, with further several sub-classifications (22).

The development of the cerebral cortex arises from the neuro-epithelial cells (NECs) at the rostral end of the neural tube. NECs are stem cells which possess epithelial features and exhibit polarity along their apical-basal axis (27). Before the onset of neurogenesis, the pool of NECs is considerably low to such an extent that they are unable to manage the massive neuron production required for the development of the cortex. In order to increase their population, they deploy the symmetrical mode of division between the GW4 and 5. This mode of division allows the self-renewal of NECs. As the NEC division progresses, their nuclei oscillate between the apical and basal surface of the ventricular zone (VZ). During G1 phase the nuclei of the NECs migrate to the basal side of the VZ, remain there till S phase, and then again migrate back to the apical surface during the G2 phase and undergo symmetric division (Figure 2-2). This mode of oscillation is termed as interkinetic nuclear migration (IKNM), which results in a profound increase in the surface area and thickness of the VZ (28,

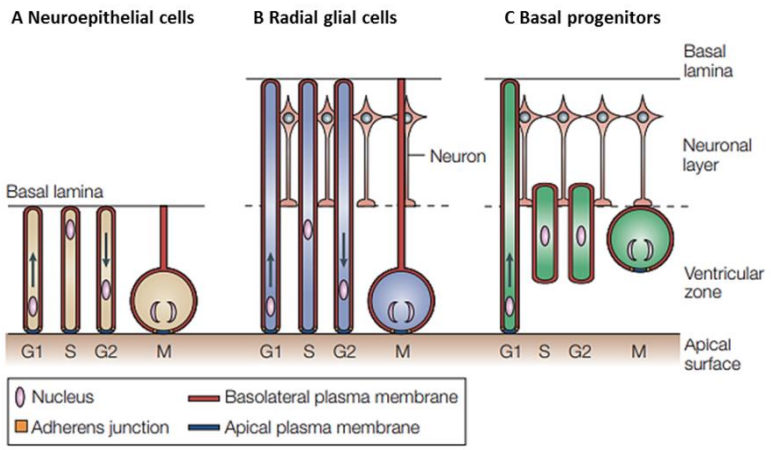

Figure 2-2 Interkinetic Nuclear Migration of neuro-epithelial cells, radial glial and basal progenitor cells.
As the cell cycle progresses, nuclei of NECs move gradually to the basal surface during G1phase, stay there till S phase completion and move back to the apical surface during the G2 phase and mitotically divide (A). Radial glial cells show the similar kinetics of IKNM except their nuclei don't migrate through the whole length of the cells (B). The nuclei of basal progenitors migrate to the basal surface during the G1 phase, and remain there till S, G2 and M phase, followed by their retraction from the apical surface of VZ (C) (26).

29). Before the onset of neurogenesis, NECs undergo various gene expression and cytological changes, and give rise to radial glial cells (RGCs) (30). Cortical neurogenesis initiates around GW 5, which is characterized by a gradual shift of RGCs to the asymmetrical mode of division. RGCs are neural progenitors which are distinct but related to NECs, as they retain neuro-epithelial features, and also exhibit the astroglial properties (30). Through asymmetric division, RGCs give rise to a combination of either RGC and a post-mitotic neuron or RGC and an intermediate progenitor cell (IPC). On the other hand, these RGCs through the junction of the apical and basal surfaces act as scaffolding surfaces for the migration of post-mitotic neurons. These post-mitotic neurons occupy their respective positions in the cortical plate (CP) giving rise to the six-layered neocortex in an "inside-out" manner (28, 30).

IPCs are of two different types: apical IPCs (aIPCs) and basal IPCs (bIPCs), and through symmetrical cell division produce neurons, constituting the population of neurogenic cells in the VZ and subventricular zone (SVZ) respectively. The SVZ in primates including humans spans the larger area comprising of inner SVZ (ISVZ) and an outer SVZ (OSVZ), separated by a delicate layer of fiber. The OSVZ also plays an essential role in the production of neurons by means of basal RGCs (bRGCs) which show the similar properties as that of apical RGCs (aRGCs) in the VZ. In humans, the massive expansion of bRGCs accounts for the larger brain size (31–33).

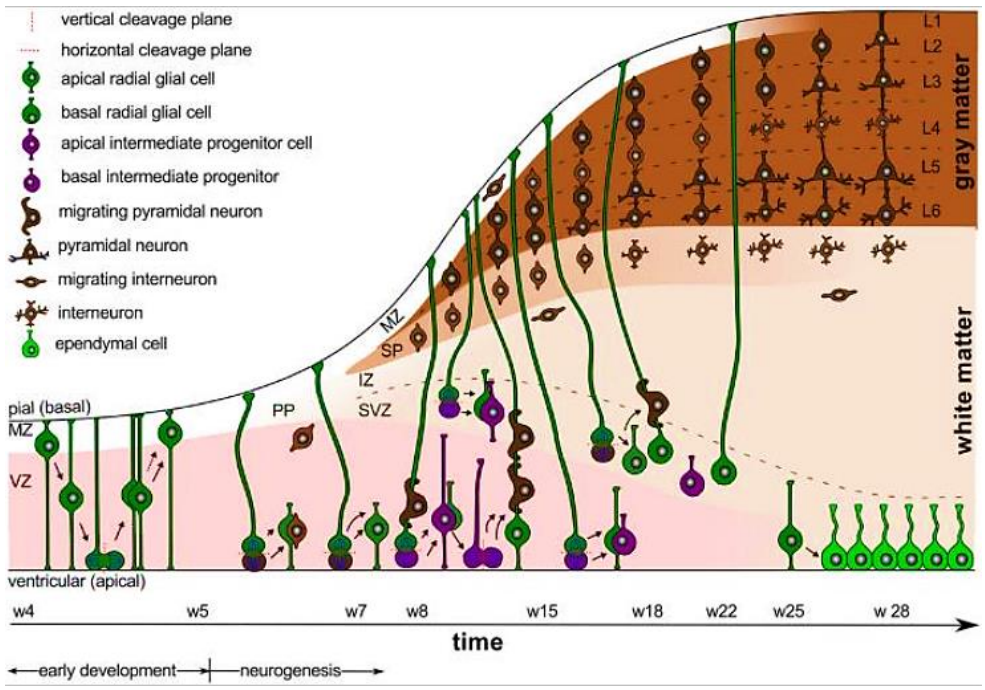

Figure 2-3 Schematic illustration of early cortex development.
Before the onset of neurogenesis, the pool of neural progenitors (NPCs) is increased by interkinetic nuclear migration (IKNM). The onset of neurogenesis is marked by the shift of NPCs from symmetric (vertical cleavage plane) to asymmetric (horizontal cleavage) division. Early born neurons form the PP, whereas subsequently born neurons form the cortex in an inside-out manner (28).

Thus, VZ and SVZ constitute the proliferating zone of pyramidal neurons which eventually migrate along the radial glial cells to form the cortical layers. The first few generated neurons

form the preplate (PP). Around GW7 the formation of the CP initiates by the radially migrating neurons of the VZ and SVZ that split the PP into an outer, marginal zone (MZ) and inner subplate (SP). The MZ transforms into the cortical layer 1 and contains tangentially migrated cells (Cajal retzius) that promote the correct inside-out development of the cortical layers. The earliest born neurons form the deepest layers of the cortex above the SP, and the last born neurons give rise to the superficial layers (28, 34).

By GW 18 the cortex develops into six laminated layers, and in the subsequent gestational weeks gyrification, gliogenesis, synaptogenesis, and myelination occur in a coordinated manner. Whereas synaptogenesis and myelination continue postnatally till adolescence to further elaborate the cerebral cortex (35).

2.1.2 Mouse Cortex Development

On account of morphology, number of neurons and convolutions (sulci and gyri) mammalian cortices differ from each other. Despite the inherent limitations in terms of the lissencephalic cortex, time-scaling of brain development, and smaller brain size than humans, still most of the research on the human cortex development is based mostly on rodent models like the mouse (*Mus musculus*). Mice share the key aspects of brain morphogenesis such as neurogenesis, neuronal migration, maturation, and organization of neuronal layers, with humans (31). Furthermore, the ease of genetic manipulation of the mouse genome conferred its wide use as an experimental animal in the field of neurosciences, that has dramatically increased our knowledge (36).

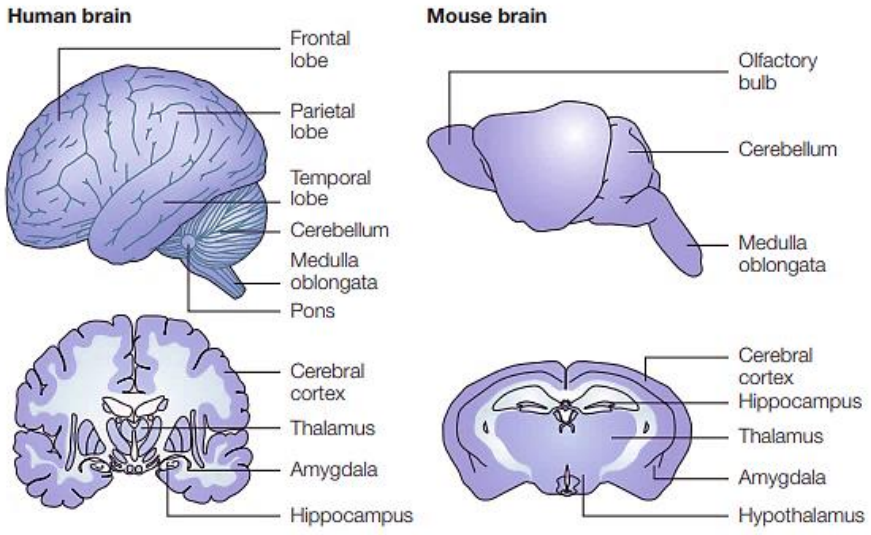

Figure 2-4 Comparison of brain anatomy between human and mouse.
Despite the difference in the size and gyrification between human and mouse brain, the overall anatomy and structural organization are evolutionarily conserved (36).

In mouse, the neural tube forms at E9-9.5, which corresponds to GW3-4 in humans (37, 38). Following the formation of the neural tube its disproportionate expansion results in the generation of the forebrain (cortex). Cortex development is initiated by symmetric divisions of NECs to amplify their pool, which is subsequently replaced by asymmetric divisions for

self-renewal and differentiation into various kinds of neurons. Around E10.5 the earliest born neurons generate a band of cells forming the PP which is located at the superficial layer of the cortex. As neurogenesis proceeds, cortical projection neurons migrate towards the PP where they settle and form the nascent cortical plate, which later gives rise to layer 2(L2) to layer 6 (L6) of the cortex. Further incoming neurons split the PP into the MZ and the SP. The MZ transforms into L1 of the cortex, whereas the SP is located below L6. The neurons of the MZ and SP are the first ones to become mature and form synapses. The morphological maturity of these neurons plays an essential role in the migration, maturity, and synaptogenesis of rest the cortical neurons. As cortex development proceeds different layers of neurons are formed in an inside-outward manner, followed by their differentiation into different subtypes corresponding to their layer location (39).

In summary, the appropriate size and folding of the cortex require an intricate balance between proliferation and differentiation of various kinds of NPCs (32). The importance of proper cortical size can be exemplified by the fact that its malformation results in various kind of neurological disorders like microcephaly with a wide range of cognitive deficits (40). The etiology of microcephaly is multifactorial, as various genetic and environmental factors like drugs, infectious agents, and IR seems to be involved with this disorder. Among genetic factors, mutation in the MCPH genes results in the development of autosomal recessive primary microcephaly (MCPH) (41). However, radiation-induced congenital microcephaly is of prime interest for this thesis (42).

2.2 Ionizing Radiation

Radiation is defined as the form of energy that travels through a medium (space or matter) in the form of particle or electromagnetic waves (43). Radiation can be categorized into two types: non-ionizing and ionizing radiation (IR). The later has the ability to create positively charged atoms or ions by removing an electron from an atom (44). However, in relevance to this dissertation and detrimental effects, only IR is discussed in detail below.

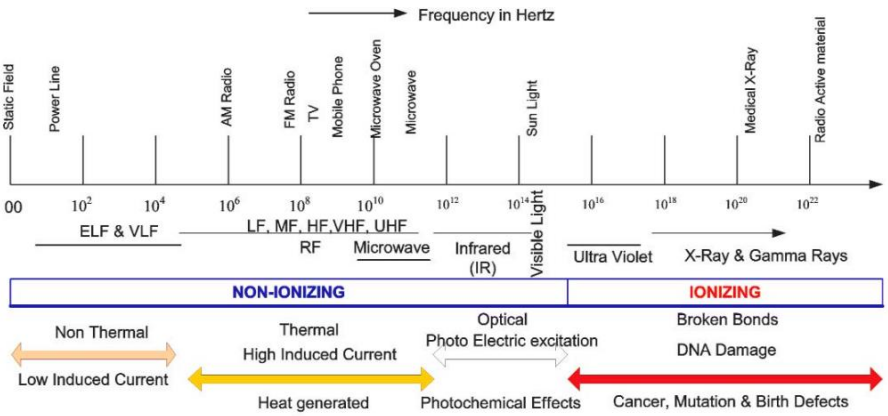

Figure 2-5 The Electromagnetic Spectrum.
Representation of electromagnetic waves frequency from left to right in ascending order. With the decreasing wavelength, the frequency, and energy of waves increases. The left part of the electromagnetic spectrum represents the non-ionizing radiation, consisting of radio frequency (RF), microwave, infrared and visible light, whereas the right part of the electromagnetic spectrum represents ionizing radiation, which comprises ultraviolet light, X and Gamma rays (44).

2.2.1 Types of Ionizing Radiation

IR can be classified into electromagnetic and particulate radiation. Electromagnetic radiation consists of photons that possess both electric and magnetic properties. This form of radiation has neither mass nor electrical charge, and it is capable of ionizing the medium indirectly with greater penetrating properties. Examples of electromagnetic radiation include X and gamma (γ) rays. Although X- and γ-rays have similar physical properties, they differ in their origin. X-rays are produced artificially by the transition of an electron from the higher to the lower electronic shell in an atom. This transition of an electron releases energy in the form of X-rays. In contrast, γ-rays are of intra-nuclear origin and produced by disintegrating nuclei of radionuclides. Both of these rays have the ability to penetrate the human body, where they can cause the disruption of biological molecules and their physiological functions (45).

On the other hand, particulate radiation possesses mass and energy and can be charged or uncharged. This radiation can cause the ionization of medium directly or indirectly. In comparison to electromagnetic radiation, particulate radiation has lower penetration power and can be stopped by a thin sheet of paper or aluminum. Examples include alpha particles, protons, neutrons, electrons, pi mesons and heavy ions (46).

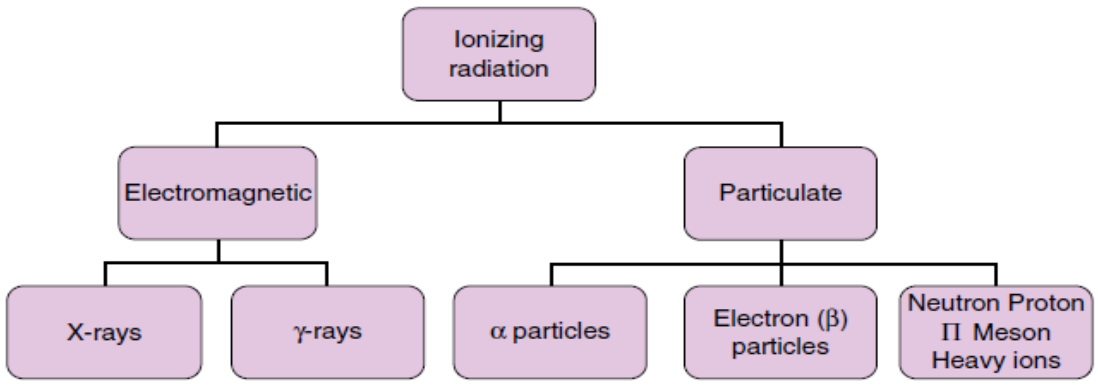

Figure 2-6 Classification of Ionizing Radiation (47).

IR can be classified further into high or low linear energy transfer (LET). LET can be described as the amount of energy delivered by IR per unit distance, and it is measured in SI units of MeV/µm. In other words, LET is the reflection of the amount of damage being produced by radiation as it passes through the medium. High LET radiations include alpha particles and neutrons, whereas X-rays, γ-rays, and beta rays come under the category of low LET radiation (48).

2.2.2 Sources of Ionizing Radiation

Throughout the daily lives, every human being is exposed to a certain level of radiation. There are two sources of radiation: natural and artificial sources. Main natural radiation sources include cosmic radiation from space and radioactive nuclides from the earth's crust, whereas

artificial sources of radiation include mostly industrial, occupational, and medical sector for various therapeutic and diagnostic purposes (43).

Worldwide the average dose of IR received by humans is approximately 2.4 Sv/year. The use of radiation in the field of medicine is increasing and it accounts for more than 14 percent of annual exposure (49). This increasing use of radiation raised concerns of scientists and has led to detail investigations about its possible damaging effects at the cellular and molecular level (43).

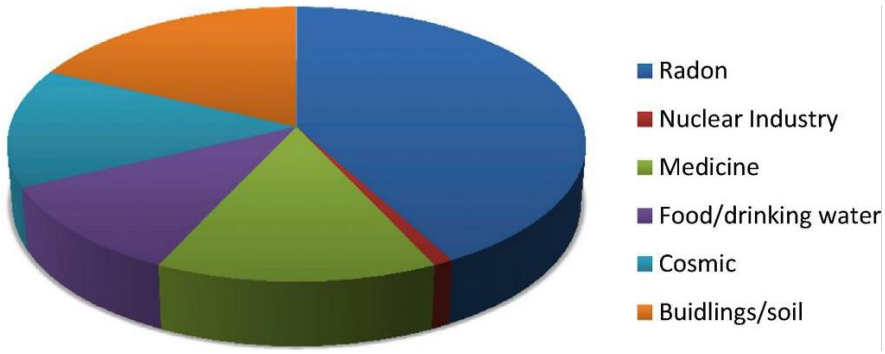

Figure 2-7 Sources of Ionizing Radiation.
Among natural sources, radon gas accounts for 85 percent of radiation exposure. Rest of the 15 percent exposure is from artificial sources, mainly from the medical sector (14%), and the nuclear industry (1%) (49).

2.2.3 Radiation Units

The SI unit for radioactivity is Becquerel (Bq), which is equal to one radioactive decay per second. With this radioactivity unit (Bq) it is somehow difficult to assess the biological impacts of radiation on the human body since such impacts depend upon the exposure time, susceptibility of exposed body parts like tissue, and the types of radiation. The absorbed energy from radiation is expressed in gray (Gy). One Gy is equivalent to one joule of radiation energy absorbed per kg of body mass, i.e. 1.0 Gy = 1.0 J/kg. However, this parameter of energy dose is still not consistent since one gray of different radiation can have different biological impacts. To make the impacts of different radiation more consistent, the energy dose gray is multiplied by a weighting factor and expressed in equivalent dose, Sievert (Sv). The weighting factor is different for each type of radiation and has been assigned by the International Commission on Radiological Protection (ICRP) (50).

2.2.4 Cellular Response to Ionizing Radiation

At cellular level radiation exposure results in the damage of various types of macromolecules, in particular, DNA (51). IR impacts the biochemical structures of cells in two different ways. In a direct manner, IR interacts with the atomic structure of biological macromolecules such as DNA, proteins, lipids, and enzymes, and alters their physiological functions. In an indirect manner, IR causes radiolysis of water which results in the production of free ions like hydroxyl ions (OH^-). Hydroxyl ion is a reactive oxygen species (ROS), and due to an

unpaired electron in its outer most shell, it is highly reactive, as it needs a pairing of the electron for stabilization. Apart from ROS, reactive nitrogen species (RNS) are also produced that further contribute to the radiation-based damage (52).

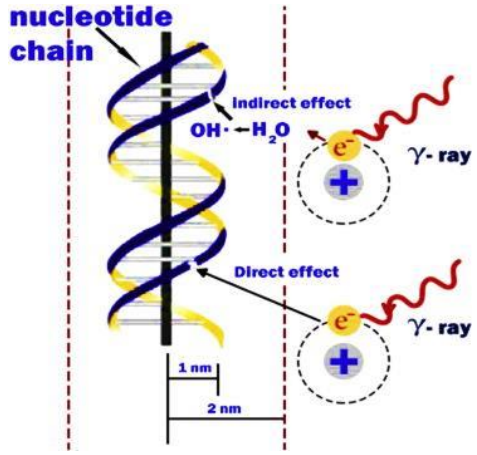

Figure 2-8 Direct and indirect effects of IR.
The IR emitted electron can damage the DNA either directly or indirectly through the radiolysis of H_2O. In an indirect manner, IR generates OH^- from the H_2O molecule, which then interacts with DNA and results in DNA damage (53).

2.2.4.1 DNA Damage and Induction of p53

DNA damage ranges from single or double-strand breaks (DSBs) to apurinic and apyrimidinic sites, base adduction and alteration of sugar moieties (54). Among such damages, DSBs are the major hallmark of IR and pose lethal consequences to the integrity of cells (55). With an increasing dose of IR, the tendency of DSBs increases, and inefficiency of cells to repair such breaks results in chromosomal aberrations, mutations, apoptosis and various forms of cancers (54). Following DNA damage, several gene expression changes occur in the cell. Most of them can be attributed to the p53 transcription factor (56).

Tp53 is the most studied genes in biomedical research, as more than 50% of the tumors in humans are due to a mutation in this gene (57, 58). Cells are continuously exposed to a wide range of endogenous and exogenous stresses which can lead to DNA damage. p53 has been described as "the guardian of the genome" because of its role in maintaining the stability by preventing and repairing genomic mutations (59).

Under normal physiological conditions, p53 expression is maintained at lower levels through its negative auto-regulator known as MDM2 (mouse double minute 2 homolog). MDM2 is a ubiquitin-protein ligase, which mediates ubiquitin/proteasome-dependent degradation of p53. Upon genotoxic stresses, p53 is converted to an active form through post-translational modifications like phosphorylation and acetylation. These modifications liberate p53 from MDM2 and result in its activation. The active form of p53 will then migrate and accumulate into the nucleus, and exert its transcriptional role through the transactivation of various downstream genes involved in cell cycle arrest, DNA repair, senescence and apoptosis (Figure 2-9) (59).

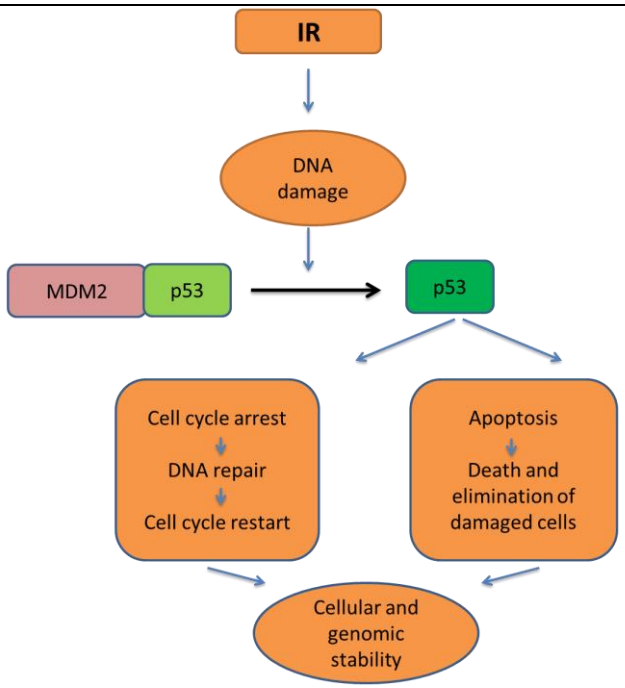

Figure 2-9 Induction of p53 following radiation exposure.
Under normal physiological conditions, p53 is inactivated by the interaction of its negative regulator, MDM2. DNA damage by IR or other cellular stresses leads to the activation of p53 through various pathways. Once activated p53 either induces a cell cycle arrest to allow repair or apoptosis to discard the damaged cells, thereby maintaining cellular and genomic stability. Adapted from (59).

In addition to its canonical functions, p53 has been shown to be involved in a wide range of other biological processes like energy metabolism, angiogenesis, cell motility, migration, differentiation, and neural development (60).

2.2.4.2 Cell Cycle Arrest & DNA Repair

To overcome the harmful effects of DNA damage and to maintain the genomic stability, cells have evolved a rescue mechanism known as the DNA damage response (DDR). DDR senses DNA damage and triggers signaling network which results in cell cycle arrest and allows cells to repair DNA damage (61). Cells activate cell cycle checkpoints which arrest the cell cycle at the G1/S and G2/M phase (Figure 2-10, A) (62). The DDR system involves a number of important proteins that are involved in the regulation of these cell cycle checkpoints. Among these proteins, MRN (Mre11, Rad50, and Nbs1) which is a nuclease complex plays an essential role in sensing, detecting and initial processing of the DSBs. The MRN complex assists in the recruitment of ATM (Ataxia Telangiectasia Mutated), which is phosphatidylinositol-3 kinase-related kinase and binds mainly to DSBs (63). The ATM then activates the p53 either directly or indirectly via the phosphorylation of checkpoint kinase2 (CHK2) (Figure 2-10, B) (64).

Activated p53 then exert its transcriptional role through the activation of many downstream genes that play a crucial role in cell cycle arrest. One of the targets of p53 is p21 (wild-type p53 activated fragment 1), which is a suppressor of cell cycle progression. Induction of p21 results in the inactivation of various cyclin-dependent kinases (CDKs). This inactivation of

CDKs inhibits the transition of the cell cycle from G1 to S phase or G2 to M phase and allows cells to repair DNA (64).

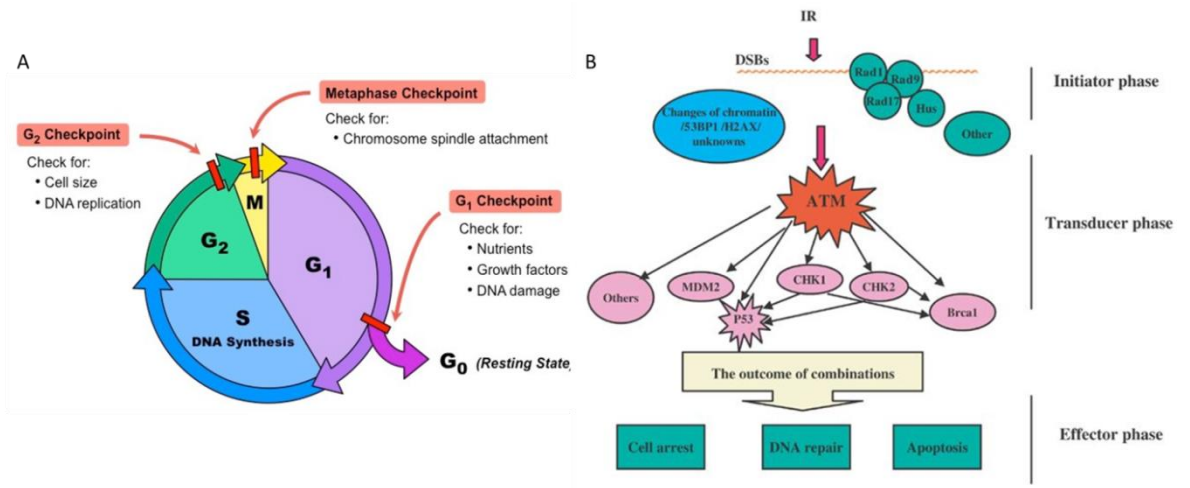

Figure 2-10 Cell cycle checkpoints (A). Schematic illustration of cellular response to DSBs (B). Different checkpoints during cell cycle ensure the genomic integrity of the cells. G1 checkpoint checks for the availability of nutrients and DNA damage prior to the DNA replication in S phase. G2 checkpoint checks for the cell size and DNA replication faults before the mitotic division of the cell, whereas metaphase checkpoint checks the mitotic spindle assembly to the centrosomes (A) (65). Various sensor proteins sense DNA damage via physical interaction at the damaged site. After sensing damage, transducer proteins amplify and transduce these damaging signals to the effector proteins of the DDR system. These effectors proteins then exert their action in the form of cell cycle arrest, DNA repair and apoptosis (B) (66).

After cell cycle arrest, the DNA repair becomes mandatory for the genomic stability and integrity. There are two types of DNA repair pathways: homologous recombination repair (HRR) and non-homologous end joining (NHEJ). Both pathways are evolutionarily conserved in eukaryotes, implicating their vital role in DSB repair. NHEJ is the dominant error-prone repair pathway in the vertebrates since this repair mechanism does not require any sequence homology, it can be observed throughout the cell cycle, particularly in G0 and G1 phase. Whereas HRR is a homologous DNA dependent, error-free repair pathway. This pathway is prevalent in S and G2 phase of the cell cycle where sister chromatids are accessible and serve as a template for homologous recombination (67).

2.2.4.3 Apoptosis

Apoptosis is a form of programmed cell death which is vital for maintaining tissue homeostasis. If DNA damage is difficult to repair, cells undergo apoptosis to prevent the transfer of the damaged DNA to their progeny and ultimately the occurrence of cancer (68). p53 plays a crucial role in apoptosis through the transactivation of several pro-apoptotic genes including the BCL-2 family of apoptosis regulators. This family comprises of two groups of regulatory genes: pro-apoptotic genes such as Bax (Bcl2-associated X protein), that promote apoptosis, and the anti-apoptotic genes such as BCL-2 that antagonizes the action of Bax (69). p53 induces Bax oligomerization and translocation into the mitochondria. In the mitochondria, Bax along with its partner Bak results in the permeabilization of its outer membrane and release of cytochrome c into the cytosol. The cytochrome c results in cleavage

and activation of caspase, which then triggers a cascade of proteolytic reactions responsible for apoptosis (70, 71).

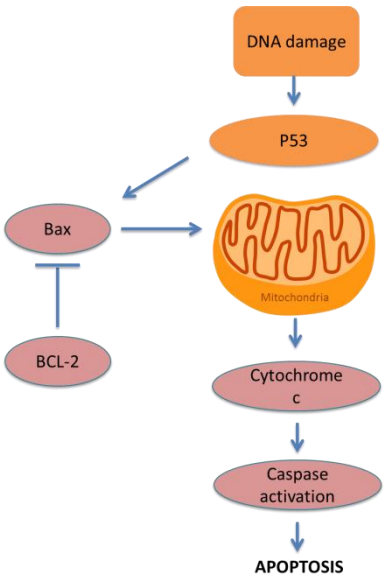

Figure 2-11 Simplified schematic illustration of p53 mediated apoptosis.
In response to irreparable DNA damage, p53 trans-activates several genes involved in apoptotic signaling pathways. Bax is upregulated by p53 and accumulates in the form of oligomers into the outer membrane of mitochondria. The oligomerization of Bax into the mitochondrial membrane results in the release of cytochrome c, which in turn activates caspase and triggers apoptosis. The pro-apoptotic role of Bax is antagonized by BCL-2. Adapted from (70).

2.3 Prenatal Radiation Exposure and Microcephaly

Microcephaly is a neurodevelopmental disorder, defined as the occipito-frontal circumference of at least 4 standard deviations below the age and sex corresponding population mean (41). Microcephaly can generally be categorized into primary and secondary types. The primary microcephaly is a non-progressive neurodegenerative disorder which is present at birth, whereas secondary microcephaly occurs postnatally and leads to progressive neurodegeneration accompanied by a drop in occipito-frontal circumference (72, 73).

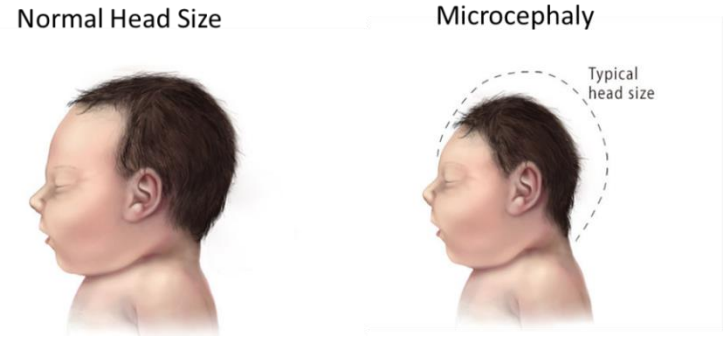

Figure 2-12 Comparison of head size in normal (left side) and microcephalic baby (right side) (74).

The etiology of primary microcephaly is complexed and multifactorial. One of the prime causal factors is a genetic mutation. Among primary microcephaly; autosomal recessive primary microcephaly (MCPH) is the most studied one. So far 12 MCPH foci, corresponding to 12 genes (MCPH1 to MCPH2) have been discovered (41). Within MCPH associated mutations, the most common mutation is conferred in MCPH5 gene which encodes for ASPM (Abnormal spindle-like, microcephaly-associated protein). ASPM plays an essential role in the proper organization of microtubules at the spindle poles and helps out in the formation of the central mitotic spindle during cell division (72, 75). A detailed study about this protein indicated that it is associated with centrosomes in the NPCs and plays an essential role in the cell cycle progression, shift from symmetric to asymmetric division and possibly in DNA repair of NPCs. Mutations in ASPM cause altered cleavage plane in NPCs, which results in their premature differentiation and reduction in number, ultimately leading towards microcephaly (32).

Apart from genetic causes prenatal radiation exposures also play a significant role in the development of microcephaly. The impacts of prenatal radiation exposure on the developing brain had been demonstrated by various epidemiological studies. Among these studies, an epidemiological survey conducted by Otake and Schull on the atomic bomb survivors of Hiroshima and Nagasaki (1945) indicated several deleterious effects of IR. Such effects include growth retardation decreased intelligence quotient (IQ), and mental retardation which was often associated with microcephaly. The propensity for such disorders increased with the increased radiation dose in a linear manner. Interestingly this study revealed that frequency of small head size (microcephaly) was more pronounced when the exposure occurred between GW8 to 15 and to a lesser extent between GW16 to 25, indicating the importance of the time point of exposure to IR (76, 77).

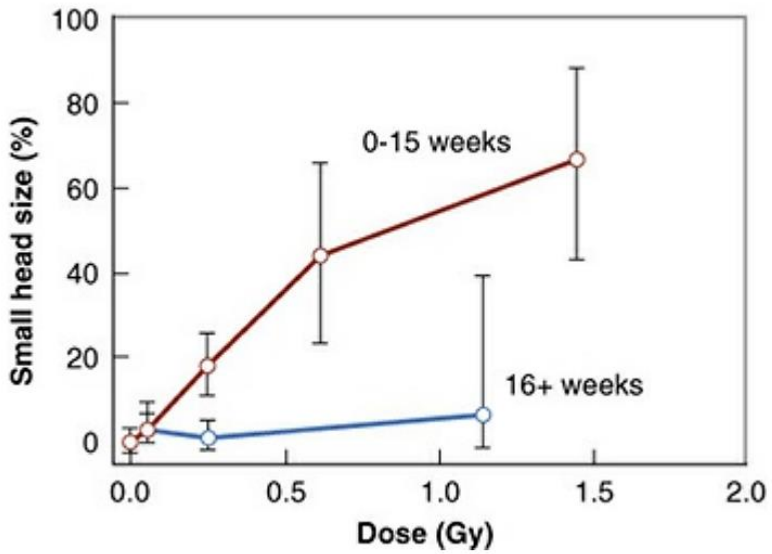

Figure 2-13 Effects of IR on the developing brain.
The frequency of small head size (microcephaly) corresponding to radiation dose in prenatally exposed survivors of Hiroshima and Nagasaki (9, 78).

Because of limited human data, various rodent models have been used to recapitulate the effects of prenatal IR exposure on the developing brain. Recent work at the Radiobiology Unit of SCK•CEN showed that radiation exposure of pregnant mice at E11 (onset of neurogenesis) resulted in the development of microcephaly in a dose-dependent manner, with a threshold level of 0.33 Gy and onwards. Furthermore, they proposed that massive apoptosis of neural progenitors and differentiating neurons might be one of the main reasons for microcephaly (10, 79). Other studies indicate that radiation exposure of mouse embryonic brain at E12 resulted in significantly decreased expression of ASPM gene in the VZ. This downregulation of ASPM promotes asymmetric division of NPCs, which leads to a reduced number of neurons ultimately causing microcephaly (75). In addition, it was demonstrated that IR exposure of the developing brain at E13.5 leads to centrosomal duplication which results in apoptosis of NPCs (80).

Apart from apoptosis, mitotic defects, a recent study (not yet published) conducted at the radiobiology unit of SCK•CEN suggests that DNA damage in NPCs leads to premature differentiation via an epithelial to mesenchymal transition (EMT) like mechanism. This early differentiation results in the depletion of NPCs resulting in microcephalic phenotype. However, whether these processes are p53-dependent is still not clear.

2.4 Research Objectives

This study is based on the previous gathered results obtained at the Radiobiology Unit of SCK•CEN. They recapitulated in pregnant mice at E11, the radiation-induced microcephaly which was observed in *in utero* exposed human survivors of the Hiroshima and Nagasaki atomic bombings. In addition, they showed that shortly after IR exposure of the pregnant mice, the gene expression changes in the brain of the embryos were mostly regulated by p53, and interestingly were similar to the $Magoh^{+/-}$ genetic mouse model of microcephaly as well as ZIKV infected hNPCS. Therefore, these findings indicate that following radiation exposure, activation of p53 and its transcriptional targets might be responsible for the small brain size later in life. To document the role of p53, mice in which p53 is genetically deleted from the dorsal forebrain (cKO mice) have been generated. The obtained results (not published) indicate that the brain size of these mice is partially rescued after exposure to IR. However, the etiology of the radiation-induced microcephaly is still not yet fully understood. Therefore, the aim of this study is to gain more insight into the cellular and molecular mechanisms underlying the radiation-induced microcephaly. More importantly, the aim is to find out whether these mechanisms are p53-dependent.

3 Chapter 3: Material and Methods

3.1 Animals and irradiation

All animal experiments were handled in agreement with the Belgian laboratory animal legislation and approved by the local SCK•CEN ethical committee (ref. 02-012). Experimental mice were housed under standard laboratory conditions with 12 h light and dark cycle. Food and water were available *ad libitum*. Female mice were coupled during a 2 h time period in the morning, at the start of the light phase (7.30 h until 9.30 h), in order to ensure synchronous timing of embryonic development.

3.1.1 Generation and characterization of dorsal telencephalon *Trp53* conditional knockout (cKO) mice

Trp53 gene was genetically deleted in the dorsal telencephalon using Cre/lox P recombination system. Briefly, mice with *Trp53* critical exons flanked by loxP sites (*Trp53*$^{fl/fl}$ mice) were coupled with mice expressing the Cre recombinase under the promotor of *Emx 1* (Emx1-Cre mice). Emx-Cre was chosen based on the expression of Emx1 by neuro-epithelial cells in the dorsal telencephalon at the early time point (E9.5-10.5), which is essential since mice will be irradiated at E11. DNA from embryonic tails was extracted and PCR genotyping was performed to confirm the knockout of the *Trp53* locus. In addition, the generation of *Trp53* cKO mice was validated by immunohistochemistry (see below).

3.1.2 Irradiation

Mice were given a single dose of whole-body radiation (1 Gy) at E11, by using an X-strahl 320 kV (0.14 Gy/min, inherent filtration: 0.21 mmAl, additional filtration: 3.8 mmAl + 1.4 mm Cu + DAP, tube voltage: 320 kV, tube current: 12 mA, source distance: 100 cm, beam orientation: vertical) in accordance to ISO 4037. Control mice were sham-irradiated. For sham irradiation, animals were taken to the radiation facility but were not placed within the radiation field. Brain samples were isolated from embryos at different time points following irradiation.

3.2 Brain dissection and morphology assessment

Brains of postnatal day 1 (P1) mice exposed to radiation *in utero* at E11 or E14 were isolated. Prior to fixation in 4% paraformaldehyde, the brains were imaged on a Leica stereo dissection microscope as previously described (81). The surface of both cortices was measured and analyzed using FIJI/ImageJ software.

3.3 Immunohistochemical (IHC) Analysis

3.3.1 Tissue Collection and IHC Staining

Embryos (E11) were dissected and fixed in 4% paraformaldehyde (PFA, Sigma® Aldrich, Germany) dissolved in phosphate buffered saline (PBS, gibco® life

technologies™, UK) at 4°C overnight. Following fixation, samples were rinsed 3 x 5 min each with PBS and stored in 70 % ethanol till further manipulations. Next, the samples were dehydrated in graded solutions of ethanol, followed by incubation in xylene and liquid paraffin. Following embedding in paraffin, seven micrometer thin coronal sections were cut using Microtome (Microm HM 340 E, Thermo Scientific, USA), and mounted on the Super frost Plus glass slides (Thermo Scientific, USA).

For IHC staining, the embedded brain sections were deparaffinized in xylene followed by rehydration in graded solutions of ethanol. After rehydration antigen retrieval was performed by boiling the sections in 1x antigen retrieval solution (Dako, USA) or citrate buffer (pH 6) for 2 x 4'40 min at a max watt, and 1x5 min at 600 watts in the microwave. These sections were cooled for 20 min at room temperature, rinsed with TBS-T for 3x 5 min, and then blocked for 1 h either with Normal Goat Serum (Invitrogen, Thermo Fisher Scientific, USA) in TNB (1:5) or PBS containing 1 % BSA (Sigma life science, USA) and 0.3 % Triton X 100 (Sigma life science, USA). The sections were then incubated overnight at room temperature with primary antibodies diluted in blocking buffer. The list of antibodies used for IHC staining is given below (**Table 3-1**). The next day, the sections were rinsed 3x5 min with TBS-T. The binding of primary antibody on the targets was detected by incubating with Alexa Fluor® (Invitrogen, Thermo Fisher Scientific, Germany) 405, 488, 568 secondary antibodies, dilution factor 1:200, for 2 h at room temperature. For CC3 immunostaining Goat anti-rabbit (Invitrogen, Catalogue # 65-6120) secondary antibody was used, followed by signal amplification with TSA Plus Cyanine 3 System (PerkinElmer, USA).

Primary Antibody	**Dilution factor**	**Company (Cat #)**	**Localization of the target**
Polyclonal rabbit anti-53BP1 (p53-binding protein 1)	1:100	Novus Biologicals (NB 100-304ss)	DNA double-strand breaks (DSBs)
Monoclonal mouse anti-βIII-tubulin (class III beta–tubulin)	1:1000	Sigma Life Science (T5076)	Mature neurons
Polyclonal rabbit anti CC3 (cleaved caspase 3)	1:100	Biovision (3015-100)	Early apoptotic cells
Polyclonal goat anti-DCX (doublecortin)	1:500	Santa Cruz Biotechnology (sc-8066)	Immature migrating neurons
Monoclonal mouse anti-p53	1:100	Santa Cruz Biotechnology (sc-126)	Tumor suppressor protein
Polyclonal rabbit anti-Pax6 (Paired box protein 6,Transcription factor)	1:200	Biolegend (901301)	Neural progenitor cells
Monoclonal rabbit anti-pH3 (Phospho-histone 3)	1:1000	Cell Signaling Technology (3377)	Proliferating cells in mitotic phase
Monoclonal mouse anti-QKi5 (Quaking5)	1:1000	Santa Cruz Biotechnology (sc-517305)	Neuro-epithelial cells

Table 3-1 List of primary antibodies used for IHC staining

Sections were counterstained for nuclei, with 4’6-diamidiono-2-phenylindole (DAPI, Sigma Aldrich, Germany) for 15 min. Finally, the slides were mounted with mowiol and images were taken by using Nikon Eclipse Ti-E inverted microscope.

3.3.2 Image analysis

Analysis of images was performed by using the ImageJ-NIH software.

3.3.3 Statistical analysis

Statistical analysis was performed using two-way ANOVA Tukey posthoc test in GraphPad Prism 7, for comparison between pairs of mean. *P* values <0.05 were considered to be statistically significant. Error bars represent SEM.

4 Chapter 4: Results

This study is based on previous research conducted at the Radiobiology Unit of Belgian Nuclear Research Centre (SCK•CEN). It was previously shown that radiation exposure of embryonic brain results in gene expression changes, which occur in a p53-dependent manner (11). Furthermore, these genes were found to be highly overlapped with those induced in Magoh$^{+/-}$ mouse, and ZIKV infected hNPCs (12). On account of these similarities, we hypothesized that radiation-induced p53-mediated gene activation leads to the development of microcephaly. In light of these findings another study at SCK•CEN demonstrated the partial brain size restoration in *Trp53* conditional knock-out (cKO) mice after *in utero* exposure to X-rays (unpublished results). These findings clearly indicate that p53 plays a vital role in the development of microcephaly after prenatal radiation exposure, and laid the foundation of our current study.

4.1 Genetic inhibition of *Trp53* partially restores the brain size after IR exposure

Mice in which the *Trp53* gene is specifically knocked-out in the dorsal forebrain (cKO mice) had been bred before. These mice were used to evaluate the brain size of prenatally (E11 and E14) irradiated mice in comparison to wild-type (WT) littermates. The obtained results indicate that when irradiated at E11 (Figure 4-1 D) or at E14 (Figure 4-1 E) the brain size of WT mice at postnatal day (P1) is reduced by 28% and 19% respectively in comparison to the non-irradiated WT mice. This shows that the radiation induced-microcephaly is more pronounced at early stages of neurogenesis, which is in agreement with the epidemiological data from atomic bomb survivors (76). In p53 cKO mice, this brain developmental defect is partially rescued after irradiation at E11 (Figure 4-1 D), and wholly rescued after irradiation at E14 (Figure 4-1 E). The difference in the E11 and E14 embryos might be partially explained by an increase in the number of p53-null cells in the E14 embryos compared to E11. Put together; these findings clearly indicate that p53 plays a role in the etiology of the radiation-induced microcephaly.

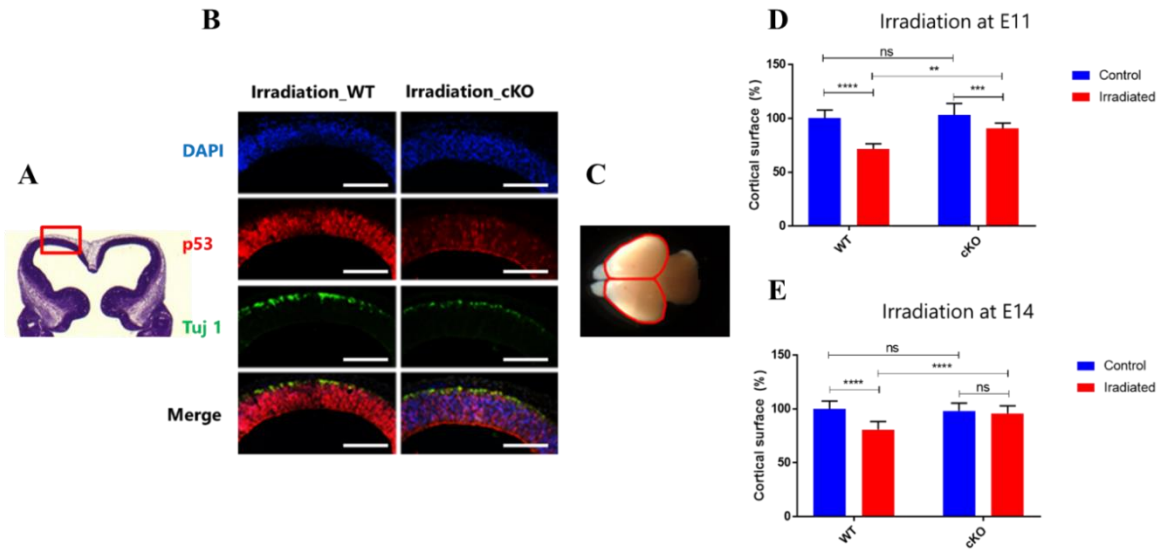

Figure 4-1 Characterization of p53 cKO mice.
Image and analysis courtesy of Andre Claude Mbouombouo Mfossa (Radiobiology Unit, SCK•CEN). **(A)** A coronal section of the E11 mouse brain, with an indication in red of the dorsal part that was used for immunohistochemical analysis. **(B)** Evidence of p53 deletion in the dorsal forebrain of cKO mice. Scale bar: 100 µm. **(C)** Schematic representation of cortical surface measurements using the ImageJ software. **(D and E)** Analysis of brain sizes indicating a reduction in irradiated WT mice which was partially or totally restored in cKO mice when irradiated at E11 **(D)** and E14 **(E)** respectively. Statistics were performed using two-way ANOVA, Tukey's multiple comparison tests. Data are expressed as percentage fold changes relative to control WT mice. **p=0.008, ***p=0.0003, ****p<0.0001. For all conditions, the pups (n) were from five different mothers (N=5), except for control E14 cKO (N=6). Control E11 WT (n=18); Control E11 cKO (n=21); Irradiated E11 WT (n=14); Irradiated E11 cKO (n=12). Control E14 WT (n=19); Control E14 cKO (n=20); Irradiated E14 WT (n=18); Irradiated E14 cKO (n=12). cKO: conditional knock-out. DAPI: 44′,6-Diamidine-2′-phenylindole dihydrochloride. E11: Embryonic day 11. ns: non-significant. Tuj1: Neuron-specific class III beta-tubulin. WT: wild-type.

4.2 Mechanism of radiation-induced microcephaly

Many studies have indicated that microcephaly could result from any perturbations that affect proper proliferation/division of neural progenitors, their differentiation into neurons, and migration to their specific layer within the cerebral cortex (82–84). These processes were assessed, with emphasis whether they are p53 dependent or not.

4.2.1 Radiation induces widespread DNA damage in the embryonic brain

It is well documented that DNA damage in response to genotoxic stress such as ionizing radiation is one of the signals if not the most critical alert in response to which cells activate a myriad of signaling pathways (85, 86). To evaluate the DNA damage in the embryonic brain, pregnant mice were irradiated at E11 with 1 Gy of X-rays. The embryonic brains were harvested at 2 h and 6 h after irradiation and stained for 53BP1, a protein which is known to relocate to the sites of DNA strand breaks in response to DNA damage (87). Our results showed at 2 h PI, a massive induction of DNA damage in the brains of irradiated WT and cKO embryos, with an average of four 53BP1 foci per nucleus (Figure 4-2 A and B). These foci were detected throughout the neocortex, indicating the DNA damage both in neural progenitors, post-mitotic as well as differentiated neurons. In contrast, non-irradiated embryos

showed only sparse foci (0.4-0.5 per nucleus) (Figure 4-2 B) which is probably due to the presence of endogenous DNA damage. Further analysis at 6 h PI, showed a reduction in the number of foci, with an average of 0.4 foci per nucleus in irradiated WT and cKO brains (Figure 4-2 A and B). As expected, there was no difference observed in the number of foci in irradiated WT and cKO mice both at 2 h and 6 h after irradiation.

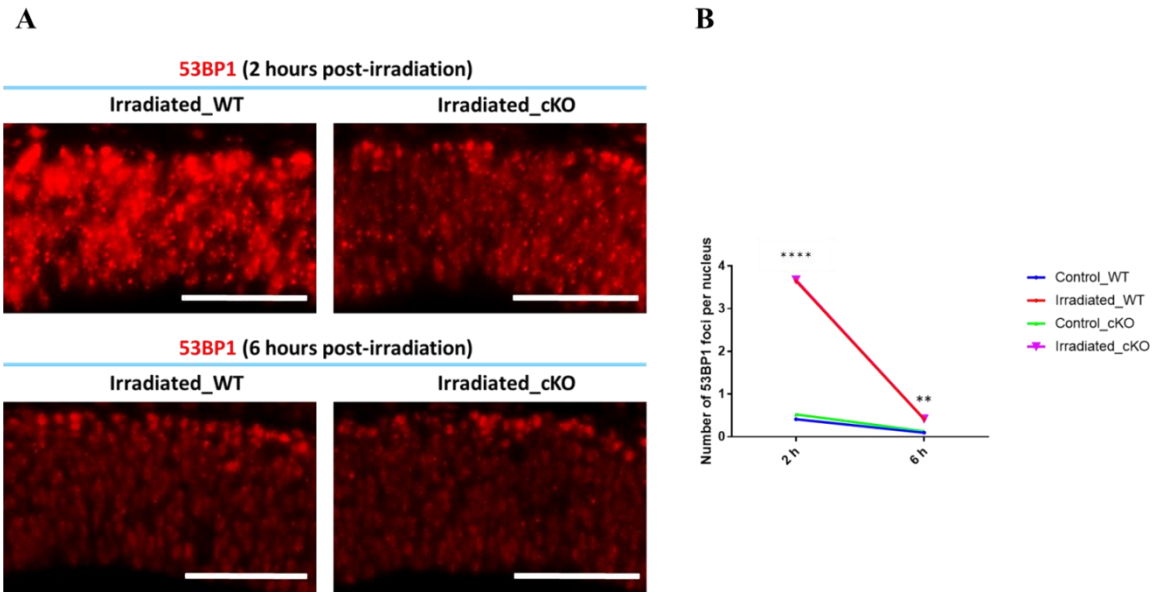

Figure 4-2 Widespread DSBs following radiation in the developing brain.
(A) DSBs visualization by 53BP1 immunohistochemistry. Scale bar: 100 µm. The diffuse and bright 53BP1 nuclear foci highly abundant at 2 h post-irradiation in both WT and cKO mice were found to be almost absent at 6 h post-irradiation. **(B)** Quantification of the 53BP1 nuclear foci indicates no difference in the number of DSBs in cKO mice in comparison to WT mice. Statistics were performed using two-way ANOVA, Tukey's multiple comparison tests. Data are expressed as mean ± SEM. ***p*=0.008. *****p*<0.0001. For all conditions, 6 embryos from at least 2 different mothers were used. 53BP1: p53-binding protein 1. cKO: conditional knock-out. DAPI: 44′,6-Diamidine-2′-phenylindole. E11: Embryonic day 11. WT: wild-type.

4.2.2 G2/M arrest following DNA Damage

In response to DNA damage, cells cycle is arrested to repair the damage, and prevent the transmission of the damage to the daughter cells (66). It is commonly accepted that radiation induces cell cycle arrest at the G2/M transition (88). To evaluate this, mouse embryos were irradiated at E11 with 1 Gy of X-rays. The embryonic brains were harvested at 2 h and 6 h after irradiation and stained for phosphorylated histone H3 (pH3), which is a marker of the G2/M transition of the cell cycle (89). The obtained results showed no difference in the number of pH3 cells between control mice both at 2 h and 6 h post-irradiation indicating that the deletion of p53 has no effect on the cell cycle. However, in response to radiation, the number of pH3 positive cells was significantly reduced in the irradiated WT brains in comparison to the non-irradiated brains. The reduction in the number of pH3 was more pronounced at 2 h following radiation, and was observed in the apical zone only (Figure 4-3 A, B, and C). Importantly, following radiation, the cell cycle arrest was attenuated (2 h post-irradiation) and was completely absent (6 h post-irradiation) in irradiated cKO mice indicating a p53-dependent component of cell cycle regulation following radiation exposure.

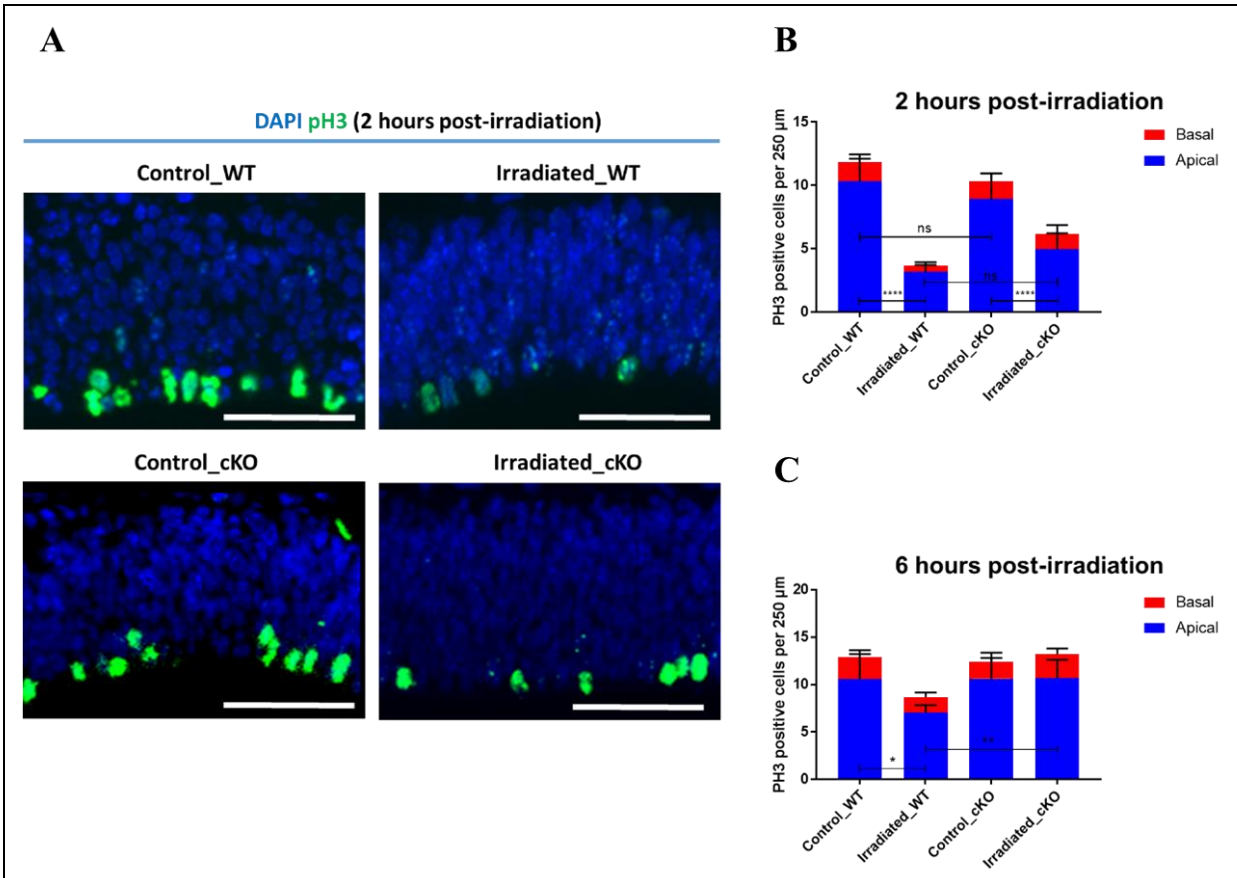

Figure 4-3 Induction of G2/M arrest following DNA damage in the brains of E11 embryos.
(A) Dividing cells in the G2/M transition phase visualized by pH3 immunohistochemistry. Scale bar: 100 µm. **(B and C)** Quantification of pH3 positive cells indicates no significant difference in the number of pH3 cells between control mice both at 2 h **(B)** and 6 h **(C)** post-irradiation. The number of pH3 positive cells was significantly reduced in the irradiated WT brains in comparison to the non-irradiated brains. The reduction in the number of pH3 positive cells was more pronounced at 2 h following radiation, and observed in the apical zones only. The cell cycle arrest was attenuated (2 h post-irradiation) and was completely absent (6 h post-irradiation) in irradiated cKO animals indicating a p53-dependent component of cell-cycle regulation following radiation exposure. Statistics were performed using two-way ANOVA, Tukey's multiple comparison tests. Data are expressed as mean ± SEM. *p=0.014, **p=0.0076, ****p<0.0001. For all conditions, 5 embryos from at least 2 different mothers were used. cKO: conditional knock-out. DAPI: 44′,6-Diamidine-2′-phenylindole. E11: Embryonic day 11. ns: non-significant. pH3: histone H3 phosphorylated at serine-10. WT: wild-type.

4.2.3 Radiation-induced apoptosis in the irradiated embryonic brain

The depletion of neuronal progenitor cells resulting from massive apoptosis is one of the mechanisms shown to lead to microcephaly (82–84). In order to elucidate the contribution of apoptosis in the development of radiation-induced microcephaly, embryos were irradiated at E11 with 1 Gy of X-rays, and then the brain sections were stained for cleaved caspase 3 (CC3), a marker of an early apoptosis (90). Our findings indicated massive cell death 6 h following radiation exposure in WT animals in comparison to control mice. Intriguingly, this was almost not the case for neural progenitor cells populating the ventricular zone (Figure 4-4 A). As expected, there was almost no cell death observed in the prefrontal cortex of irradiated cKO mice in comparison to irradiated WT mice, whereas ganglionic eminence still showed massive apoptosis. These results indicate that radiation-induced cell death is p53 dependent, therefore could partially explain the rescue of the brain size of cKO animals after irradiation.

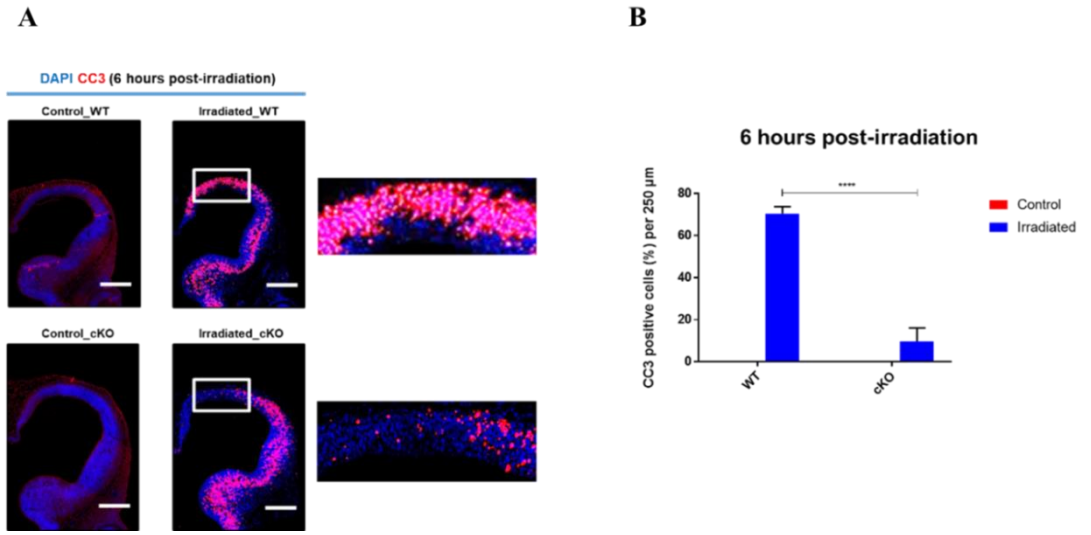

Figure 4-4 Selective apoptosis in the mouse neocortex following DNA damage.
(A) Immunohistochemistry for cleaved caspase 3 (CC3) was carried out on coronal sections of embryonic brain irradiated with 1 Gy of X-rays. Scale bar: 500 µm. **(B)** Quantification of CC3 positive cells 6 hours following radiation exposure showed in irradiated WT mice, a massive cell death which was found to be restricted to the upper part of the neocortex. These observations were less obvious in irradiated cKO mice, indicating a p53-dependent cell death following radiation. Statistics were performed using two-way ANOVA, Tukey's multiple comparison tests. Data are expressed as mean ± SEM. ****$p<0.0001$. For all conditions, 6 embryos from at least 3 different mothers were used. cKO: conditional knock-out. DAPI: 44′,6-Diamidine-2′-phenylindole. WT: wild-type. E11: Embryonic day 11.

4.2.4 Premature differentiation of neural progenitor cells in response to DNA damage

A previous study suggested that IR exposure of embryonic brain results in p53-mediated premature neuronal differentiation, which might lead to the development of microcephaly (11). To verify this, embryos were exposed at E11 to 1 Gy of X-rays. 6 h and 24 h after irradiation, brain samples were on the one hand stained for Pax6, a marker of radial glial cells (91) (Figure 4-5 A) and on the second hand for doublecortin (DCX) a marker of immature migrating neurons (92) (Figure 4-5 D). Our results showed, both at 6 h (Figure 4-5 B) and 24 h (Figure 4-5 C) post-irradiation a significant depletion of radial glial cells in irradiated WT embryos in comparison to control embryos. Interestingly we observed no difference in the number of Pax6 positive cells in irradiated cKO mice compared to control mice (Figure 4-5 B, C). Concerning the expression of DCX, this was restricted to cells in the preplate. However, exclusively in irradiated WT embryos, ectopic immature neurons were found in the apical zone of the cortex (Figure 4-5 D and E). In addition, based on a previously unpublished result which indicates that DNA damage might induce premature neuronal differentiation via an EMT like mechanism, we next investigated whether this process was p53 dependent. In this respect, using similar samples as for DCX immunostaining, we performed staining for Quaking5 (QKi5) (Figure 4-5 F), which is a known repressor of neuronal differentiation (93). The obtained results showed a significant reduction in the number of QKi5 expressing cells in irradiated WT brains in comparison to controls. Interestingly we observed no difference in the number of QKi5 positive cells in irradiated cKO brain compared to control mice (Figure 4-5 G). Put together, our results indicate a p53-dependent neuronal differentiation in response to

DNA damage which is accompanied by the reduction of the pool of neural progenitor and the abundance of immature neurons toward the apical zone of the neocortex.

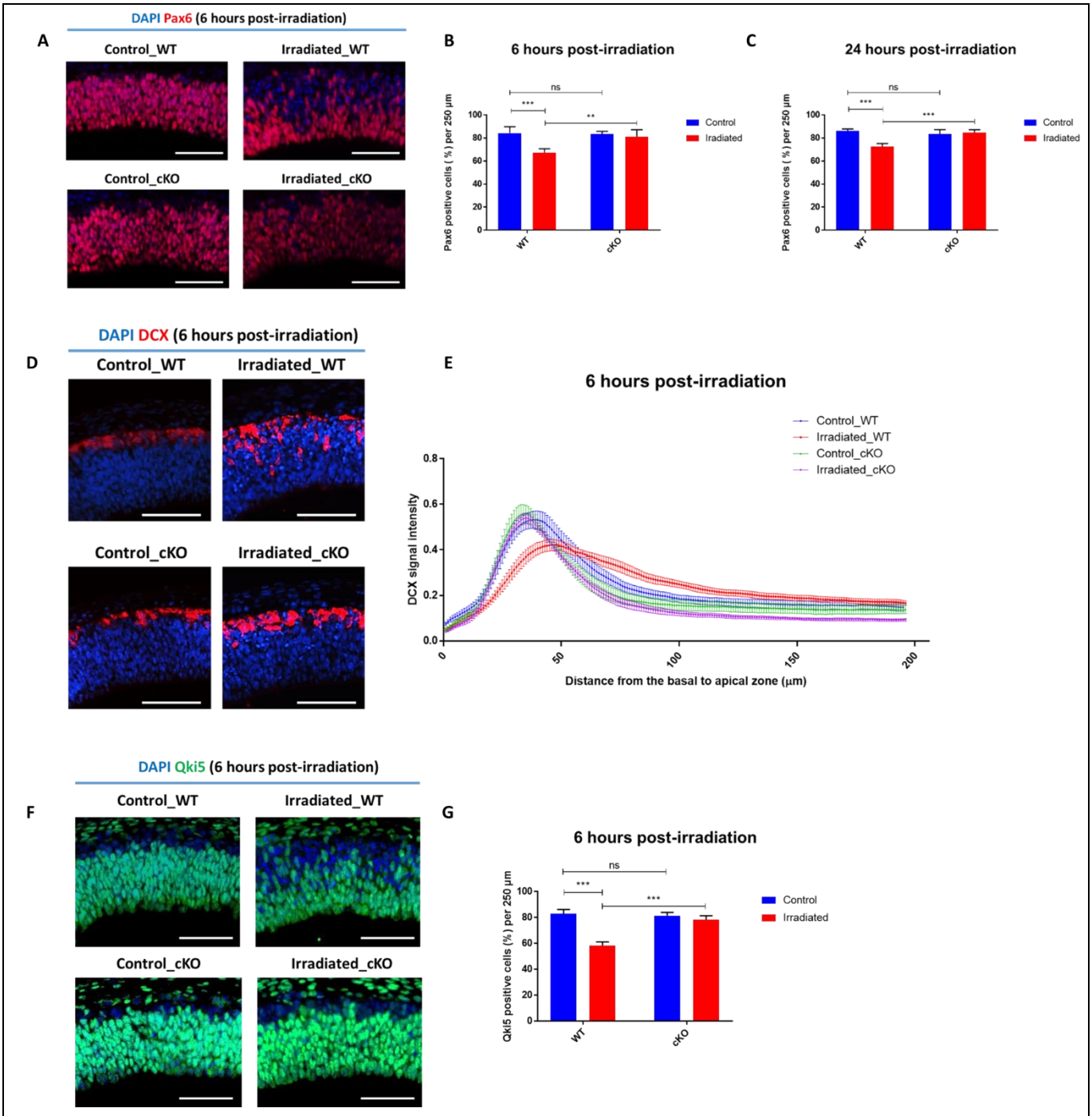

Figure 4-5 DNA damage induces premature differentiation of neural progenitors.
(A, B and C) The visualization of Pax6 positive cells permits to quantify the pool of progenitor cells in response to DNA damage. In comparison to control WT mice, the number of neural progenitor cells was significantly reduced in irradiated WT mice both at 6 h **(B)** and at 24 h **(C)** after irradiation, no difference was found in irradiated cKO mice in comparison to the control mice. DCX immunostaining **(D)** and quantification **(E)** indicating the abundance of immature migrating neurons in the apical zone of irradiated WT mice. Like Pax6, Qki5 immunostaining **(F)** and quantification **(G)** indicates the reduction of Qki5 expression in the neocortex of irradiated WT mice in comparison to control WT mice. No difference was observed between the irradiated cKO mice and controls mice. Scale bar: 100 µm. Statistics were performed using two-way ANOVA, Tukey's multiple comparison tests. Data are expressed as mean ± SEM. $^{**}p<0.003$ $^{***}p<0.0005$. For all conditions, 5 embryos from at least 2 different mothers were used. cKO: conditional knock-out. DAPI: 44′,6-Diamidine-2′-phenylindole. E11: Embryonic day 11. ns: non-significant. WT: wild-type.

5 Chapter 5: Discussion

In this study, we examined the potential role of p53 in the underlying cellular and molecular mechanisms that lead to the development of radiation-induced microcephaly. To evaluate the role of p53 in the development of microcephaly, we first examined and compared the brain size of p53 cKO mice with WT mice. We observed a partial and full brain size restoration in irradiated cKO mice after *in utero* radiation exposure at E11 and E14 respectively (Figure 4-1 D and E). Interestingly the brain size of control cKO mice was comparable to the control WT mice. Altogether our results indicate that p53 plays an important role in the development of radiation-induced microcephaly, and more importantly its deletion from the dorsal forebrain does not perturb the normal brain development. Next, we assessed the implications of p53 in the underlying cellular and molecular mechanisms that lead to the development of radiation-induced microcephaly.

The association of various DNA repair deficiency syndromes with microcephaly indicates that DNA damage is the one of the main underlying causes of microcephaly (83, 84). Indeed we also observed a massive DNA damage at 2 h PI in the irradiated WT and cKO brains which was reduced at 6 h PI (Figure 4-2 A and B). Interestingly, irrespective of the genotype, DNA damage was observed in both irradiated cKO and WT brains. Altogether our results show that following radiation exposure, embryonic DNA damage is evident and independent of p53 expression. The DNA damage triggers various cellular responses that further contribute to the development of microcephaly.

The immediate cellular response to DNA damage is the cell cycle arrest which occurs in a p53–dependent or independent manner, thereby allowing cells to repair the damage (94). However, the cell cycle arrest varies between different phases, and following radiation exposure it has been shown to occur in the G2/M phase (95, 96). In comparison to control WT brains, we observed a significant cell cycle arrest in the NPCs of irradiated WT brains at 2 and 6 h PI (Figure 4-3 B and C). Despite the deletion of p53, we observed an attenuated cell cycle arrest in the irradiated cKO brains at 2 h PI (Figure 4-3 B) which we assume partly to be p53-independent at this time point. Our assumption seems to correlate with a gene expression analysis conducted on *TrP53* null mice, where they showed a significant downregulation of cell cycle genes after radiation exposure (97). However, at 6 h PI, irradiated WT brains showed the persistent cell cycle arrest which was completely absent in the irradiated cKO brains, indicating a p53-dependent cell cycle arrest (Figure 4-3 C). Overall our findings indicate that following radiation exposure, p53 plays a significant role in the cell cycle arrest of NPCs to repair the DNA damage.

The microcephaly has generally been attributed to apoptosis, as observed in various genetic mouse models of microcephaly (98, 99). Indeed at 6 h PI, we observed a massive apoptosis in the differentiating and mature neurons of irradiated WT brains, which was almost absent in the irradiated cKO brains (Figure 4-4 A and B). Our findings are in the agreement with a previous study where they reported the radiation-induced microcephaly as a consequence of massive apoptosis of differentiating and mature neurons (10, 79). On a broad aspect, this finding can further be reinforced on account of another study which demonstrated that

deletion of p53 prevented the apoptosis, and consequently the fetal brain abnormalities after radiation exposure (100). So altogether our results indicate that the pro-apoptotic activity of p53 is one of the key cellular mechanisms that lead to the development of radiation-induced microcephaly. Furthermore, p53-mediated neural apoptosis in ZIKV infection is also suggested as one of the leading cause of microcephaly (101). Despite all these similarities, previous studies and law of Bergonié and Tribondeau propose that proliferating NPCs are more sensitive to IR as compared to post-mitotic neurons, hence more prone to apoptosis (96, 102, 103). However we observed apoptosis in differentiating and mature neurons. This observed anomaly can be explained on account of an insensitive G2/M arrest of NPCs following DNA damage. NPCs having less than 10-20 DSBs often undergo partial G2/M arrest, which is released before the completion of DSBs repair (104). NPCs with such persistent DSBs are then differentiate and migrate to the PP/CP, whereas in PP/CP these post-mitotic cells show high sensitivity to even a low level of persisting DSBs and undergo apoptosis (105). We assume that insensitive G2/M arrest as a consequence of less than 10 DSBs (Figure 4-2 B) in irradiated WT brains might be leading reason of observed apoptosis in the differentiating and mature neurons.

In conjunction with apoptosis, premature neuronal differentiation has also been evident as one of the underlying mechanisms of microcephaly in various mouse models, ZIKV infected hNPCs and brain organoids (82, 99, 106). A previous study suggested that prenatal irradiation-induced p53-mediated gene activation results in premature neuronal differentiation which leads to the microcephalic phenotype (11). We also observed premature neuronal differentiation which was evident by depletion of radial glial cells in irradiated WT brains, followed by the emergence of ectopic immature neurons (Figure 4-5 A-E). By contrast, this premature differentiation was well prevented in irradiated cKO brains. Taken together our findings propose that following radiation exposure, p53-mediated premature neuronal differentiation leads to the aberrant neurogenesis, and likely contribute to microcephaly by the depletion of NPCs. The implication of p53 in eliciting premature differentiation has not been elucidated in many studies. However, our findings are analogous to a study in which they proposed radiation-induced neuronal differentiation in C17.2 mouse neural stem cells, to be p53-dependent (107). For future studies, it will be of interest to know the exact cause of premature neuronal differentiation. Generally, mitotic defects such as altered spindle orientation, and centrosome duplication in NPCs have been associated with premature differentiation (108–110).

Ectopic neuron production had also been observed in the prenatally exposed survivors of Hiroshima and Nagasaki. This ectopic neuron production was suggested due to defects in neuronal migration. However as per our findings, we assume this ectopic neuron production is primarily due to the premature neuronal differentiation, and as evidence, we observed a significantly reduced expression of QKi5 in irradiated WT brains (Figure 4-5 F-G). The QKi5 is an epithelial marker which regulates epithelial to mesenchymal transitions (111). QKi5 is highly expressed in the NPCs, and functions to repress their differentiation. As the neuronal differentiation proceeds, the expression of QKi5 decreases and allows NPCs to differentiate (93). The altered expression of this epithelial marker possibly indicates an EMT like mechanism, as a driving factor in this premature differentiation of NPCs. EMT is a common

mechanism which is implicated in a wide range of biological processes such as embryogenesis, organogenesis, neural development and cancer metastasis (112, 113). Getting more insight by exploring other epithelial markers, and more importantly, their co-relation with p53 will further reinforce our proposition of radiation-induced premature neuronal differentiation via an EMT like mechanism.

6 Chapter 6: Conclusion and Future Perspectives

The results of our study showed the potential role of p53 in the development of radiation-induced microcephaly. Our study revealed that following embryonic DNA damage, pro-apoptotic activity of p53 is one of the key cellular mechanisms that lead to the development of microcephaly. A remarkable new finding is a radiation-induced p53-mediated premature neuronal differentiation. We indicated that this premature neuronal differentiation might involve an EMT like mechanism. The further in-depth study will help out in dissecting the interplay between p53 and premature neuronal differentiation. Thus altogether our study provided a new insight into the acute cellular and molecular mechanisms following radiation exposure, which might be important mediators in the development of radiation-induced microcephaly later in life.

In future, this study will further document the use in clinics of transient pharmacological inhibitors of p53 to prevent the detrimental effects of IR. Furthermore, on account of convergent similarities with ZIKV infection, the outcome of this research might be able to propose therapeutic interventions for ZIKV infection during pregnancy which also results in congenital microcephaly (114). Our research group is also working on the human iPSC-derived brain organoids which are a better candidate to represent the complexity of human brains in comparison to the rodent's brain (115). The obtained results from brain organoids will further help out in understanding the etiology of radiation-induced microcephaly.

References

1. Rodier PM. 2004. Environmental causes of central nervous system maldevelopment. Pediatrics 113:1076–83.

2. Rice D, Barone S, Jr. 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 108 Suppl:511–33.

3. Williams PM. 2010. Health Effects of Prenatal Radiation Exposure.

4. Schull WJ, Norton S, Jensh RP. 1990. Ionizing radiation and the developing brain. Neurotoxicol Teratol 12:249–260.

5. Otake M, Schull WJ. 1998. Radiation-related brain damage and growth retardation among the prenatally exposed atomic bomb survivors. Int J Radiat Biol 74:159–71.

6. Shi Y, Kirwan P, Smith J, Robinson HPC, Livesey FJ. 2012. Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. Nat Neurosci 15:477–486.

7. Wertelecki W, Yevtushok L, Zymak-Zakutnia N, Wang B, Sosyniuk Z, Lapchenko S, Hobart HH. 2014. Blastopathies and microcephaly in a Chornobyl impacted region of Ukraine. Congenit Anom (Kyoto) 54:125–49.

8. Kodama K, Mabuchi K, Shigematsu I. 1996. A Long-Term Cohort Study of the Atomic-Bomb Survivors. J Epidemiol 6:95–105.

9. Otake M, Schull WJ. 1993. Radiation-related Small Head Sizes among Prenatally Exposed A-bomb Survivors. Int J Radiat Biol 63:255–270.

10. Verreet T, Rangarajan JR, Quintens R, Verslegers M, Lo AC, Govaerts K, Neefs M, Leysen L, Baatout S, Maes F, Himmelreich U, D'Hooge R, Moons L, Benotmane MA. 2016. Persistent Impact of In utero Irradiation on Mouse Brain Structure and Function Characterized by MR Imaging and Behavioral Analysis. Front Behav Neurosci 10:83.

11. Quintens R, Verreet T, Janssen A, Neefs M, Leysen L, Michaux A, Verslegers M, Samari N, Pani G, Verheyde J, Baatout S, Benotmane MA. 2015. Identification of novel radiation-induced p53-dependent transcripts extensively regulated during mouse brain development. Biol Open 4:331–44.

12. Quintens R. 2017. Convergence and divergence between the transcriptional responses to Zika virus infection and prenatal irradiation. Cell Death Dis 8:e2672–e2672.

13. Gabriel E, Ramani A, Karow U, Gottardo M, Natarajan K, Gooi LM, Goranci-Buzhala G, Krut O, Peters F, Nikolic M, Kuivanen S, Korhonen E, Smura T, Vapalahti O, Papantonis A, Schmidt-Chanasit J, Riparbelli M, Callaini G, Krönke M, Utermöhlen O, Gopalakrishnan J. 2017. Recent Zika Virus Isolates Induce Premature Differentiation of Neural Progenitors in Human Brain Organoids. Cell Stem Cell 20:397–406.e5.

14. Devhare P, Meyer K, Steele R, Ray RB, Ray R. 2017. Zika virus infection dysregulates human neural stem cell growth and inhibits differentiation into neuroprogenitor cells. Cell Death Dis 8:e3106.

15. Pavlidis NA. 2002. Coexistence of pregnancy and malignancy. Oncologist 7:279–87.

16. Andersson TM-L, Johansson AL V., Fredriksson I, Lambe M. 2015. Cancer during pregnancy and the postpartum period: A population-based study. Cancer 121:2072–2077.

17. Luis S, Christie D, Kaminski A, Kenny L, Peres M. 2009. Pregnancy and radiotherapy: Management options for minimising risk, case series and comprehensive literature review. J Med Imaging Radiat Oncol 53:559–568.

18. Kal HB, Struikmans H. 2005. Radiotherapy during pregnancy: fact and fiction. Lancet Oncol 6:328–333.

19. DeFelipe J. 2011. The Evolution of the Brain, the Human Nature of Cortical Circuits, and Intellectual Creativity. Front Neuroanat 5:29.

20. Stiles J, Jernigan TL. 2010. The basics of brain development. Neuropsychol Rev 20:327–48.

21. Back SA, Plawner LL. 2012. Congenital Malformations of the Central Nervous System, p. 844–868. *In* Avery's Diseases of the Newborn. Elsevier.

22. Harris J, Tomassy GS, Arlotta P. 2015. Building blocks of the cerebral cortex: from development to the dish. Wiley Interdiscip Rev Dev Biol 4:529–44.

23. Brain Regions and Functions | Ask A Biologist.

24. Rakic P. 2009. Evolution of the neocortex: a perspective from developmental biology. Nat Rev Neurosci 10:724–35.

25. Lui JH, Hansen DV, Kriegstein AR. 2011. Development and Evolution of the Human Neocortex. Cell 146:18–36.

26. Götz M, Huttner WB. 2005. The cell biology of neurogenesis. Nat Rev Mol Cell Biol 6:777–788.

27. Laguesse S, Peyre E, Nguyen L. 2015. Progenitor genealogy in the developing cerebral cortex. Cell Tissue Res 359:17–32.

28. Budday S, Steinmann P, Kuhl E. 2015. Physical biology of human brain development. Front Cell Neurosci 9:257.

29. Florio M, Huttner WB. Neural progenitors, neurogenesis and the evolution of the neocortex.

30. Laguesse S, Peyre E, Nguyen L. Progenitor genealogy in the developing cerebral cortex.

31. Lui JH, Hansen D V, Kriegstein AR. 2011. Development and evolution of the human neocortex. Cell 146:18–36.

32. Sun T, Hevner RF. 2014. Growth and folding of the mammalian cerebral cortex: from molecules to malformations. Nat Rev Neurosci 15:217–32.

33. Dehay C, Kennedy H, Kosik KS. 2015. The Outer Subventricular Zone and Primate-

Specific Cortical Complexification. Neuron 85:683–694.

34. Molnár Z, Métin C, Stoykova A, Tarabykin V, Price DJ, Francis F, Meyer G, Dehay C, Kennedy H. 2006. Comparative aspects of cerebral cortical development. Eur J Neurosci 23:921–34.

35. Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ. 2013. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Prog Neurobiol 106–107:1–16.

36. Cryan JF, Holmes A. 2005. The ascent of mouse: advances in modelling human depression and anxiety. Nat Rev Drug Discov 4:775–790.

37. Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ. 2013. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Prog Neurobiol 106–107:1–16.

38. Gray J, Ross ME. 2011. Neural Tube Closure in Mouse Whole Embryo Culture. J Vis Exp.

39. Kwan KY, Sestan N, Anton ES, Gruss P. 2012. Transcriptional co-regulation of neuronal migration and laminar identity in the neocortex. Development 139:1535–46.

40. Sun T, Hevner RF. 2014. Growth and folding of the mammalian cerebral cortex: from molecules to malformations. Nat Rev Neurosci 15:217–32.

41. Faheem M, Naseer MI, Rasool M, Chaudhary AG, Kumosani TA, Ilyas AM, Pushparaj P, Ahmed F, Algahtani HA, Al-Qahtani MH, Saleh Jamal H. 2015. Molecular genetics of human primary microcephaly: an overview. BMC Med Genomics 8 Suppl 1:S4.

42. Rodier PM. 2004. Environmental causes of central nervous system maldevelopment. Pediatrics 113:1076–83.

43. Grupen C, Rodgers M. 2016. Is Radioactivity Everywhere?, p. 61–78. *In* Radioactivity and Radiation. Springer International Publishing, Cham.

44. Zamanian A, Hardiman C. Electromagnetic Radiation and Human Health: A Review of Sources and Effects.

45. Chang DS, Lasley FD, Das IJ, Mendonca MS, Dynlacht JR. 2014. Interactions of Electromagnetic Radiation with Matter, p. 35–41. *In* Basic Radiotherapy Physics and Biology. Springer International Publishing, Cham.

46. Chang DS, Lasley FD, Das IJ, Mendonca MS, Dynlacht JR. 2014. Interactions of Particulate Radiation with Matter, p. 43–54. *In* Basic Radiotherapy Physics and Biology. Springer International Publishing, Cham.

47. Beyzadeoglu M, Ozyigit G, Ebruli C. 2010. Radiation Physics, p. 1–70. *In* Basic Radiation Oncology. Springer Berlin Heidelberg, Berlin, Heidelberg.

48. Sutherland BM, Bennett P V, Schenk H, Sidorkina O, Laval J, Trunk J, Monteleone D, Sutherland J. 2001. Clustered DNA damages induced by high and low LET radiation, including heavy ions. Phys Med 17 Suppl 1:202–4.

49. Thomas GA, Symonds P. 2016. Radiation Exposure and Health Effects - is it Time to Reassess the Real Consequences? Clin Oncol (R Coll Radiol) 28:231–6.

50. Grupen C, Rodgers M. 2016. What Are Radioactivity and Radiation?, p. 7–25. *In* Radioactivity and Radiation. Springer International Publishing, Cham.

51. Spitz DR, Hauer-Jensen M. 2014. Ionizing radiation-induced responses: where free radical chemistry meets redox biology and medicine. Antioxid Redox Signal 20:1407–9.

52. Azzam EI, Jay-Gerin J-P, Pain D. 2012. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett 327:48–60.

53. Desouky O, Ding N, Zhou G. 2015. Targeted and non-targeted effects of ionizing radiation. J Radiat Res Appl Sci 8:247–254.

54. SHIKAZONO N, NOGUCHI M, FUJII K, URUSHIBARA A, YOKOYA A. 2009. The Yield, Processing, and Biological Consequences of Clustered DNA Damage Induced by Ionizing Radiation. J Radiat Res 50:27–36.

55. Lomax ME, Folkes LK, O'Neill P. 2013. Biological Consequences of Radiation-induced DNA Damage: Relevance to Radiotherapy. Clin Oncol 25:578–585.

56. Senturk E, Manfredi JJ. 2013. p53 and cell cycle effects after DNA damage. Methods Mol Biol 962:49–61.

57. Ozaki T, Nakagawara A. 2011. Role of p53 in Cell Death and Human Cancers. Cancers (Basel) 3:994–1013.

58. Dolgin E. 2017. The most popular genes in the human genome. Nature 551:427–431.

59. Ozaki T, Nakagawara A. 2011. Role of p53 in Cell Death and Human Cancers. Cancers (Basel) 3:994–1013.

60. Menendez D, Inga A, Resnick MA. 2009. The expanding universe of p53 targets. Nat Rev Cancer 9.

61. Bekker-Jensen S, Mailand N. 2010. Mini-review Assembly and function of DNA double-strand break repair foci in mammalian cells. DNA Repair (Amst) 9:1219–1228.

62. Wang B. 2014. Analyzing Cell Cycle Checkpoints in Response to Ionizing Radiation in Mammalian Cells, p. 313–320. *In* . Humana Press, New York, NY.

63. Vignard J, Mirey G, Salles B. 2013. Ionizing-radiation induced DNA double-strand breaks: a direct and indirect lighting up. Radiother Oncol 108:362–9.

64. Menon V, Povirk L. 2014. Involvement of p53 in the repair of DNA double strand breaks: multifaceted Roles of p53 in homologous recombination repair (HRR) and non-homologous end joining (NHEJ). Subcell Biochem 85:321–36.

65. Cell Checkpoints | BioNinja.

66. Fei P, El-Deiry WS. 2003. P53 and radiation responses. Oncogene 22:5774–5783.

67. Bassing CH, Alt FW. 2004. The cellular response to general and programmed DNA

double strand breaks. DNA Repair (Amst) 3:781–796.

68. Bates S, Vousden KH. 1999. Mechanisms of p53-mediated apoptosis. C Cell Mol Life Sci 55:28–37.

69. Brady CA, Attardi LD. 2010. p53 at a glance. J Cell Sci 123:2527–32.

70. Amaral JD, Xavier JM, Steer CJ, Rodrigues CM. 2010. The Role of p53 in Apoptosis. Discov Med 9:145–152.

71. Little JN, Dwyer ND. p53 deletion rescues lethal microcephaly in a mouse model with neural stem cell 1 abscission defects 2 3 4 5.

72. Woods CG, Bond J, Enard W. 2005. Autosomal recessive primary microcephaly (MCPH): a review of clinical, molecular, and evolutionary findings. Am J Hum Genet 76:717–28.

73. Von M, Hagen D, Pivarcsi | Mark, Liebe J, Horst |, Bernuth V, Didonato N, Julia |, Hennermann B, Christoph |, Uhrer B€, Wieczorek D, Angela |, Kaindl M, Kaindl AM. 2014. DEVELOPMENTAL MEDICINE & CHILD NEUROLOGY Diagnostic approach to microcephaly in childhood: a two-center study and review of the literature.

74. Facts about Microcephaly | Birth Defects | NCBDDD | CDC.

75. Fujimori A, Yaoi T, Ogi H, Wang B, Suetomi K, Sekine E, Yu D, Kato T, Takahashi S, Okayasu R, Itoh K, Fushiki S. 2008. Ionizing radiation downregulates ASPM, a gene responsible for microcephaly in humans. Biochem Biophys Res Commun 369:953–957.

76. Otake M, Schull WJ. 1998. Radiation-related brain damage and growth retardation among the prenatally exposed atomic bomb survivors. Int J Radiat Biol 74:159–71.

77. Schull WJ. Brain Damage Among Individuals Exposed Prenatally to Ionizing Radiation: A 1993 Review.

78. Hall EJ, Giaccia AJ. Radiobiology for the radiologist.

79. Verreet T, Quintens R, Van Dam D, Verslegers M, Tanori M, Casciati A, Neefs M, Leysen L, Michaux A, Janssen A, D'Agostino E, Vande Velde G, Baatout S, Moons L, Pazzaglia S, Saran A, Himmelreich U, De Deyn PP, Benotmane MA. 2015. A multidisciplinary approach unravels early and persistent effects of X-ray exposure at the onset of prenatal neurogenesis. J Neurodev Disord 7:3.

80. Shimada M, Matsuzaki F, Kato A, Kobayashi J, Matsumoto T, Komatsu K. 2016. Induction of Excess Centrosomes in Neural Progenitor Cells during the Development of Radiation-Induced Microcephaly. PLoS One 11:e0158236.

81. Bekaert S, Derradji H, Meyer T De, Michaux A, Buset J, Neefs M, Mergeay M, Jacquet P, Van Oostveldt P, Baatout S. 2005. Telomere shortening is associated with malformation in p53-deficient mice after irradiation during specific stages of development. DNA Repair (Amst) 4:1028–1037.

82. McMahon JJ, Miller EE, Silver DL. 2016. The exon junction complex in neural development and neurodevelopmental disease. Int J Dev Neurosci 55:117–123.

83. Zhou Z-W, Tapias A, Bruhn C, Gruber R, Sukchev M, Wang Z-Q. 2013. DNA damage response in microcephaly development of MCPH1 mouse model. DNA Repair (Amst) 12:645–655.

84. O'Driscoll M, Jeggo PA. 2008. The role of the DNA damage response pathways in brain development and microcephaly: Insight from human disorders. DNA Repair (Amst) 7:1039–1050.

85. Iyama T, Wilson DM. 2013. DNA repair mechanisms in dividing and non-dividing cells. DNA Repair (Amst) 12:620–636.

86. Chatterjee N, Walker GC. 2017. Mechanisms of DNA damage, repair, and mutagenesis. Environ Mol Mutagen 58:235–263.

87. Ward IM, Reina-San-Martin B, Olaru A, Minn K, Tamada K, Lau JS, Cascalho M, Chen L, Nussenzweig A, Livak F, Nussenzweig MC, Chen J. 2004. 53BP1 is required for class switch recombination. J Cell Biol 165:459–464.

88. Deckbar D, Jeggo PA, Löbrich M. 2011. Understanding the limitations of radiation-induced cell cycle checkpoints. Crit Rev Biochem Mol Biol 46:271–83.

89. Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranalli T, Brinkley BR, Bazett-Jones DP, Allis CD. 1997. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106:348–360.

90. Kashiwagi H, Shiraishi K, Sakaguchi K, Nakahama T, Kodama S. 2018. Repair kinetics of DNA double-strand breaks and incidence of apoptosis in mouse neural stem/progenitor cells and their differentiated neurons exposed to ionizing radiation. J Radiat Res 59:261–271.

91. Englund C, Fink A, Lau C, Pham D, Daza RAM, Bulfone A, Kowalczyk T, Hevner RF. 2005. Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J Neurosci 25:247–51.

92. Ayanlaja AA, Xiong Y, Gao Y, Ji G, Tang C, Abdikani Abdullah Z, Gao D. 2017. Distinct Features of Doublecortin as a Marker of Neuronal Migration and Its Implications in Cancer Cell Mobility. Front Mol Neurosci 10:199.

93. Shu P, Fu H, Zhao X, Wu C, Ruan X, Zeng Y, Liu W, Wang M, Hou L, Chen P, Yin B, Yuan J, Qiang B, Peng X. 2017. MicroRNA-214 modulates neural progenitor cell differentiation by targeting Quaking during cerebral cortex development. Sci Rep 7:8014.

94. Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW, Vogelstein B. 1997. 14-3-3sigma is a p53-regulated inhibitor of G2/M progression. Mol Cell 1:3–11.

95. Deckbar D, Jeggo PA, Löbrich M. 2011. Understanding the limitations of radiation-induced cell cycle checkpoints. Crit Rev Biochem Mol Biol 46:271–283.

96. Gatz SA, Ju L, Gruber R, Hoffmann E, Carr AM, Wang Z-Q, Liu C, Jeggo PA. 2011. Requirement for DNA ligase IV during embryonic neuronal development. J Neurosci

31:10088–100.

97. Verheyde J, de Saint-Georges L, Leyns L, Benotmane MA. 2006. The Role of Trp53 in the Transcriptional Response to Ionizing Radiation in the Developing Brain. DNA Res 13:65–75.

98. Silver DL, Watkins-Chow DE, Schreck KC, Pierfelice TJ, Larson DM, Burnetti AJ, Liaw H-J, Myung K, Walsh CA, Gaiano N, Pavan WJ. 2010. The exon junction complex component Magoh controls brain size by regulating neural stem cell division. Nat Neurosci 13:551–8.

99. Lizarraga SB, Margossian SP, Harris MH, Campagna DR, Han A-P, Blevins S, Mudbhary R, Barker JE, Walsh CA, Fleming MD. 2010. Cdk5rap2 regulates centrosome function and chromosome segregation in neuronal progenitors. Development 137:1907–17.

100. Kubota, S. Takahashi, X.-Z. Sun, H. Y. 2000. Radiation-induced tissue abnormalities in fetal brain are related to apoptosis immediately after irradiation. Int J Radiat Biol 76:649–659.

101. Teng Y, Liu S, Guo X, Liu S, Jin Y, He T, Bi D, Zhang P, Lin B, An X, Feng D, Mi Z, Tong Y. 2017. An Integrative Analysis Reveals a Central Role of P53 Activation via MDM2 in Zika Virus Infection Induced Cell Death. Front Cell Infect Microbiol 7:327.

102. Hoshino K, Kameyama Y. 1988. Developmental-stage-dependent radiosensitivity of neural cells in the ventricular zone of telencephalon in mouse and rat fetuses. Teratology 37:257–262.

103. Vogin G, Foray N. 2012. The law of Bergonié and Tribondeau: A nice formula for a first approximation The law of Bergoni é and Tribondeau: A nice formula for a fi rst approximation. Artic Int J Radiat Biol 1–7.

104. Deckbar D, Jeggo PA, Löbrich M. 2011. Understanding the limitations of radiation-induced cell cycle checkpoints. Crit Rev Biochem Mol Biol 46:271–83.

105. Gatz SA, Ju L, Gruber R, Hoffmann E, Carr AM, Wang Z-Q, Liu C, Jeggo PA. 2011. Requirement for DNA ligase IV during embryonic neuronal development. J Neurosci 31:10088–100.

106. Gabriel E, Ramani A, Karow U, Gottardo M, Natarajan K, Gooi LM, Goranci-Buzhala G, Krut O, Peters F, Nikolic M, Kuivanen S, Korhonen E, Smura T, Vapalahti O, Papantonis A, Schmidt-Chanasit J, Riparbelli M, Callaini G, Krönke M, Utermöhlen O, Gopalakrishnan J. 2017. Recent Zika Virus Isolates Induce Premature Differentiation of Neural Progenitors in Human Brain Organoids. Cell Stem Cell 20:397–406.e5.

107. Eom HS, Park HR, Jo SK, Kim YS, Moon C, Kim S-H, Jung U. 2016. Ionizing Radiation Induces Altered Neuronal Differentiation by mGluR1 through PI3K-STAT3 Signaling in C17.2 Mouse Neural Stem-Like Cells. PLoS One 11:e0147538.

108. Pilaz L-J, McMahon JJ, Miller EE, Lennox AL, Suzuki A, Salmon E, Silver DL. 2016. Prolonged Mitosis of Neural Progenitors Alters Cell Fate in the Developing Brain. Neuron 89:83–99.

109. Shimada M, Matsuzaki F, Kato A, Kobayashi J, Matsumoto T, Komatsu K. 2016.

Induction of Excess Centrosomes in Neural Progenitor Cells during the Development of Radiation-Induced Microcephaly. PLoS One 11:e0158236.

110. Insolera R, Bazzi H, Shao W, Anderson K V, Shi S-H. 2014. Cortical neurogenesis in the absence of centrioles. Nat Neurosci 17:1528–35.

111. Conn SJ, Pillman KA, Toubia J, Conn VM, Salmanidis M, Phillips CA, Roslan S, Schreiber AW, Gregory PA, Goodall GJ. 2015. The RNA binding protein quaking regulates formation of circRNAs. Cell 160:1125–34.

112. Kalcheim C. 2015. Epithelial-Mesenchymal Transitions during Neural Crest and Somite Development. J Clin Med 5.

113. Kalluri R, Weinberg RA. 2009. The basics of epithelial-mesenchymal transition. J Clin Invest 119:1420–8.

114. Gabriel E, Ramani A, Karow U, Gottardo M, Natarajan K, Gooi LM, Goranci-Buzhala G, Krut O, Peters F, Nikolic M, Kuivanen S, Korhonen E, Smura T, Vapalahti O, Papantonis A, Schmidt-Chanasit J, Riparbelli M, Callaini G, Krönke M, Utermöhlen O, Gopalakrishnan J. 2017. Recent Zika Virus Isolates Induce Premature Differentiation of Neural Progenitors in Human Brain Organoids. Cell Stem Cell 20:397–406.e5.

115. Gabriel E, Gopalakrishnan J. 2017. Generation of iPSC-derived Human Brain Organoids to Model Early Neurodevelopmental Disorders. J Vis Exp.