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Dissecting the Role of CLASP1 in Mammalian Development

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"Uma boa recordação talvez seja cá na Terra mais autêntica do que a felicidade."

Alfred de Musset

Dedicada à minha avó.

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Aims of the work

CLIP-associated proteins (CLASPs) are microtubule-associated proteins that accumulate at the microtubule plus-ends. These proteins are highly conserved in eukaryotes and play important roles both in interphase and mitosis.

In *Drosophila*, yeast, *Xenopus* and *C. elegans*, CLASPs were shown to be important in mitosis for chromosome segregation, maintenance of spindle bipolatity and regulation of microtubules at the kinetochore level. CLASPs are also implicated in a variety of cellular processes in interphase cells, namely in microtubule stabilization and cell polarity.

Mammals have two paralogues, CLASP1 and CLASP2, whose functions have been considered partially redundant and little is known about their individual roles. The recent generation of *Clasps* KO mammalian models will further improve our knowledge about these proteins in a physiological context. In this work we took advantage of a *Clasp1* KO mouse model to study the CLASP1 role in mammalian physiology.

Abstract

Mitosis is a complex cellular event through which cells correctly divide two copies of their DNA content to daughter cells. This process is mediated by a cellular machine called mitotic spindle, a bipolar structure built by microtubules (MTs). MT dynamics is regulated by MT-associated proteins (MAPs), such as MT plus-end tracking proteins (+TIPs). CLIP-associated proteins (CLASPs) belong to this family and are highly conserved among eukaryotes. These proteins interact with MTs regulating mitotic spindle bipolarity, chromosome segregation and MT behavior at the kinetochore. Thus, CLASPs have been described as essential factors for genetic integrity during cell division.

A *Clasp1* knockout (KO) mouse strain, previously generated in our lab, is an ideal tool to uncover the physiological role of CLASP1 in mammals. All *Clasp1* KO mice display a lethal phenotype, dying a few minutes after birth, with an acute lung failure. Upon histological analysis, we found that KO lungs exhibit a morphogenic delay. However, molecular analysis of lung late maturation markers have shown that type I and type II pneumocytes, the cells responsible for the gas-exchange, are differentiated in KO mice at the moment of their birth. Nevertheless, an underlying lung defect cannot yet be excluded. High amounts of glycogen in KO pulmonary parenchyma were found, which might be, not only a sign of lung immaturity, but also deficiencies in surfactant lipid component. Regarding CLASP1 expression, we observed that this protein is transiently expressed among different organs throughout mouse development, being particularly strong in brain, a fact that might explain its roles in neuronal biology. CLASP1 is also ubiquitously expressed in adult mice, suggesting that this protein is also important in mature tissues. Moreover, it is not clear whether the morphological delay observed in KO lungs may explain the newborn lethality observed in *Clasp1* KO mice. At this stage, the biological meaning of CLASP1 in mammals' physiology is not clear. So far, no *Clasp1* KO mice was able to survive *ex uterus*, suggesting that this protein is important during late developmental stages in mammals.

Key words: CLASP1, knockout, lungs and development.

Sumário

A mitose é o evento celular, através do qual uma células transmite uma cópias do seu DNA às células filhas. Este processo é mediado pelo fuso mitótico, o qual consiste numa rede bipolar microtubulos. A dinâmica dos microtubulos é regulada por proteínas associadas a estes (MAPs – Microtubule-Associated Proteins), tais como as proteínas associadas às extremidades positivas dos microtubulos (+TIPs – Plus-ends Tracking proteins). As proteínas associadas às CLIPs (CLASPs – CLIP-associated proteins) pertencem a esta família e estão altamente conservadas nos eucariotas. Estas interagem com os microtubulos regulando o fuso mitótico, a segregação dos cromossomas e o comportamento dos microtubulos ao nível do cinetocoro. Assim, as CLASPs têm sido descritas como essenciais à manutenção da integridade genética durante a divisão celular.

Um modelo animal *knockout* para o gene *Clasp1* é uma ferramenta indispensável à descoberta do papel da CLASP1 a nível fisiológico. Nos animais *knockout* foi observado um fenótipo letal, no qual 100% dos recém-nascidos morreram poucos minutos após o nascimento, no decurso de falência respiratória. Após análise histopatológica, observamos que os pulmões dos animais *knockout* apresentam um atraso no desenvolvimento. Porém, a análise da expressão de marcadores de diferenciação celular, mostrou que os pneumócitos tipo I e II estão presente e diferenciados nos animais *knockout* aquando do seu nascimento. No entanto, um defeito primário a nível pulmonar ainda não pode ser excluído. Níveis elevados de glicogénio no parênquima alveolar dos animais *knockout* sugerem imaturidade pulmonar ou deficiente produção do líquido surfactante. Adicionalmente, ainda não está esclarecido de que forma pode este atraso explicar a letalidade observada nos recém-nascidos *knockout*. Verificamos também que expressão de CLASP1 é transiente ao longo do desenvolvimento, sendo particularmente elevada no cérebro, o que pode explicar o seu papel já descrito na biologia dos neurónios. A CLASP1 é ubiquamente expressa em mamíferos adultos, o que sugere que esta proteína é também importante em tecidos diferenciados. Nesta fase, o significado biológico da CLASP1 em mamíferos ainda não foi descortinado. No entanto, nenhum animal *knockout* para *Clasp1* foi capaz de sobreviver *ex uterus*, o que sugere um papel fundamental desta proteína na fase final do desenvolvimento dos mamíferos.

Palavras-chave: CLASP1, *knockout*, pulmões e desenvolvimento.

Índex

EXPERIMENTAL WORK

GENERAL INTRODUCTION

CHAPTER I - General Introduction

All cells are the product of the cell division of a pre-existent cell (*omnis celllula e cellula*, Rudolf Virchow, 1859). The earliest mentions about cell division are from 1766, when Trembly observed for the first time the process in *Diatom Synedra*. Since then, cell division has fascinated the scientific world, not only because it is the key event which perpetuates biogenesis, but also because it triggers the most complex changes in all cell cycle. Since Walter Fleming's assumptions, in which he describes mitosis with a remarkable detail, this cellular process has been the target of a huge scientific investment in the cellular biology niche. However, despite all the advances in this field, a lot of molecular and biophysical events related to the regulation of the distribution of the genetic material through the daughter cells remain unknown.

1. The Eukaryotic Cell Cycle

The eukaryotic cell cycle is a highly regulated process, in which each cell ensures the transmission of the correct copy of its genome to each daughter cell. It is divided in two main parts: the interphase, which is the most prolonged period of all cell cycle, and the M phase, that includes mitosis (nuclear division) and cytokinesis (cytoplasm division). Additionally, there are four fundamental events during the cell cycle that are particularly important for the maintenance of the correct DNA content, which are: cell growth, DNA duplication, chromosome segregation and cell division (Morgan, 2007). Interphase is a period of growth and DNA replication divided in three stages: gap phase 1(G1 phase), synthesis phase (S phase) and gap phase 2 (G2 phase). These gap phases are transition periods in which an important portion of the regulatory machinery of the cell controls the cell cycle. G1 phase is the period prior to the synthesis of DNA. In this phase, the cell increases in mass and prepares itself to undergo cell division. In S phase DNA is synthesized and duplicated. Lastly, G2 phase is the period between DNA synthesis and the start of M phase, in which the cell synthesizes proteins and continues to increase in size. In late interphase, the cell still has nucleoli present, the nucleus is bounded by a nuclear envelope and chromosomes have duplicated but not condensed. (J. M. Mitchison, 1971; Murray, 1993).

Despite the valuable contribution of Walter Fleming to the knowledge of the cell division, his assumptions were only proved when it became possible to observe the movement of chromosomes by live cell microscopy (T. J. Mitchison & Salmon, 2001). In vertebrates, mitosis encompasses five stages: prophase, prometaphase, metaphase, anaphase and telophase (Gorbsky, 1992). Prophase is the first mitotic event visible at the microscope and encloses chromosome condensation, as well as centrosome separation and the begining of the microtubular spindle assembly. The spindle consists in a complex bipolar structure of MTs, which are an important component of the cytoskeleton and ubiquitously present in all eukaryotic cells (Morgan, 2007). During prometaphase, nuclear envelop breaks down and migration of the chromosomes to the metaphase plate occurs. Metaphase is a very short mitotic event, which corresponds to the alignment of all chromosomes at the metaphase plate until anaphase onset. Anaphase is one of the most complex and dramatic events during mitosis. It involves cohesin disruption, a protein that holds sister chromatids together, and migration of each set of chromatids to the poles, in a process known as chromosome segregation. The last step of mitosis is telophase. In this phase, chromosomes and other nuclear elements are re-packaged in two new nuclei, spindle starts to disassemble and there is only one centrosome for each set of chromosomes (Morgan, 2007).

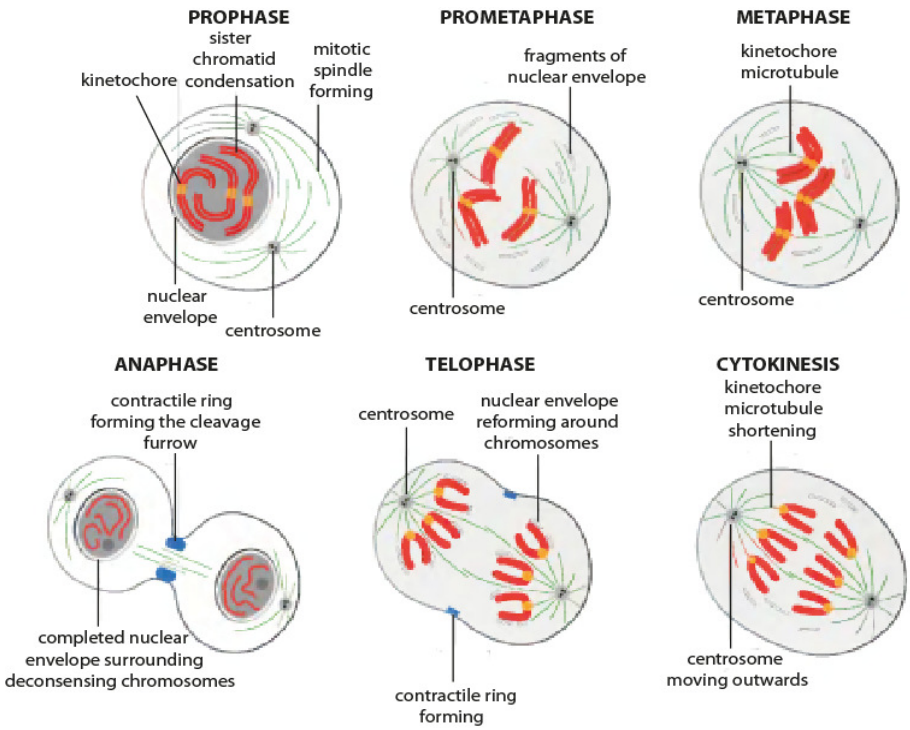

Fig. 1 – Events of mitosis in vertebrate cell. Prophase (P), prometaphase (PM) and metaphase (M) are the cellular events of the early mitosis while anaphase (A), telophase (T) and citokinesis (C) belong to the late M phase (adapted from Morgan – The Cell Cycle - Principles of Control © 2007 New Science Press).

2. The Cell-Cycle Control System

Cell cycle is a highly controlled phenomenon regulated by a complex network of effectors, which control the order and timing of cell-cycle events. A series of regulatory proteins active specific effectors, while others are inhibited. However, cell-cycle regulators work as single units in a complex orchestra, in which the perfect synchrony is required for the efficiency of this fundamental biological process.

This complex set of players, which is active at a specific point, allows cells to know when it is time to divide, or to maintain in a sleeping stage until extracellular signals trigger cell division.

The most important regulators of the cell cycle are the cyclin-dependent kinases (Cdks), which upon activation by the respective cyclin, activates a set of downstream pathways, which regulate cell-cycle events. Cdks concentrations is constant throughout cell cycle, but its phosphorylation status changes as a consequence of oscillations of their cyclin partners. These proteins bind specifically to each Cdk, triggering its catalytic activity. Different Cdks are activated at each point of the cell cycle, while others are silent. The efficiency of these phosphorylation switches is a fundamental requirement to ensure the correct sequence of events during cellcycle and cell division (Morgan, 2007).

Focusing only on the cyclins that regulate Cdks activity involved in cell cycle regulation, we may enclose them in four main classes - G1/S cyclins, S cyclins, M-cyclins – which are directly involved in control of cell cycle events- and G1 cyclins, the family involved in control of cell-cycle entry in response to extracellular signals.

To ensure the correct distribution of the genetic material to their progeny, cells make use of regulatory steps termed checkpoint controls. When cells sense some errors, checkpoints delay cell cycle progression, preventing the transmission of compromised genome integrity. The first checkpoint called Start occurs when the regulatory machinery checks if all conditions are ideal for cell proliferation, G1/S and S phase cyclin-Cdk complexes are active and trigger DNA replication, centrosome duplication, as well as other early cell-cycle events. Ultimately, G1/S and S- active Cdks promote the activation of M-Cdks, which drive progression to the next checkpoint. The second checkpoint occurs prior to cell entry into mitosis and is termed G2/M checkpoint. This regulatory step responds to DNA damage (Pearce & Humphrey, 2001) or other injurious influences (Mikhailov, Cole, & Rieder, 2002), acting by inhibiting the master mitotic regulator, the cyclin B/CDK1 complex. The third checkpoint controls the metaphase-to-anaphase transition. The

satisfaction of this point of control promotes sister chromatid segregation, completion of mitosis and cytokinesis. This checkpoint counts on Cdc20 and Cdh1, as well as Mad and Bud family proteins present on unattached, but not on attached kinetochores to prevent the activation of the anaphase-promoting complex (APC) (Vigneron, *et al*., 2004; Zhou, Yao, & Joshi, 2002). The metaphase-to-anaphase checkpoint is also termed spindle assembly checkpoint (SAC) because it monitors if all kinetochores are attached to the spindle MTs. Once all kinetochores are bioriented at the metaphase plate and attached to the spindle MTS, SAC is satisfied, anaphase onset takes place and cells start exiting mitosis (Rieder & Maiato, 2004). The APC is a large ubiquitin-ligase that targets securin (Nasmyth, Peters, & Uhlmann, 2000) and cyclin B (Hagting, *et al*., 2002) to proteolysis. The respective degradation of both proteins allows chromosome segregation and completion of mitosis (telophase). Cyclins degradation leads to the inactivation of Cdks in the cell, which allows phosphatases to dephosphorylate Cdks substrates. Those dephosphorylations are required for spindle disassembly, completion of mitosis and cytokinesis. Recently, a new checkpoint has been proposed, which delays cytokinesis in the presence of lagging chromosomes. This point of control is mediated by Aurora B/Ipl1 kinase activation in the presence of chromatin at the midzone in posttelophase stages, which leads to furrow regression and thereby preventing aneuploidy (Mendoza, *et al.*, 2009; Steigemann, *et al.*, 2009)

3. The Mitotic Apparatus

During mitosis, chromosome segregation is carried out by a complex machine known as mitotic spindle. This structure pulls each set of chromosomes apart toward the poles of the cell during chromosome segregation. The mitotic apparatus consists of: microtubules (MTs), centrosomes (or MT self-organizing centers when cells are not provided of centrosomes), kinetochores and MT-associated motor and non-motor proteins that organize them into two antiparallel arrays of MTs.

3.1. Microtubules

MTs are the main structural component of the mitotic spindle, whose basic building block is a heterodimer of α and β-tubulin (fig. 2; a). Stable α/β-tubulin heterodimers are

aligned in a polar head-to-tail fashion to form protofilaments (Akhmanova & Steinmetz, 2008) (fig. 2; a). Typically, a MT is made up of thirteen protofilaments, which associate laterally to each other to form a hollow tube about 25 nm in diameter (fig. 2; b) (Akhmanova & Steinmetz, 2008; Alberts, 2002; Morgan, 2007).

A molecule of guanosine triphosphate (GTP) binds to each unit of α-tubulin and β-tubulin. According to the model currently accepted, the GTP of β-tubulin is hydrolyzed by the moment that a new heterodymer subunit is incorporated at the growing end of the growing MT. However, the GTP molecule of the β-tubulin that is exposed at the growing tip remains intact, forming a structure called GTP-cap (McNally, 1999). β-tubulin GTP hydrolysis results in a change of conformation of the heterodimer, which plays a significant role in the dynamic turnover of MTs, an essential property for their fast growth and shrinkage. The fast exchange from one state to another is called MT instability (fig. 2; c) (Desai & Mitchison, 1997; Morgan, 2007).

The rearrangement of MT subunits results in two opposite MT ends with different characteristics. The end that exposes the β-tubulin GTP is called plus end, while the opposite end, called minus end, exposes the α-tubulin subunit. So, MTs have two clearly distinct ends and the regulation of the dynamics of both ends occurs independently of each other. The fact that MTs are polar structures, rearranged in a head-to-tail fashion, allows them to act like rail to motor molecules, which are peculiar molecular organizers of the intracellular space and cytoskeleton (Morgan, 2007). The MT plus ends have the capacity to grow fast *in vitro* and, *in vivo* and are provided of a fascinating ability to exchange fast between periods of growth and shrinkage. (Akhmanova & Steinmetz, 2008). The MT dynamic instability model, based on *in vitro* experiments, dictates that MT exist in persistent phases of either growth or shortening, with abrupt transition between both states (Desai & Mitchison, 1997). These abrupt exchanges are termed catastrophe, when occurs the switching from growing to shortening, and rescue, when the opposite happen (fig. 2; c). During polymerization, MT ends often have a sheet-like extension in which some protofilaments have grown longer than others (fig. 2; c1). On the other hand, when MT depolymerize, individual protofilaments peel away from the polymer lattice (Desai & Mitchison, 1997) (fig. 2, c3). As it was previously mentioned, The GTP-cap is the molecular key of the MT dynamics. Shortening MTs have lost its GTP-cap, exposing GDP-tubulin subunits at the end of the MT, which dissociates fifty times faster than GTP-tubulin, resulting in a rapid depolymerization (Morgan, 2007). In a very simple away,

catastrophe occurs when MT start to lose its GTP cap, while rescue requires the GTP-tubulin re-cap of the end of a shortening MT (Morgan, 2007).

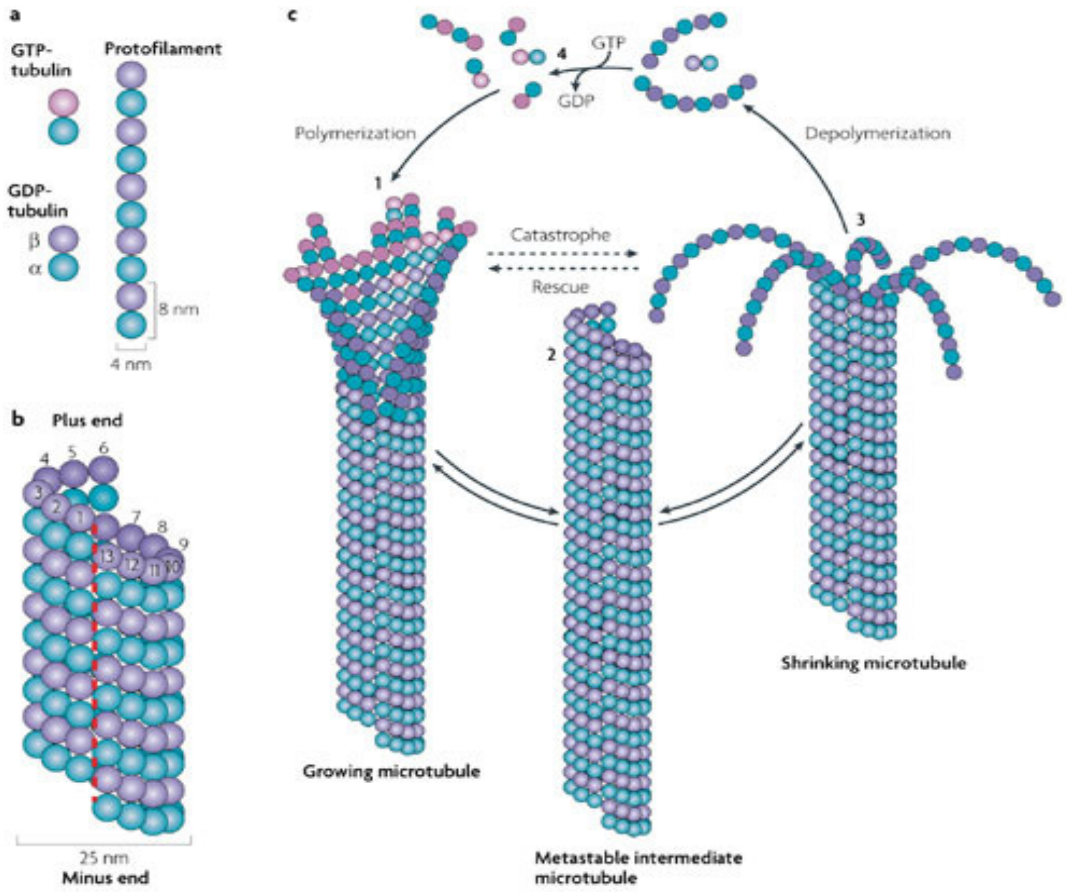

Fig. 2 – Microtubules structure and dynamics. Microtubules (MTs) are composed of stable α/β-tubulin heterodimers that are aligned in a polar head-to-tail fashion to form protofilaments (a) Typically, 13 parallel protofilaments form a cylindrical and helical microtubule wall (b). MT instability (c) is driven by the binding, hydrolysis and exchange of a guanine nucleotide on the β-tubulin monomer. Polymerization is typically initiated from a pool of GTP-loaded tubulin subunits (c - 1), while shorinking MT have lost its GTP-cap, exposing GDP-tubulin subunits at the end of the MT, which dissociates faster than GTP-tubulin, resulting in a rapid depolymerization (c - 3).

3.2. The Centrosomes

To ensure correct chromosome segregation, the best way is to separate each set of chromosomes to an opposite position inside the cell. The way that cells accomplish that is through the assembly of a bipolar spindle, a phenomenon termed bi-orientation. The bilateral symmetry is critical to the ability of the spindle to efficiently pull apart sister chromatids and it has been reported that any disarrangement of bi-orientation can cause

potentially lethal errors in chromosome segregation (Morgan, 2007; Wadsworth & Khodjakov, 2004).

Although all normal spindles are bipolar, the structure from which MTs nucleate may differ between some organisms. The centrosome is the primary microtubule-organizing centre (MTOC) in animal cells, which regulates cell motility, adhesion and polarity in interphase, and facilitates the organization of the spindle poles during mitosis (Bettencourt-Dias & Glover, 2007). However, some cell types, such as the ones existing in higher plants and oocytes of many vertebrates, are not provided with centrosomes and the organization of the mitotic spindle depends on the self-organizing capacity of the MTs. So, the acentrosomal pathway relies on many MT-associated proteins to generate two spindle poles (Gadde & Heald, 2004; Morgan, 2007).

The centrosome consists of two centrioles surrounded by an electron-dense matrix, the pericentriolar material (PCM) (fig. 3; A). Each centriole is composed by 9 MT triples with ~0,5µm in length and ~0,2 µm in diameter, positioned orthogonally one to another and localized in the cytoplasm, just outside the nuclear envelope (NE) (Bettencourt-Dias & Glover, 2007; McGill, Highfield, Monahan, & Brinkley, 1976). In this PCM are localized a large amount of γ-tubulin ring complexes (γ-TuRCs). γ-tubulin is highly conserved in all eukaryotes (Oakley & Oakley, 1989) and is the main MT nucleation center at the MTs minus-ends, allowing the plus-ends to grow outwards (Moritz & Agard, 2001; Moritz, Braunfeld, Guenebaut, Heuser, & Agard, 2000; Moritz, Braunfeld, Sedat, Alberts, & Agard, 1995). In the acentrosomal pathway, like in plant cells and frog oocytes, γ-TuRCs are not organized at the vicinity of centrioles, but are rather scattered around the sister chromatids (Caudron, Bunt, Bastiaens, & Karsenti, 2005).

The life cycle of centrosomes is slightly ahead of the chromosomes. Centrosome duplication begins at G1, when they also lose their orthogonal orientation and split slightly apart. Each old centriole – the mother centriole - provides a pre-existing mold upon which a daughter centriole is built. During S phase, daughter centrioles elongate and form a right angle to the old centrioles. Dispite centrosome duplication is complete by the beginning of mitosis, the new centriole is not completely finished until later in mitosis. When cells enter mitosis, centrosomes migrate to opposite poles of the cell and spindle MTs form between them (fig. 3; B). After chromosome segregation and cytokinesis, each newborn G1 cell is provided of a single centrosome, which will be duplicated in the next cell cycle (Morgan, 2007).

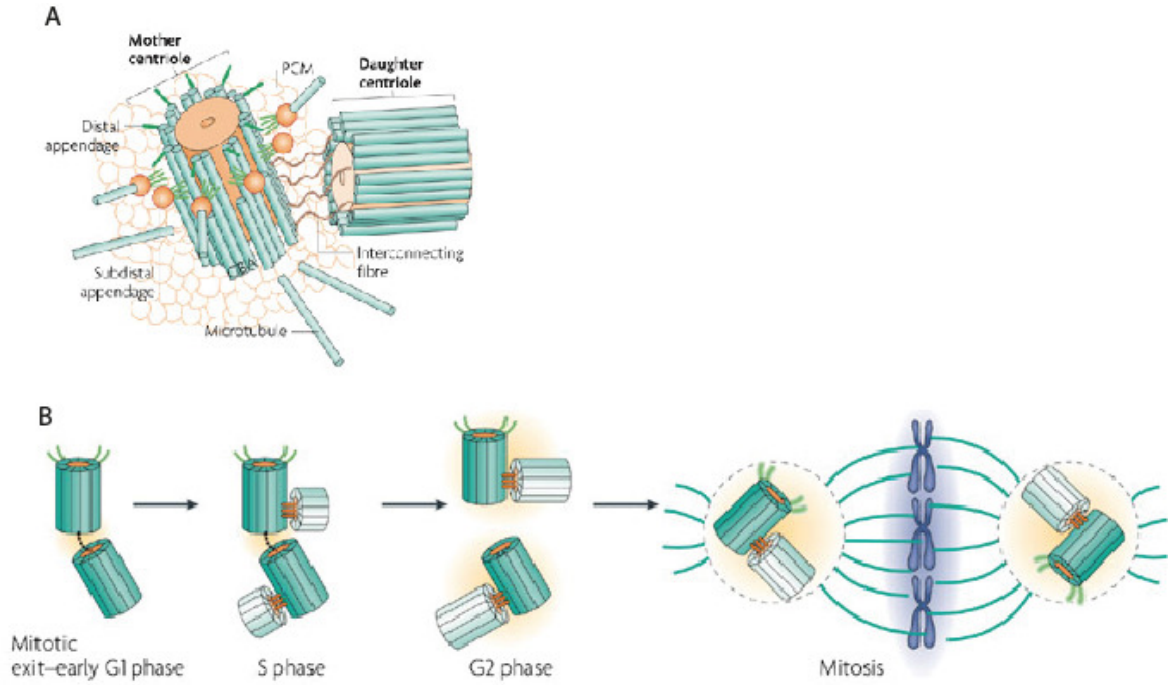

Fig. 3 - The centrosome structure and cycle. (A) Schematic view of the centrosome. The centrosome consists of two centrioles orthogonally positioned to each other and surrounded by an electron-dense pericentriolar matrix. (B) The centrosomes life cycle in *Caenorhabditis elegans*. Despite differences in the structure, the centriole cycle of this specie seems to be regulated in a similar way to the humans. Nucleation of daughter centrioles happens in S phase and elongate during G2 phase and mitosis. When cells enter mitoses, centrosomes migrate to opposite poles of the cell and spindle MTs form between them (Bettencourt-Dias & Glover, 2007).

3.3. The Kinetochore

Early cytologists observed that metaphase and anaphase cells had an achromatic region or constriction that was localized within individual chromosomes. They also found out that those regions were constantly establishing connections with the spindle (Agar, 1912). Throughout the following years, these regions were also termed "*spindle attachment region*" by Metzner in 1845 (Shrader, 1944) and "kinetic bodies" by Nawaschin (L. Sharp, 1934). Later on, J. A. Moore called "kinetochores" to this structures that had the ability to move the chromosomes (L. Sharp, 1934). The first ultrastructural studies of the kinetochore showed that these bodies were 0.2-0.3 µm trilaminar disk adjacent to the chromatin (B. Brinkley & Stubblefield, 1970; B. R. Brinkley & Stubblefield, 1966; Jokelainen, 1967). McEwen described kinetochore morphology as a structure composed by an inner plate of 20-40 nm granular material and an outer plate of 30-40 nm structure of irregular and regular 10-20 nm thick fibrillar components (Maiato & Sunkel, 2004; McEwen, Arena, Frank, & Rieder, 1993). Such different composition

suggests different functional domains. Between both plates, there is an intermediate region of 15-35 nm wide of loosely organized fibrillar material that appears clear in electron micrographs (Maiato & Sunkel, 2004)

In eukaryotes, accurate chromosome segregation requires each chromosome to interact appropriately with spindle MTs, upon which chromosome segregation occurs. This interaction is mediated by the kinetochore, a macro-molecular complex composed by more than 90 proteins that assembles at the centromeric region of each chromosome only during mitosis (Gascoigne & Cheeseman, 2011) However, cells treated with antibodies against kinetochore antigens (Moroi, Peebles, Fritzler, Steigerwald, & Tan, 1980; Tan, *et al*., 1980) stained discrete nuclear spots along the decondensed chromatin, suggesting that there is a "kinetochore organizer" or "presumptive kinetochore" in interphase chromatin (Brenner, Pepper, Berns, Tan, & Brinkley, 1981; Rieder, 1982). True kinetochore pairs are only visible at prophase as a "spheric ball" (0.6-0.8 μm in diameter) of fibrillar material inserted into a dense "cup" (Rieder, 1982). As soon as cells enter prometaphase, MTs attach to the kinetochores, their conformation changes from a "ball and cup" structure to a plate-like structure and the lighter staining corona becomes visible (fig. 4).

Centromere epigenetic marks, such as the presence of centromere associated protein A (CENP-A) containing nucleosomes, chromatin structure, and DNA sequence properties mark the site for kinetochore formation (Dalal & Bui, 2010). CENP-A is a histone H3 variant that occurs predominantly in centromeres and is required for kinetochore assembly (Gascoigne & Cheeseman, 2011). Additional proteins are also found constitutively at the human centromere throughout the cell cycle, in particular a group of 15 proteins known as Constitutive Centromere Associated Network (CCAN). Together, these proteins form a stable base for dynamic kinetochore assembly, as well as promote the recruitment of new CENP-A (Hori, *et al*., 2008). The outer kinetochore plate and fibrous corona assemble upon entry into mitosis, and contain proteins with direct microtubule binding activity (Cheeseman & Desai, 2008). Among the outer kinetochore proteins with MT binding activity, there is the KMN (KNL1, Mis12 and Ndc80) network (Cheeseman & Desai, 2008), ska1 complex ((Welburn, *et al*., 2009) and CENP-E (Wood, Sakowicz, Goldstein, & Cleveland, 1997), as well as other transient factors. The master player of the outer kinetochore is the Ndc80 complex, which is formed by four conserved proteins, Ndc80/Hec1, Nuf2, Spc24 and Spc25 (DeLuca, et al., 2005). Together with Dam1 complex, a ten-subunit complex, and other MT-regulating and motor proteins, are essential

for MT attachment and regulation of MT plus-end behavior (Miranda, De Wulf, Sorger, & Harrison, 2005).

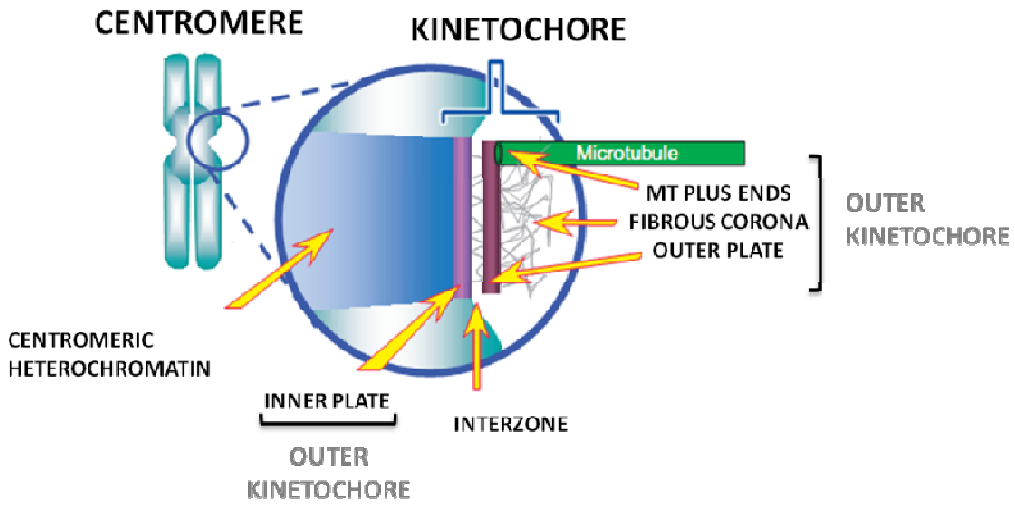

Fig. 4 - Organization of the animal kinetochore. Mature kinetochores are plate-like structures that assemble in specific sites of the centromeric region. It consists of an inner plate that assembles in the centromere, a outer plate and the fibrous corona that contain proteins with direct microtubule binding activity. Between both plates, a loosely organized fibrilar region constitute the interzone (Maiato, DeLuca, Salmon, & Earnshaw, 2004)

3.4. MT-associated proteins

A complex repertoire of MT-associated proteins (MAPs) is required to orchestrate spindle dynamics and architecture. MAPs are divided in two fundamental classes, according to its ability to move along the spindle. MT dynamic instability depends on a broad range of proteins that promote MTs stabilization or destabilization. Usually, these proteins are associated to the MT plus or minus ends, but do not move along the spindle – non-motor MAPs. Kinesin-13 family is an important group of non-motor proteins, which induces catastrophe events by triggering conformational changes that disrupt lateral interactions between protofilaments. The inverse phenomenon is promoted by stabilizing factors, such as XMAP215 family (TOG in humans) that bind to the plus ends, thus blocking the binding of destabilizing factors, such as kinesin-13 family (Gadde & Heald, 2004). Stabilizing proteins are associated not only to MT growth, but they are also involved in the association of the plus ends to another cellular structures, such as the cell cortex. Within the stabilizing proteins group, are also found CLIP-associated proteins (CLASPs), which will be highlighted in this thesis.

Additionally, there is another group of MAPs able to travel along the MTs – motor MAPs. Through hydrolyzis of ATP, motor proteins generate force and movement of the spindle. Most kinesins or kinesins-related protein motors move along MTs towards the plus ends. However, some members of this family move in the opposite direction. Local regulation of MTs is driven by motor proteins. These mechanochemical ATPases can move MTs unidirectionally towards the plus or minus ends, transporting a molecular cargo or linking MTs into force-generating arrays, such as spindle (Gatlin & Bloom, 2010). The first motor protein described was dynein, a minus-end-directed motor protein (Gatlin & Bloom, 2010). Some plus-end directed kinesins localize to chromosome arms (chromokinesins) contribute to chromosome attachment and movement towards the metaphase plate, while cytoplasmic dynein in the cortex can contribute to astral MTs organization (Gadde & Heald, 2004; Gatlin & Bloom, 2010; Morgan, 2007). Several plus-end-directed proteins, such as Eg5, Mklp1/CHO1 and chromokinesis/KIF4, interact with MTs, promoting MT antiparallel sliding that drives spindle pole separation during anaphase B. In general, kinesin-15 works to promote increased spindle length (outward directed forces), whereas minus end motors, such as dynein and kinesin-14 function to promote spindle shortening (inwards directed forces) (T. J. Mitchison, *et al.*, 2005; Saunders, Lengyel, & Hoyt, 1997; D. J. Sharp, *et al.*, 2000). Closer to the poles, where parallel MT orientation is more prevalent, dynein mediated minus end clustering forces dominate (Braun, Drummond, Cross, & McAinsh, 2009).

The mechanisms that shape the spindle start to be clarified, especially concerning the motors associated to the kinetochores. Nevertheless, a long avenue has to be explored to get the full picture of where and how these proteins interact with MTs and their partners to control such a complex set of molecular events.

4. The Spindle Assembly

The mitotic spindle assembly occurs in early mitosis and faces two important challenges: to build a bipolar structure and to properly attach chromosomes in order to establish a MT array with the correct bi-orientation. In mammals, the bipolar spindle is highly dependent on the correct positioning of the centrosomes, which are motor-dependent pulled apart to by motor proteins the opposite sides of the prophase nucleus.

During this mitotic stage, the nuclear envelope breakdown (NEB) allows MTs to invade the nuclear space and contact with chromosomes.

The absence of centrosomes does not obstruct the formation of a bipolar spindle in acentrosomal systems. In these cases, spindle assembly relies on MT self-organization, whereby MTs form in the vicinity of sister chromatids and then become organized by motors into a bipolar array (Gatlin & Bloom, 2010) Eventually, spindle acquires by itself a fusiform architecture with interdigitated plus ends near the chromosomes and minus ends focused at the poles (Gaglio, Dionne, & Compton, 1997; Heald, Tournebize, Habermann, Karsenti, & Hyman, 1997). However, it was previously observed that chromosome-directed nucleation is not exclusive to the acentrosomal pathways. It was also found that animal somatic cells also nucleate MTs near chromosomes (Maiato, Rieder, & Khodjakov, 2004).

An evolutionary question involves the biological purpose of the centrosomes, since the acentrosomal pathway is able to efficiently self-organize a bipolar spindle. In fact, the presence of centrosomes seems to facilitate MT nucleation because they provide a MT bipolar mold upon which they polymerize. In addition, centrosomes nucleate astral MTs, which are an advantage in terms of connection of the spindle to the cell cortex and spindle positioning within the cell (Wadsworth & Khodjakov, 2004).

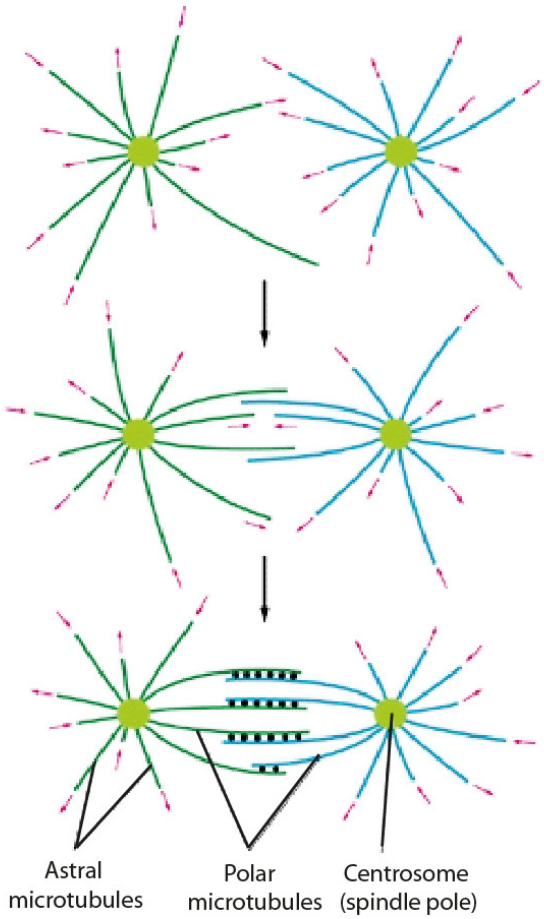

Fig. 5 – The bipolar mitotic spindle. New microtubules grow out in random directions from two nearby centrosomes. The microtubules are anchored to the centrosome by their minus ends and their plus ends are "dynamically unstable", switching between catastrophe and rescue periods. When two microtubules from opposite centrosomes interact in an overlap zone, microtubule-associated proteins are thought to cross-link the microtubules together in a way that caps their plus ends, stabilizing them by decreasing their probability of depolymerizing.

Spindle assembly and function is regulated by a huge amount of proteins. Among them, Ran is an important regulatory protein involved in activation of numerous spindle assembly-promoting factors around the chromosomes. Ran is a GTPase, whose activity depends on GTP binding, carried out by an activation protein called RanGAP and a guanine-nucleotide exchange factor called RCC1. Inside the nucleous, Ran-GTP binds to importin, a nuclear transporter protein, that inhibits several proteins involved in spindle assembly. RCC1, which is highly concentrated near chromosomes, activates Ran-GTP in the immediate vicinity (Maiato, DeLuca, *et al*., 2004; Morgan, 2007). Ran-GTP triggers dissociation of importin from regulatory proteins, allowing spindle formation around chromosomes (Kalab, Pu, & Dasso, 1999; Kalab, Weis, & Heald, 2002; Morgan, 2007). Ran GTpases exists in a concentration gradient around mitotic chromosomes (Carazo-Salas

et al., 1999), which promps spindle assembly through two mechanisms. One involves the stimulation of MT nucleation by chromatin (Heald, 2006), while the other works through the generation of a local concentration of MT stabilizing factors near the chromosome that promote the capture of MTs. Besides Ran-GTP gradient, it was shown in *xenopus* extracts that aurora B kinase, together with its partners, Inner Centromere Protein (INCENP), borealin and survivin, forms the Chromosomal Passenger Complex (CPC), interacts with chromosomes in early mitosis, promoting local stability of MTs by inhibiting the catastrophe factor centromere-associated kinesin (MCAK) (Klein, Nigg, & Gruneberg, 2006; Sampath, *et al*., 2004). CPC is a key factor in attachment error correction before anaphase onset which will be discussed below (Adams, Carmena, & Earnshaw, 2001; Gassmann, *et al.,* 2004).

4.1. Kinetochore-MT attachment

In 1986, Kirschner and Mitchison proposed an explicative model of the process by which MTs attach to kinetochores. According to this model, upon NEB in animal cells, highly dynamic MTs invade the tree-dimensional cytoplasmic space until find a kinetochore. Once MTs encounter a kinetochore, become stabilized, whereas those that do not, soon depolimerize (Hayden, Bowser, & Rieder, 1990; T. Mitchison, Evans, Schulze, & Kirschner, 1986). The kinetochore-microtubule interface is highly fluid and kinetochore itself is remarkably dynamic, changing its makeup upon MT attachment. Indeed, prophase spherical shaped kinetochores turn into a plate-like structure upon MT binding (Rieder, 1982)

Probably, the foremost question regarding kinetochore-microtubule binding is how can MTs remain firmly attached to the kinetochores when they are continuously growing and shrinking, allowing chromosomes to move back and forth on the spindle. Several studies have revealed that this complex is crucial for the stable kinetochore-microtubule attachments, required to sustain the centromere tensions involved in the achieving of the proper chromosome alignment in higher eukaryotes (DeLuca, Moree, Hickey, Kilmartin, & Salmon, 2002; Howe, McDonald, Albertson, & Meyer, 2001). Until recently, no evident direct interaction between Ncd80 complex and the MTs was found. Thus, another protein would connect both. In yeast, kinetochore-microtubule attachment requires Dam1 complex (also known as DASH complex) because some members of this complex bind directly to MTs, while others bind to Ncd80 complex (Westermann, *et al.*, 2003). Dam1 seems to

work as an adaptor between kinetochores and MTs. The most appealing hypothesis suggests that Dam1 forms a ring surrounding the MT close to the plus tip, serving as a collar around MT that is anchored to the inner kinetochore through Ndc80 complex. However, the ring is not necessary to drive motion. Therefore, MTs plus ends at the kinetochore can be lost or added without being released from the kinetochore (fig. 6) (Westermann, *et al*., 2003).

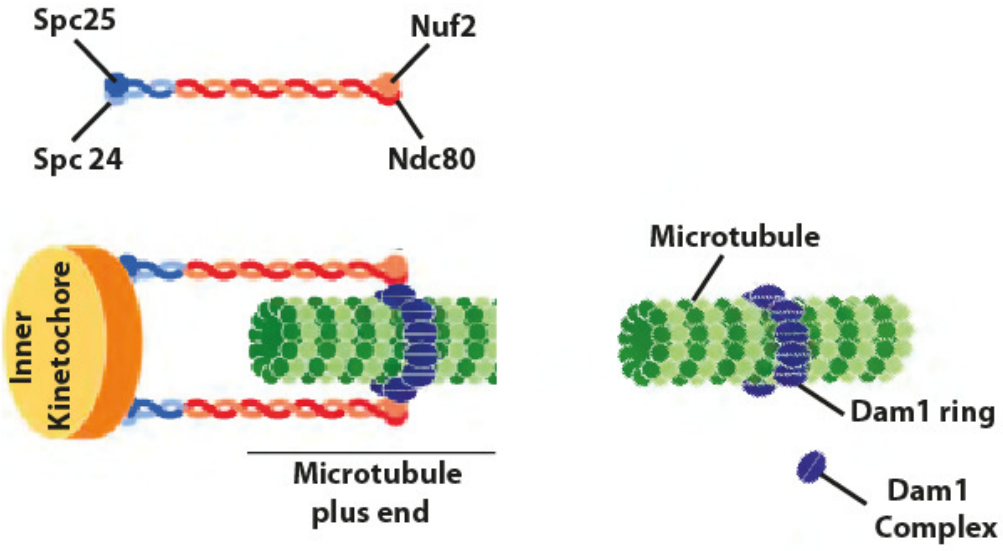

Fig. 6 – a possible mechanism for dynamic kinetochore-microtubule attachment. The Daml ring may provide a sliding microtubule collar that is anchored in the kinetochore by the Ndc80 complex. The exposed microtubule plus end can interact with regulatory factors that influence microtubule polymerization and depolymerization (adapted from Morgan – The Cell Cycle - Principles of Control © 2007 New Science Press).

Another protein complex important for kinetochore-microtubule attachment is the Ska complex (Ska1, Ska2 and Sk3/Rama1) that localizes to the outer kinetochore and along spindle MTs (Gaitanos, *et al*., 2009; Hanisch, Sillje, & Nigg, 2006). Ska complex depletions has been reported to cause severe chromosome alignment defects, due to the incapacity to form stable kinetochore-microtubule attachments in eukaryotes and Ska complex should be considered among the essential pieces of this process (Gaitanos, *et al*., 2009). Like Dam1, Ska binds to MTs and couple bead movement to depolimerize their ends (Welburn, *et al.*, 2009). Stable attachment of MTs to kinetochores also depends on motor proteins in the kinetochore, such as plus-end-directed kinesin-7 motor CENP-E and the minus-end-directed dynein, which are anchored to components of the outer kinetochore (Lombillo, Nislow, Yen, Gelfand, & McIntosh, 1995; McEwen, *et al.*, 2001; Putkey, *et al*.,

2002). These proteins interact mostly with MTs to promote attachment and generate force to move chromosomes along the spindle. Although these two proteins are not essential to MT-kinetochore attachment, dynein, which is released from kinetochores upon MT attachment (Hoffman, Pearson, Yen, Howell, & Salmon, 2001), has been reported as an important factor required for the inactivation of the SAC, whereas CENP-E located in the fibrous corona, is implicated in the initial encounter between kinetochores and MTs during prometaphase (Cooke, Schaar, Yen, & Earnshaw, 1997; Yao, Anderson, & Cleveland, 1997). CENP-E is also involved in anchoring kinetochores to shortening MT *in vitro* (Lombillo, Stewart, & McIntosh, 1995) and MT efficient binding to its kinetochores (McEwen, *et al*., 2001; Putkey, *et al*., 2002).

4.2. Error correction in kinetochore-microtubule attachment

During prometaphase, MTs search and capture kinetochores and attachment errors are common. However, cells are provided of molecular machinery that senses certain incorrect kinetochore-microtubule attachments, delaying activation of APC/C and, therefore, anaphase onset. During this mitotic stage, chromosomes can be mono-oriented by one (monotelic attachment) or both sister kinetochores (synthelic attachment) (fig. 6) (reviewed by Maiato *et al*., 2004). Over the years, two conditions have been proposed to be keys for SAC signaling: lack of kinetochore-microtubule attachment and lack inter-kinetochoreo tension (Nezi & Musacchio, 2009). Thus, SAC senses the lack of kinetochore ocupancy in monotelic attachments, as well as the low tension at the kinetochores in syntelic attachments (Nicklas & Koch, 1969; Nicklas, Waters, Salmon, & Ward, 2001). The accurate chromosome segregation requires an amphitelic attachment, where one sister kinetochore is attached to MTs solely from one pole, whereas the other is attached to MTs solely from the opposite pole (reviewed by Maiato *et al*., 2004). (fig. 6). Sometimes, it may happen that one or both sister kinetochores has MT attachments to both poles (fig. 6). This kind of error is known as merotelic attachment and escape from checkpoint because the level of kinetochore occupancy is high, as well as the tension exerted at the kinetochores (fig. 7) (Cimini, Fioravanti, Salmon, & Degrassi, 2002; Cimini, et al., 2001). Over the last few years, some investigators have tried to find a connection between tension-based error correction pathway and the SAC response (reviewd by Nezi & Musacchio, 2009). A few studies have shown that Aurora B/Ipl1 kinase is a crucial element of the correction system (Lampson, Renduchitala, Khodjakov, & Kapoor, 2004; Tanaka, *et al*., 2002) and has been

reported that syntelic and merotelic attachments are not corrected when Aurora B is inhibited (Bakhoum, Thompson, Manning, & Compton, 2009; Lampson, *et al*., 2004)

One key factor in error correction before anaphase onset is CPC, which is involved in several places during mitosis (Ruchaud, Carmena, & Earnshaw, 2007) In the beginning of mitosis, CPC localizes at the chromosome arms and centromere and is required for the recruitment of several SAC proteins to the kinetochore (A. Carvalho, Carmena, Sambade, Earnshaw, & Wheatley, 2003; Lens & Medema, 2003; Lens, *et al*., 2003; Murata-Hori & Wang, 2002; Vigneron, *et al*., 2004). In anaphase, CPC leaves kinetochores and relocalizes at the midzone (Kelly & Funabiki, 2009). Aurora B is probably the most important player in error correction. In vertebrates, this protein is required for mediating proper attachments (Hauf, *et al*., 2003) and loss of its activity results in a remarkable increase in the overall stability of kinetochore MTs (Cimini, Wan, Hirel, & Salmon, 2006; Zhang & Walczak, 2006), suggesting that Aurora B is promping detachment of incorrect attached microtubules by changing dynamics of kMTs. The exact mechanism by which Aurora B is able to adjust the incorrect attachments is not yet clear, but has been proposed that Aurora B corrects kinetochore–MT mal-attachment through regulation of MCAK and Ndc80/Hec1 (Cheeseman *et al*., 2002, 2006; Andrews *et al*., 2004; Lan *et al*., 2004; Ohi *et al*., 2004; Deluca *et al.*, 2006). An emerging "attachment-only" hypothesis proposes that SAC satisfaction is the only crucial parameter monitores by SAC (Nezi & Musacchio., 2009). This model implies that tension-dependent errors correction mechanisms depends on Aurora B that generates unattached kinetochores, which are sensed by the SAC (Pinsky, Kung, Shokat, & Biggins, 2006). This model conceptualizes an error correction pathway, of which Aurora B/Ipl1 is the most authoritative representative, as being crucial for bi-orientation, but also as being physically and functionally distinct to SAC. Under this concept, Aurora B is exclusively implicated in the correction of improper kinetochore-microtubule attachments and is not a SAC component because it is not required for SAC signaling *per se* (Nezi & Musacchio., 2009).

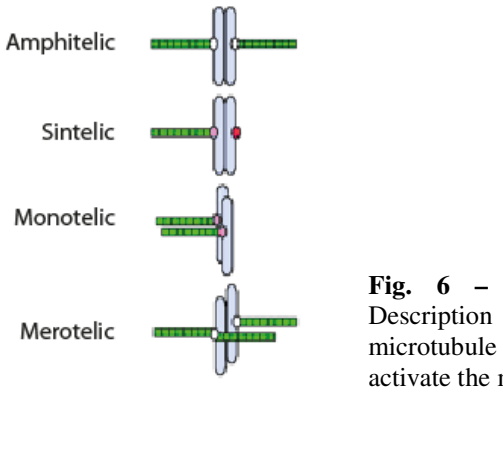

Fig. 6 – Kinetochore attachment errors. Description of the most common kinetochore-microtubule attachment errors and their ability to activate the mitotic checkpoint

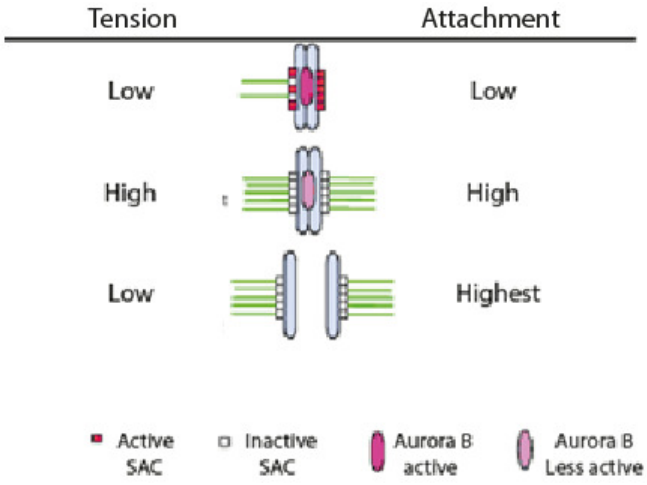

Fig. 7 –Model showing the predict activity of Aurora B at centromeres under different conditions of microtubule occupancy.

5. Mechanisms of Chromosome Movement

5.1. Chromosome congression

Before alignment at the metaphase plate in vertebrates, sister chromatids dramatically oscillate during prometaphase, moving towards the poles and away from them. At some point, the pulling and pushing forces exerted by the MTs at the kinetochores drive the chromosomes to the spindle equator. The phenomenon through which animal sister-chromatids align at the center of the spindle in metaphase is known as chromosome congression (Morgan, 2007).

Two main forces act on chromosomes to move them back and forth during chromosome congression. The first generated by the plus-end depolimerization of the MTs attached to the kinetochore (Khodjakov, Cole, Bajer, & Rieder, 1996; Khodjakov & Rieder, 1996; Skibbens, Skeen, & Salmon, 1993). The mechanism through which this force occurs is not yet fully understood, but it has been suggested that depolimerizing plus-ends at the kinetochore, forms a outward curling structure of the MT that generates a force that pushes against the collar toward the poles, while remaining attached to the kinetochore (Khodjakov, *et al*., 1996; Khodjakov & Rieder, 1996; Skibbens, *et al.*, 1993). The depolimerization of the plus-ends at the kinetochores generates poleward movement creating high tension between sister chromatids, which is counterbalanced by the switch to a polymerization state, that moves the chromosome away from the poles, decreasing tension between sister chromatids. This equilibrium between pushing and pulling forces generated at the kinetochores is known as directional instability (Skibbens, *et al*., 1993). Additionally, a second type of forces acts at the chromosome arms. A force known as "polar ejection" or "polar wind" pushes chromosome arms away from poles (Rieder *et al*., 1986; reviewed by Ault *et al*., 1994; Rieder *et al*., 1994; and Inoue *et al*., 1995). The mechanism of the "polar ejection" forces are not well established, but it has been suggested that non-kinetochore MTs act at the chromosome arms, pushing them away from the poles. These pulling and pushing forces work together in a very coordinated manner in order to place the chromosomes at the spindle equator at metaphase.

5.2. Chromosome Segregation

The movement of each sister-chromatid towards the poles during anaphase is known as chromosome segregation. This phenomenon occurs mostly through the action of two types of forces. Both entail kinetochore microtubules (kMTs) depolimerization, which may occur at the plus-end of the MTs attached to kinetochores, a process known as "pacman" (Gorbsky, Sammak, & Borisy, 1987, 1988), or at the minus end at the poles (Desai, Maddox, Mitchison, & Salmon, 1998; Maddox, Desai, Oegema, Mitchison, & Salmon, 2002). In the poleward flux mechanism, tubulin is incorporated into the plus ends of the kinetochore MTs, which are translocated in the direction of the poles and finally dissociate from the minus-ends of the MTs (G. C. Rogers, Rogers, & Sharp, 2005). The contribution of each mechanism to the chromosome segregation varies between cells. In

vertebrates, approximately 70% of chromatid migration occurs via "pacman" and only 30% is due to flux (T. J. Mitchison & Salmon, 1992)

The activity of molecular motor proteins at kinetochores is also crucial for chromosome segregation. Dynein is a key factor to the flux mechanism because it delivers components of the flux mechanism, such as Kif2A, a depolimerizing kinesin. Members of the kinesins-13 family are also implicated in depolimerization of MTs at the kinetochore in *Drosophila*, therefore contributing for "pacman" (S. L. Rogers, Wiedemann, Hacker, Turck, & Vale, 2004).

CLASPs, that will be discussed in more detail below, have been described as important players in the incorporation of tubulin at the kinetochore, contributing to flux. Depletion of CLASP in *Drosophila* blocks flux because kinetochore MTs do not incorporate tubulin (Maiato, Khodjakov, & Rieder, 2005).

6. CLIP-associated proteins – CLASPs

6.1. Microtubule-plus end tracking proteins

+TIPs are specialized MAPs conserved among species, which characteristically accumulate at MT plus ends (Mimori-Kiyosue, Shiina, & Tsukita, 2000; Perez, Diamantopoulos, Stalder, & Kreis, 1999; Schuyler & Pellman, 2001). These proteins typically bind to the MT growing ends, but not to the shrinking ones. The first described +TIP was the cytoplasmic linker protein of 170 KDa (CLIP-170) (Perez, *et al*., 1999). +TIPs are a structurally and functionally diverse group of proteins. The dynamic +TIP interacting activity relies on a limited number of protein modular and linear sequence motifs, such as calponin homology domain (CH), end-binding homology domain (EBH), CAP-Gly domain, the acidic-aromatic C-terminus EEY/F sequence motif that is found in α-tubulin and CLIP-170, and SxIP motifs. These sequences allow the interaction between each other and MTs and typically display affinities in a low molecular range (Gupta, et al., 2009; Mishima, et al., 2007; Weisbrich, et al., 2007).

Presently, more than twenty families of +TIPs have been described, whose structure and function may be very different. In some cases, they may even have contradictory biological behaviors (for example, MT stabilizing versus destabilizing factors, such as

CLASPs and OP18, respectively) However, in some other cases, they share characteristic features of +TIPs (Akhmanova & Steinmetz, 2008; Morgan, 2007).

Although the majority of the +TIPs interact with the growing MTs, some can bind to depolymerizing MTs, such as Dam1/DASH complex (Salmon, 2005) and XMPA215 (Brouhard, *et al*., 2008). The most presumptive explanation for +TIPs accumulation at the growing ends relies on structural differences between the growing tips and the remaining tube. The presence of the GTP-cap is probably one of the most important molecular peculiarities in the core of this process. A few hypotheses have been proposed to explain the plus-tracking mechanism. Among them, some +TIPs have been claimed to recognize tubulin sheets and bind to the MT lattice seam (Sandblad, *et al*., 2006). Other +TIPs, such as CLIPs, co-polymerize with tubulin and are gradually released from the older lattice (Browning, Hackney, & Nurse, 2003; Rickard & Kreis, 1990). This type of interaction assumes transiently immobilization. Dissociation of these +TIPS occurs by spontaneous dissociation or by conformational changes in the MTs, a mechanism known as "treadmilling" (Perez *et al*., 1999). Unlike "treadmilling", a mechanism termed processive transport implies long distance motor-based movement along the cytoskeleton filament without dissociation. Proteins such as XMAP215, MCAK and dynactin seem to track growing MT ends processively, remaining at the same MT end during multiple rounds of tubulin subunit addition (Brouhard, *et al*., 2008). Several +TIPs are transported to growing MT ends by plus-end-directed motors proteins. The MT targeting by association with MT-binding partners is known as "hitchhiking", which is a common plus-tracking mechanism for many +TIPs (P. Carvalho, Tirnauer, & Pellman, 2003).

Due to attachment to the plus ends, +TIPs influence the structure of the MTs and accessibility for interaction with other proteins. A majority of MT stabilization is promoted by +TIPs, which act either by reducing the catastrophe levels, either by promoting rescue (Akhmanova, *et al.,* 2005).

6.2. Structure of CLASPs

Within the scope of this thesis, only the specific +TIPs CLASPs will be described in more detail. CLASPs *Drosophila* homologue, Mast/Orbit (from Multiple Asters), was firstly described in a genetic screening developed in flies, from which was identified and characterized (Lemos, *et al.,* 2000). Mast/Orbit homologues were also found and described in *Xenopus* (Xorbit) (Hannak & Heald, 2006), in *C. elegans* (CLS-2) (Cheeseman,

MacLeod, Yates, Oegema, & Desai, 2005), *S. cerevisiae* (Stu1p) (Pasqualone & Huffaker, 1994) and *S. pombe* (Peg1) (Grallert, et al., 2006). The CLASP1 and CLASP2 paralogues were found in mammals as molecular partners of CLIPs-115 and 170 (Akhmanova, et al., 2001).

Human CLASP1 and CLASP2 are encoded by chromosome 2 and 3, respectively. In mice *Clasp1* gene localizes in chromosome 1 and *Clasp2* localizes in chromosome 9. Alternative splicing may occur originating several CLASPs isoforms. So far, only one biologically active isoform for CLASP1 has been described, known as CLASP1α (~170 KDa), while CLASP2 counts with three active isoforms, including CLASP2α (~170 KDa), CLASP2β (~140 KDa) and CLASP2γ (~140 KDa) (Akhmanova, et al., 2001). The two paralogues share approximately 77% of homology, albeit their expression varies between different cells and tissues (Akhmanova, *et al*., 2001).

Structurally, CLASPs are highly conserved proteins that contain several serine and arginine enriched sequences, which confer flexibility to the protein (fig. 8). These sequences are predicted to be flexible and mediate interaction with MTs and EB proteins. The domains responsible for the interactions with CLIPs, kinetochores, Golgi apparatus and cell cortex belong to the C-terminus region (Galjart, 2005; Mimori-Kiyosue, et al., 2005). At the N-terminus, CLASPs contain a Dis1/TOG-like domain, except for CLASP2β because this isoform has a N-terminus palmitoylation motif that is important to the ancoring of the protein to the membrane, The Dis/TOG-like domain is associated with binding to tubulin and MTs (Gard, Becker, & Josh Romney, 2004). However, TOG-like domains function within CLASPs biological behavior is not yet established.

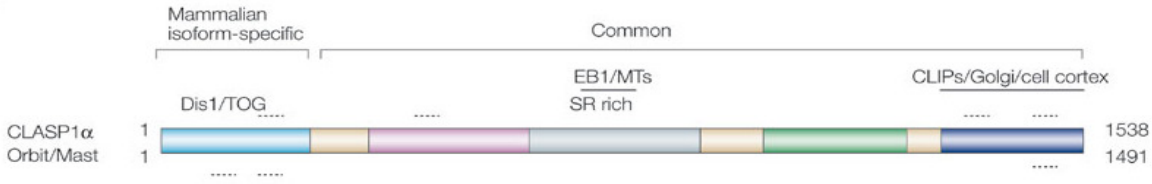

Fig. 8 - Domain structure and interactions of CLASP1α/Orbit/Mast. Mammalian CLASP1 and CLASP2 genes encode isoforms of 140–170 kDa, which differ at their N-terminus. The longest isoform (CLASP1α) is depicted, as this isoform is most similar to Orbit/Mast, the domain structure of which is also represented. Conserved domains are indicated in different colors. Potential HEAT repeats (stippled lines) are detected in CLASP1α (indicated above the protein) and Orbit/Mast (indicated below the protein), one of which is embedded in a region that has similarity to the Dis1/TOG family of microtubule-stabilizing proteins. A Ser- and Arg-rich (SR rich) region contains the domain that is responsible for the interaction of CLASPs with end-binding protein-1 (EB1) and with microtubules (MTs). The C termini of CLASPs interact with CLIPs, the Golgi apparatus or the cell cortex (Galjart, 2005).

6.3. Cellular distribution of CLASPs

In interphase cells, CLASPs localize to centrosomes and the Golgi apparatus, as well as in growing MT plus ends (Akhmanova, *et al*., 2001). Studies have proven that CLASPs co-localize with CLIPs, enforcing the previous assumptions about their partnership (Akhmanova, *et al*., 2001). However, it was also reported that CLASPs do not only bind to MTs through CLIPs, but also through EB1-related proteins (Mimori-Kiyosue, *et al*., 2005). Moreover, in *D. melanogaster,* Mast seems to conserve the same interaction with CLIPs (Mathe, Inoue, Palframan, Brown, & Glover, 2003; S. L. Rogers, et al., 2004). However, the role of CLASPs on MT dynamics appears to be independent of CLIPs, since *in vitro* studies have shown that CLASPs can bind tubulin *per se* (Grallert, *et al*., 2006; Lansbergen, *et al.*, 2006).

During mitosis, mammalian CLASPs, as well as its *Drosophila* homologue Mast localize at centrosomes, kinetochores, spindle midzone and midbody (Lemos, *et al*., 2000; Maiato, *et al*., 2003; A. L. Pereira, *et al*., 2006). CLASPs also tip-track astral MTs (Maiato, *et al.*, 2003) and CLASP1 localizes to the fibrous corona at the kinetochore in a MT independent manner (Maiato, *et al*., 2003).

6.4. Functions of CLASPs.

The localization of CLASPs in key structures within the cell gives a clue about their presumable functions, regarding the regulation of MT dynamics in the mitotic spindle and interphase MTs (Bratman & Chang, 2008; Gard, *et al.*, 2004). During interphase, CLASPs have been reported to mediate the selective stabilization of MTs in fibroblasts (Akhmanova *et al*., 2001). This finding was further supported by RNAi for CLASP1 and CLASP2, which resulted in a decreased MT stability, accompanied by a significant reduction in MT density, a phenotype that was not observed in single depletion for CLASP1 and CLASP2 (Mimori-Kyosue *et al.,* 2005). Together with the similar localizations of both CLASPs, the hypothesis of a functional redundancy in mammalian cells during interphase came over. Additionally, it was demonstrated that CLASPs act as stabilizing factors at the edge of the cell, either by reducing catastrophe, or by promoting rescue (Mimori-Kiyosue *et al.,* 2005). Also in interphase cells, CLASPs interact with the cell cortex through a complex with LL5β by interaction with IQGAP1 (Watanabe *et al.,* 2009: Lansbergen *et al*., 2006). Importantly, CLASPs have been shown to play an important part in cell polarization,

through the polarization of cytoplasmic arrays of MTs in migrating cells, towards the leading edge of the cell (Baas & Qiang, 2005).

It was also shown that CLASP2 is negatively regulated by glycogen synthase kinase 3β (GSK3β), which modulates CLASP-microtubule association and lamella MT attachment (Kumar, *et al*., 2009). In recent years, CLASP1 and CLASP2 were shown to interact through the plus-ends with the actin filaments, functioning like crosslinkers in interphase cells (Tsvelov *et al*., 2007). CLASPs also mediate MT nucleation from the Golgi apparatus by interacting with the trans-Golgi network protein GCC185, leading to the asymmetry of the MT array nucleated at the Golgi (Efimov *et al*., 2007). Studies in neurons reported a very active role of CLASPs in this cell population (Jaworski, Hoogenraad, & Akhmanova, 2008). Furthermore, CLASPs display a role in intracellular transport (Baas, Vidya Nadar, & Myers, 2006), neuronal development including migration (Kholmanskikh, *et al*., 2006), formation, growth and guidance of axons (Lee, *et al*., 2004).

CLASPs are also known to play important roles in mitotic cells. The pioneer studies regarding CLASPs role in mitosis were performed in *Drosophila*, where it was proven their essential role. CLASPs depletion by RNAi lead to failure in chromosome segregation and formation of multipolar spindles, as well as monopolar spindles caused by shortening of kinetochore MTs, due to spindle collapse (Lemos *et al*., 2000; Maiato *et al*., 2002; Maiato *et al*., 2005). These results were also supported by CLASP1 inhibition with anti-CLASP1 antibodies, which resulted in the generation of monopolar spindles (Maiato *et al*., 2003). Fluorescence recovery after photobleaching (FRAP) experiments showed that CLASP depletion blocked poleward flux (Maiato *et al*., 2005). In yeast, the CLASP homologue Stu1p was shown to be associated with β-tubulin at the kinetochore level, which allows flux in mature kinetochore microtubules (Yin *et al.,* 2002). Recently, it was shown in U2OS and Hela cells that CLASP1 forms complexes with Kif2b and Astrin at the outer kinetochores. CLASP1-Kif2b localizes at the outer kinetochore during prometaphase, being replaced by CLASP1-Astrin in metaphase (Manning, *et al*., 2010). It was also shown that Astrin stabilizes MTs at the outer kinetochore, while Kif2b destabilizes them in prometaphase, but not in metaphase (Manning, *et al.*, 2010) The mutually exclusive localization of CLASP1-Kif2b and CLAS1-Astrin complexes might be used as a switch to regulate the temporal changes in kMTs stabilization during prometaphase-to-metaphase transition (Manning, *et al*., 2010). Together, these findings suggest that CLASPs are required, not only for the assembly of functional kinetochore-microtubule attachments, but

also to maintain spindle bipolarity through the regulation of MT dynamics at the kinetochore of the mitotic cells (reviewed by Maiato *et al*., 2004).

Double RNAi experiments in Hela cells have shown that depletion 70% of CLASPs is sufficient to cause mitotic defects, such as multipolar and monopolar spindles, among others (Mimori-Kiyosue *et al*., 2005; (A. L. Pereira, et al., 2006). Interestingly, the single depletion of CLASP1, did not cause such mitotic defects, which may suggest a redundancy between CLASPs also during mitosis. However, the literature so far only describes cellular phenotypes and functions regarding both mammalian CLASPs. In order to shed light on the physiological roles of these proteins, as well as to discriminate in more detail their cellular roles, knockout (KO) mouse models for CLASP1 and CLASP2 were made. So far, it was shown that *Clasp2* KO animals are viable, but the develop maturation/differentiation defects, dying early in life (Pereira, 2009). In this thesis we intend to initiate the characterization of a *Clasp1* KO mouse model in order to uncover its role in mammals' physiology.

7. Aneuploidy

The mitotic defects in small proportions of *Clasp2* KO MEFs, are likely to be in the basis for the observed chromosomal instability in these cells (A. L. Pereira, et al., 2006). Despite the mitotic defects observed in these cells, they are viable, but the mitotic fidelity is strongly compromised. Over time, the cumulative effects due to the absence of *Clasp2* KO during embryonic development may contribute to chromosomal instability in adults. Considering the previously suggested partial redundancy between CLASP1 and CLASP2, one possible consequence of CLASP1 depletion in mammals would be the malignant transformation and cancer.

In many cancers, mutations are accompanied by numerical and structural chromosomal instability (CIN). Karyotypic abnormalities have been extensively recognized has one of the main hallmarks of solid tumors. However, it is not clearly established if CIN is a cause or a consequence of oncogenic transformation. CIN is associated with problems during mitotic chromosome segregation, which is highly correlated with a compromised SAC (Kops, Weaver, & Cleveland, 2005). Actually, it would be expected that inactivation of mitotic checkpoint genes would directly lead to miss-segregation events. However, loss-of-function mutations in mitotic genes appear to be

rare in human cancers (Draviam, Xie, & Sorger, 2004; Weaver & Cleveland, 2006). Furthermore, targeted inactivation of mitotic checkpoint genes in mouse has been associated with embryonic lethality, rather than cancer and partial loss of function generally results in very mild tumorigenesis phenotypes. In fact, is more predominant a premature senescence than cancer.

Although mitotic checkpoint robustness is essential to the accuracy of chromosome segregation, sometimes it cannot detect errors, such as merotelic attachments. The efficient transmission of the right number of chromosomes also depends on correct centrosome duplication, establishment of spindle polarity and correct chromosome-MT attachment via kinetochores.

The accurate duplication of centrosomes and its migration to opposite poles is a geometrical requirement for the spindle bipolarity. Depletion or mutation of proteins involved in centrosome biogenesis, leads to the formation of multi-polar mitotic spindles, gross defects in chromatin disjunction and numerical CIN. Moreover, many solid tumors have abnormal centrosome numbers (Duensing & Munger, 2001; Nigg, 2006). Another condition for accurate chromosome segregation is bi-orientation. It means that a pair of sister chromatids bind to MT emanating from opposite poles. Attachment of chomatids to MTs is regulated by kinetochores. It has been reported that components of kinetochores CENPs exhibit abnormal expression in human cancer, suggesting that abnormal kinetochore composition might be related to tumorigenesis (Yuen, Montpetit, & Hieter, 2005).

As it was previously mentioned, CLASP1 localizes also at the outer kinetochore, as well as, at the centrosomes. The role of CLASP1 in kinetochore and centrosomes is not clear yet, but it might be actively preventing mitotic defects. Since mono and multipolar spindles have been observed in *Clasp2* KO MEFs, we could predict that mutations in *Clasps* genes may lead to aneuploidy and, ultimately, to cancer.

8. Mouse models of embryonic development

8.1. Mouse models

Although *in vitro* studies have proven to be an essential tool to the comprehension of the behavior of CLASPs inside the cell, their functions, particularly CLASP1, have

never been accessed in a complex living organism, such as mammals. Mice are, par excellence, the chosen model to reproduce a given perturbation of a living system, either by inducing exogenously a lesion/disease, or by changing its genome such is the case of transgenic and KOs.

On the other hand, it has been widely accepted that *in vitro* and *in vivo* studies are not always coincident and the studies based only cellular models do not always reproduce what is happening in a living organism. On explanation relies on the huge amount of extracellular signaling factors, secreted not only by a single cell type, but also by thousands of different cell types, whose secretion is regulated by the endocrine system. Such amounts of factors that are present in a physiological environment are not possible to be gathered in a cell culture medium. To understand the physiological role of CLASP1 in mammals, a KO model for it was generated (Pereira, 2009).

8.2. *Clasp1* knockout mouse model

A KO mouse is a genetically engineered mouse in which a given gene is replaced or disrupted with an artificial piece of DNA. The loss of gene activity often causes changes in a mouse phenotype, which includes appearance, behavior and other observable physical and biochemical characteristics. The *Clasp1* KO mouse was not generated by the ablation of the gene, but rather by interruption of the gene by insertion of a specific cassette that prevent gene transcription. The targeting protocol and the *Clasp1* KO generation of this animal model is beyond the scope of this thesis.

9. Mouse development

Mouse embryonic development is highly similar to humans. For this reason, many researchers have been studying the embryology of the mouse in order to understand what happens throughout embryonic and fetal development in humans.

Mouse development comprises five fundamental stages: The first one, termed pre-embryonic development, involves fertilization, in which a male spermatocyte penetrates the cellular membrane of a female oocyte. From nuclear fusion of both gametocytes results the zygote, which undergoes mitosis. Cleavage of the zygote continues until it becomes a morula (a growing mass of undifferentiated cells) after the second day. On the third day,

morula becomes a blastocysts with 16 to 40 cells surrounding a blastocoele (a fluid filled cavity). On day four occurs the implantation of the blastocyst and 24 hours after the blastocysts implantation a placenta forms. The second stage is the elongation period, which starts by day five, the blastocyst elongates and forms an egg cylinder. The placenta that has started to grow begins to provide maternal blood to the embryo. Inside the placenta, the egg starts to differentiate into early embryonic and extra-embryonic regions. By the end of the sixth day, gastrulations, or true differentiation starts (Tam & Rossant, 2002). Gastrulation is the third step of mouse embryonic development. A fold starts to form at the tail end by the seventh day. This structure will turn into the coelemic fold, and later the end of the gastrointestinal canal. Within the next 24 hours forms the foregut, an early precursor of the stomach. At the same time, a very simple delineation of the cardiovascular system appears, which includes branchials, or gill-like structures used to breathe in uterus. Optic pits, which will give raise to the optical system start to form. Organogenesis occurs at day ten to twelve and is characterized by many morphogenical changes that drive the growing embryo to the fetus stage (Tam & Rossant, 2002). At the organogenesis stage, the early brain continues to develop and grow, as well as the optic pits. Hind and forelimb buds and, a short while later, hand plates (the precursors of feet) become visible. Mandibles and maxilla begin to develop. Nasal pits form below the pit. The tail elongates and the foregut extends into a slightly more developed digestive tract. Also, sex determination occurs during this phase (Tam & Rossant, 2002). The last stage of mouse's life in uterus is termed the post-embryonic development. Here, the fetus becomes a fully formed newborn mouse. At this stage, the development of the signs of individual digits occurs on the front and hind limbs. The pinna (cartilaginous portion of the outer ear) becomes discernible and develops at a right angle to the head. The cardiovascular system develops further to include a heart and lungs. The lungs in mammals are not fully functional until after birth, when they are exposed to air for the first time. In the few days prior to birth, skin thickens and whiskers mature (Tam & Rossant, 2002).

9.1. Lung development and physiology

Mammalian lungs consist of a highly branched network containing thousands to millions of airways arrayed in intricate patterns that are essential for respiration. The lungs arise from the foregut endoderm at Embryonic Day 9.5 (E9.5). At this stage, the primary buds consist of an inner, apparently unpatterned, epithelium surrounded by loosely packed

mesenchyme and a thin mesothelial layer (Rawlins, 2008). The lung buds undergo repeated rounds of branching and outgrowth. During the pseudoglandular stage (~E10.5- E14.5), the conductive airways are formed and lined with a mixture of secretory (Clara cells), neuroendocrine (NE) and basal cells (Rawlins, 2008). Blood vessels and lymphatics extend along the tubules (deMello, Sawyer, Galvin, & Reid, 1997) and smooth muscle cells differentiate and coat the bronchial tubules (Mailleux, *et al.*, 2005). Nerves can also be distinguished side to side to the vascular structures, giving rise to the primordial lung nerve system. Cartilage precursor tissue also starts to differentiate along the ventral region of trachea and bronchi. The unpatterned distal buds of the lungs then elongate at the cannalicular stage (~E14.5-E16.5) and ultimately give rise to the terminal sacs containing type I and II epithelial cells (Rawlins, 2008; Whitsett, Wert, & Weaver, 2010). The saccular stage, which occurs between E17.5 to fifth day of postnatal development in mice, is a particular lung morphogenesis stage in which lung switch from a fluid-filled to an air-filled structure, upon which survival depends following birth. This stage is marked by dilatation of peripheral airspaces, resembling sacs, differentiation of the respiratory epithelium, increasing vascularity and surfactant synthesis. Undifferentiated cuboidal cells turn into type I and II epithelial cells. Type II epithelial cells (or type II pneumocytes) contain lamellar bodies, which are intracellular storage units of surfactant (Mercurio & Rhodin, 1976). Some authors have suggested that type II pneumocytes appear earlier than type I. Thus, Type II pneumocytes might give rise to type I pneumocytes (Mercurio & Rhodin, 1976). Type II pneumocytes are crucial for normal lung development and function as they are the producers of surfactant and its secretion is essential for lung function after birth. Type I pneumocytes line the greater part of the alveolar surface and are the responsible for the gas-exchange. While type II pneumocytes are round cells without extensions and contain “lamellar inclusions”, type I partners exhibit long cytoplasmic extensions (Campiche, Gautier, Hernandez, & Reymond, 1963).

Postnataly, the primitive saccules enlarge and are subdivided into smaller units by a process of septation called “alveolization” (Maeda, Dave, & Whitsett, 2007; Warburton, Gauldie, Bellusci, & Shi, 2006). Alveolization occurs in late gestation in humans and postanatally in mice. This greatly increases the surface area, and lung compliance.

The pulmonary surfactant system is one of the latest systems developed in lung maturation. Surfactant is miscellaneous substance composed by phospholipids (80%), neutral lipids (12%), and proteins (8%). The protein part of the surfactant system is made by surfactant protein – A (SP-A), B (SP-B), C (SP-C) and D (SP-D). However, all

surfactant proteins have their specific roles along gestation, The more important surfactant proteins for the life after birth are B and C partners. Surfactant is secreted to the alveolar lumen and coats the intra-alveolar wall decreasing the tension at the air-liquid interface and so facilitating expansion of the alveoli during respiration (Boyden, 1977).

9.1.1. Lung defects

The first, and probably the biggets extrauterine challenge, is the first breath of air. When pups are being born, they become cyanotic at the first couple of minutes. This cyanosis corresponds to an interval between ceasing of the placental oxygen support and the time that breathing takes over. After a few minutes, normal pups recover their pinkish color. However, any abnormality that affects different physiological system might be life-threatening.

Partial or total lung collapse is one of the most common hallmarks of respiratory diseases. However, it may occur in the absence of a primary lung defect, but due to other morfophysiological system defects, such as cardiovascular defects, neuromuscular defects, skeletal defects, among others (Turgeon & Meloche, 2009). Primary lung defects that have associated lung collapse are usually related to insufficient maturation of the lung tissue after delivery. In humans, the most common cause of pulmonary syndrome distress in newborns relies on insufficient synthesis of surfactant or genetic mutations in one surfactant protein B (Nkadi, Merritt, & Pillers, 2009). Type II pneumocytes may not be mature enough by the time that newborns have to breathe by their own, and surfactant synthesis and secretion is not sufficient to allow the proper lung expansion. In what concerns lung maturation, a large amount of gene disruption may lead to lung collapse. *Nfib, Pdn* and *Nds1* knockout mice, albeit make visible efforts to breathe, inflation is not sufficient to provide the proper gas-exchange and these mutants die within a few minutes after birth. *Pdn* deficient mice have abnormal distal saccules with blockade in type I pneumocytes differentiation. However, surfactant is high, which indicates that maturation of type II does occur normally (Ramirez, *et al*., 2003). On the contrary, type II pneumocytes differentiation seems to be compromised in *Nds1* deficient animals, resulting in impaired surfactant production. These mice also develop lung failure within ten hours after delivery and die (Fan, *et al*., 2000). A huge amount of genetic defects, even though not directly related with lung tissue, result in lung collapse. Thus, it becomes clear that respiration is a physiological phenomenon that counts, not only with lungs *per ser*, but also

with other organs/tissues that work together to achieve the proper breathing movements and gas-exchange.

EXPERIMENTAL WORK

CHAPTER I

Characterization of *Clasp1* Knockout Mice

1 . Introduction

Cellular models have brought important insights for the understanding of many particularities of many proteins, such as CLASPs, especially concerning their intracellular localization, which provided several clues about their potential functions.. The most significant insights about the role of CLASPs came from studies in yeast, *Carnorhabditis elegans, Drosophila*, and *Xenopus*, which only have one single CLASP orthologue that is essencial for mitosis and organism viability (Gonczy, *et al*., 2000; Hannak & Heald, 2006; Inoue, *et al.*, 2000; Lemos, *et al.*, 2000; Pasqualone & Huffaker, 1994). *Clasp2* KO mouse embryonic and adult fibroblasts (MEFs and MAFs, respectively) have revealed an increase of chromosome segregation defects, namely monopolar and multipolar spindles and lagging chromosomes (Pereira, *et al.*, 2006). Although these cells are viable, their mitotic fidelity is compromised. Interestingly, partial normal phenotype was recovered upon ectopic expression of both GFP-CLASP1 and GFP-CLASP2. Moreover, the rescue efficiency was higher upon ectopic expression of GFP-CLASP2 (Pereira, *et al*., 2006). Together, these finding support the hypothesis that both CLASP1 and CLASP2 cooperate to ensure mitotic fidelity, being partially redundant.

The outcomes from cellular models do not always corroborate the behavior of proteins within a living organism. The physiological environment is a complex system of molecular signaling, which cannot be reproduced in a petri dish. The first attempt to understand the role of CLASPs *in vivo* involved *Clasp2* KO animals. Although these animals are viable, they frequently dye early within the first six months of life exhibiting maturation/differentiation defects in high-proliferative tissues, such as the hematopoietic and reproductive systems (Pereira, 2009). Particularly, absence of CLASP2 impairs erythropoietic differentiation. *Clasp2* KO mice have shown a huge reduction in number of progenitors in bone marrow, which means that depletion of CLASP2 plays an important role at the early progenitors level.

Our group is taking the first steps to uncover the physiological role of CLASP1 in a mammalian model. As it was aforementioned, we generated a *Clasp1* KO mouse model, which is the ideal tool to understand the physiological impact of the absence of this protein in mammals.

2. Materials and Methods

2.1. Animals

C57Bl/6 background animals were bred in the animal facility of the Institute for Molecular and Cell Biology, according to protocols approved by the Ethics Committee of the mentioned institution. Animal were housed in groups of 2-4 animals per cage, with free access to distillated tap water and fed *ad libitum*. Rooms were acclimatized and room temperature was surrounding 20-24º C; 45-65% humidity; ventilation of 15-20 changes/hour and light/dark cycles of 12/12 hours.

2.2. Breeding and detection of KO mice

Breeding entailed housing one heterozygous male with 2 heterozygous females overnight. This breeding resulted in littermates with WT/HT/KO proportions non-concordant with the expected ratio of 1:2:1. These proportions did not obey rule and also oscillated among litters. Mating was ascertained in the next morning by the presence of a vaginal plug and this time was designated embryonic day 0,5. All offspring were routinely genotyped for *Clasp1* gene using tail tissue. Genotyping was performed by PCR using the following conditions primers:

Gen1: GTCCGCCACTCTCTCCTTTATTGCC
Gen3: CCTCATGTCCTTCCCCCATAACC
#261: CGGCATCAGAGCAGCCGATTG
Annealing temperature: 55ºC

2.3. Delivery and newborn handling

In average, pregnancy of C57/Bl6 takes between 18.5-20 days. Pups that were born by normal delivery were considered postnatals. Some litters were removed from the females by c-section.

2.4. Overall analysis of the newborn pups

Newborn pups were closely monitored during their birth and evaluated for injuries caused by the delivery process. Once detected any cannibalistic intention, pups were carefully separated from the mother. Pups were carefully handled and observed, with special attention paid to the color and texture of the skin, breathing movements, gasping, reflex stimuli, posture and movement of the limbs and weight.

2.5. Water-floating test

This test consisted of releasing the lung samples into the saline solution/water and analyze their floating capacity (whether they were inflated or collapsed, respectively).

2.6. Histological analysis

Adult mice were anesthetized with volatile isoflurane (Merck) and then sacrificed by cervical dislocation Histological samples were taken and fixed in 10% neutral buffered formalin for 24-48h, at room temperature. Specimens were dehydrated in a graded series of ethanol and cleared with Clear-Rite (Merk). Finally, samples were embedded in paraffin wax (Merk). The formalin-fixed and paraffin-embedded (FFPE) samples were sectioned with 3 µm thick and mounted in glass slides coated with 3-aminopropyltriethoxysilane (APES). Sections were deparaffinized in xylene and rehydrated in a graded series of ethanol until water. Representative samples of all organs and tissues were routinely stained for hematoxylin and eosin. Embryos/fetus and postnatals were sacrificed by decapitation. The whole body integrity was preserved by fixation with 10% neutral buffered formalin for 48 hours, at room temperature. The skin was removed in order to ensure the proper fixation. After fixation, transversal, longitudinal and coronal sections of the whole body were made for routine staining in FFPE samples with 3 µm thick.

2.7. Histochemistry and Immunohistochemistry

FFPE tissue section 3µm thick were deparaffinized in xilene and subjected to trichromic staining techniques to highlight the connective tissue. Less porous tissues, such as muscle, were stained by acid fucsin, while the more porous, such as collagen, were stained by aniline blue. Glycogen content were also identified by periodic acid Shiff (PAS), an histochemical technique which consists primarily of oxidizing the free aldehyde groups with periodic acid. Additional FFPE tissue sections 4 µm thick were used to immunohistochemical techniques to specifically identify lung markers of late distal differentiation. Sections were rehydrated in a graded series of ethanols until water. Endogenous peroxidase was blocked by 4% of hydroxide peroxide diluted in methanol for 40 minutes, at room temperature. HIER was the chosen method for antigen retrieval. This method included heating samples in citrate buffer pH 6 around 90-100ºC, for 5 minutes using a microwaves at the maximum power. Unspecific reactions were blocked with 3% BSA plus 10% FBS in PBS for 1 hour, at room temperature. Primary antibodies used were monoclonal Syrian hamster anti-T1α, (1:200, Developmental Studies Hybridoma Bank) and polyclonal rabbit anti-pro surfactant protein C (1:4000, Chemicon). Sections were incubated with the primary antibodies overnight, at 4ºC. To identify the pro surfactant protein C, a peroxidase polymer system detection was used after incubation with the primary antibody. T1α was identified by the use of a biotinilated goat anti-Syrian hamster secondary antibody for 30 minutes, followed by detection with avidin-biotin complex (ABC, Vector). The reaction was developed using 3-3'-Diaminobenzidine (DAB) (Sigma). Qualitative analysis was preceded using an optical microscope (Olympus) and representative fields were photographed.

2.8. Western blot analysis

Whole tissue extracts were prepared by tissue homogenization and run on 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted on nitrocellulose membranes (Protran, Wattman), using a transfer apparatus (Bio-Rad). Blots were blocked in 5% MILK in PBS with 0,05% Tween20.

T1α and pro-surfactant protein C were detected by incubation with monoclonal syrian hamster antibody against T1α (1:500, Developmental Studies Hybridoma Bank) and polyclonal rabbit pro-surfactant protein C (1:1000, Chemicon), respectively. Secondary goat antibodies against Syrian hamster-biotinilated and rabbit-HRP were used. To detect

T1α, one additional step entailed the use of avidin-biotin complex (ABC, Vector). The amount of protein was detected by conjugation with ECL (Amersham).

3. Results

3.1. *Clasp1* KO mice are not viable

Upon careful monitoring of the delivery process, it was observed that the majority of pups are born with slight cyanosis, which corresponds to the period of time between the arrest of placental oxygenation and the moment when respiration takes over. Once pups started to breathe by their own, they acquired their characteristic pinkish color. However, some pups did not recover from that cyanosis and dyed within a few minutes (less than 30 min) after periods of gasping respiration (fig. 11). Beside cyanotic, these mice had also less frequent respiratory-like movements, mainly happening upon manual stimulation. Additionally, body size and weight comparison showed that these mice smaller and lighter (approximately 20% less) than the other healthy littermates (fig. 12). After genotyping, it was confirmed that this phenotype was exclusive of the *Clasp1* KOs, while their WT and heterozygous (HT) littermates were perfectly healthy, displaying a very characteristic pinkish skin color and rhythmic breathing movements.

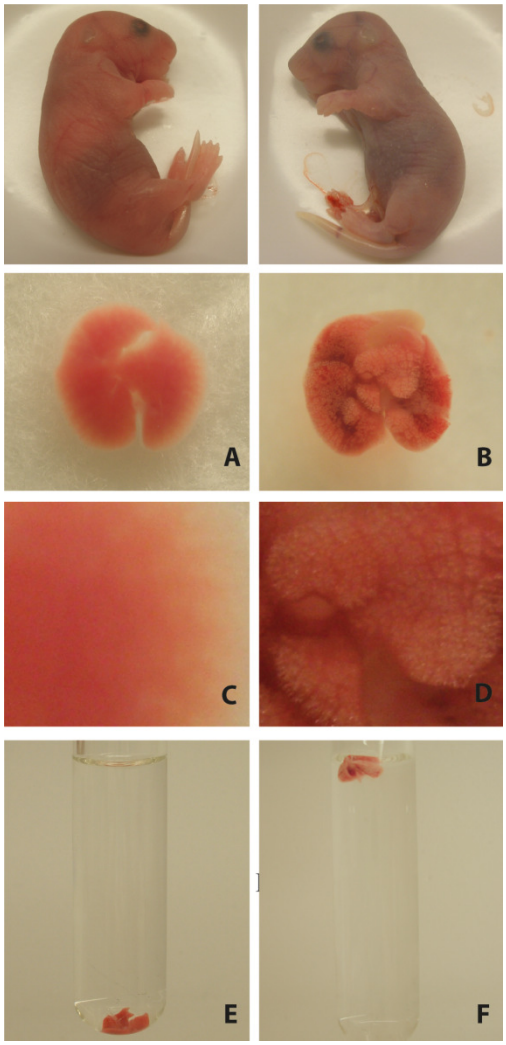

Fig. 11 – Phenotypic characterization of *Clasp1* KO mice. (A and B) Photographs of newborn mice with the respective lung macroscopic features and water-floating test result (bottom). Note the bluish color (cyanosis) of knockout (KO) pups (B) comparing to the pinkish color of the wild-type (WT) littermates (A). The foamy aspect of the WT lungs is missing in KO lungs, indicating the absence of air inside the alveoli. At the bottom, the water-floating test confirm the lung collapse of KO animals, whose

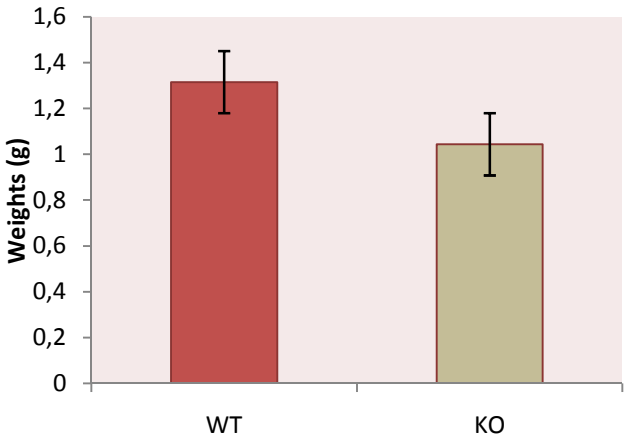

Fig. 12 – Weight analysis of *Clasp1* knockout mice. This graphic depicts the differences in weights between wild-type and knockout mice for *Claps1*. By the time of their birth, *Clasp1* knockout mice have a reduction in their weight of approximaly 20%, comparing to their littermates.

Table I – Weight measurements by genotype, of postnatal (E19.5) mice. On average, *Clasp1* knockout mice have a decrease of, approximately, 20% of their weight, comparing to the wild-type littermates

Number of animals	Postnatal (E19.5)	
	Wild-type	Knockout
1	1,69	1,104
2	1,167	0,906
3	1,164	1,083
4	1,238	1,042
5		1,083
Mean	**1,31475**	**1,0436**

Cyanosis, together with the lack of frequent and rhythmic breathing movements, is suggestive of lung failure. Further macroscopic analysis of the lungs revealed a less "foamy" structure of the KO lungs due to the absence of air, a finding confirmed by the water-floating test, in which the KO lungs immediately sunk to the bottom of the recipient (fig. 11). Histological analysis of the lung of both *Clasp1* KO and WT controls revealed a lung collapse, a frequent consequence of pulmonary syndrome distress in newborns (fig 13).

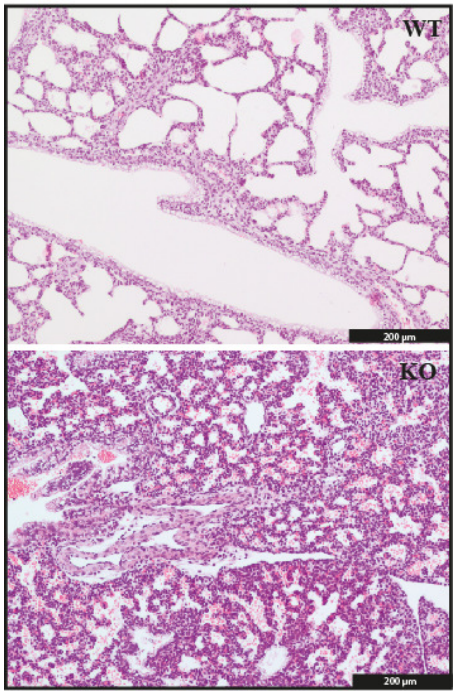

Fig. 13 – Histopathological analysis of postnatal *Clasp1* knockout mice. Hematoxylin and eosin staining of FFPE lung samples from wild-type and *Clasp1* knockout postnatals, showing a clear lung collapse in KO lungs, charaterized by alveolar spaces not inflated with air.

3.1. Clasp1 KO newborns have a delay in lung morphogenesis

Upon histological analysis, it was observed that KO lungs apparently have normal structure and architecture when compared with the WT littermates. Nevertheless, after the E15.5, a slight delay started to be noticed (fig 14; C,D). This phenotype was persistent until birth, upon which all KO mice did not survive and display lung failure (fig. 14). Despite this delay, all basic histological structures were present at the upper and lower respiratory tree. At E14.5 it may be observed a lung in pseudoglandular stage, which is characteristically associated to a glandular-like structure embedded in a mesenchymal bed. At this stage, lungs somewhat histologically resemble exocrine glands. No significant differences were noticed between KO and controls (fig. 14; A, B). By the E15.5, lungs started to display a different structural rearrangement typically characterized by cannalicular morphology (fig. 11; C,D). The lumina of the bronchi and terminal bronchioles become larger, and the lung tissue becomes highly vascularized. At the end of the cannalicular stage (E17.5) some thin-walled terminal sacs (primordial alveoli) have developed at the ends of the respiratory bronchioles and the lung tissue is well vascularized. Until now, we have never seen any mouse with normal embryonic development, to survive *ex uterus* at this stage. This result was not unexpected considering that its respiratory, as well as other systems are still relatively immature. Interestingly, upon E15.5, a slight delay began to be perceptible that became even more evident towards the end of this period (until E17.5) (fig. 14; C, D, E, F, G, H). The saccular stage begins at the E18.5 and extends until a few days after mouse's birth. During this period, many more terminal sacs or saccules develop and their epithelium becomes very thin (fig. 14; I, J, K, L). Capillaries begin to bulge into these sacs (developing alveoli). The intimate contact between epithelial and endothelial cells establishes the blood-air barrier, which permits adequate gas exchange for survival of the fetus if it is born prematurely. By the end of this stage, which occurs after birth in mice, the terminal sacs are lined mainly by squamous epithelial cells of endodermal origin-type I pneumocytes across which gas exchange occurs (analyzed in more detail below). In *Clasp1* KO mice, it was also possible to observe this morphological evolution. However, a lung collapse specimen must be carefully analyzed because the histological architecture of a collapsed lung may be a potential histopathological pitfall. The capillary network proliferates rapidly in the mesenchyme around the developing alveoli. Scattered among the squamous epithelial cells are rounded secretory epithelial cells-type II pneumocytes, which secrete pulmonary surfactant (fig. 14;

I, J, K and L and fig. 17). Interestingly, the sooner it was possible to observe WT mice to survive and breathe by their own was at E18.5 (data not shown).

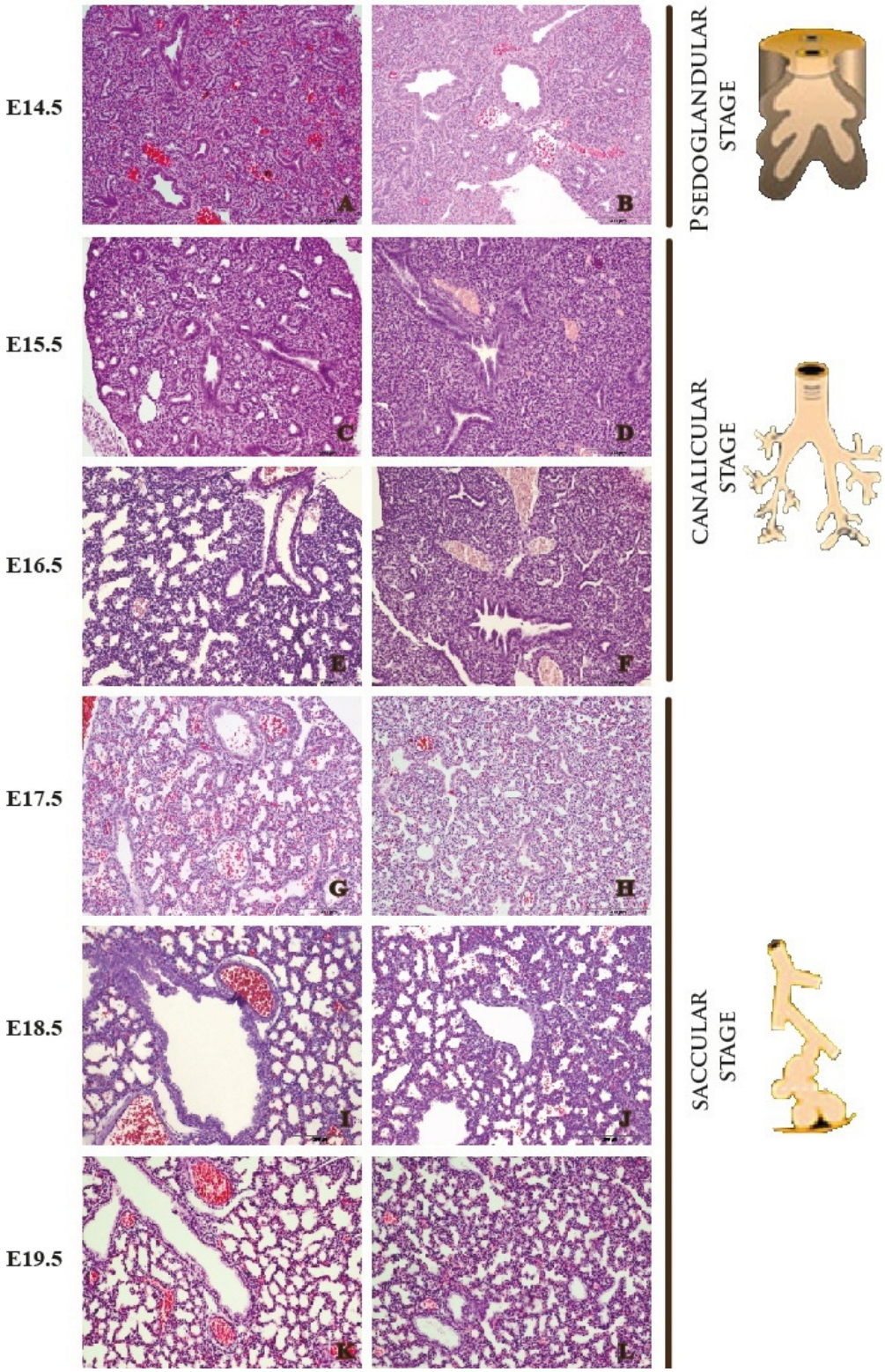

Fig. 14 – Histological analysis of Clasp1 KO lungs. Hematoxylin and eosin staining of FFPE lung samples of WT (A, C, E, G, I and K) and KO (B, D, F, H, J and L) animals from E14.5 to E19.5 without lung inflation. The right schemes depict the lung structure characteristic of each stage of lung development.

3.2. Respiratory muscles and connective tissue defects are absent in *Clasp1* KO animals

Taking advantage of the biochemistry of each tissue, it is possible to highlight different histological structures. Masson trichrome is a histochemical staining frequently used to highlight connective tissue. With this staining, it was possible to rule out defects on connective tissues (data not shown). This staining also favors the muscle histological analysis because muscle fibers stain in red, contrasting to the blue staining of the surrounding connective tissue. According to our results, no evident differences between both WT and KO diaphragm were noticed (fig 15).

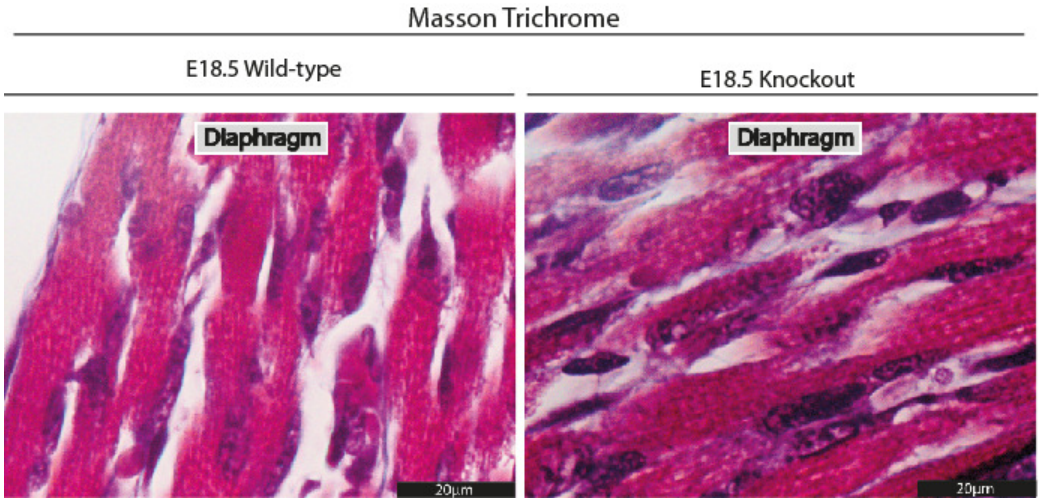

Fig. 15 – Histological analysis of diaphragm of Clasp1 knockout mice and wild-type littermates. Masson trichrome staining of FFPE dyaphragm samples of wild-type (left) and knockout (right) E18.5 animals.

3.3. Glycogen accumulation are higher in *Clasp 1* knockout lungs

To further investigate the cause of lung failure/collapse of the *Clasp1* KO newborns we histochemically assessed the glycogen content in the pulmonary parenchyma (not fully maturated alveolar epithelium) of E18.5 pups. In lungs, mucins (PAS positive) are secreted by the glandular cells lining the bronchiole walls, while the developing alveolar parenchyma of embryo lungs stores glycogen. Higher amounts of PAS positive substances were observed in KO pulmonary parenchyma, which might have a physiological impact (fig.16).

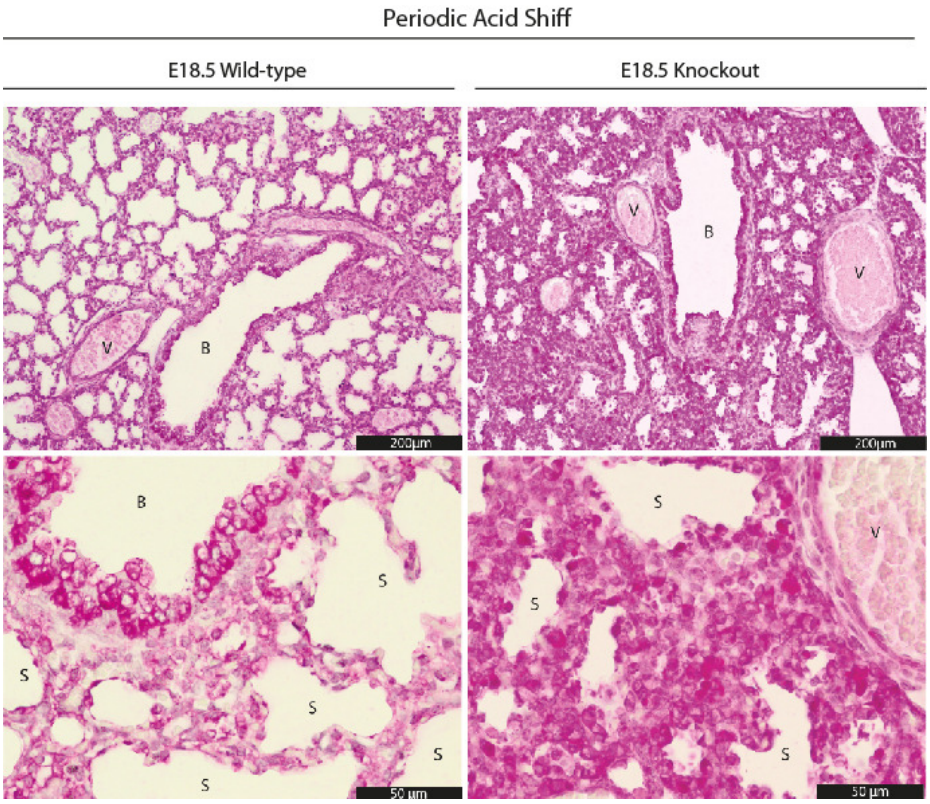

Fig. 16 – Histological analysis of lungs of Clasp1 KO mice and WT littermates. Periodic acid Shiff staining of FFPE lung samples of WT (left) and KO (right) postnatals. The lower panel corresponds to a higher magnification of lungs, showing the pulmonary parenchyme in more detail. B – bronchiole; S – saccule; V – vessel.

3.4. Alveolar epithelial cells responsible for the gas-exchange are differentiated in *Clasp1* knockout lungs at birth

Next, we assessed the level of distal maturation by staining lung samples with type I and II pneumocytes differentiation markers, T1α and pro surfactant protein C, respectively. In pulmonary cell population, only type I pneumocytes express T1α, while pro surfactant protein C is exclusive to the type II pneumocytes. According to the immunostaining results, both alveolar epithelial cells are differentiated in the moment of the KO mice birth. Both in KO mice and controls, type I pneumocytes are differentiated and lining the alveolar sacs, while type II pneumocytes, a smaller cell population, are scattered all over the alveolar parenchyma (fig. 17). Western blot analysis has shown that both markers are already expressed in lungs at E16.5 and are persistently expressed since then (fig. 18). Expression profile of both markers did not vary significantly between WT and KO animals. Regarding these findings, it is reasonable to assume that KO lungs have the proper alveolar epithelium content to take over respiration upon the discontinuation of the placental oxygen supply.

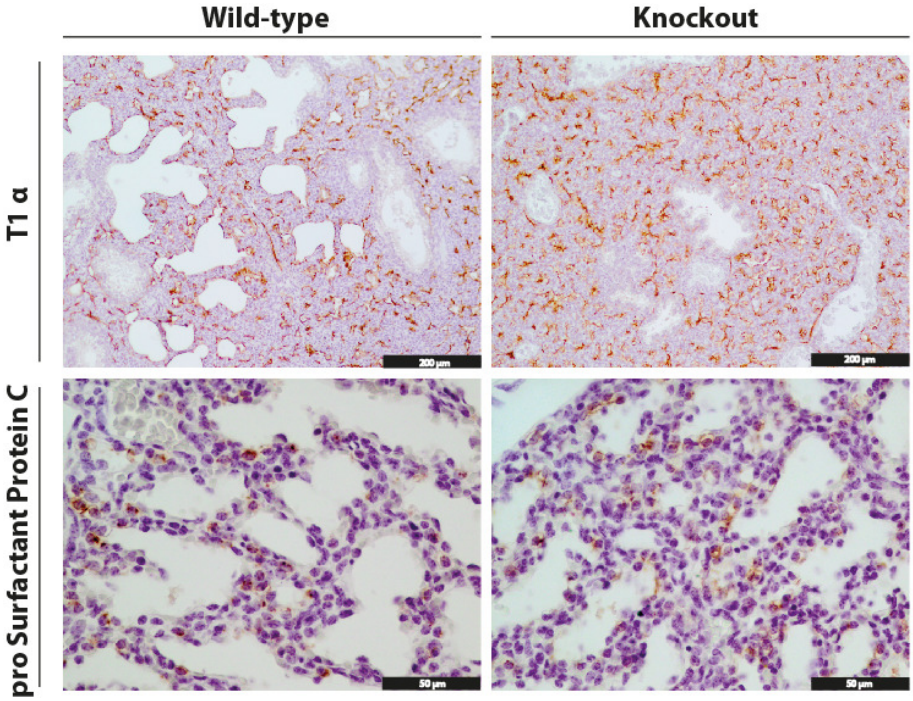

Fig. 17 – Tissue expression profile of T1α and pro surfactant protein C. Immunohistochemical staining of FFPE lung samples of Wild-type (left) and Knockout (right) postnatals. T1α expression is specific of differentiated type I pneumocytes, lining the alveolar lumina. Pro surfactant protein C is expressed in type II pneumocytes, a smaller population of cells that secrete and produce

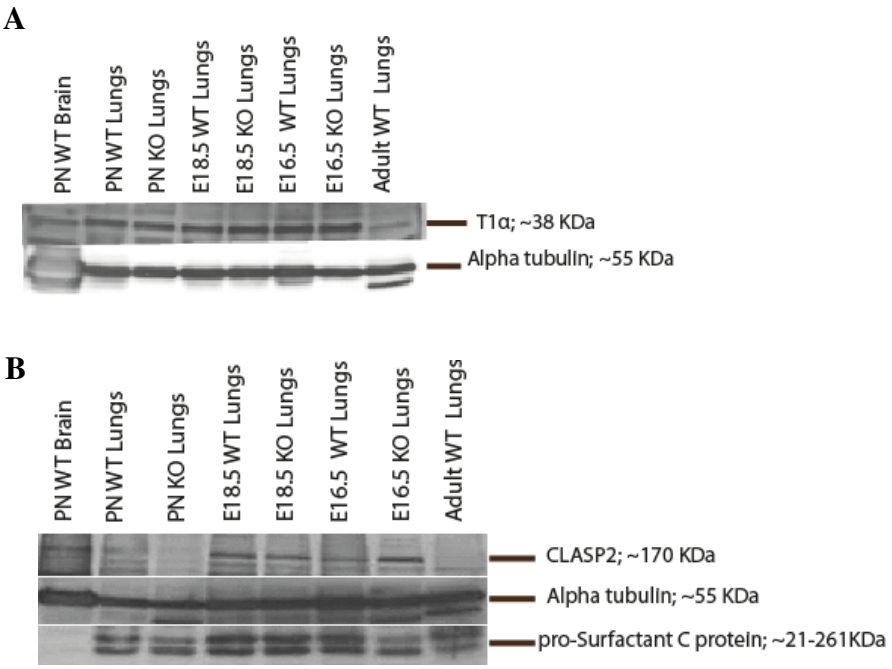

Fig. 18 – T1α, pro surfactant protein C and CLASP2 expression profile at different embryonic stages. Western blot analysis on extracts from lung tissue of wild-type (WT) and knockout (KO) mice. (A) and (B) show the molecular expression profile of T1α (A) and CLASP2 and pro surfactant protein C (B). Both in (A) and (B), α-tubulin was used as a loading control.

4. Discussion

4.1. CLASP1 is required for life after birth

The initial characterization of *Clasp1* KO mice involved the establishment of the death time point. The careful monitoring of the delivery process allowed us to observe that the animals are alive until birth, but they cannot survive longer than a few minutes. Cannibalistic behavior of mothers in the presence of defects of their offspring is not unusual, leading to the absence of *Clasp1* KO newborns, which is why it was initially suspected that the fetuses were dying in an early embryonic stage. The cyanosis of the mice and absence of normal breathing movements were clear manifestations of a lung failure. The absence of a "foamy" aspect of the KO lungs, together with the results from the water-floating test, in which the KO lungs immediately sunk to the bottom of the recipient upon being released into the saline solution, we confirmed that a lung collapse was happening in *Clasp1* KO mice. Although the water-floating test is a less accurate technique, it allowed us to confirm our first suspect. Histological evaluation of the lungs revealed an unquestionable lung collapse, which, histologically, corresponds to a lung whose alveolar spaces are present, but not inflated with air, confirm the results from the histological analysis. Together with the absent breathing movements, it is reasonable to assume that those lungs have never been inflated with air.

An interesting aspect of the *Clasp1* KO mice was the remarkable constant phenotype. All *Clasp1* KO mice suffered from lung failure, a condition that was never seen in the WT or HT littermates. At this point, it is possible to state that CLASP1 plays an essential role during development, whose absence impairs life after birth, with a remarkable 100% penetrance.

4.1. *Clasp1* KO lungs display normal lung cellular structure required for respiration

Breathing is a physiological function which is not confined only to the lungs. The movement of air into the lungs and out them is the final result of a perfect synchrony of many anatomical structures and physiological pathways. Because of that, lung failure is a frequent secondary manifestation of defects in other systems, namely neuronal, cardiovascular, renal, muscular and skeletal (Turgeon & Meloche, 2009).

The respiratory muscles are extremely important for ventilation movements (Turgeon & Meloche, 2009). So, defects in diaphragm, the master muscle of respiration, could be a reason of lung failure, as well as defects in connective tissue. So, by staining postnatal lung samples with Masson trichrome, it was possible to rule out structural muscle and connective tissue defects in *Clasp1* KO mice. Nevertheless, we must note that more quantitative measurements must be performed to confirm and validate this result.

Shortly before birth, alveolar type II cells must be able to efficiently secrete surfactant. This material is a complex mixture of lipids and proteins essential to reduce the surface tension at the air/liquid interface. The lack of sufficient amounts of surfactant is one of the main causes of pulmonary syndrome distress in premature newborns. The most abundant lipid specie in surfactant is phosphatidylcholine (PC) with dipalmitoyl-PC being the surface-active reagent. Undifferentiated pulmonary epithelium has large glycogen stores, which are depleted as the surfactant starts do be synthesized (Brehier & Rooney, 1981; Goerke, 1998). Moreover, these glycogen accumulations are absent in fully differentiated type II cells (Ridsdale, Tseu, Wang, & Post, 2001). Biochemically, temporal relationships between glycogen and phospholipids have suggested that glycogen serves as carbon source for surfactant lipids. However, this relationship has not yet been proved. Glycogen content was assessed by PAS. Although it is not specific for glycogen it is a very cheap and was used as a screening technique to detect differences in glycogen storage between KOs and WT controls. The first perception upon PAS staining was that KO had higher amounts of glycongen/mucins in the pulmonary parenchyma. However, the lower specificity of the technique and the higher number of cells per area due to the lung collapse must be carefully analyzed. Considering that glycogen may serve as surfactant lipid backbone during the lung development, a higher accumulation of glycogen in KO lungs may stand for a delay in lung development, confirming the outcome obtained by previous histological evaluation. One alternative explanation for higher amounts of glycogen in KO lungs may be associated with a blockage on the surfactant lipid synthesis from the glycogen stores.

Having this first clue for type II penumocytes immaturity, we immunostained lung samples for pro surfactant protein C, to rule out surfactant secretion deficiency. Both KO and control lung samples exhibit the same lung expression pattern, which means that type II pneumocytes are differentiated in postnatals. T1α expression profile was also assessed to exclude defects in type I pneumocytes maturation and, once again, no significant differences were found between *Clasp1* KOs and controls. The molecular expression

profile of both markers was also evaluated by Western blotting and the outcome was concordant with the immunohistochemistry results. Although the histological analyzes seem to indicate a morphological delay in *Clasp1* KO lung development, the alveolar epithelial cells, intervenient in the gas-exchange, are differentiated. However, it is not clear whether this slight delay impairs type I and II pneumocytes function. Moreover, the effect of CLASP1 depletion in alveolar epithelial cells has never been assessed. So, it is not known in which extent CLASP1 absence may deregulate their biological behaviour.

CHAPTER II

CLASP1 Expression Profile Throughout Development

1. Introduction

Is has been shown that CLASP homologues are not only important, but essential for yeast, *Drosophila* and *C. elegans* viability. However, *Clasp2* KO mice are viable, which suggests that CLASP1 may partially cover CLASP2 absence. CLASP1 expression profile has already been assessed in wild-type adult mice and it has been shown that CLASP1 is ubiquously expressed in adult mice, while CLASP2 is mostly expressed in brain (A. L. F. Pereira, 2009). Regarding the lethal phenotype observed in our *Clasp1* KO mice model, we evaluated CLASP1 expression profile throughout embryonic development in order to find any relationship between the expression profile and the newborns' phenotype.

2. Materials and Methods

2.1. Western blot analysis

Whole tissue extracts were prepared by tissue homogenization and run on 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted on nitrocellulose membranes (Protran, Wattman), using a transfer apparatus (Bio-Rad). Blots were blocked in 5% MILK in PBS with 0,05% Tween20.

T1α and pro-surfactant protein C were detected by incubation with monoclonal syrian hamster antibody against T1α (1:200, Developmental Studies Hybridoma Bank) and polyclonal rabbit pro-surfactant protein C (1:4000, Chemicon), respectively. Secondary goat antibodies against Syrian hamster-biotinilated and rabbit-HRP were used. To detect T1α, one additional step entailed the use of avidin-biotin complex (ABC, Vector). The amount of protein was detected by conjugation with ECL (Amersham).

3. Results

3.1. CLASPs Expression throughout mouse development

To describe the normal CLASP1 expression profile in murines, we performed Western blotting using a specific rat monoclonal antibody against the c-terminus of CLASP1 (1A6). A band of ~170 KDa, corresponding to the α-CLASP1 (Akhmanova, et al., 2001), was detected in various tissues of WT mice (fig 19). The most striking observation considering α-CLASP1 expression is its variable expression throughout the late stages of embryonic development, except in brain, where CLASP1 expression is higher throughout embryonic development and in adulthood. As it is shown in figure 11, CLASP1 is clearly expressed in several organs, including brain, lungs, heart and kidneys at E16.5 and E18.5. Nevertheless, half a day later, only brain and heart were clearly expressing CLASP1. CLASP2 expression was also evaluated at E19, being only detected in brain. When pups are ready to be born, at the E19.5, only brain remains expressing CLASP1 and CLASP2, while their expression in other organs is shut down.

Interestingly, CLASP1 expressed again in adulthood, namely in lungs, heart, liver, diaphragm, spleen, kidneys and ovaries (fig. 19). As it was previously reported, a second, lower molecular weight band (<150 KDa) was observed in several organs from adult WT mice (fig. 19) (A. L. F. Pereira, 2009). Among the different embryonic stages, consistently in heart, a lower weight band was detected. Taking into account that the lower band was detect using a specific monoclonal antibody against the C-terminus of α-CLASP1, it is suggestive that *Clasp1* gene undergoes alternative splicing, resulting in a possible alternative variant of CLASP1, similar to what was described for CLASP2 (Akhmanova, *et al*., 2001). Additionally, the same antibody was also able to recognize a higher weight band in several adult organs, including liver, spleen, kidneys and ovaries. Comparing both CLASP1 and CLASP2 in adult female tissues, CLASP1 seems to be predominant comparing to CLASP2. While CLASP1 is expressed in the majority of the organs, CLASP2 expression is restricted to the brain, lungs and ovaries (among the tissues where CLASPs expression was evaluated).

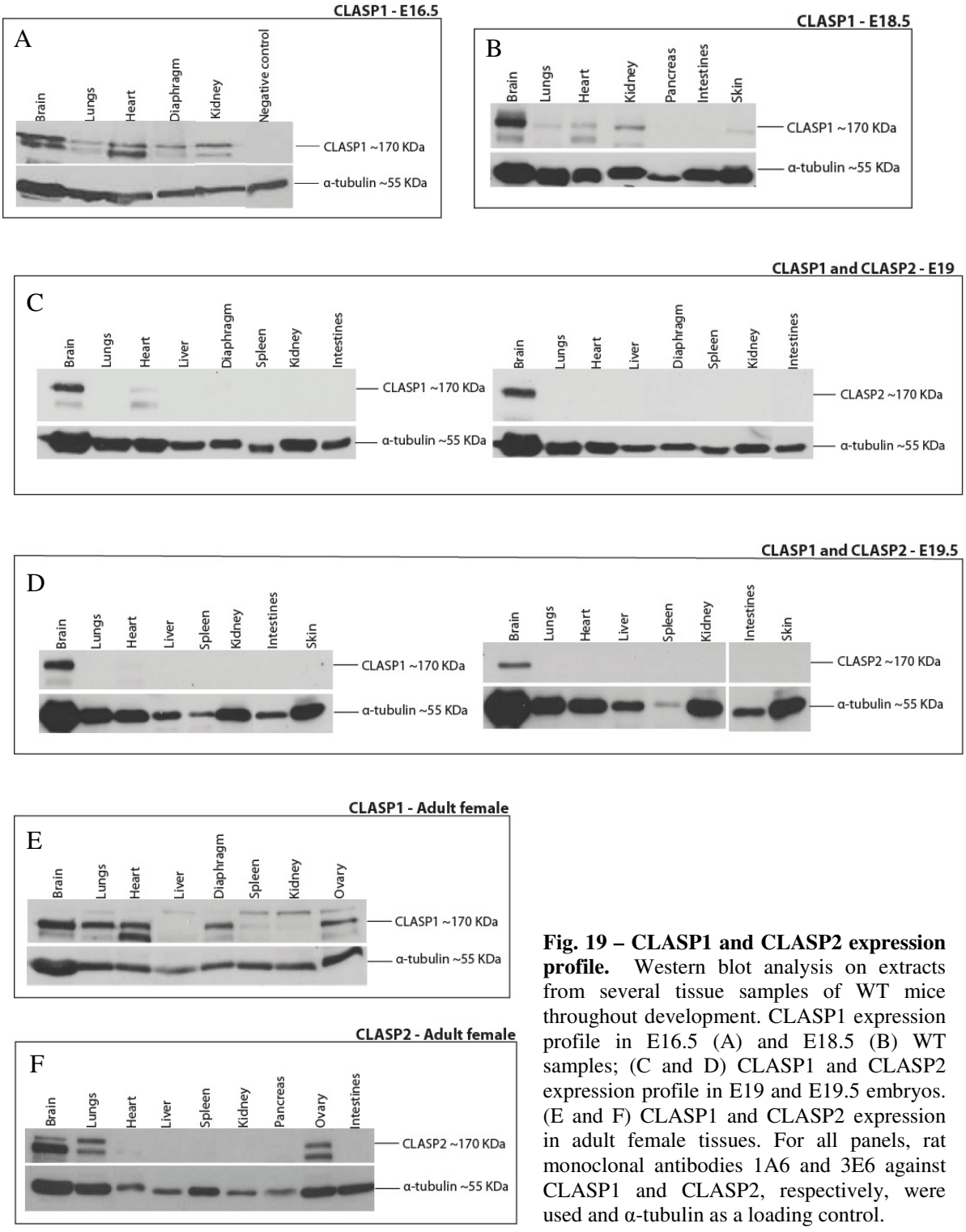

Fig. 19 – CLASP1 and CLASP2 expression profile. Western blot analysis on extracts from several tissue samples of WT mice throughout development. CLASP1 expression profile in E16.5 (A) and E18.5 (B) WT samples; (C and D) CLASP1 and CLASP2 expression profile in E19 and E19.5 embryos. (E and F) CLASP1 and CLASP2 expression in adult female tissues. For all panels, rat monoclonal antibodies 1A6 and 3E6 against CLASP1 and CLASP2, respectively, were used and α-tubulin as a loading control.

4. Discussion

4.1. CLASP1expression is variable throughout development

CLASP1 expression throughout mouse development seems to be transient among different organs namely lungs, heart, diaphragm and kidneys. In the brain, CLASP1 and CLASP2 are strongly expressed from the early embryonic stages to adulthood, being the organ with the higher expression of this protein. +TIPs have been intensively studied in neurons. Neurons are highly differentiated cells specialized in signal transduction, sometimes through long distance and requires a well-organized cytoskeleton. CLASPs, as well as other +TIPs, have been reported to regulate cellular polarity (Baas, Karabay, & Qiang, 2005; Baas, *et al*., 2006) and intracellular transport in which MTs act as railways for motor-based transport. They have also been shown to be important during several stages of neuronal development including migration (Kholmanskikh, *et al*., 2006), formation, growth and guidance of axons (Lee, *et al*., 2004). The chemical signaling requires proper communication between mature neuronal cells, which is also dependent on +TIPs, due to their role in positioning of organelles, receptors and channels (Gu, *et al*., 2006). The strong expression of CLASPs in brain may explain the wide range of roles that both proteins might play in neurons. However, a low expression is not meaningless. For instance, in *Clasp2* KO mice, hematopoiesis is affected, regardless of a low CLASP2 expression in bone marrow extracts.

CHAPTER III

Optimization of CLASP1 Immunostaining for Histological Samples

1. Introduction

The use of antibodies to specifically detect proteins began with Albert H. Coons in 1941, who described a revolutionary technique to visualize tissue components using an antibody labeled with a fluorescent dye (John D. Bancroft, 2008). Since then, stainings based on antigen-antibody interaction (immunostainings) have boosted cellular biology. Presently, it is possible to localize a given protein (or a group of antigens) within a cell, as well as track its translocations and interactions with other molecular partners (John D. Bancroft, 2008). Immunohistochemistry refers to the immunostainings applied to tissue samples. Using mono or polyclonal antibodies, it is possible to detect antigens and assess the morphological detail at the same time.

Polyclonal antibodies have been used to detect the expression of CLASP1. However, the use of polyclonal antibodies may arise drawbacks regarding the specificity of the immunostainings. Polyclonal antibodies are generated by immunizing an animal, whose humoral system activates numerous clones of plasma cells (polyclonal). Each clone will produce specific immunoglobulins, which react with specific epitopes. The smallest variation between immunoglobulins of an anti-serum, may generate cross-reactivity between the protein of interest and similar proteins (John D. Bancroft, 2008). One way to increase the specificity of an immunostaining, is to use monoclonal antibodies, by using hybridoma cell lines (Eichmann, 2004; John D. Bancroft, 2008). This method involves the fusion of plasma cells with an immortalized myeloma cell line. This hybrid cell line only produces on type of immunoglobulin. The result is a constant, more specific and reliable source of pure antibodies, with higher specificity. Monoclonal antibodies against CLASP1 and CLASP2 were generated and well characterized previously (Maffini, *et al*., 2009; Pereira, 2009). These antibodies have shown to be highly specific for immunofluorescence techniques in fixed cultured cells and western blot. However, the staining of CLASPs in FFPE histological samples has not been successfully achieved. The characterization of the recently generated *Clasp1 and Clasp2* KO mice strains have lead us to optimize these

technique for histological samples, since it is mandatory to correlate the morphological features observed in the mice models with expression of CLASPs in different organs and tissues.

Performing immunostainings in FFPE samples is less straightforward and requires a few additional steps comparing to the same technique applied to cell models. Tissue preservation requires longer fixation, dehydration, clearing and wax embedding in order to obtain a well preserved architecture of the tissues, as well as very thin sections. However, tissue processing may change the conformation of the epitopes, which may block the antibodies' binding to the respective epitopes. Formalin fixation has been extensively implicated in this masking of epitopes. It has been described that formalin creates intermolecular and methylene bridges and weak Schiff bases forms intramolecular cross-linkadges along proteins, which may prevent the antibody-antigen specific recognition (John D. Bancroft, 2008). In order to bypass this problem, antigen retrieval steps have been developed and added to the immunohistochemistry protocols. A few antigen retrieval methods have increased the quality of the immunohistochemistry over the last decades, which have been essential, not only for investigation, prognosis and treatment of diseases. Antigen retrieval allows the disruption of the weaker Schiff bases created along the processing steps, preserving tissue architecture. The most widely used antigen retrieval method in routine histopathology and investigation is the heat induced epitopes'e retrieval (HIER), which consists of incubating histological samples in specific buffered solutions at high temperature. Although other methods have been used, namely enzymatic treatment and treatment with acid solutions, HIER is the most standardized, quick, clean and associated with less background. Still, sometimes antigen retrieval methods are worthless and it is impossible to recover the antigen epitopes. In these cases, different methods of tissues' preservation must be chosen. The alternative is to cryopreserve tissues and avoid paraffin processing. In fact, frozen sections are the gold standard when evaluating and assessing new antibodies. When evaluating an immunostaining result in FFPE samples it can be compared with the results achieved in frozen sections. Even though frozen sections remain an important histological tool, they contain inherent disadvantages, including poor morphological detail, limited retrospective studies and storage of material (John D. Bancroft, 2008). In this work it was only possible to obtain a few outcomes related to CLASP1 detection in mice tissue samples. The monoclonal antibody against CLASP1 (named 1A6) available for this study is incompatible with the paraffin processing, but it was possible to specifically detect CLASP1 in frozen sections. Although this technique

requires further improvements, it was possible, for the first time, to detect CLASP1 in histological samples.

2. Materials and Methods

2.1. Frozen Sections Preparation

Adult mice were anesthetized with volatile isoflurane (Merck) and then sacrificed by cervical dislocation. Brain samples were taken and fixed in 10% neutral buffered formalin for 24-48h, at room temperature. Specimens were embedded in a 50% (m/v) sucrose solution until they sunk to the bottom of the recipient. The same procedure was performed using a 70% (m/v) sucrose solution. Samples were embedded in a gel-like medium consisting of water-soluble glycols and resins. An optimum cutting temperature (OCT) compound (Sakura) was used to provide a convenient specimen matrix for cryostat sectioning at temperatures of aproximately -25º C. Samples were cryosectioned with 10 µm thick and mounted in glass slides coated with APES (SIGMA).

2.2. Immunostaining of CLASP1 detection in cryopreserved tissues

Cryosections of 10 µm thick were melted at room temperature and washed in PBS 0.05% Triton X-100 (). Endogenous peroxidase was blocked by 4% of hydroxide peroxide diluted in methanol for 40 minutes, at room temperature. Unspecific reactions were blocked with 3% BSA plus 10% FBS in PBS for 1 hour, at room temperature. The primary antibody used was the 1A6 rat monoclonal anti-mouse CLASP1, with 1:5 dilution in 3% BSA plus 10% FBS. CLASP1 was identified by the use of a Biotinylated Rabbit Anti-Rat IgG (1:50, SIGMA) for 30 minutes, followed by detection with ExtrAvidin Peroxidase (1:100, SIGMA). The reaction was developed using DAB (SIGMA). Qualitative analysis was preceded using a NIKON (ver ataf) and representative fields were captured.

2.3. Immunostaining of CLASP1 detection in paraffin sections

FFPE tissue sections 4 µm thick were used to evaluate CLASP1 expression in wild type brain tissue. Sections were rehydrated in a graded series of ethanols until water. Endogenous peroxidase was blocked by 4% of hydroxide peroxide diluted in methanol for 40 minutes, at room temperature. Two antigen retrieval methods were tested in FFPE tissue samples: HIER and treatment with an acid solution. HIER protocol included heating samples in citrate buffer pH 6 around 90-100ºC, for 5 minutes using a microwaves at the maximum power. The acid treatment protocol was performed be incubating samples in a 10% HCL solution for 30 minutes, at room temperature, followed by neutralization with 1M Bórax, for 5 minutes. Unspecific reactions were blocked with 3% BSA plus 10% FBS in PBS for 1 hour, at room temperature. The following steps were the same as it was previously described for the immunostaining of CLASP1 in frozen sections. (inverter a ordem de 2.2 e 2.3)

3. Results

3.1. CLASP1 immunostaining using 1A6 monoclonal antibody against CLASP1

After the first approach using FFPE tissue samples, in which different antigen retrieval methods were used, as well as broad range of antibody's dilutions, one possible assumption is that, either formalin fixation, or paraffin processing, damage the antigen epitopes in such an extensive way that is impossible to recover them. In FFPE samples it was never possible to detect CLASP1 expression in positive controls (brain samples). However, a satisfactory outcome was achieved in cryosections without requiring additional steps for antigen retrieval. Detection of CLASP1 expression in a few very specific brain cell populations supports the specificity of the antibody. Moreover, even after such a prolonged formalin fixation (24-48 hours), frozen section did not require antigen retrieval to satisfactorily stain for CLASP1. Hence, one possible reason for the negative results in FFPE tissue samples is the irreversible destruction of the antigenic determinants during the paraffin processing.

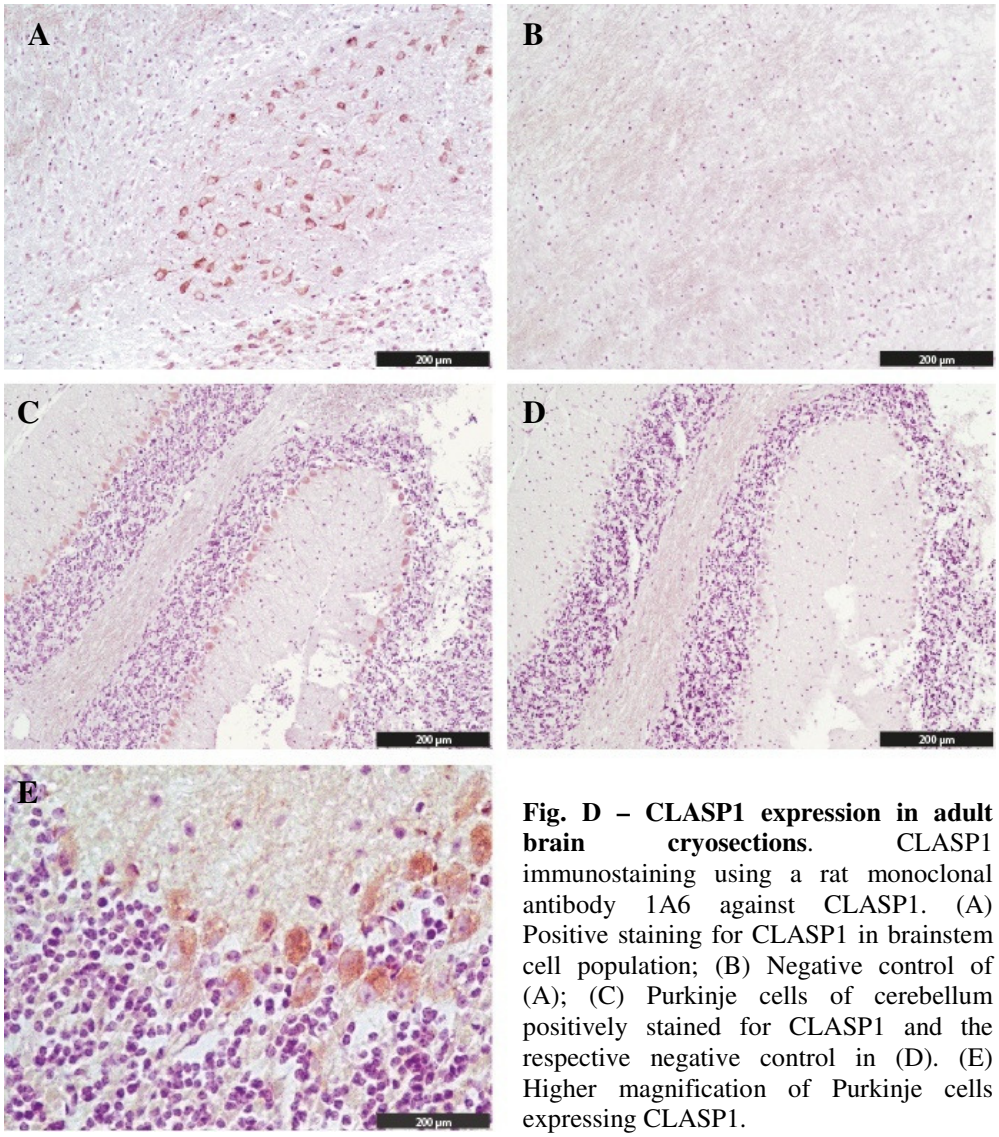

Fig. D – CLASP1 expression in adult brain cryosections. CLASP1 immunostaining using a rat monoclonal antibody 1A6 against CLASP1. (A) Positive staining for CLASP1 in brainstem cell population; (B) Negative control of (A); (C) Purkinje cells of cerebellum positively stained for CLASP1 and the respective negative control in (D). (E) Higher magnification of Purkinje cells expressing CLASP1.

4. Discussion

4.1. 1A6 rat monoclonal antibody against CLASP1 is incompatible with paraffin processed tissues

After several attempts to detect CLASP1 expression in tissues using the monoclonal antibody 1A6, a satisfactory outcome was achieved in cryopreserved tissues. Formalin is known to mask antigen epitopes, blocking the antigen-antibody binding. Although these samples have been previously fixed with 10% buffered formalin, they did not require any antigen retrieval procedure to satisfactorily stain for CLASP1. So, one possible reason for the negative results in FFPE tissue samples is the irreversible destruction of the antigenic determinants during the paraffin processing.

4.2. CLASP1 expression in adult brain

Regarding CLASP1 expression profile in adult brain, it is specifically expressed in a few populations of the brainstem and Purkinje cells. Its negative staining in other areas of the brain confirms the specificity of the antibody. Interestingly, the neuronal command of breathing movements localizes in the brainstem in a few specific populations termed respiratory centers. It is possible, since CLASP1 is strongly expressed in brainstem, that its absence might impair the neuronal functions that control respiration. This hypothetic situation is compatible to the absence of air in the KO lungs because, in a situation of absence of neuronal stimuli, respiratory muscles do not contract or relax properly to let the air enter into the lungs.

GENERAL DISCUSSION

General Discussion

CLASPs are highly conserved proteins among species, which have been described to play a role in microtubule dynamics, both in interphase and mitosis. During the last few years, CLASPs orthologues have been studied in a few organisms (Grallert, *et al*., 2006; Hannak & Heald, 2006; Inoue, *et al*., 2000; Lemos, *et al*., 2000; Pasqualone & Huffaker, 1994), including mammals (Akhmanova, *et al*., 2001; Inoue, *et al*., 2000; Lemos, *et al*., 2000). However, while in the first three there is one single CLASP orthologue, in mammals two paralogues have been identified (Akhmanova, *et al*., 2001; Inoue, *et al*., 2000; Lemos, *et al*., 2000). Depletion of CLASP in organisms with a single orthologue is incompatible with life. However, it has been shown that *Clasp2* KO mice are viable (Drabek, *et al*., 2006; Pereira, 2009). One possible explanation for the survival of mammals with only CLASP1 expression might be related to the separation of the functions of one single protein by two paralogues and previous work in cellular models has already pointed toward a partial redundancy between both CLASPs (Pereira, *et al*., 2006). However, their specific roles, both in cellular and physiological level, require further investigation. So far, *Clasp2* KO mice are viable, but develop what seems to be maturation-related dysfunctions, mainly in the hematopoietic system. These mice display bone marrow aplasia with severe anemia and dye early in life (six months on average) when compared with WT littermates. Absence of CLASP2 seems to impair the proper erythroblast differentiation, which means that depletion of CLASP2 plays an important role at the early progenitors level. CLASP2 depletion was also described to cause maturation problems in the reproductive system (*Clasp2* KO are infertile) (Drabek, *et al*., 2006; Pereira, *et al*., 2006; Pereira, 2009). Interestingly, CLASP2 depletion affects mostly tissues with high cellular turnover, which might also be related to the role of CLASPs in mitosis. Thus, CLASP2 is suggested to play a role in cellular differentiation and tissue maturation in mammals. However, contrary to what is observed with *Clasp2* KO, our *Clasp1* KO model is not viable after birth. To uncover CLASP1 specific functions at the physiological level, a *Clasp1* KO mouse model was generated (A. L. F. Pereira, 2009).

Until now, it was thought that *Clasp1* KO mice were dying during embryonic development, due to the absence of KO animals in the offspring (A. L. F. Pereira, 2009). Cannibalistic behavior was observed in the females after delivery of KOs. Because of that, we closely monitored the delivery process and pups were separated from the female if any

cannibalistic intention was noticed. Afterwords, we observed that some pups, which were turning cyanotic immediately after birth, were also dying within a few minutes, while others were apparently healthy. After genotyping, it was possible to conclude that all and only *Clasp1* KO mice were displaying perinatal lethality. In fact, *Clasp1* KO mice are viable until birth, although, they cannot survive *ex uterus*. Phenotype resulting from mutations can be very challenging. This is especially true for mutants dying perinatally, which represent a substantial proportion of lethal phenotypes in the mouse. Unless the gene is expressed in a tissue-specific manner or is conditionally inactivated in specific tissues, determining which specific physiological defects is responsible for lethality may require considerable effort.

Cyanosis is a hallmark of lung failure, which is also one of the most frequent complications in human preterm newborns, as well as low birth weight that was also observed in *Clasp1* KO pups. So, the next step was be macro and microscopic analysis of *Clasp1* KO lungs. Histological analysis revealed a lung collapse, which was confirmed by the water-floating test. The absence of clear alveolar spaces, together with the absence of air, suggest that *Clasp1* KO pups were not able to inflate their lungs. Lung collapse, or pneumothorax, may be a result of an underlying lung disease, but defects in other organs or systems may also be a cause. Histological analysis of lung samples from the E14.5 E14.5 to the E19.5 revealed a slight delay in lung development in *Clasp1* KO animals. Regarding this delay, the next step was to determine if *Clasp1* KO mice would be functionally prepared to undergo gas-exchange after after.

Respiratory tract consists of a remarkable, highly branched, tubular structure that leads to millions of alveolar sacs. The alveolar surface is lined by type II and type I alveolar epithelial cells that are in direct contact with respiratory gases, creating collapsing forces at the air-liquid interface. To maintain lung expansion, these surface forces are mitigated by the presence of pulmonary surfactant that is synthesized and secreted onto the alveolar space by type II epithelial cells. Lack of pulmonary surfactant in preterm infants with respiratory distress syndrome causes atelectasis leading to respiratory failure. Considering CLASP2 role in cell differentiation and the *Clasp1* KO mice lung failure phenotype, our first hypothesis would be defects in lung maturation. Thus, we checked if *Clasp1* KO lungs were functionally prepared to undergo respiration. The presence of mature type I and type II pneumocytes would tell if *Clasp1* KO lungs were prepared to undergo gas-exchange. Then, we searched for molecular markers of type II and type I pneumocytes and stained for pro surfactant protein C and T1α, respectively, to check if

these cells were, actually, differentiated in KO lungs. In fact, these markers were present not only in WT lungs, but also in KOs. We also stained for pro surfactant protein C in early embryonic stages and we observed that this protein starts to be clearly expressed in WT lungs by the E15.5, but KO animals only show a clear expression by the E16.5 (data not shown). This means that, in fact, there is a delay in lung development of, at least, ~1 gestation day. However, this one day of delay (premature) would not be enough to explain the lethal phenotype because WT E18.5 embryos survive upon premature extraction from the mother by C-section. This means that, physiologically, this lung failure represents more than a delay of 1 gestation day. A closer observation of the histological architecture of KO lungs, allowed us to note that this delay was becoming more evident along the later gestation steps. For instance, KO lungs at the E16.5 were more alike to the WT littermates at the E15.5, but some the lungs of E19.5 KOs were more similar to the WT controls at the E17.5.

In late gestation, glycogen-laden type II pneumocytes shift their metabolic program toward the synthesis of surfactant, of which phosphatidylcholine (PC) is, by far, the most abundant lipid component of surfactant. So, abnormal high accumulation of glycogen in lungs would suggest that both type II pneumocytes immaturity or defects in glycogen metabolism, could lead to insuficient surfactant production. PAS staining results in E18.5 lung samples have shown an apparent higher accumulation of PAS positive substances in the KO alveolar parenchyma (excluding glandular cells with PAS positive neutral mucins). However, this histochemical procedure is not totally specific for glycogen and is not a direct measurement of the lipidic component of surfactant. Moreover, we only tested for pro surfactant protein C, while the protein component of surfactant are composed by surfactant A, B, C and D proteins.

Regarding CLASP1 expression profile during development, the most striking observations were its strong and consistent expression in brain throughout development and its variable expression among the other organs. At the early embryonic stages (E16.5 and E18.5), CLASP1 was expressed in many organs, such as brain, lungs, heart and kidneys. Nevertheless, by the time of birth, only brain was clearly expressing CLASP1 and CLASP2, while their expression in the others organs is shut down. In adult mice, CLASP1 is ubiquously expressed. These results do not allow us to directly compare the amount of proteins expressed in each organ/tissue due to differences in the loading. Protein extraction procedures are less efficient in tissues samples. The amount of protein isolated is usually lower, comparing to cell extracts. Moreover, tissues with high content of connective tissue

and muscle are harder to disrupt. Thus, further improvements in protein extraction techniques are one of the future prospects of this work.

Collectively, this work has given the first steps to uncover CLASP1 role in living mammals. So far, we were able to show that *Clasp1* depletion causes death after birth in mice. *Clasp1* KO mice are, indeed, viable until the moment of their birth, but they cannot survive more than a few minutes after it. Although an underlying lung defect is not completely ruled out, we proved that KO lungs express molecular markers of late lung morphogenesis. Thus, *Clasp1* KO lungs seem to be ready to undergo gas-exchange after birth, even exhibiting a delay in lung architecture comparing to control littermates. We also cannot exclude defects in surfactant production yet. The apparent higher amounts of glycogen corroborates the histological findings, which suggests lung immaturity. Muscular and ribs defects were apparently absent in KO animals. However, taking into account the technical limitations of the histochemical techniques, this part of the work requires further investigation.

In a near future, it will be important to explore, in more detail, other systems directly related to respiration. Neuronal defects have been described as cause of a very similar phenotype to the *Clasp1* KO animals. Defects in neurons controlling respiration may block the signal transmission to the respiratory muscles, impairing the proper rhythmic movements and shutting down ventilation. Considering the aforementioned role of CLASPs in neuronal biology, an underlying neuronal cause may be shutting down respiration, leading to the *Clasp1* KO mice death right after birth. So far, we have characterized the *Clasp1* KO phenotype and some possible causes of lung failure have been ruled out. Although we have shown that *Clasp1* KO mice cannot inflate their lungs, the primary physiological failure remains unclear. Nevertheless, the most important conclusion regarding CLASP1 impact in the physiology environment of mammals is that CLASP1 is, unquestionably, required for mammals' life.

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