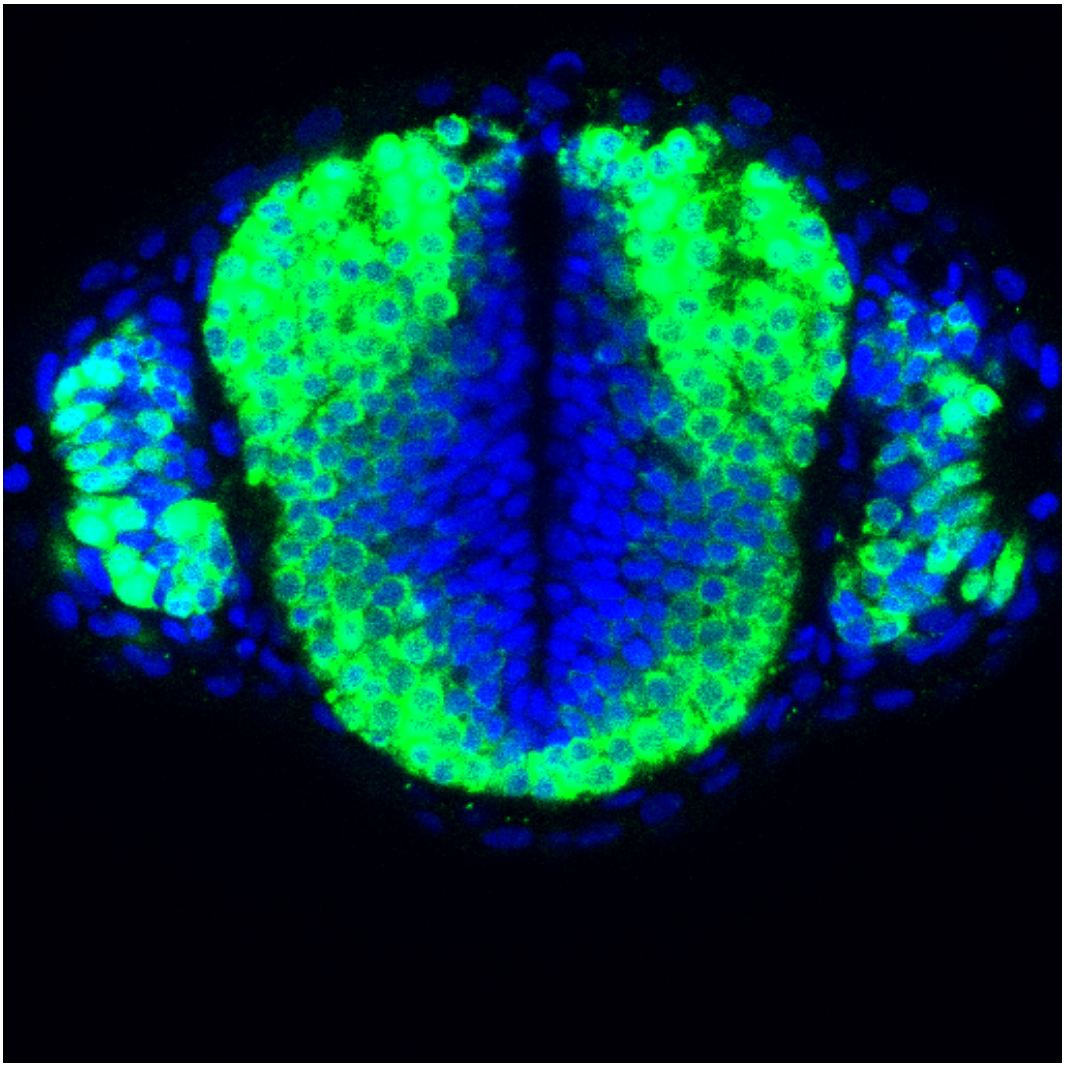

CDK5RAP2 function during Zebrafish neurogenesis.

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Farmacologia Aplicada. O trabalho foi realizado sob a orientação científica da Investigadora Doutora Paula Alexandre (*Institue of Child Health – University College London*) e supervisão da Professora Doutora Cláudia Margarida Gonçalves Cavadas (Faculdade de Farmácia da Universidade de Coimbra).

Setembro 2014

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On the front page:
Optical section of zebrafish telencephalon with all nuclei stained with DAPI (blue) and neurons expressing HuC/D (green).

CDK5RAP2 function during Zebrafish neurogenesis.

Tiago Filipe Mendes Martins

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Universidade de Coimbra
2014

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Aim of Study

The aim of the present work is to investigate the role of CDK5RAP2 on brain development and modulation of neuronal differentiation in zebrafish embryos.

Our specific aims were:

1) To determine the expression of *zCdk5rap2* during zebrafish development (by immunohistochemistry and in situ hybridization)
2) To knockdown *Cdk5rap2* function by injecting anti sense oligo morpholinos and determine their effect in production of microcephalic phenotypes, cell polarity, and neurogenesis.
3) To validate morpholino and custom made antibody specificity.

Abstract

Primary Microcephaly is a human brain disease that develops during gestation and it is characterized by a small brain phenotype. Several centrosome-associated proteins have been associated with this disease (*Cdk5rap2, Aspm, Wdr62*, etc). They are associated with regulation of many characteristics of cell division. It has been suggested that microcephalies can arise from disruption of progenitors divisions and cell cycle exit. The aim of the present work was to investigate the role of CDK5RAP2 on brain development and modulation of neuronal differentiation in zebrafish embryos.

To abrogate *Cdk5rap2* function antisense oligo morpholinos predicted to block protein translation and splicing between exon 4 and 5 were injected. Brain size was measured; neurogenesis rate and apical/basal progenitor divisions were evaluated in telencephalon of zebrafish embryos injected with control and CDK5RAP2 morpholinos. The validations of CDK5RAP2 antisense oligo morpholinos and antibody specificity were also undertaken.

Preliminary results show that CDK5RAP2 reduced function disrupts brain growth, cell proliferation and neurogenesisin zebrafish embryos. Microcephaly does not result from an increase in cell death caused by morpholino injection neither from a disruption of polarity proteins. We could not observed an increase in neurogenesis as previously reported. In telencephalon of zebrafish embryos, with morpholinos injection, neurogenesis and cell proliferation are decreased. These results might suggest that progenitors maybe arrested at some point in the cell cycle or in increased cell cycle length. Further live-imaging experiments are required to confirm these observations. So far, the present work suggests that decrease on CDK5RAP2 function causes microcephalies and prevents neuronal differentiation.

Resumo

Microcefalia de origem primária é uma doença caracterizada por afectar o desenvolvimento cerebral. *Cdk5rap2, Aspm* e *Wdr62* são algumas das proteínas centrossomáis que têm sido descritas como sendo associadas a microcefalias. As proteínas associadas aos centrossomas são evidenciadas como reguladoras da divisão celular e tem sido sugerido que a saída prematura do ciclo celular e a interferência com o tipo de divisão de células progenitoras pode causar microcefalias. O objetivo deste estudo consistiu em investigar o papel de *Cdk5rap2* no desenvolvimento cerebral e na modulação da diferenciação neuronal em embriões de peixe zebra.

A disfunção de *Cdk5rap2* foi conseguida através da injeção de *oligoantisense morpholinos* que estão desenhados de forma a bloquear a tradução proteica e o *splicing* entre o exão 4 e 5 que, teoricamente, levaria à formação de uma proteína truncada. De forma a avaliar os efeitos da injeção dos *morpholinos* contra CDK5RAP2 e *morpholinos* controlo, foi medido o tamanho do cérebro e foram contadas o número células presentes no telencéfalo. A validação do *oligoantisense morpholino* para CDK5RAP2 e a especificidade do anticorpo desenhado contra CDK5RAP2 de peixe zebra foram também estudadas.

Os resultados obtidos sugerem que a redução da função de CDK5RAP2 afeta o crescimento do cérebro, a proliferação celular e a neurogénese no peixe zebra. As microcefalias observadas não são resultantes de um aumento da morte celular ou de algum tipo de interferência causada nas proteínas de manutenção de polaridade possivelmente resultante da injeção do *morpholino*. A diminuição da expressão de Cdk5rap2 não induziu um aumento da neurogénese, como descrito anteriormente em mamíferos. No entanto, a injeção de *morpholino* induziu uma diminuição da neurogénese. Quando foi injetado *morpholino* para *Cdk5rap2,* foi observado uma diminuição da proliferação celular. Desta forma os resultados obtidos podem sugerir que existe um bloqueio do ciclo celular, em alguma das suas fases, ou até o aumento do tempo em que a célula permanece em divisão. Estes resultados deverão ser confirmados realizando ensaios de imagem em tempo real (*live-imaging*) de modo a avaliar uma mudança no destino previsto da célula entre a diferenciação ou a proliferação.

Os resultados obtidos no presente trabalho sugerem existir uma diminuição na diferenciação neuronal e um desenvolvimento de microcefalias com a injeção do *morpholino* contra CDK5RAP2

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Abbreviated Terms

Anti-aPKC	Monoclonal antibody against atypical protein kinase
Anti-HuC/D	Monoclonal antibody against Elav family members HuC, HuD and Hel-N1 neuronal proteins
Anti- PH3	Monoclonal antibody against phosphohistone-H3
Anti-ZO1	Monoclonal antibody against zona occludens-1
ANOVA	Analysis of variance
AP	Alkaline phosphatase
ASPM	Abnormal spindle-like, microcephaly associated
ATG MO	Cdk5rap2 translation-block antisense oligo morpholino
bp	Nucleic base pairs
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CDK5	Cyclin-dependent kinase 5
CDK5R1	Receptor 1 of Cyclin-dependent kinase 5
CDK5RAP2	Cyclin-dependent kinase 5 regulatory associated protein 2
CENPJ	Centromeric protein J
CMO	Standard control morpholino
CNN	Centrin
CNS	Central Nervous System
CPAP	Cell-cycle regulated protein that controls centriole length
DAPI	4',6' diamino-2-phenylindole
DNA	Deoxyribonucleic acid
dpf	Days post-fertilization
ELISA	Enzyme-Linked Immunosorbent Assay
g	Gram
h	Hours

hpf	Hours post-fertilization
kDa	Kilodaltons
l	Liter
MCPH	Autosomal recessive primary microcephaly
MCPH3	Autosomal recessive primary microcephaly type 3
mg	Miligram
MINDBOMB	Ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta
MIS MO	Cdk5rap2 translation-block mismatch antisense oligo morpholino
mT	Meting Temperature
min	Minutes
ml	Milliliter
mM	Millimolar
MO	Antisense Oligo Morpholino
mRNA	Mature Ribonucleic Acid
NEDD1	Neural precursor cell expressed, developmentally down-regulated 1
ng	Nanogram
OFC	Occipital-frontal head circumference
o/n	Overnight
PAR3	Polarity protein Par3
PBS	Phosphate buffered saline
PBT	Phosphate buffered saline with 0.1% of tween-20

PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pg	Pictogram
PK	Proteinase K
Pre-mRNA	Precursor mRNA (immature single strand of messenger ribonucleic acid)
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Real Time Polymerase chain reaction
SB MO	Cdk5rap2 splice-block antisense oligo morpholino
SS	Somites number
Stil	Centrosomal proteins Stil
Wdr62	Centrosomal protein Wdr62
w/v	Weight per volume
ZO1	Zona occludens-1
µg	Microgram
µl	Microliter
µm	Micrometer

Chapter I

Introduction

Brain development

The central nervous system (CNS) of vertebrates is specified during early embryonic development and is derived from the ectodermal layer. The neural plate is formed by neuroepithelial cells (also called neural progenitors) and later generates the neural tube that is already patterned along the anteroposterior axis in brain and spinal cord. The telencephalon is the most anterior part of the neural tube and in mammalian systems has the highest rate of cell proliferation and growth. Neuroepithelial cells (also known as radial glia in mammalian cortex) initially proliferate symmetrically to generate two other neuroepithelial cells. This increases the neural progenitor pool and determines final brain size. Later in embryonic development, neuroepithelial cells start differentiating by undergoing asymmetric divisions that generate a radial glia cell and a neuron, which will migrate to different parts of the brain. For this reason, neuroepithelial cells are often called the mother cells of all brain cells. During the embryonic development, by extending axons and dendrites neurons will establish communications through the synapses with other neurons. Small perturbations on different aspects of brain development can lead to several deformities such as anencephaly, spinal bifida, microcephalies and many other developmental brain disorders. (Mochida 2008; Lizarraga *et al.* 2010 and Woods *et al.* 2005)

Primary microcephalies

Microcephaly is a rare disease characterized by small brain phenotype determined by significant reduction in the occipital-frontal head circumference (OFC) compared with age and sex matched controls. Individuals suffering from this disease may suffer from intellectually disability, epilepsy, and others (Mochida 2008). If microcephaly develops during embryonic development is called primary microcephaly and it is detectable prior to 36 weeks of gestation, while secondary microcephaly develops in general after birth.

The exact cause(s) of microcephaly are unknown and there are currently no therapies. However, have been many genetic and environmental factors linked to

microcephaly, such as alcohol consumption by mother prior to birth. Genetic factors are usually associated with failure or decrease in neurogenesis, loss of progenitors population, cell cycle arrest, cell death, neuronal degeneration and/or defects on neuronal migration have been linked to small brain phenotype in several organisms (Issa *et al.* 2013b). The present thesis will focus on the genetic disorders at the origin of primary microcephalies.

Primary microcephaly have been clinically divided in to four subtypes: 1) primary microcephaly with dwarfism – it is important to distinguish between proportionate growth retardation where the OFC is reduced to a greater extent than length and weight; 2) primary microcephaly, with dysmorphic features and/or congenital anomalies; 3) primary microcephaly with additional clinical features such as mitochondrial and metabolic disorders which are usually predominant; and lastly 4) the 'autosomal recessive primary microcephaly' (MCPH) phenotype which is usually diagnosed in a child presenting with developmental delay of mild–moderate degree with a normal brain scan but no other abnormal growth, and dysmorphic or neurological features (Woods *et al.* 2013). The present work will focus on primary microcephalies that result from centrosomal proteins misfunction. This will include microcephalies subtypes 1 (*Pericentrin*) and 4 (*Nedd1, Aspm, Wdr62, Cdk5rap2, CenpJ, Ninein*).

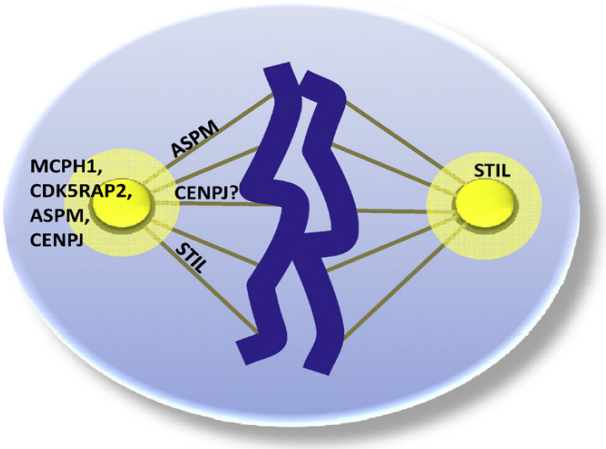

Figure 1: Localization of MCPH proteins in dividing cell. MCPH proteins are localized to the centrosome and/or mitotic spindle poles. Adapted by A.M. Kaindl *et al.* 2010.

Centrosomal proteins and primary microcephalies.

So far, every gene product found implicated in MCPH has been reported to display centrosomal localization. Centrosomes are small organelles that regulate many aspects of cell division. The centrosome, a key microtubule organizing centre, is composed of centrioles, embedded in a protein-rich matrix. Centrosomes control the internal spatial organization of somatic cells, and as such contribute to cell division, cell polarity and migration. Centrosomal genes are targeted by mutations in numerous human developmental disorders, ranging from diseases exclusively affecting brain development, through global growth failure syndromes (Chavali *et al.* 2014). Disruptions of centrosomal proteins function, such as; PERICENTRIN, NEEDd1, ASPM, WDR62, NINEIN CENPJ and CDJ5RAP2, have been reported as cause of neurogenesis defects.

PERICENTRIN localizes to the pericentriolar protein matrix and functions in ciliogenesis, centrosomal recruitment, and mitotic spindle assembly. PERICENTRIN recruits CDK5RAP2 to the centrosome to support its critical role in the maintenance of the progenitor pool during corticogenesis (Buchman *et al.* 2010).

NEED1 is another protein that plays a role in mitosis through its interaction with gamma-tubulin. Its known that depletion of NEDD1 causes impaired centrosome and chromatin microtubules assembly that results in the failure of microtubule nucleation and prevents proper spindle formation (Haren *et al.* 2006).

The *Aspm* gene possesses multiple functions essential to the differentiation and proliferation of neural stem and progenitor cells in developing of central nervous system. The function of the ASPM protein in neural progenitor cell expansion, as well as the localization in mitotic spindle and centrosome, suggests that regulates brain development by a cell division-related mechanism. In addition, a loss of ASPM proteins causes a massive loss of germ cells, resulting in a severe reduction in the size of the testes and ovaries, reducing fertility (Fujimori *et al.* 2013).

So far, 25 mutations of the *Wdr62* gene have been reported in 27 families or individual patients worldwide, most of them predicted to produce truncated proteins. *Wdr62* knockdown in cortical progenitors reduced their proliferative capacity, caused spindle orientation defects, decreased the integrity of centrosomes and displaced them

from spindle poles, and delayed mitotic progression (Farag *et al.* 2013).

NINEIN, is important for positioning and anchoring the microtubules in epithelial cells and was demonstrated that NINEIN associates with the mother centrioles only. Have been reported that multiple alternatively spliced transcript variants that encode different isoforms of Ninein exist (Hong *et al.* 2000).

CenpJ encodes the centromere protein CPAP, which is present at centrosomes and also in the cytoplasm of proliferating cells (Tang *et al.* 2009). Truncated mutations in *CenpJ* cause primary microcephaly and are associated with Seckel syndrome, a rare autosomal recessive disorder characterized by intrauterine and postnatal growth delay; microcephaly with mental retardation; and facial dysmorphisms (Bond *et al.* 2005). CENPJ has been shown to contribute to centriole location and formation by generating overly long centrioles (Shi *et al.* 2014).

CDK5RAP2 (cyclin-dependent kinase 5 regulatory associated protein 2) is a centrosomal protein that can cause autosomal recessive primary microcephaly, a disorder caused by defective proliferation and cell-fate determination of neural progenitors during neurogenesis and the disruption of CDK5RAP2 function results in the disorganization of interphase microtubules and the formation of disrupted mitotic spindles. CDK5RAP2 plays an essential role in the organization of microtubules by centrosomes (Jia *et al.* 2013). The present thesis focuses on CDK5RAP2 function.

MCPH Type 3 (MCPH3) and CDK5RAP2 function

In 2005, MCPH type 3 (MCPH3) was established to be caused by homozygous mutations of the *Cyclin-dependent kinase 5 regulatory subunit-associated protein 2* (*Cdk5rap2*) gene (Bond *et al.* 2005). So far, seven different mutations have been identified in MCPH families (see table 1). They include homozygous mutations in 2 Pakistani families (Bond *et al.* 2005; Hassan *et al.* 2007); a truncating homozygous mutation was identified in a six year old girl born of consanguineous Somali parents (Pagnamenta *et al.* 2012). Compound heterozygous mutations have also been identified in two patients of Caucasian origin (Lancester *et al.* 2013; Tan *et al.* 2013). The *Cdk5rap2* mutations range from a nonsense mutation in exon 4, an A to G transition in intron 26 introducing a new splice acceptor

site, a frame shift, a premature stop codon, and a nonsense mutation in exon 8. These three mutations have been proposed to lead to a truncated protein and loss of CDK5RAP2 function (Issa *et al*. 2013a).

Table 1 - Mutations identified in the Cdk5rap2 gene in MCPH3 patients

Family Origin	Mutation	Protein change	Reference
Pakistani	c.243T>A	p.S81X	Bond *et al.* 2005
Pakistani	c.246T>A	p.Y82X	Hassan *et al.* 2007
Somali	c.700G>T	p.E234X	Pagnameta *et al.* 2012
Caucasian	c.4546G>T	p.E1516X	Lancastrer *et al.* 2013
Caucasian	c.4672C>T	p.R1558X	Lancaster *et al.* 2013
Caucasian and Cherokee ancestry	c.524_528 del	p.Q175Rfs.Ter42	Tan *et al.* 2013
Caucasian and Cherokee ancestry	c.4005-1G>A	IVS26AS, G>A-1	Tan *et al.* 2013

The *Cdk5rap2* gene, for non-progressive phenotypes in humans, controls MCPH3. Neurodevelopmental studies of MCPH have shown that microcephaly begins to show evidence of decreased of cerebral cortex volume by the 24th week of gestation. Recent works suggest that this small brain phenotype results from the reduction of progenitor pool and a premature cell cycle exit and neuronal differentiation. Neither neuroimaging nor neuropathological studies have been reported to date for humans MCPH phenotype (Issa *et al*. 2013b).

CDK5RAP2 (cyclin-dependent kinase 5 regulatory associated protein 2)

- **CDK5RAP2 structure**

Nagase *et al. (2000)* first cloned human *Cdk5rap2* (designed as KIAA1633) through sequencing clones obtained from a size-fractionated fetal brain cDNA library (Negase *et al.* 2000). The human full length contains 1893 amino-acids and a 215kDa protein product (NP_060719.4). CDK5RAP2 has a predicted N-terminal interaction site with the gamma-

tubulin ring complex and two predicted structural maintenance-of-chromosomes sites known to be important for chromatid cohesion and DNA recombination during meiosis and mitosis. It also contains a Ser-rich motif for interaction with plus-end binding protein EB1, an interaction site with pericentrin, a C-terminal Cnn Motif 2 domain for Golgi complex interaction and binding to calmodulin and a C-terminal interaction site with CDK5R1, an activator or modulator of CDK5 (Revenkova *et al.* 2001; Evans *et al.* 2006; Hirano 2005; Fong *et al.* 2009; Wang *et al.* 2010; Kraemer *et al.* 2011).

Four isoforms produced by alternative splicing have been reported thus far: Isoform 1, (Q96SN8-1) is the full- length form; Isoform 2 (Q96SN8-2) is missing amino acids 702–733 (exon 19); Isoform 3 (Q96SN8-3) is missing amino acids 1009–1049 aa (exon 23); and Isoform 4 (Q96SN8-4) does not contain amino acids 1576–1654 (exon 32) (Kraemer *et al.* 2011). Further isoforms exist in other species as well, such as zebrafish, mice, rat, and others, depending on the age and organ studied (Kraemer *et al. 2011)*.

- **CDK5RAP2 expression**

CDK5RAP2 is widely expressed in mouse and human tissue with high levels detected in the CNS during early embryonic development (Ching *et al.* 2000; Bond *et al.* 2005; Buchman *et al.* 2010). *Cdk5rap2* is described as the gene with slowest rate of evolution in MCPH genes group (Rauf and Mir, 2013), and is strongly down-regulated with brain maturation. *Cdk5rap2* mRNA is widely expressed in human and in embryonic mouse tissue. There is some evidence that gene expression and/or protein isoform synthesis changes throughout murine brain development (Buchman *et al.* 2010). In humans, moderate to high expression was detected in all adult tissues and specific brain regions (thalamus, corpus callosum, substantia nigra, hippocampus and caudate nucleus) examined by quantitative RT-PCR ELISA with the highest levels in skeletal muscle, fetal liver, brain, kidney and ovary (Nagase *et al.* 2000; Ching *et al.* 2000; Bond *et al.* 2005). In mice, *Cdk5rap2* is detected in the most brain proliferative regions (Bond *et al.* 2005; Fong *et al.* 2008; Buchman *et al.* 2010).

- **Cdk5rap2 cellular localization**

Cdk5rap2 has been reported to be associated with the golgi and centrosome. Consistent with being a centrosomal protein the subcellular localization of *Cdk5rap2*

changes during the cell cycle. *Cdk5rap2* is down regulated at centrosomes during interphase by a microtubule-independent mechanism. CDK5RAP2 levels are increased in the prophase and remain high throughout mitosis until telophase, when the level drops again to interphase levels (Barrera *et al.* 2010). It has been shown that the golgi-localization of CDK5RAP2 is dependent of centrosome function and energy supply, but independent of microtubules (Wang *et al.* 2010).

- **CDK5RAP2 cellular function**

CDK5RAP2 seems to regulate several aspects of centrosome duplication in different species. All of which might affect cell division and lead to cell cycle arrest and cell death. Alternatively, the observed brain defects might have a connection to the regulation of cell cycle, DNA damage checkpoints, and thus proliferation and apoptosis (Jiang *et al.* 2005 and 2009). This could definitely impair tissue growth. During embryonic development studies it has become clear that CDK5RAP2 has an influence on brain size regulation. A shift in symmetric to asymmetric proliferation of progenitors may lead to a premature neuronal differentiation and cause brain reduction; there is a possibility that CDK5RAP2 is involved in cell cycle control and plays a role in regulating this shift, this is one of the hypothesis of MCPH phenotype caused by CDK5RAP2 (Megraw *et al.* 2011; Kraemer *et al.*2011; Issa *et al*. 2013b).

Symmetric and Asymmetric Divisions

There is evidence that controlling the balance of symmetric and asymmetric modes of division may regulate brain growth. Mouse studies revealed that when neural epithelia cells undergo an extra round of symmetric proliferative divisions instead of asymmetric mode of division significantly increased brain size (Setsuko and O'leary 2009). If neural epithelia cells start dividing asymmetrically from early on this will lead to the depletion of progenitors pool. Therefore, premature shift from symmetric to asymmetric neural progenitor cell divisions with a subsequent depletion of the progenitor pool and a reduction in the final number of neurons is one of the hypotheses believed to cause primary microcephalies (Megraw *et al.* 2011; Kraemer *et al.* 2011; Issa *et al*. 2013b). However evidence for this is still missing.

Several mechanisms such as cleavage orientation and asymmetric inheritance of cell fate determinants during cell division have been proposed to regulate the mode of cell divisions and cell fate decisions in vertebrates brain.

- **Cleavage orientation**

In vertebrates, the cleavage plane orientation during neural progenitors divisions is believed to alter neurogenesis and daughter cell fate decisions (Fish *et al.* 2006; Konno *et al.* 2008, Morin *et al.* 2011). It has been hypothesized that cleavage planes perpendicular to the ventricle surface, potentiate the symmetric mode divisions that generate for example two progenitors. While small deviations in cleavage orientation might be enough to disrupt the symmetric mode of divisions and promote asymmetric mode of division that could generate a neuron and a progenitor for example (Huttner and Kosodo, 2005; Zhong and Chia, 2008). Although this still needs to be proved. So far there is little experimental evidence supporting the role of cleavage plane orientation and modes of division. For example, Alexandre *et al.* 2010 demonstrated asymmetric divisions can result from perpendicular cleavage plane (Alexandre *et al.* 2010). Suggesting that orientation of cleavage planes alone might not influence the cell fate decisions in the vertebrate brain.

- **Asymmetric inheritance**

It is known from non-vertebrates studies that asymmetric divisions result from asymmetric inheritance of cell fate determinants. In vertebrates, similar mechanisms have been recently described. For example, asymmetric inheritance of Notch signaling regulator Mindbomb between the two daughters during neural progenitors division is dependent of the polarity protein Par3 function and might influence the fate decision between the two cell daughters (Dong *et al.* 2012). In addition, the asymmetric inheritance of Par3 protein and junction domain expressing ZO1 were described by Alexandre et al. 2010 and Konno et al. 2008, respectively, to correlate with asymmetric cell fate decisions. Less well understood is the asymmetric inheritance of mature centrosomes by the mother cell and/or stem cells while neurons inherit the young centrosome (Wang *et al.* 2009) and how centrosome could regulate cell fate decisions.

Have been shown that a switch in *Cdk5rap2* isoform expression might be specific to post-mitotic cells, as they persist in expression following the end of neurogenesis. It is

established that apical progenitors have the capacity to self-renew and maintain the progenitor pool during neurogenesis. In contrast, the vast majority of basal progenitors undergo a single terminal neurogenic division (Noctor *et al.* 2008). Thus, any decrease in the tendency of apical progenitors to undergo self-renewing divisions would be predicted to lead to a rapid depletion of the progenitor pool. On the other hand, an increase in the proportion of basal progenitors would lead to a transient increase in neuronal output. *Cdk5rap2* knockdown alters the progenitor pool itself, decreasing the relative portion of apical progenitors and increasing the relative proportion of basal progenitors. This shift is also supported by the increase in non-surface mitoses observed following *Cdk5rap2* knockdown (Buchman *et al.* 2010).

The decrease in progenitors population and premature neuronal differentiation is the main cause of decrease in brain size in mammalians caused by CDK5RAP2 misfunction. However, we still are unaware whether symmetric and asymmetric modes of division are affected.

Evolutionarily, the later cortical neuronal layers formation of mammalian cortex only appeared after mammals diverged from reptiles (Nieuwenhuys, 1994), and the formation of the superficial layers has been proposed to be primarily responsible for the disproportionate expansion of the cerebral cortex comparative with the mammals body size (Cheung *et al.* 2007). The essential roles of CDK5RAP2 protein in progenitor proliferation might reflect its relevance to the evolutionary expansion of the cerebral cortex (Kriegstein *et al.* 2006). MCPH in males are correlated with a decrease in cortical area, a finding that agrees with a primary effect of *Cdk5rap2* knockdown on proliferation of radial glia (Rimol *et al.* 2010).

Animal Model

Danio rerio (zebrafish) has become in the last years one of the most popular experimental model organisms to study the development of vertebrates. Zebrafish embryos have been shown to offer unraveled advantages compared to other vertebrate systems to study neural development and neuronal differentiation in vivo (Clarke 2009; Park and Appel 2003). Zebrafish produces a large clutch of eggs; embryos develop externally, amenable to genetic manipulation (allows the use of antisense oligo morpholinos and targeted mutations using CRispr Cas9 approaches). Their transparency (as embryos) is the biggest reason for their attractiveness to study biological processes at cellular and subcellular level *in vivo*.

Zebrafish embryogenesis is classified into distinct morphological stages, against which experimentation can be described to allow repeatable studies (figure 2). At 24 hours post fertilization (hpf), embryos develop the organ primordia, allowing rapid assessment of cell fate after previous genetic manipulation (via microinjection at early stages of embryonic development). As a vertebrate model, zebrafish embryonic development is in many aspects comparable to other vertebrate models. Zebrafish research so far has allowed putative findings from non-vertebrates models to be extrapolated to other classes, with the ultimate aim of understanding the fundamental (and pathological) developmental mechanisms of *Homo sapiens* (Halbritter *et al.* 2013; Mitchison *et al.* 2012; Beales *et al.* 2007).

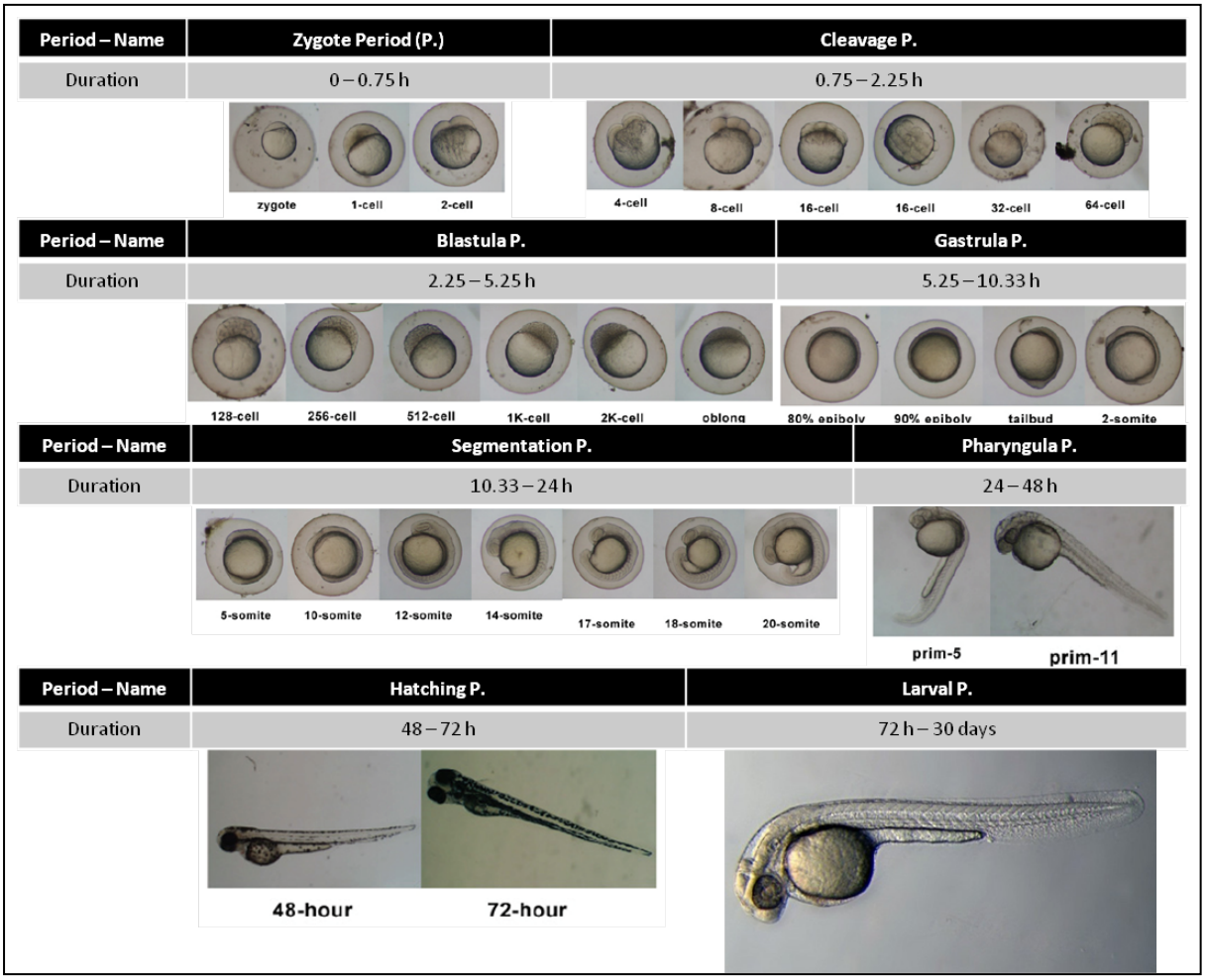

Figure 2: Timeline and Stages of Embryonic Development. The morphological staging observed here allows maximum reproducibility. For microinjection of morpholino, was used the one-cell stage embryos, this way the morpholino is homogeneously spread all over the components. For a mosaic labeling injection, embryos were injected in later stages, 64 – 128 cells (Kimmel *et al.* 1995).

Chapter II

Materials and Methods

Animal care

Wild type adult AB strain fish *Danio rerio* provided by the Institute of Child Health fish facility (UCL, London) were used in this study. The fishes were maintained on a 12h light/dark automatic cycle at 28.5°C under continuous flow of water. Fishes were feed with a special fish food: small granula (Special Diets Services, UK) 3 times daily and supplemented with brine shrimp (Interpet, UK) the night before embryo collection. For embryo collection, an adult male and 2 adult female fish, were placed inside one-liter fish tank with the inner mesh and divider. Males and females were kept in separate compartments during the night. Embryos were obtained by natural breeding by removing the divider. On collection, embryos were kept in fish water at 28°C in a cooling incubator (LMS, UK) (Westerfield *et al.* 2009). For some experiments, embryos were incubated at 24 hour post-fertilization (hpf) in N-Propytthiouracie 0.2mM (Sigma-Aldrich, USA) to block pigmentation development. All fish were anesthetized using Tricaine (Ethyl 3-aminobenzoat methanesulfonate, Sigma) 0.2mM.

All experiments involving zebrafish were conducted according to the UK Home Office rules. The research protocols outlined here comply with internationally recognized guidelines for zebrafish experimentation in biomedical research.

Antisense oligo-morpholinos (MO) and microinjection.

To abrogate CDK5RAP2 function, we designed antisense oligo-morpholinos (MO) to block translation (ATG MO) and the splicing of zebrafish CDK5RAP2 between Exon 3 and 4 (SB MO). CDK5RAP2 splice-block morpholino is predicted to retain the intron between Exons 3 and 4 and insert a premature STOP codon to produce protein truncation that would mimic one of the human mutations. As a control we designed Mismatch CdK5RAP2 ATG MO (MIS MO) that has 5 nucleotides substitution compared to the original sequence and a Standard Control MO (CMO).

All MOs were obtained from Gene Tools, LLC, USA. The MOs were dissolved to 4mM in sterile nuclease-free water (Life Technologies, USA) and stocks stored at -20°C.

For microinjection, MOs were diluted to 0.5mM (ATG MO and MIS MO), 0.6mM (SB MO) and 0.8mM (CMO) in nuclease-free water (Life Technologies, USA), and injected into one-cell stage embryos with a microinjector (Picospritzer III, Intracel, UK) with pressurized air aiding transport of MO from a glass needle into cell cytoplasm. Pressure was calibrated in each instance to ensure drop size (measured on incremented oil drop slide-graticule) represented the required amount of morpholino.

Cdk5rap2 MO sequences:

- CDK5RAP2 ATG MO: 5'-TCCTCTCCGACCACAGAATCCATTC-3'.
- CDK5RAP2 SB MO: 5'-CTGTGATTTGC TGCAAGGGAAACAT-3').
- Mismatch CDK5RAP2 ATG MO lower case letters indicate mismatch bases (5'-TCCTCTaCcACCAaAGAtTaCATTC-3')
- Standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3')

Embryos were then staged according to hours post-fertilization (hpf) and somite number (SS) (Kimmel et al., 1995). The MOs injected embryos were incubated in fish water at 28°C. Their embryos development was evaluated at distinctive time points (20hpf, 36hpf, 48hpf).

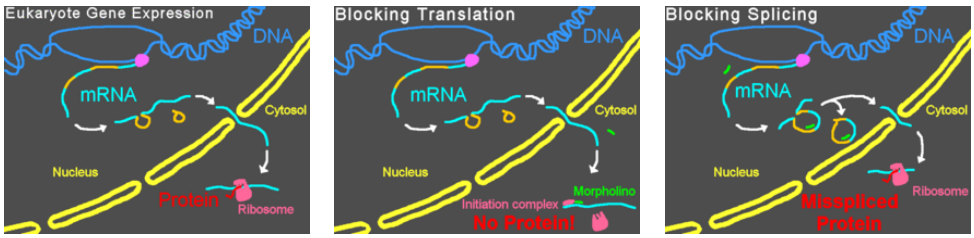

Figure 3: Morpholino function. A) Eukaryote gene expression. B) Blocking translation by morpholino injection. C) Blocking splicing by morpholino injection. Image adapted from Jon D. Moulton of Gene Tools, LLC.

Whole-Mount Immunohistochemistry

Embryos injected with CMO, CDK5RAP2 ATG MO, CDK5RAP2 SB MO and non-injected embryos were fixed in paraformaldehyde (PFA) 4% (w/v) (Sigma-Aldrich, USA) in PBS (Sodium chloride 8g/l; Potassiumchloride 0.2g/l; Di-sodium hydrogen phosphate 1.15 g/l and Potassium dihydrogen phosphate 1.1g/l) (Thermo Scientific, UK) for 2h at room temperature (RT) after 20hpf, 36hpf and 48hpf. Embryos were washed 3x 5min in PBS + 0.1% Tween-20 (PBT) and incubated in Blocking Solution (10% Sheep Serum, 0.3% Tween in 1x PBS) for 1h at RT. To enhance the primary antibody penetration in older embryos (36 and 48hpf), embryos were given 2 cryogenic shock treatments in cryogenic solution (5% Sheep serum, 8% Sucrose, 1% Tween in 1x PBS) at -20°C, 10min each. Embryos were washed 3x 5min each, in PBT. The cryogenic treatment was followed by Proteinase K treatment (PK 1mg/ml in PBT) 30min at RT. Embryos were washed 3x 5min each, fixed in 4% PFA for 10min to inactivate the Proteinase K and washed 3x 5min. Embryos were then incubated with primary antibodies (see table 2) in Blocking solution overnight (o/n) at 4°C. On the second day of whole-mount immunohistochemistry, embryos were washed 10x 10min each, in PBT to remove the excess of primary antibody, incubated in blocking solution for 1h at RT, and left with the correspondent secondary antibodies (see table 2) diluted 1:500 and DAPI (4',6-Diamidino-2-Phenylindole) 1:10000 (Life Technologies, USA) in blocking solution o/n at 4°C. After washing 20x 10min each, in PBT, embryos were mounted in 1.5% low-melting point agarose. Images were acquired in laser scan confocal microscope (Zeiss LSM 710, Germany) and analyzed by ImageJ software.

Frozen embryo sections Immunohistochemistry

Zebrafish embryos were fixed in 4% PFA in PBS for 2h. After washing in PBS, the embryos were cryopreserved by placing in 20% (w/v) sucrose in PBS o/n at 4°C. The embryos were embedded in a Mount Solution (Gelatin 7.5% / Sucrose 20% in PBS) and stored at -80°C. The frozen blocks were cut using a cryostat (Leica CM 1900 UV, Leica, Germany) into 12μm thick sections collected on SuperFrost Plus glass slides (Menzel-Glaser, Germany). Glass slides were dried for 2h and stored at -20°C.

Frozen sections were thawed for 2h at RT. After hydrating the sections 3x 10min each, in PBT, the sections were blocked in 1% BSA, 10% Sheep Serum and 0.1% Tween-

20 solution for 30min in a humidified atmosphere at RT. Sections were then placed o/n at 4°C in a humidified atmosphere with primary antibodies (see table 2) in block solution. After several washes in PBT, slides with sections were incubated with the corresponding secondary antibody (1:500) (see table 2) in block solution o/n at 4°C in a humidified atmosphere. The nuclei were stained with DAPI (1:10000). After PBT washes, cryostat sections were mounted using Fluor Save Reagent mounting medium (Calbiochem, Germany) with coverslip (Scientific Laboratory Supplies LTD, UK). Image acquisition occurred on a laser scanning confocal microscope (Zeiss LSM 710, Germany) and Images were later analyzed with ImageJ free software.

Table 2 – Antibodies used in immunofluorescence labeling

Antibody	Species	Supplier	Dilution used
Primary antibodies			
Anti - ZO-1	Mouse	Life Technologies, USA	1:300
Anti - HuC/D	Mouse	Life Technologies, USA	1:200
Anti - aPKC	Rabbit	Santa Cruz Biotechnology	1:300
Anti – PH3	Mouse	Milipore	1:2000
Secondary antibodies			
Alexa Fluor® 568 anti-mouse IgG	Rabbit	Life Technologies, USA	1:500
Alexa Fluor® 488 anti-rabbit IgG	Mouse	Life Technologies, USA	1:500
Alexa Fluor® 488 anti-sheep IgG	Rabbit	Life Technologies, USA	1:500
Alexa Fluor® 488 anti-mouse IgG	Mouse	Life Technologies, USA	1:500

ZO-1, Zona occludens-1. HuC/D, Elav family members HuC, HuD and Hel-N1 neuronal proteins. aPKC, Atypical protein Kinase C. PH3, P hosphohistone-H3.

Probe synthesis and *In-situ* Hybridization.

To determine the expression pattern of Cdk5rap2 mRNA in zebrafish, two distinct probes (P1 and P2) were synthesized. The primers sequence can be found in Table 3. cDNAs fragments corresponding to P1 and P2 Cdk5rap2 in situ hybridization probes have been amplified by Polymerase chain reaction (PCR) from a cDNA library of 24hpf zebrafish. PCR amplified products were run on 1% Agarose Gel by electrophoresis. Bands corresponding to 400 base pairs (bp) were cut from the gel and DNA purified using a Qiagen gel extraction kit. DNA concentration was measured on the Nanodrop (Thermo scientific, USA). DNA fragments were cloned by T/A cloning into an expression vector (PROMEGA pGEM-T Vector systems). Ligation occurred o/n at 4°C. Ligation products were transformed into XL10 bacteria by heat-shock. Transformed bacteria were then plated in LB agar plates with ampicillin. Colonies of ampicillin resistant bacteria were growth overnight in LB medium plus ampicilin at 37°C. Plasmids were purified using Miniprep kit (Qiagen, USA). Cdk5rap2 P1 and P2, antisense and sense were synthesized using RNA DIG labeling probes.

24hpf embryos were fixed in 4% PFA o/n at 4°C. Embryos were washed in PBT 3x 10min each, and dehydrated progressively in Methanol. Embryos were stored in 100% Methanol at -20°C. For in situ hybridization, embryos were re-hydrated with 75%, 50% and 25% Methanol/PBT solutions, 5min each. They were washed 4x 5min each and incubated with Hyb Mix solution (50% Formamide, 1.3x SSC20x, 5mM EDTA, 50μg/ml Yeast RNA, 0.2% Tween-20 and 100μg/ml Heparin in water) for 30min at RT, and then 1h at 67°C. Embryos were incubated with P1 and P2 sense and antisense probes over-night, 0.5; 1 or 1.5ng/μl in hybridization solution, at temperatures tested ranging from 55 to 67°C.

On the second day of in situ hybridazation the excess of unbound RNA probe was washed away by successive washes of formamide based solutions at high temperatures. Embryos were then incubated o/n at 4°C with anti digoxigenin antibody coupled to Alkaline phosphatase. On the third day of in situ, the excess of antibody was washed away with 1x MABT buffer (0.1M maleic acid, 150mM NaCl and 0.1% Tween-20). Finally, embryos were incubated for 10min in an alkaline solution suitable for Alkaline Phosphatase (AP) functioning (pH 8.5). Alkaline Phosphatase converts NBT/BCIP

substract into a blue precipitate. This precipitate will stain cells that contain RNA DIG labeled probes.

Table 3 – Primers sequence and melting temperature (mT).

Oligo Name	Sequence	mT °C
Cdk5rap2 P1 Forward	5´-GCAGATTGGAGCAGAAAACC	63.7
Cdk5rap2 P1 Reverse	5´-GTTGCTCCAGCTCTTGTTCC	63.4
Cdk5rap2 P2 Forward	5´-GCCTTGGATGAGAAGTTTGC	63.7
Cdk5rap2 P2 Reverse	5´-GCTGATCTTTGCTGTGATG	63.7

Embryos Imaging

For live-imaging, embryos were injected with mRNAs coding for fluorescent tagged proteins such as membrane fused to Green or Red Fluorescent protein (GFP or RFP) at 1 to 64 cells stage. Embryos were left to grow at 28°C until they reached 24hpf. At this point embryos were manually dechorionated. Detection of adequate fluorescence was assessed using a lower power light microscope with fluorescent filters. Where appropriate, embryos were segregated by phenotypic severity under light microscopy. Embryos were treated with N-Propytthiouracie 0.2mM to prevent development of pigmentation and they were anesthetized in Tricaine (0.2mM) to block their movements. Embryos were then mounted in 1% low melting-point agarose (Sigma) in a small microscope petri dish with frontal aspect of telencephalon facing down. Images were obtained using 40x water-immersion objectives in Zeiss LSM 710 laser scan confocal microscope. During imaging embryos were maintained at 28°C in fish water containing N-Propytthiouracie and Tricaine (Tawk et al, 2007, Alexandre et al. 2010). To produce

detailed time-lapse movies series of Z-stacks, 2.50μm apart, were captured every 5min for several hours (Tawk *et al.* 2007, Alexandre *et al.* 2010).

Zebrafish embryos labeled by immuno-histochemistry, were mounted in low-melting point agarose as described above, maintained in 1x PBS and imaged in Zeiss LSM 710 confocal microscope with 40x water-immersion objective. Z-stacks, 2.50μm apart, were captured in series down the front-caudal axis to generate digital reconstructions.

To measure brain size, embryos were anaesthetized with Tricaine (0.2mM) and immobilized in 1% low melting-point agarose (Sigma). Images were taken from lateral view using a dissecting scope and camera Leica DFC490.

Image analysis

To build and analyze the time-lapses images, count the total number of neurons and progenitors in telencephalon and measure brain sizes, images were analyzed in free software ImageJ and FiJi.

Statistical treatment.

Images were exported from Zeiss LSM 710 confocal microscopy software as lsm format files and stacked/analysed using ImageJ, to generate data. These data were then analysed using graphical and statistical functions in Microsoft Excel, GraphPad Prism and SigmaStat.

Once raw data was collated from defining and counting mitotic positions and neuronal and progenitor cells in zebrafish telencephalon, they were exported from Microsoft Excel into Graphpad Prism and Sigma Stat for statistical analysis.

Chapter III

Results

Cdk5rap2 gene expression in zebrafish embryos.

Cdk5rap2 is expressed in human and mouse CNS (Buchman et al. 2010; Ching et al., 2000). To determine the mRNA expression pattern of *Cdk5rap2* in zebrafish embryos, a whole mount *in situ* hybridization has been performed in 24hpf zebrafish embryos. Two distinct antisense mRNA *in situ* probes were designed for the N-terminal region of *zCdk5rap2*. Unfortunately, both probes were non-specific. Different hybridisation temperatures and probe concentrations were used with no success. Some examples can be observed in figure 2. Future plans include new probes design around the C-terminal of *zCdk5rap2*.

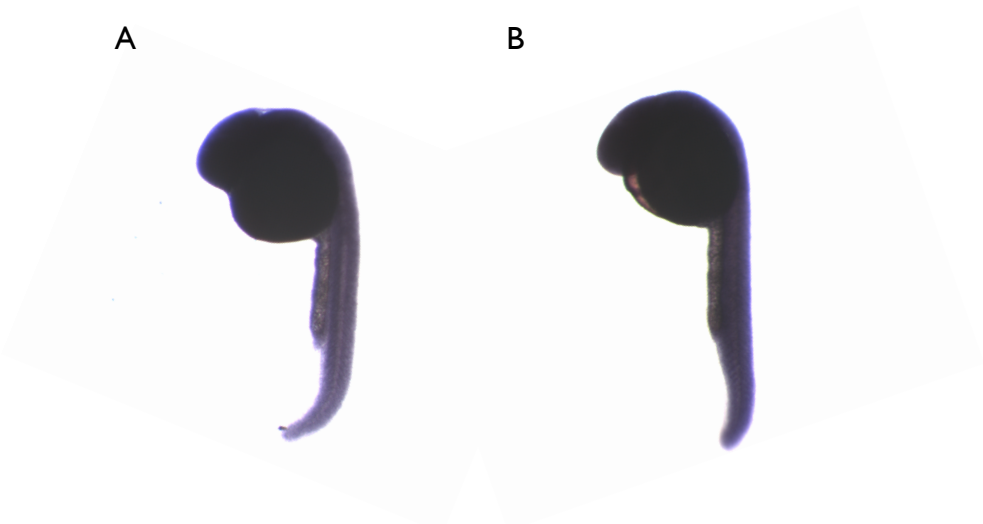

Figure 4: Non-specific detection of zCDK5RAP2 mRNA expression by *in situ* hybridization. Lateral view of 24hpf zebrafish embryos. *In situ* hybridisation was performed in zebrafish embryos with two different probes by *in situ* hybridisation technique (A – anti-sense designed probe; B – sense designed probe). The detection revealed non-regionalised patterns of z*Cdk5rap2* mRNA expression typical for non-specific probes. The images were obtained under dissecting scope and camera Leica DFC490 with 4X magnification.

Microcephaly in zebrafish embryos is induced by injection of CDK5RAP2 antisense oligo-morpholinos.

Cdk5rap2 misfunctions have been associated with human microcephalies and small brain phenotypes (Woods *et al.* 2005). In order to understand the role of CDK5RAP2 on brain size, CDK5RAP2 antisense translation-block (ATG MO) and splice-block (SB MO) oligo-morpholinos were injected in one-cell stage zebrafish embryos. Brain sizes, length and width, were measured at 2 days post-fertilization (dpf) and 5dpf (figure 5).

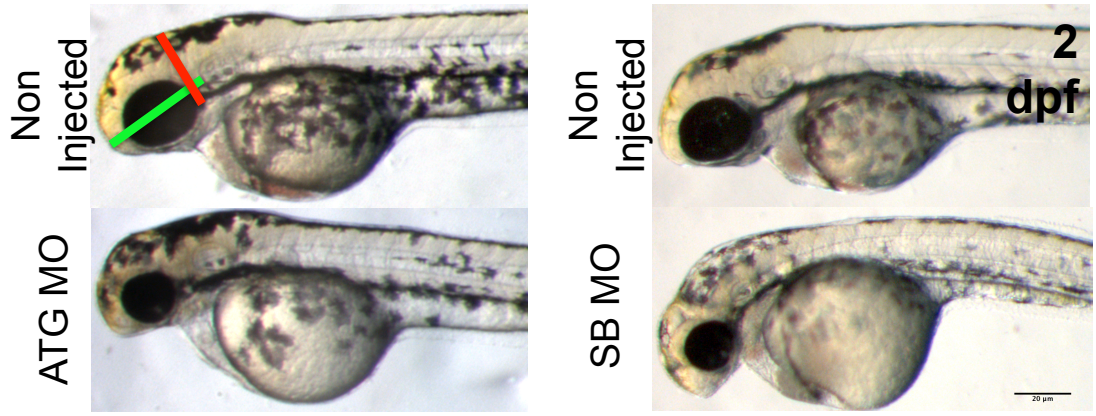

Figure 5: CDK5RAP2 morpholino injection produces small brain phenotypes. Lateral view of non-injected (on top), translation-block morpholino injected zebrafish embryo (on bottom left) and splice-block morpholino injected zebrafish embryo (on bottom right). Red and green lines represent brain width and length, respectively. Images representative of CDK5RAP2 translation-block (ATG MO) and splice-block (SB MO) morpholino injection experiments. Scale bar: 20 μm.

Injection of CDK5RAP2 translation-block antisense oligo (ATG MO) is predicted to reduce the translation of the coding region of the targeted *Cdk5rap2* gene and to induce microcephalic phenotype in zebrafish embryos.

We observed that both translation-block and splice-block antisense morpholinos (ATG MO and SB MO) induce microcephalic phenotype in zebrafish embryos when compared to the control groups (figure 6 and 7). No significant differences in brain size were detected between the control groups (non-injected and standard control morpholino injected embryos). This suggests that morpholino injection on one-cell stage embryos does not promote a decrease on the brain size.

The CDK5RAP2 translation-block morpholino exerts a dose dependent effect on the brain growth (figure 6). Microcephalic phenotypes produced by lower concentrations

of translation-block morpholino are rescued by 5dpf (figure 6D), which can be explained by the earlier depletion of antisense morpholino. In most experimental conditions undertaken, zebrafish embryonic brain grew between the 2nd and 5thdpf, however, higher concentrations of CDK5RAP2 translation-block morpholino lead to brain growth arrest. This indicates that higher concentrations of morpholino can efficiently block brain development. The CDK5RAP2 translation-block morpholino also show a dose dependent way in brain effects (figure 6).

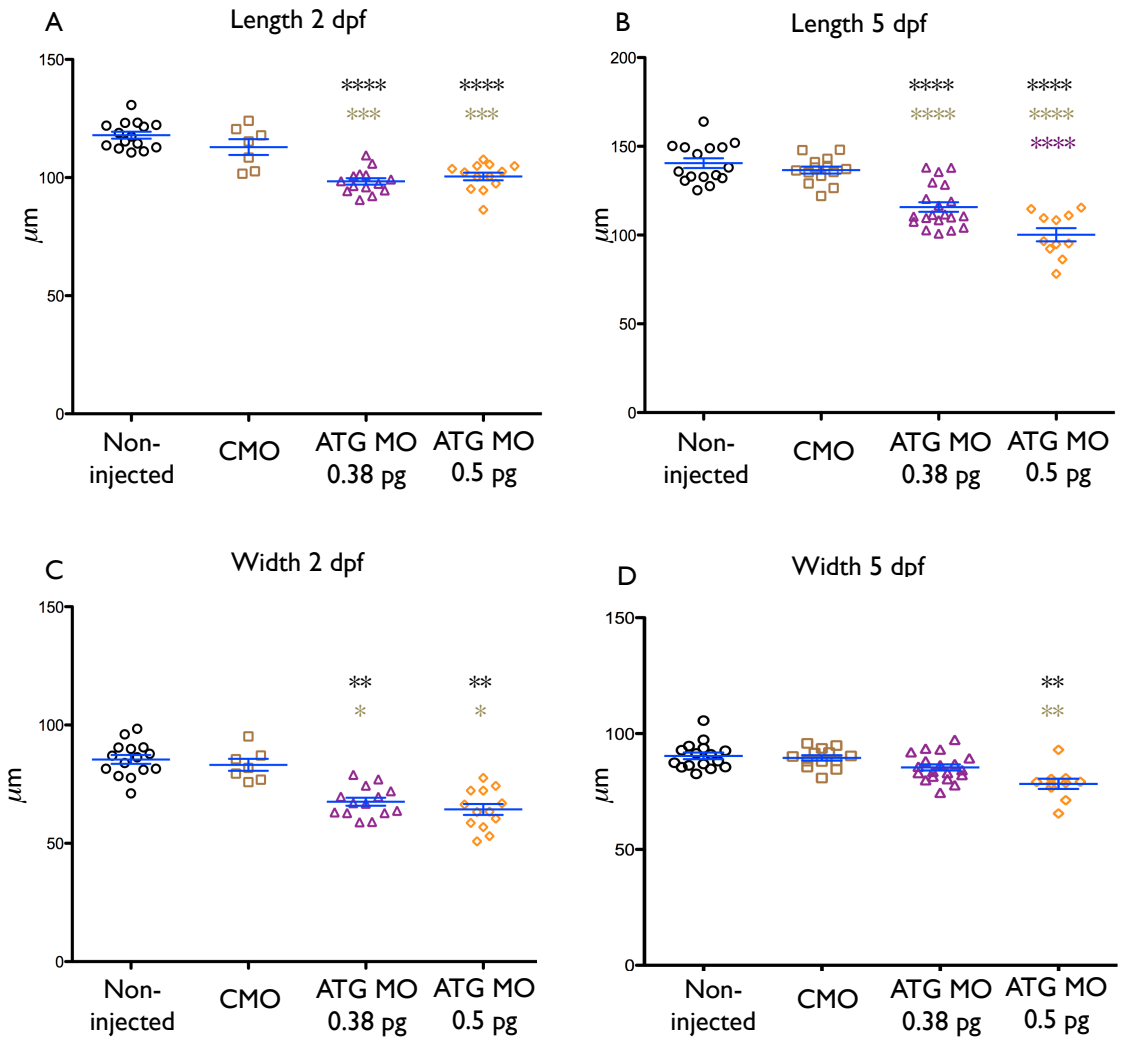

Figure 6: Zebrafish embryos injected with CDK5RAP2 translation-block antisense morpholino have smaller brains. Brain length (A, B) and width (C, D) of non-injected and Control Morpholino (CMO) with Translation-block Morpholino (ATG MO) 2dpf (A, C) and 5dpf (B, D) zebrafish embryos. (A, B, C, D) ATG MO injection significantly decreases brain sizes at 2dpf and 5dpf compared to the non-injected and CMO with similar ages. In A) or B) the injection of 0.5pg of translation-block morpholino significantly decreased brain size when compared with the embryos injected with 0.38pg. Asterisks in black compare brain sizes of non-injected embryos with ATG MO injected embryos while brown asterisks compare CMO injected embryos and purple stars analyses compare to the embryos injected with 0.38pg of

ATG MO. Two way ANOVA of group's length and width, followed by Holm-Sidak multiple comparison post test, *p<0.05; **p<0.01; ***p<0.005 and ****p<0.001.

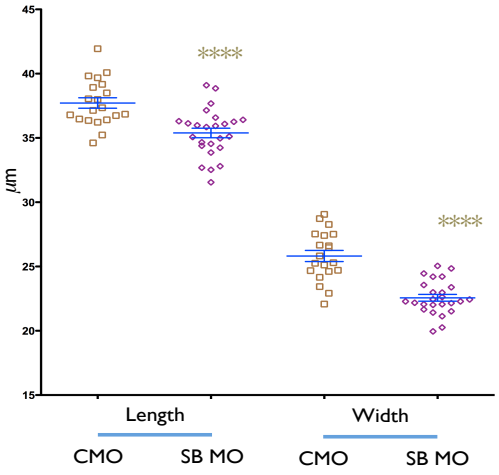

Figure 7: Brain size decreases with CDK5RAP2 splice-block MO injection in zebrafish embryos. Brain length and width at 2dpf of: Control Morpholino (CMO) and Splice-block Morpholino (SB MO) injected zebrafish embryos. SB MO injection also significantly decreases the length and width of brain of zebrafish embryos. The brown stars are the analyses compare to the CMO injected embryos. One way ANOVA of groups; length followed by Holm-Sidak's multiple comparison test, and width followed with kruskal-wallis and Dunn's multiple comparison post test ****p<0.001.

The zCDK5RAP2 splice-block morpholino (SB MO) was designed to create a truncated protein that mimics *Cdk5rap2* mutations found in humans. Splice-block morpholinos interfere with pre-mRNA processing stages and prevent splicing by blocking the splice-directing small nuclear ribonucleoproteins complexes from binding to their targets at the borders of introns on a strand of pre-mRNA.

These morpholino injection experiments revealed that both splice-block and translation-block morpholinos affects brain development and growth. However, these observations have to be confirmed by validating morpholino function and producing a zebrafish *Cdk5rap2* mutant.

We tried to validate translation-block MO function and specificity by injecting a five base mismatch morpholino (MIS MO) predicted not to recognize the target sequence neither have an effect in brain development. However, mismatch MO injected embryos presented a more severe microcephaly than embryos injected with translation-block morpholino (figure 8). The MIS MO decreased the width of brain size similar to higher concentration of translation-block morpholino ($p<0.05$) (figure 8). Later at 5dpf, the translation-block morpholino starts losing the effect, while mismatch morpholino still

reduce width of brain size ($p<0.001$). All embryos injected with higher concentrations of mismatch morpholino did not develop until 5dpf (figure 8). These unexpected observations demonstrated that mismatch MO may still recognise the target RNA or this morpholino may be non-specific and recognise multiple target sequences. In future, we will distinguish between these two possibilities by rescuing morpholino effects with the injection of zCdk5rap2 mRNA.

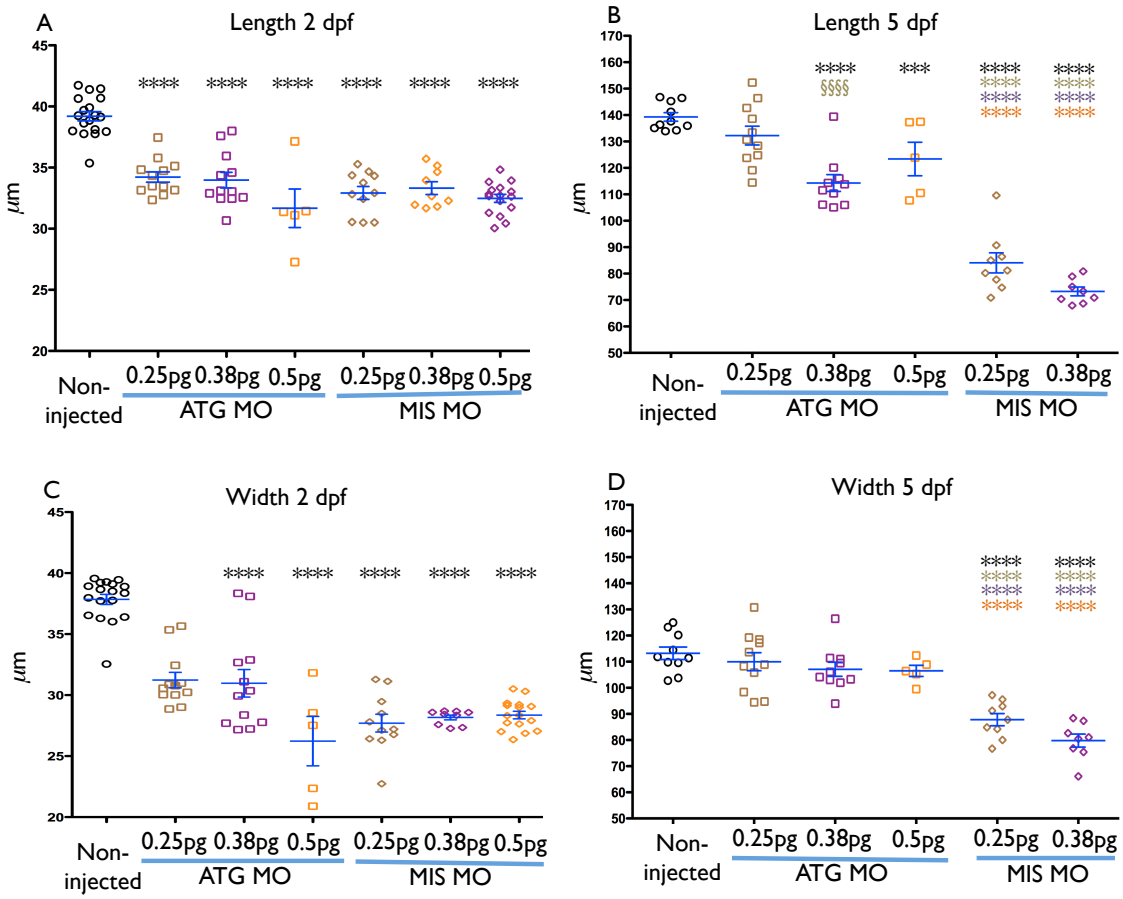

Figure 8: Cdk5rap2 Mismatch Morpholino promotes a severe microcephalic phenotype. Zebrafish embryos were analysed in different groups: Non-injected, Translation-block (ATG MO) and Mismatch Morpholino (MIS MO) injected at different concentrations. A) Brain length of zebrafish embryos, at 2dpf. ATG MO and MIS MO injections significantly decrease brain length at 2dpf. B) Brain length of zebrafish embryos, at 5dpf. The higher concentrations of ATG MO significantly decrease brain length at 5dpf compare with the lower concentration of 0.25pg and with non-injected embryos. MIS MO injection significantly decreases the brain lengths at 5dpf when compared to the non-injected embryos. C) Brain width of zebrafish embryos, at 2dpf. ATG MO injection (0.38 and 0.5pg) significantly decreases the brain width when compared with non-injected embryos. MIS MO significantly decreases brain width when compared with non-injected embryos. D) Brain width of zebrafish embryos, at 5dpf. MIS MO significantly decreases brain width when compared with all the other conditions. The embryos injected with 0.5pg of MIS MO did not develop until 5dpf. The black stars analyses compare to the non-injected embryos, brown stars analyses compare to the CMO injected embryos; purple stars analyses compare to the embryos injected with 0.38pg of ATG MO; orange stars analyses compare to the embryos injected with 0.5pg of ATG MO. Statistical significant difference was analysed by One way ANOVA of group's width at 2dpf (with

Kruskal-Wallis and Dunn's method); width at 5dpf (with Holm-Sidak's method); length at 2dpf (with Holm-Sidak's method); width at 5dpf (with Kruskal-Wallis and Dunn's method) ***p<0.005 and §§§§ or ****p<0.001.

The concentration of 0.6pg of control and splice-block morpholinos, and 0.5pg of translation-block morpholino were chosen for the following experiments. These morpholinos show a dose dependent phenotype and these concentrations led to a small brain phenotype (translation-block and splice-block morpholino) with less or no tissue death (grey appearance) visible under the microscope.

Apoptotic cell death does not explain the small brain phenotype caused by the injection of antisense oligo-morpholinos.

With a gross observation of tissue grey discoloration of cells, an increase in cell death was observed with increasing concentrations of antisense oligos morpholinos. In order to investigate whether small brain phenotypes could be induced by an increase in apoptotic cell death, we used acridine orange a nucleic acid-selective metachromatic stain that stains death cells. (Yuan et al. 2014)

After the morpholino injection, embryos were stained, imaged and analysed. An increase in acridine orange labelling was observed on both control and splice-block morpholino injected embryos when compared to non-injected embryos (figure 9). The cell death is generally associated to the injection of oligonucleotides.

To investigate whether the increase in cell death could explain the microcephalic phenotype caused by CDK5RAP2 morpholino injection we injected translation-block morpholino in $p53^{-/-}$ mutant background embryos. The p53 knockout zebrafish mutant is known to have inhibited apoptotic cell death.

Following morpholino injection, embryos were imaged and head length/width were measured and compared with control embryos. As shown in figure 8, CDK5RAP2 injected $p53^{-/-}$ embryos had both significantly reduced head length and width when compared with control morpholino injected $p53^{-/-}$ embryos ($p<0.0001$) (figure 10).

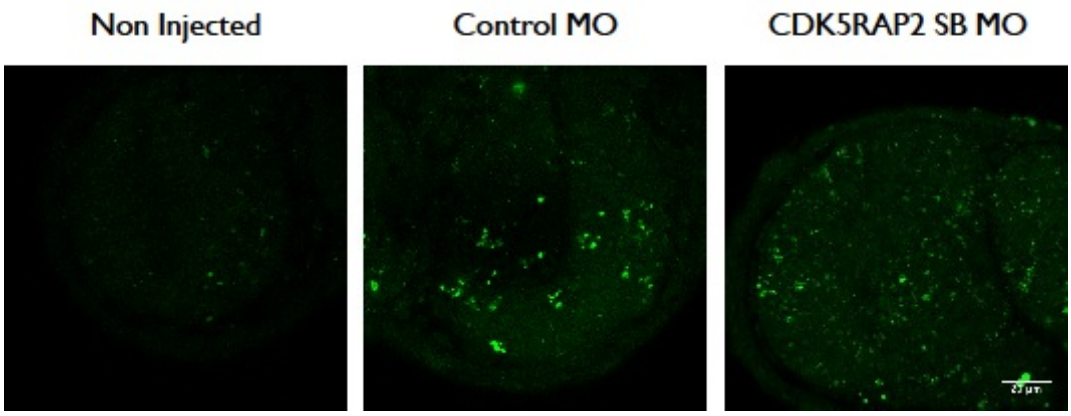

Figure 9: Cell death by acridine orange staining does not increase with morpholinos injection. Acridine orange staining (green) on telencephalon of zebrafish embryos (non-injected and control morpholino or splice-block morpholino injected embryos) at 24hpf. Scale bar: 20 μm.

Aside from the reduced head size, no other significant phenotypic features were noted. This suggests that microcephalies induced by CDK5RAP2 morpholino do not result from an increase in apoptosis.

Although it is not possible to rule out the role of other cell death pathways in microcephaly from these results alone, it is possible to state that the role of p53-related apoptosis, a major cell death pathway, is not relevant for the microcephalic phenotype. Investigations into the role of CDK5RAP2 abrogation in microcephaly were continued with the knowledge that incidences of apoptosis were not part of any underlying mechanisms.

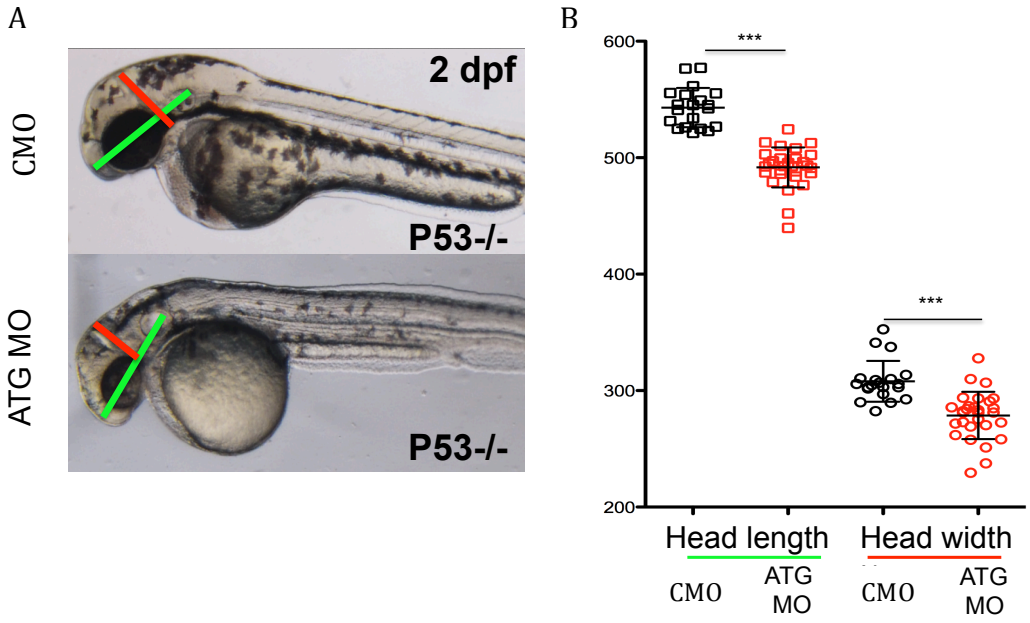

Figure 10: Brain size remains decreased with translation-block morpholino injection. A) Lateral view of representative zebrafish, Control morpholino (CMO) and Translation-block morpholino (ATG MO) in p53$^{-/-}$ background at 2dpf. B) Brain length and width at 2dpf of: CMO and ATG MO injected zebrafish embryos. ATG MO injection decreases significantly the length and width of brain of zebrafish embryos. Stastistical significant difference was analysed by Unpaired two-tail t-test: *** p<0.001.

Epithelia cell polarity is not altered in embryos injected with CDK5RAP2 translation-block morpholino.

The neuronal progenitor division happens mainly at the apical surface and soon after the division daughter cell reform apical and basal attachments to the apical and basal surfaces. Apical cell polarity is important in determining the mode of neural progenitors division (symmetric and asymmetric) that occurs at the apical surface of the neuroepithelium (Alexandre *et al,* 2010; Huttner and Kosodo, 2005).

In order to test whether a decrease in growth caused by CDK5RAP2 MO injection depends on the apical polarity protein malfunction, we detected aPKC and ZO1 (polarity markers) by immunostaining in control and CDK5RAP2 morpholino injected embryos. We found no differences between CDK5RAP2 and control morpholino injected embryos, suggesting that microcephalies induced by translation-block morpholino do not result from apical proteins mislocalisation (figure 11).

This suggests that apical polarity remains unaffected and that the microcephalic phenotype may be induced by other molecular mechanisms.

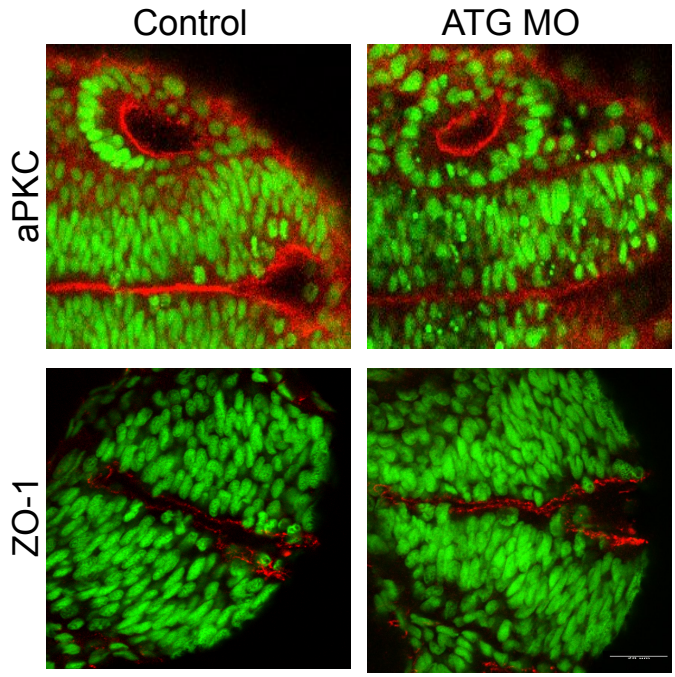

Figure 11: Apical polarity markers are present in embryos injected with translation-block morpholino. Nucleus stained with DAPI (green) and the apical polarity stained with aPKC and ZO-1 antibodies (red). Dorsal (aPKC) and transversal (ZO-1) views of zebrafish hindbrain at 24hpf. Control morpholino (on left column) and translation-block morpholino (on right column) demonstrated that both polarity markers are present in embryos injected with CDK5RAP2 morpholino. Scale bar: 20 μm.

CDK5RAP2 morpholino injection decreases neurogenesis rate (N/P) in zebrafish embryos.

Our work suggests that cell death and cell polarity were unlikely the causes of small brain phenotypes observed in zebrafish embryos injected with CDK5RAP2 antisense oligo morpholinos. It has been shown in mammalian systems that CDK5RAP2 promotes premature neuronal differentiation and premature depletion of stem cell populations. To determine the rate of neurogenesis, zebrafish embryos were injected with control morpholino and CDK5RAP2 morpholinos (translation-block morpholino and splice-block morpholino). We distinguished neurons and progenitors by performing HuC/D antibody staining and counterstaining with DAPI. To determine if CDK5RAP2 regulates neurogenesis, we counted the total number of neurons (N) (HUC/D positive) and progenitors (P) (HUC/D negative) in telencephalon and analysed the neurogenesis rates (N/P) in control and experimental embryos (figure 12A).

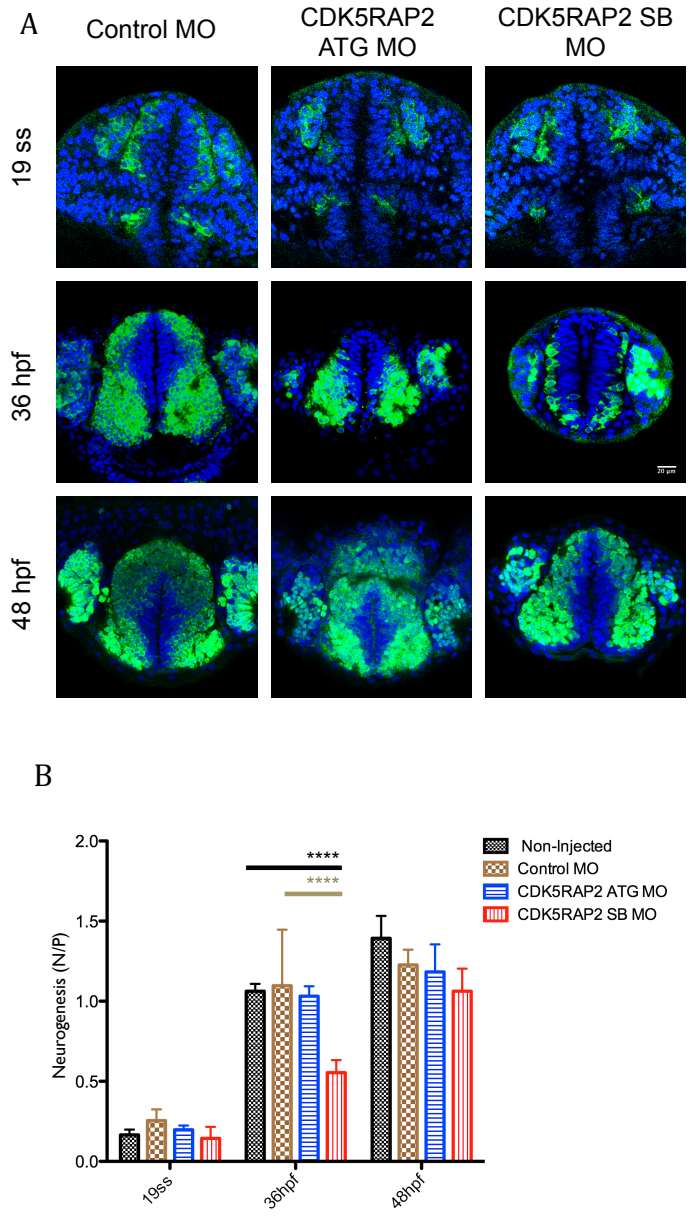

Figure 12: Neurogenesis (N/P ratio) decreases with injection of Cdk5rap2 splice-block morpholino. Neurogenesis rates (N/P ratio) in control, translation-block and splice-block morpholino injected zebrafish embryos at 20, 36, 48hpf. A, B) The neurogenesis was quantified on zebrafish telencephalon counting the neurons (N) labelled with HuC/D antibody (green) and the progenitors (P) labelled only with DAPI (blue). A) Optical section of zebrafish telencephalon with all nuclei stained with DAPI (blue) and neurons expressing HuC/D (green). B) Neurogenesis increased from the early to late stages of embryonic development. In embryos injected with splice-block morpholino, neurogenesis significantly decreased when compare with non-injected and control morpholino injected embryos **** $p<0.001$. Statistical significant difference was analysed by One-Way ANOVA followed by Holm-Sidak multiple comparison post test. Scale bar: 20 μm.

We observed that during zebrafish embryonic development (from 19 somites (ss) to 48hpf) neurogenesis increased in telencephalon in all experimental conditions. In embryos injected with splice-block morpholino, neurogenesis is significantly reduced at

36hpf when compared to the control conditions (figure 12B) ($p<0.001$). This observation is surprising and contradicts previous mammalian data that suggested microcephalic phenotypes are due to an increase in neuronal differentiation at early stages of embryonic development (these mechanisms have been thoroughly reviewed Kraemer *et al.* 2011 and Megraw *et al.* 2011). Our data also reveals that CDK5RAP2 splice-block morpholino has a stronger effect than translation-block morpholino to decrease neurogenesis.

Injection of CDK5RAP2 morpholinos decrease the total number of progenitor divisions.

To determine whether cell proliferation is affected in CDK5RAP2 morpholino injected embryos and whether this could explain the unexpected reduction in neurogenesis, we counted the number of dividing cells in mitosis phase (labelled by P-Histone 3 antibody staining) at the apical and basal surfaces of neural epithelium.

Injection of translation-block morpholino showed a significant decrease on the proportion of apical divisions in zebrafish embryos at 36hpf when compared to control embryos ($p<0.001$). Zebrafish embryos injected with splice-block morpholino showed a similar reduction on the proportion of apical divisions at earlier (19SS) stages of embryonic development when compared to the control ($p\leq0.005$). However, splice-block morpholino injected embryos later showed (at 48hpf) an increase in basal divisions ($p<0.01$) (figure 13). All together this data suggests that apical divisions tend to decrease in embryos injected with CDK5RAP2 morpholinos. We also observed that at 48hpf there was a significant increase on the proportion of basal divisions in embryos injected with ATG morpholino when compared to the control cases ($p<0.001$). However, these divisions are in very low number (1 in 15) and therefore the alterations in this population of progenitors are very difficult to address.

Our data suggests that the decrease on the proportion of cell divisions might depend on cell numbers that are reduced. This suggests that alternative mechanisms such as cell cycle arrest or an increase of cell cycle length may account for the decrease in neuronal populations and small brain phenotypes.

At the later stages of embryonic development, in embryos injected with CDK5RAP2 MO the proportion of cells in proliferation appears to catch up with wild type embryos (figure 14). Zebrafish brain is highly proliferative and from our results it

seems to be recovering either from a possible cell cycle arrest or an increase of cell cycle length during early stages.

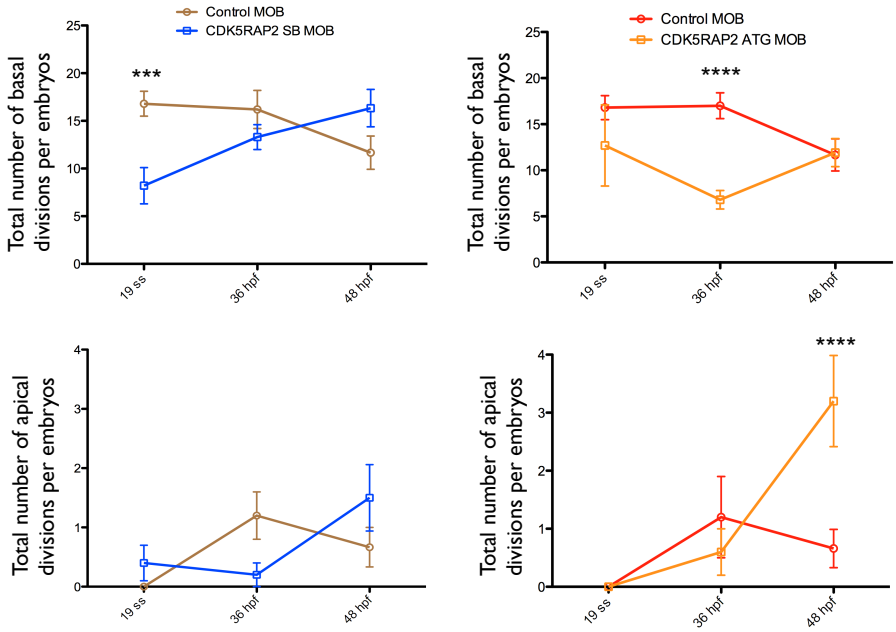

Figure 13: Injection of CDK5RAP2 morpholinos decreases the proportion of mitosis. The number of cells in mitosis, pH3 positive, in all telencephalon of control and CDK5RAP2 morpholinos injected embryos was determined. Embryos injected with splice-block morpholino had a significant decrease on the proportion of apical divisions at 19 somite's stage embryos. Embryos injected with translation-block morpholino showed a significant decrease on the proportion of apical divisions in 36hpf and an increase in basal divisions at 48hpf embryos when compared to control embryos. Statistical significant difference was analysed by two way ANOVA followed by Holm-Sidak multiple comparison post test; ***p<0.005 and ****p<0.001.

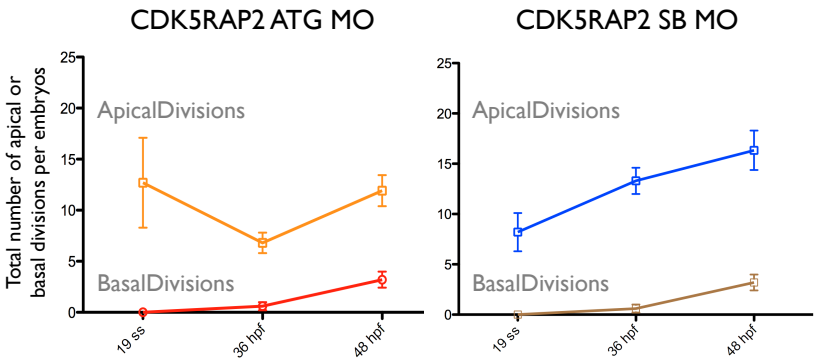

Figure 14: Rescue or natural catch up of proliferation at later stages. At the later stages of development a rescue appear to start or a natural catch up of cell proliferation is observed at 36hpf till 48hpf. Zebrafish is known as a higher proliferative system and it seems to be recovering from a possible disruption of cell proliferation caused by CDK5RAP2 morpholinos injection.

Validation of CDK5RAP2 morpholino and antibody.

To validate CDK5RAP2 morpholinos functions, we tested a customer-made antibody against zebrafish CDK5RAP2 by Western blot. The zCDK5RAP2 protein is predicted to have two isoforms, one with 300kDa and the other with 150kDa. However, this antibody recognized bands corresponding to 5 different sizes. Three of them (300kDa, 150kDa and 100kDa) were faint when we injected CDK5RAP2 splice-block morpholino but not translation-block morpholino (figure 15). To confirm that these three bands truly correspond to *zCdk5rap2*, we performed Mass Spectrometry analyses and we are still awaiting the results.

Unfortunately translation-block morpholino did not have any effects in bands intensity suggesting that this morpholino it may be non-specific. This also reveals the importance to repeat some of our initial experiments with antisense oligo splice-block morpholino and urgently produce a Crispr/Cas9 mutant to validate our current observations.

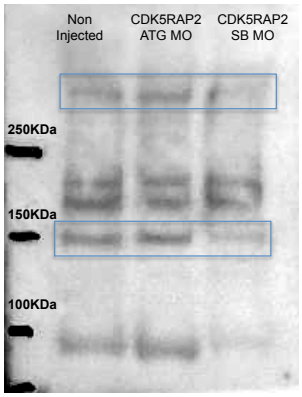

Figure 15: CDK5RAP2 decrease in zebrafish embryos injected with splice-block morpholino. We observed by western blot analysis of custom-made antibody against zebrafish CDK5RAP2 recognizes 5 bands; and two of them have the predicted size for zebrafish CDK5RAP2 (300 and 150kDa) (blue square). The staining confirms a decrease on CDK5RAP2 expression when we injected with splice-block morpholino but not translation-block morpholino.

In summary, CDK5RAP2 morpholino injection, induce a small brain phenotype during the embryonic development. However, this microcephalic phenotype is not caused by an increase of cell death or disruption of polarity proteins.

Neurogenesis and cell proliferation might be decreased with CDK5RAP2 morpholino. This could be caused by either an increase of cells in cell cycle arrest or cell cycle length.

Although the validation of our zCDK5RAP2 antibody is still in progress and we are not able to confirm the specificity of our antibody. In order to confirm all our achievements a Cdk5rap2 mutant background is being produced.

Chapter IV

Discussion

The major focus for this project was to understand the function of *Cdk5rap2* in the development of zebrafish telencephalon. In microcephaly studies, it is suggested that *Cdk5rap2* influences brain size during development (Woods *et al.* 2005). Zebrafish is a well-characterized model which is particularly useful in studies involving genetics and cellular biology due to its genetics, transparency and easy visualization of deep tissue. In the same way as *c. elegans, drosophila* and cell culture, zebrafish is a good model to analyze gene function and theoretically, to understand the origin of MCPH3. Using this model, this study revealed that CDK5RAP 2 abrogation disrupts the brain size by preventing cell proliferation, which go against the previous findings in mammalian systems thought to increase neurogenesis.

To validate these observations and correctly gauge the impact of the decrease in *Cdk5rap2* function, we used CDK5RAP2 morpholino injected cells and transplantation approaches to analyzed the expression of gamma-tubulin around the centrosome and cell behaviours. If we had more time, this transplantation approach would have allowed us to compare progenitors depleted of *Cdk5rap2*, integrated close to wild-type progenitors in the same embryo. Given that the intensities of immunofluorescence greatly varied between samples, the ability to detect differences of gamma-tubulin in wild type and CDK5RAP2 morpholino injected progenitors in the same embryo would have overcome this problem. Unfortunately, using this technique did not allow us to confidently detect differences in gamma-tubulin intensities, or in cell behaviour. Gamma-tubulin intensities varied depending on the phase of cell cycle, while embryos prepared for live-imaging encountered problems of embryo misorientation and wrong age. This cell transplantation approach could also be used to show that cell polarity in CDK5RAP2 morpholino injected embryos are intact.

We were unable to detect *Cdk5rap2* mRNA expression by in situ hybridization with two distinct probes. After several attempts of optimization of the protocol and preparation of solutions, the *in situ* hybridization worked for the control probe Her4 but not for *Cdk5rap2*. There could have been an issue with probes specificity. New *in situ* hybridization probes in C-terminal region of CDK5RAP2 sequence should be designed.

Our data obtained with injection of morpholinos supports the findings obtained in mammalian systems where the loss of CDK5RAP2 function results in a decrease in brain size. However, our results contradicts the mammalian data, in the sense that knockdown of *Cdk5rap2* in zebrafish resulted in a decrease in neurogenesis and decrease of cells in proliferation. To prove that zebrafish microcephalies may be caused by different molecular mechanism that the one described in mammalian system, we will produce and confirm these observations using *Cdk5rap2* mutant.

It has been shown that a 24-hour delay in the switch from symmetric divisions to asymmetric divisions leads to an increased in proportion of progenitors in mice. The seemingly minor alteration leads to a significant increase in brain size of affected mice (Setsuko and O'Leary 2009). Taking this into account, it is an absolute requirement in the microcephaly field to demonstrate that microcephalies result from the disruption of symmetric mode of divisions. Using the zebrafish as a model it would be possible to follow the behavior of individual neural progenitors at the neuroepithelium by doing live imaging as described in Alexandre et al (2010). This would allow observation and quantification of changes in the proportion of symmetric and asymmetric division or the number of cells moving to the basal surface or cell cycle arrest. Our ultimate goal is to demonstrate for the first time that cell fates are altered in *Cdk5rap2* mutants and morpholino injected embryos by following early cell divisions and cell fate decisions *in vivo*.

We also observed a decrease in the total number of cell divisions, which may be due to either an increase in cell cycle arrest or cell cycle length. To determine which one may be happening, we could use cell cycle markers such as, PCNA-GFP that labels transition between G2 and M (Leung et al. 2011) or FUCCI line (which is a zebrafish Fucci system), which would permit us to determine the proportion of progenitors in different phases of cell cycle in wild type and compare it to mutants (Bouldin and Kimelman (2014). This would establish whether *Cdkrap2* knockdown could lead to progenitor pool depletion by driving neural progenitors toward terminal neurogenic divisions as a result of increased cell cycle length (Buchman et al. 2010). To this end, more experiments should be conducted to consider the hypothesis of disruption in cell cycle.

We were disappointed with morpholino technology and to discover that mismatch morpholino used to validate the function of translation-block morpholino does not work. This morpholino with 5-mismatched base pairs should not recognize the targeted sequence and should prevent CDK5RAP2 protein translation. However, the injection of the mismatch morpholino gave us the same microcephalic phenotype as well as the translation-block morpholino. This indicates two things, either that mismatch morpholino recognizes the targeted sequence or that this morpholino is non-specific. In order to validate the function of translation-block morpholino we will have to rescue its function by injecting the full length of mRNA.

It would also be interesting to investigate the extent to which divisions need to be disrupted for a microcephalic phenotype to occur. It therefore seems logical to determine whether a premature switch in division mode leads to a decrease in brain size and the extent to which this prematurity must occur to cause a significant alteration in cortical size.

Manifestation of microcephaly in MCPH patients is established during embryonic growth with a head circumference that is below average (Woods et al. 2005). The majority of MCPH patients do not exhibit severe growth defects. Primary microcephaly is the most notable common feature of these diseases. Our data complement current hypotheses about the origins of MCPH that a defect in neural progenitor proliferation can account for the manifestation of primary microcephaly (Bond and Woods, 2006; Fish et al. 2006; Woods et al. 2005). We optimized that the injection of CDK5RAP2 morpholinos at early time points during neurogenesis in the telencephalon is a suitable strategy for the study of MCPH3 as it allows us to target the cell population thought to be most involved in the manifestation of MCPH within the most severely affected region of the CNS. However, the Cdk5rap2 mutant background shall be the best model to investigate the effects of Cdk5rap2 knockdown and accurately determine the role of Cdk5rap2 in MCPH research.

Chapter V

Conclusions

The results obtained in this work have shown that *Cdk5rap2* plays an important role in brain growth in the zebrafish. Reduction of Cdk5rap2 function results in a microcephalic brain phenotype with an increase in neurogenesis and a reduction of cells in division. These findings imply that *Cdk5rap2* is essential to restrict the normal development in zebrafish brain. This observation suggests that CDK5RAP2 is fundamental for maintenance of many properties of cell division. Further studies are needed to confirm the present conclusions.

Chapter VI

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