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Tumor suppressor function of the deubiquitinating enzyme BAP1 and its substrate γ-tubulin in regulation of cell cycle and genome stability

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DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Department of Laboratory Medicine Lund, Translational Cancer Research, Lund University, Sweden.

To be defended at Medicon Village, Building 302 Lecture Hall, Lund.

Wednesday 27th of May 2015 at 9:00.

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LUND UNIVERSITY
Faculty of Medicine

To my grandparents

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1. List of Papers

Papers included in thesis

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I Nuclear localization of γ-tubulin affects E2F transcriptional activity and S phase progression

Greta Höög, [1] **Reihaneh Zarrizi**, [1] Kristoffer von Stedingk, Kristina Jonsson, and Maria Alvarado Kristensson

[1]These authors contributed equally to this work

FASEB J. 2011 Nov;25(11):3815-27

II Deubiquitination of γ-tubulin by BAP1 prevents chromosome instability in breast cancer cells

Reihaneh Zarrizi, Julian Menard, Mattias Belting and Ramin Massoumi

Cancer Research J. 2014

III BAP1 induces cell cycle arrest and cell death in neuroblastoma

Reihaneh Zarrizi and Ramin Massoumi

Manuscript

Paper not included in thesis

Association of nuclear-localized Nemo-like kinase with heat-shock protein 27 inhibits apoptosis in human breast cancer cells.

Gina Shaw-Hallgren, Katarzyna Chmielarska Masoumi, **Reihaneh Zarrizi**, Ulf Hellman, Per Karlsson, Khalil Helou and Ramin Massoumi

PLoS One *J*. 2014

2. Abbreviations

BAP1	BRCA1 associated protein 1 protein
BARD1	BRCA1-associated RING domain protein 1
BCL2	B-cell lymphoma 2
BRCA1	Breast cancer susceptibility protein
CDK	Cyclin – dependent kinase
CCNE	Cyclin E gene
DNA	deoxyribonucleic acid
EMT	Epithelial to Mesenchymal Transition
E2F	E2 promoter binding factor protein
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor
NLS	Nuclear localisation signal
PR	Progestron receptor
PARP	Poly ADP-ribose polymerase
Rb	Retinoblastoma tumor suppressor protein
TUBG	γ-tubulin protein
UCH	Ubiquitin C-terminal hydrolase

3. Introduction

Precise cellular machinery is needed to duplicate the eukaryotic cell. Cell division is composed of two consecutive processes: the interphase, which involves DNA replication, and mitosis, which accurately segregates the replicated chromosomes into the daughter cells [1]. The cell cycle process produces two cells with identical genetic content to that of the parent cell. The smallest part of the overall cell cycle length in cell reproduction is mitosis. The cell spends most of its time in interphase. Cell cycle progression is a tightly regulated process, which involves multiple checkpoints that monitor extracellular growth signals, cell size and DNA integrity. A common feature of human cancer is cell cycle deregulation, which is recognised by the unscheduled proliferation, genomic instability and chromosomal instability [2]. The molecular analysis of human tumours has shown that various components of the cell cycle regulatory system are mutated, overexpressed or eliminated in human cancers [3]. As the rate of mitosis increases, the chances of further DNA damage increases, and the growth of cells becomes completely unregulated. Thus, the result is uncontrolled cellular mitosis, which may lead to cancer. Therefore, understanding the molecular mechanisms of the deregulation of cell cycle progression in cancer can provide important insights into how normal cells become tumorigenic and how new cancer treatment strategies can be designed.

4. The cell cycle

A cell reproduces by performing a series of events, which results in the duplication of its contents, leading to its division into two identical daughter cells. This cycle of duplication and division is known as the cell cycle. The cell cycle is tightly regulated and has evolved a complex network of regulatory proteins [2-4]. In eukaryotic cells, the cell cycle is divided into four distinct phases: the Gap I, Synthesis, Gap II and Mitosis phases. The first gap, which is also named the growth phase (Gap I), initiates at the end of the previous M phase and continues until the beginning of DNA synthesis. A high rate of biosynthetic activities in the cell is observed during this phase, resulting in an increased supply of proteins and number of organelles, and the cell grows in size [5-7] During the synthesis (S) phase, which occurs between the Gap I and Gap II phases, the complete DNA is duplicated. The second gap (Gap II) occurs between DNA synthesis and mitosis to ensure that everything is ready to enter the M phase [7]

Typically, in a mammalian cell cycle, it takes about 10–12 hours for DNA duplication or S phase to occur, and during the M phase, the chromosomes segregates and the final cell division takes place. During the M phase, the nuclear components and the replicated chromosomes are divided into two identical daughter cells by the last step in mitosis, cytokinesis (Figure 1).

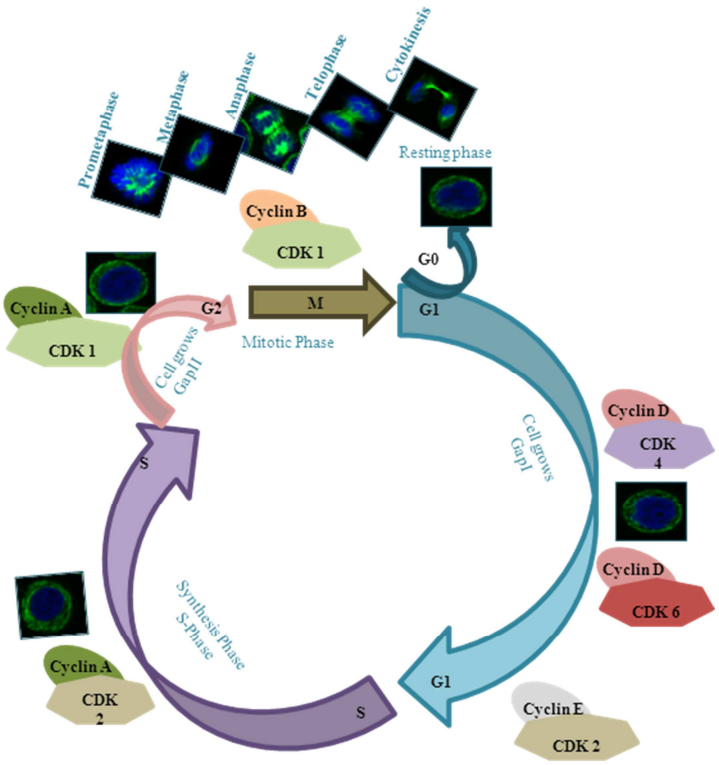

Figure 1. The cell cycle. G0 (dark aqua) is a resting phase. Some cells stop dividing, which might be a temporary resting period or more permanent. Cells increase in size in GI (aqua) phase. Cellular contents like RNA and protein, except chromosomes, are duplicated. Chromosomes are replicated during S phase (purple). In GII (pink), the cell double-checks the duplicated DNA for errors, and makes any needed repairs. Finally, in M phase (olive), the cell divides into two daughter cells. Immunofluorescence images showing DNA (blue) and microtubules (green) throughout the cell cycle in human breast cell lines.

The cells monitor the internal and external environment to ensure that conditions are suitable before the cell commits to cell division. Extra gap phases are inserted into cell cycle. The Gap I phase is inserted between the M and S phases. The Gap II phase is between the S phase and mitosis. The length of the GI phase can vary depending on the microenvironmental signals [5].

Unfavourable extracellular conditions lead the cells to enter a specialised resting state, known as G0, in which they can remain for a long time before resuming proliferation. If the extracellular conditions are favourable, cells in early GI or G0 progress through a commitment point near the end of GI, known as the restriction point [8, 9]. After passing this point, cells are committed to divide [7]. DNA replication and cell cycle progression can be halted by cellular stress, such as DNA damage. [8, 9]. Disturbed checkpoints, resulting in uncontrolled cell proliferation, have been linked to many forms of cancer [9-11].

4.1 Regulation of the cell cycle

Progression of the cell cycle is controlled by cyclically activated protein kinases, known as cyclin-dependent kinases (CDKs), which consist of Cdk1, Cdk2, Cdk4/Cdk6[2, 12-15].

The activity of these kinases differs through the cell cycle, which leads to cyclical changes in the phosphorylation of intracellular proteins that initiate or regulate the major events of the cell cycle [1-3]. The activity of CDKs is controlled by other proteins known as cyclins, which have no protein kinase activity [16]. Cyclins undergo a cycle of synthesis and degradation in each cell cycle, which results in the cyclic assembly and activation of the cyclin-CDK complexes, and this activation in turn triggers cell cycle events. There are four classes of cyclins, defined by the stage of the cell cycle in which they bind to the CDKs [16, 17]. The D-type cyclins form a complex with Cdk4 or Cdk6, and complex activity is are required during the GI phase [18]. Before S phase, just after the GI checkpoint, the E-type cyclins bind to Cdk2, which is required for the transition from the GI phase to the S phase [3, 16, 19]. The synthesis of A-type cyclins is

promoted by proteins required for S-phase entry[20]. As the cell enters the S phase, A-type cyclins replace the E-type cyclins as the partner of Cdk2 [21-24]. The A-type cyclins exchange partners from Cdk2 and form a complex with Cdk1 in S phase [25]. The B-type cyclins are known as mitotic cyclins, which bind to Cdk1, and the activity of cyclin B-Cdk1 triggers many of the events during mitosis [26]. The cell cycle progression depends on the balance between CDK activation and inactivation, which in turn, depends on the expression level and availability of the different cyclins [16] (Figure 1).

Tight regulation of cell cycle progression is critical for the normal development of organisms and the prevention of cancer. There are numerous factors involved in the cell cycle regulation processes. The retinoblastoma family of proteins plays an essential role in the regulation of the cell cycle by controlling the activity of E2F [27-32]. The expression of factors involved in cell cycle regulation is also regulated at the level of transcription, post-translational modification and protein stability. [27, 33].

4.2 Retinoblastoma

The product of the retinoblastoma tumour suppressor gene (RB) is a key regulator of entry into cell division. pRB acts as a signal transducer connecting the cell cycle clock with the transcriptional machinery, [34, 35] and allows the cell to check the expression of genes that mediate cell cycle progression from the GI to the S phase.[34, 36].

The retinoblastoma family of proteins plays an essential role in the regulation of the cell cycle by controlling the activity of E2F [27, 37, 38].

pRB belongs to the pocket protein family, which also includes p107 and p130, and they all bind to the transcription factor family E2F through their highly conserved carboxy-terminal domain, *i.e.* the pocket domain [39-43]. The domain structure of pRB consists of an N-terminal domain (RbN), a central pocket domain, and a C-terminal domain (RbC), which is disordered except for a short sequence that adopts a structure upon E2F-binding.

There are also several conserved consensus CDK phosphorylation sites. The pocket domain of pRB binds to the E2F transactivation domain (TD), and the RbC binds the E2F-DP marked box domains. [44].

4.3 The E2 promoter binding factor family

In higher eukaryotes, the E2 promoter binding factor (E2F) proteins consist of a family of eight members that function as transcription factors. The E2F family was initially divided into two groups of activators and repressors. Each E2F can exert a variety of cellular effects, some of which represent opposing actions. The activity of specific E2Fs depends on the cellular context. The main functional output E2Fs is the transcriptional activation or repression of their target genes associated with a variety of cellular processes, such as inducing or inhibiting cell proliferation, and enhancing or inhibiting apoptosis. This complexity reflects the importance that these transcription factors have on a cell's fate [45-47].

In most members of the E2F protein family, several evolutionally conserved domains were found, including a DNA binding domain, a dimerization domain, which determines the

interaction with the differentiation-regulated transcription factor proteins (DP), a transactivation domain that is enriched in acidic amino acids, and a tumour suppressor protein–association domain, which is embedded within the transactivation domain [48, 49].

The E2F1 protein and another two members, E2F2 and E2F3, have an additional cyclin-binding domain. E2F1 binds preferentially to retinoblastoma protein pRB in a cell cycle–dependent manner. E2Fs can mediate both cell proliferation and apoptosis [50-52].

5. Mitosis and the mitotic spindle

Mitosis is the process of dividing chromosomes during cell division in eukaryotic cells. An intimate relationship between the cell cycle and the chromatin architecture has been observed in mitotic cells [53, 54]. The usual method of cell division is characterised by the compaction of the chromatin into mitotic chromosomes, which is needed to ensure the fidelity of separating identical genetic information into two daughter cells during mitosis [53, 55]. While mitosis is happening, there is no cell growth and all of the cellular energy is focused on cell division. The final stages of cell cycle begin during mitosis, which is conventionally divided into five important stages, prophase, prometaphase, metaphase, anaphase and telophase [53, 55, 56].

During prophase, chromosome condensation initiates, and chromosomes become shorter and fatter as the process progresses. The duplicated centrosomes separate to opposite ends of the cell. The sister chromatid pairs become visible at the later stages of prophase. During prometaphase, the nuclear envelope breaks down, and the condensed chromosomes spill into the cytoplasm.

During metaphase, the spindle apparatus fully develops, and the condensed chromosomes align at the metaphase plate. During anaphase, the duplicated chromosome pairs are pulled apart to the opposite poles of the cell by mitotic spindle elongation. As the two genetically identical daughter chromosomes reach the opposite poles of the cell, telophase begins (Figure 2). The chromatin decondenses and the nuclear envelope reforms when the mitotic spindle distributes the duplicated chromosomes into two daughter cells. Mitotic exit is then completed by mitotic spindle disassembly. [57].

Errors in mitosis may lead to changes in the genetic material, which can potentially result in genetic disorders [58, 59]. The successful completion of mitosis requires correct functioning of the mitotic spindle throughout mitosis [60, 61]. The mitotic spindle is composed of microtubules that extend from the two opposing centrosomes (known as the microtubule-organising centres or MTOC) [62, 63]. Diverse changes in the microtubule network have been identified and characterised in a wide variety of cancers. Alterations in the chromosome number and structures cause massive genetic instability, which is a hallmark of many cancers [64-66].

The molecular mechanism behind genome instability includes the chromosome mis-segregation during mitosis by the timely spindle assembly or disassembly is unknown[61].

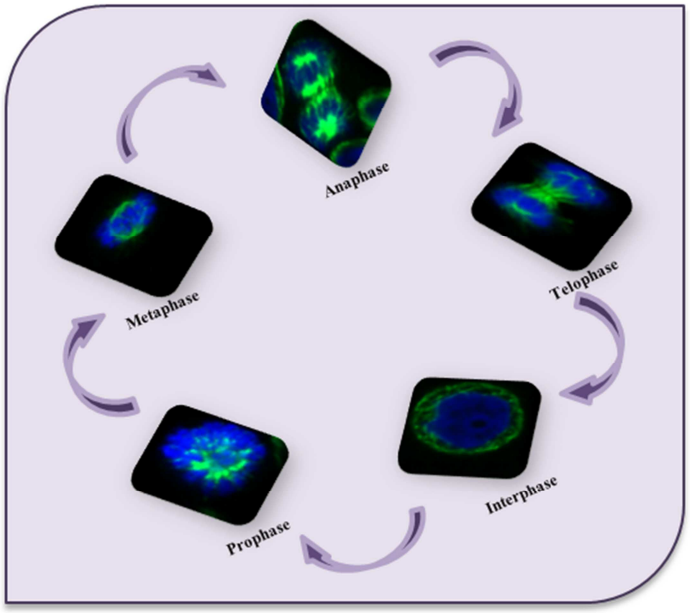

Figure 2. Mitosis in a human breast cell line. The progression of mitosis through the canonical morphological stages is shown. Interphase – the DNA is still contained in the loosely coiled chromatin; Prophase – the chromatin with the replicated DNA is visible; Metaphase – the chromosomes arrange themselves in the centre of the cells; Anaphase – the two chromatids are separated; Telophase – the chromosomes move away from the centre of the cell. Immunofluorescence images showing DNA (blue) and mitotic spindle (green) throughout the mitosis in a human breast cell line.

5.1 The microtubule regulatory protein γ-tubulin

The main constituents of the spindle apparatus during cell division are microtubules, which are filamentous polymers of the protein tubulin, in all eukaryotic cells[67, 68]. The formation of a

new microtubule from free tubulin dimers begins with the process of nucleation[69]. Microtubule nucleation requires another member of the tubulin family, γ-tubulin, which is a highly conserved protein in all eukaryotes [67, 70-74]. γ-Tubulin is one of the best-characterised components of the microtubule-organising centre, which is involved in the initiation of microtubule nucleation, centrosome duplication and mitotic spindle formation. [71, 75-80]. γ-Tubulin is found in two main complexes: the γ-tubulin ring complex (γ-TuRC), which is involved in the promotion of nucleation of microtubules, and the γ-tubulin small complex (γ-TuSC) [81-84]. The closer a cell is to the onset of mitosis, the more γ-tubulin accumulates at the centrosome to support spindle formation [67, 85-88].

The precise duplication of the centrosome is a highly regulated process during the cell cycle, which is initiated by the assembly of daughter centrioles during the late GI/S phase [85, 89-92]. The activities of Cdk2–cyclin E and Cdk2–cyclin A, the two kinase complexes that drive the cell into the S phase, are important for controlling the centrosome duplication [93-97].

γ-tubulin is considered the most important protein for the regulation of centrosome number and function. The centrosome is the major microtubule-organizing center (MTOC) in most vertebrate cells, and its function is important for establishing functional bipolar mitotic spindle during mitosis [77, 98, 99].

Centrosome-mediated spindle assembly provides a pathway to ensure high fidelity of chromosome segregation, and thus, failure to properly control the centrosome number can cause

the formation of an abnormal mitotic spindle, leading to chromosome abnormalities. Previous studies have shown that ubiquitin interacts with γ-tubulin, suggesting that this modification may be involved in the maintenance of centrosome number and prevent genome instability [77].

A wide range of centrosome abnormalities has been frequently found in early-stage lesions of human tumours derived from breast and other tissues [90, 100]. Our recent data also indicate that deubiquitination of γ-tubulin might be important for preventing abnormal mitotic spindle formation, and thus may direct and ensure the correct segregation of chromosomes during the cell cycle [101].

5.2 The chromosome and genome instability

The fundamental goal of mitosis is to accurately duplicate the genome and to produce two genetically identical daughter cells. Daughter cells must have the exact copies of their parent cell's genome, and so any kind of failure to achieve this purpose, or an abnormally high frequency of errors during this process, leads to various forms of genomic alteration in the daughter cells, which is a major driving force of tumourigenesis. Genomic alterations may lead to cell cycle retardation, imbalance between cell growth, death and cancer [102, 103]. An increased rate of DNA alteration in tumour cells leads to chromosomal genomic instability[104]. Genomic instability may occur either from increased rates of damage, from which normal repair systems will not be able to restore genomic integrity, or defective repair systems being unable to cope with normal rates of damage [103, 105-108]. Chromosomal instability *i.e.* an increased rate of chromosome mis-segragation in mitosis arises from a failure to maintain the correct chromosomal complement[109, 110]. Chromosomal instability can be caused by inappropriate

chromosome segregation, including a weakened or over-activated mitotic spindle assembly checkpoint, sister chromatid cohesion defects, increased kinetochore–microtubule attachments or the presence of extra centrosomes, bipolar spindle assembly and recombination[111, 112]. A hallmark feature of nearly all solid tumours is an unstable genome and this instability occurs early in tumour progression [105, 113].

6. The ubiquitination pathway

One of the most versatile post-translational modifications is ubiquitination, which has roles in the regulation of many essential cellular processes by targeting the proteins for assembly into complexes, transport and degradation[114-117]. The ubiquitination pathway promotes covalent attachment of highly conserved polypeptide ubiquitin to protein substrates through the sequential action of three enzymes named ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3), which provides specificity. First, the E1 enzyme activates ubiquitin in an ATP-dependent manner and forms a high-energy thioester linkage with the carboxyl group of ubiquitin. The activated ubiquitin is then transferred to the E2 enzyme. Finally, ubiquitin is conjugated to the ε-amino group of an internal lysine residue in the substrate protein with the help of an E3 ligase enzyme [118](Figure 3). The protein ubiquitination can be either a mono- or polyubiquitination, and the type of ubiquitin modification determines the function of the modified protein [114, 119-124]. Ubiquitination can regulate protein stability, cellular localisation, DNA repair and cell cycle progression [125-128].

6.1 The deubiquitination pathway

Ubiquitination is a reversible post-translational modification. The cleavage of covalently attached mono- or polyubiquitin chains from the substrate protein, catalysed by deubiquitinating enzymes (DUBs), is important to regulate the abundance or functional activity of target proteins [129-133]. Deubiquitinases are proteases that play fundamental roles in the ubiquitin system based on their ability to specifically deconjugate ubiquitin from pro-proteins or target proteins [134-138]. The deubiquitination process is also involved in numerous cellular functions, such as cell cycle regulation, proteasome- and lysosome-dependent protein degradation, gene expression, DNA repair and kinase activation [134, 135, 139-142].

DUBs have multiple key roles in the regulation of cellular events. Firstly, activation of the ubiquitin pro-proteins after translation may be regulated by the activity of DUBs. Secondly, DUBs are essential for the recycling of the ubiquitin molecules by cleaving them from the substrates Thirdly, DUBs influence the stability of proteins by rescuing them from degradation before they are recognised by the degradation machinery. Finally, DUBs can also affect the binding affinity of the substrate to its interactor protein by removing the ubiquitin molecule from its target and thereby regulate downstream processes [133, 134, 140].

The human genome encodes approximately 95 DUBs, which have been divided into five major classes: ubiquitin-specific proteases (USP), ubiquitin C-terminal hydrolases (UCH), ovarian tumour proteases (OTU), Josephins, and the Jab1/MPN/MOV34 metalloenzymes (JAMM, also

known as MPN1) [143-145]. The large number of gene families and individual members suggests that they exhibit a significant degree of substrate specificity (Figure 3). Similar to ubiquitination, deubiquitination is a highly regulated process, and dysregulation of components involved in the ubiqutin or deubiquitin pathway have been associated with many different human diseases, including cancer [146-150].

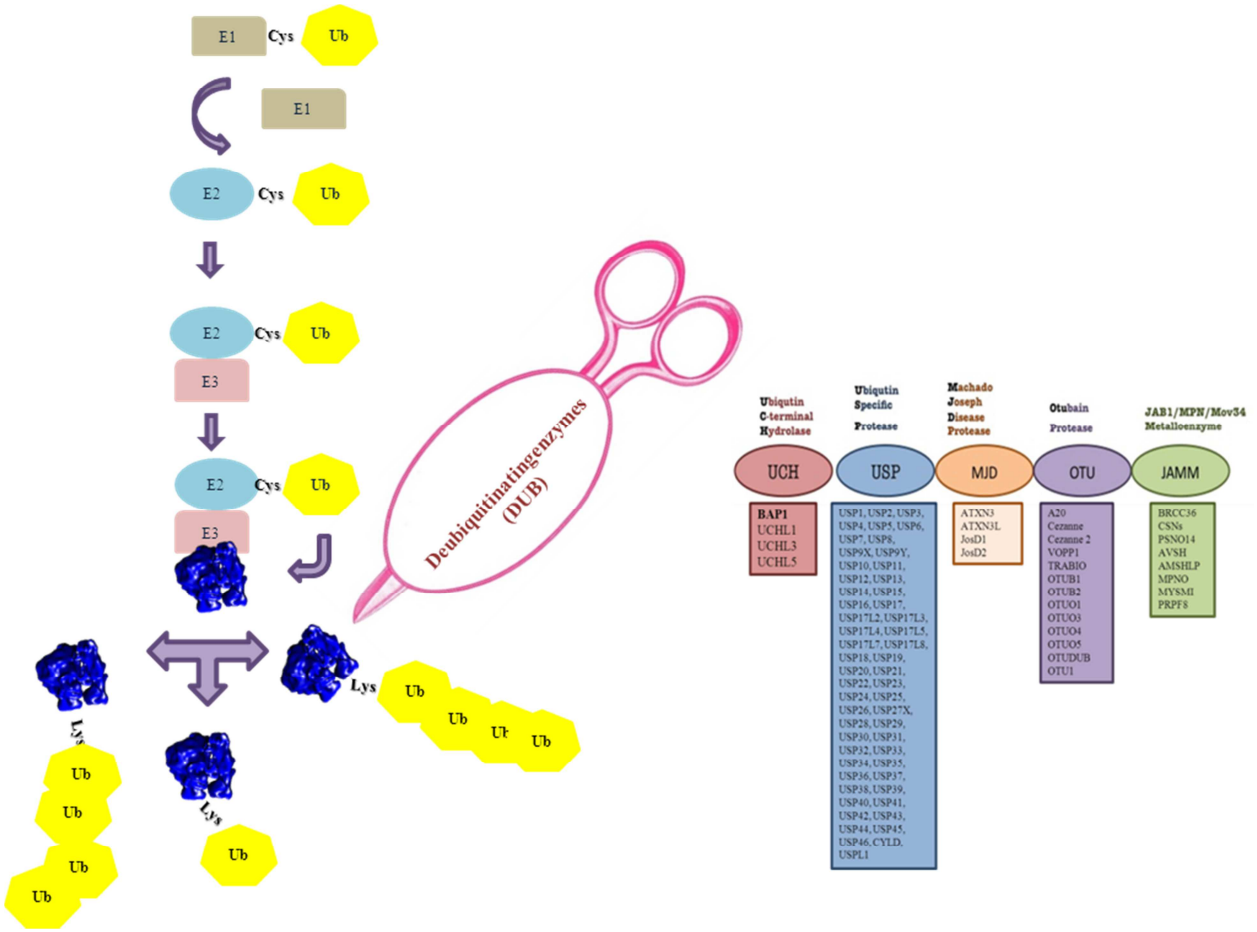

Figure 3. The ubiquitination pathway. Ubiquitin (Ub; yellow) is activated for conjugation by E1 (ubiquitin-activating enzyme; grey) then transferred to E2 (ubiquitin-conjugating enzyme; blue). From there, ubiquitin is transferred to a substrate (dark blue). This process is catalysed by an E3 (ubiquitin ligase; pink) enzyme. In some cases, substrates receive a single ubiquitin, whereas in others, they receive multiple ubiquitins linked together in different ways. Ubiquitination is a reversible process. Deconjugation of mono- or polyubiquitin is performed by the action of DUBs, which generate monomeric ubiquitin from a specific substrate.

6.2 The deubiquitination enzyme BRCA1-Associated Protein 1

BRCA1-Associated Protein 1 (BAP1) belongs to the ubiquitin C-terminal hydrolase (UCH) family of deubiquitinating enzymes (See Figure 3). The UCH family consists of four members: UCHL1, UCHL3, UCHL5 and BAP1[151, 152]. Although most UCH enzymes are relatively small, BAP1 is a large protein with a C-terminal domain that contains two nuclear localisation signals, binding domains for BRCA1 and BARD1, and an N-terminal UCH catalytic domain [153, 154]. BAP1 was initially discovered by the yeast two-hybrid technique as a protein that binds to the breast cancer type 1 susceptibility protein (BRCA1) via the RING finger domain of the latter. BAP1 is a tumour suppressor gene, and earlier studies have shown that the BAP1 gene is deleted or mutated in various human cancer types, including breast cancer, lung cancer, renal cell carcinoma, metastatic uveal melanomas and malignant pleural mesotheliomas [153, 155-165]. The tumour suppressor property of BAP1 is dependent on its nuclear localisation and deubiquitin activity [166]. BAP1 can suppress the growth of non-small-cell lung carcinoma NCI-H226 cells in culture and as solid tumours in athymic nude mice [155]. We showed recently that BAP1 deubiquitinates γ-tubulin, which was required to prevent abnormal mitotic spindle formation and genome instability in breast cancer cells [101].

Previous studies have shown that BAP1 plays key roles in several different cellular processes, including regulation of transcription, regulation of cell cycle progression and the response to DNA damage [166]. It has been reported that BAP1 forms multiprotein complexes with several chromatin-associated proteins, notably the host cell factor 1 (HCF-1), and regulates transcription

[151, 164, 167]. BAP1 can regulate cell cycle progression by influencing the expression of E2F1 target genes in uveal melanoma cells. Furthermore, it has been found that BAP1 is involved in the DNA damage response by mediating rapid poly(ADP-ribose)-dependent recruitment of the polycomb deubiquitylase complex PR-DUB to sites of DNA damage [168, 169]. Phosphorylation of BAP1 at S592 is also an important regulatory mechanism to dissociate BAP1 from chromatin and to regulate specific genes during DNA replication and repair [170].

7. Breast cancer

Breast cancer is one of the most common forms of cancer in women in the Western world, accounting for approximately 30% of all cancer diagnoses in Sweden (Socialstyrelsen, cancer incidence in Sweden, 2011). The breast cancer incidence has increased over the last 20 years, but this high incidence rate is being tempered by a decline in mortality (Socialstyrelsen, cancer incidence in Sweden, 2011). This might be due to the recent advances in disease detection and adjuvant therapeutic management [171]. Multiple factors, with different levels of significance, have been linked to the risk of developing breast cancer. These include both hereditary and non-hereditary factors. Established risk factors include: early menarche and late menopause; late age at first childbirth; hormone-replacement therapy; lifestyle and dietary choices; lack of physical activity and high body mass index; high alcohol and coffee consumption; smoking; and exposure to ionizing radiation to the chest area at a young age [172-175]. The majority of breast cancers are sporadic and non-familial. Germ-line mutations of BRCA1 and BRCA2, associated with a high risk of developing breast cancer, are only detected in 15–20% of cases in families with a history of breast cancer [176-178].

Breast cancer is a heterogeneous disease with a high degree of diversity between tumours. The traditional classification of breast cancer based on histology has been processed with a novel molecular classification system based on the gene expression patterns. Advances in technologies, such as gene expression profiling of breast cancer tissue, have provided new insights into the heterogenic molecular composition of breast cancer, and have allowed us to identify molecular

subgroups with prognostic implications [179, 180]. Microarray-based gene expression analysis and unbiased hierarchical clustering have identified five major molecular subtypes. A majority of breast cancers are classified into two luminal subtypes (luminal A and luminal B) with high ER expression. The luminal A and B tumours can be identified by deregulation of genes involved in the ER signalling pathway from other subtypes [181-183]. The luminal B tumours express a higher level of the proliferation marker Ki67, show increased proliferation and have a worse prognosis compared to luminal A tumours [181, 182, 184, 185]. The HER2 subgroup is enriched with tumours with amplification of the ERBB2 gene and displays a poor prognosis. The normal-like subtype gene expression profile resembles normal breast epithelial cells and displays an intermediate prognosis [181, 182, 186]. The basal-like subtype is associated with poor prognosis and lack of ER, PR and HER2 expression; these tumours are called triple negative. The claudin-low subtype, which is mainly triple negative tumours, shows decreased expression of adhesin molecules, such as E-cadherin and claudin-3, -4 and -7, along with having similarities to stem cells and epithelial-to-mesenchymal transition (EMT) gene signatures[187, 188].

8. Neuroblastoma

Neuroblastoma (NB) is the most frequent solid tumour of early childhood arising in the developing sympathetic nervous system, and results in approximately 15% of cancer-related deaths in infants [189-191]. Neuroblastoma is the most common extra-cranial solid tumour in children and tumours can develop in any location where sympathetic ganglia are found, typically in the adrenal gland or paraspinal ganglia, and therefore NB tumours may occur in the neck, chest, abdomen or pelvis [189-193]. Neuroblastoma is known for its genotypically and phenotypically heterogeneous nature with a remarkable variation in clinical behaviour, ranging from localised tumours that can spontaneously regress to widespread metastasis that shows relentless progression [189, 194].

Neuroblastoma is divided into different stages according to the International Neuroblastoma Staging System (INSS), which can be used for prognostic purposes and for treatment planning [195]. Localised tumours are divided into stages I, II and III, and often display good outcome, while patients over the age of 1 year with distant involvement are categorised as stage IV and have a worse outcome [195-198]. The last group is called IV-S, which includes patients with a specific metastatic pattern of tumours to the skin, bone marrow and liver; these patients, diagnosed before the age of 1 year, are associated with a favourable prognosis and spontaneous regression [199, 200]. The favourable biological features of most localised neuroblastomas

(Stage I to III) are observed in low-risk patients who are successfully treated with surgery alone, whereas in patients with intermediate-risk, surgery is combined with chemotherapy [195]. Patients harbouring extensive regional or metastatic disease are treated with surgery and chemotherapy together with radiotherapy [189, 190, 200].

9. Present investigation

9.1 Aims

- To examine the role of nuclear γ-tubulin in controlling cell cycle progression

- To identify the function of γ-tubulin deubiquitination by BRCA1 Associated Protein 1 (BAP1) in normal and cancer cells

- To characterize tumor suppressor function of BAP1 in breast cancer and in neuroblastoma

9.2 Results and discussion

9.2.1 Paper I

γ-Tubulin is a member of the tubulin family, which is required for interphase αβ-tubulin nucleation, spindle formation and centrosomal duplication. In this study, we investigated the mechanism that allows the centrosome and microtubule-regulating protein γ-tubulin to moderate E2F transcriptional activity. We found that γ-tubulin contains a C-terminal signal that results in its translocation to the nucleus during late G1 to early S phase of the cell cycle. In the nucleus, γ-tubulin interacts with the transcription factor E2F1 and forms a complex during G1/S transition, when E2F1 is transcriptionally active (Figure 4). The binding of γ-tubulin to E2F1 reduces E2F

transcriptional activity during S-phase entry. Furthermore, we found that, in the absence of γ-tubulin, increased E2F activity elevates E2F-mediated expression of RB, which leads to a delay in S-phase entry. In summary, we propose that at the G1/S phase transition, when RB releases E2Fs, the E2F–γ-tubulin complex ensures a transient transcription of genes necessary for entry into the S phase of the cell cycle (Figure 4).

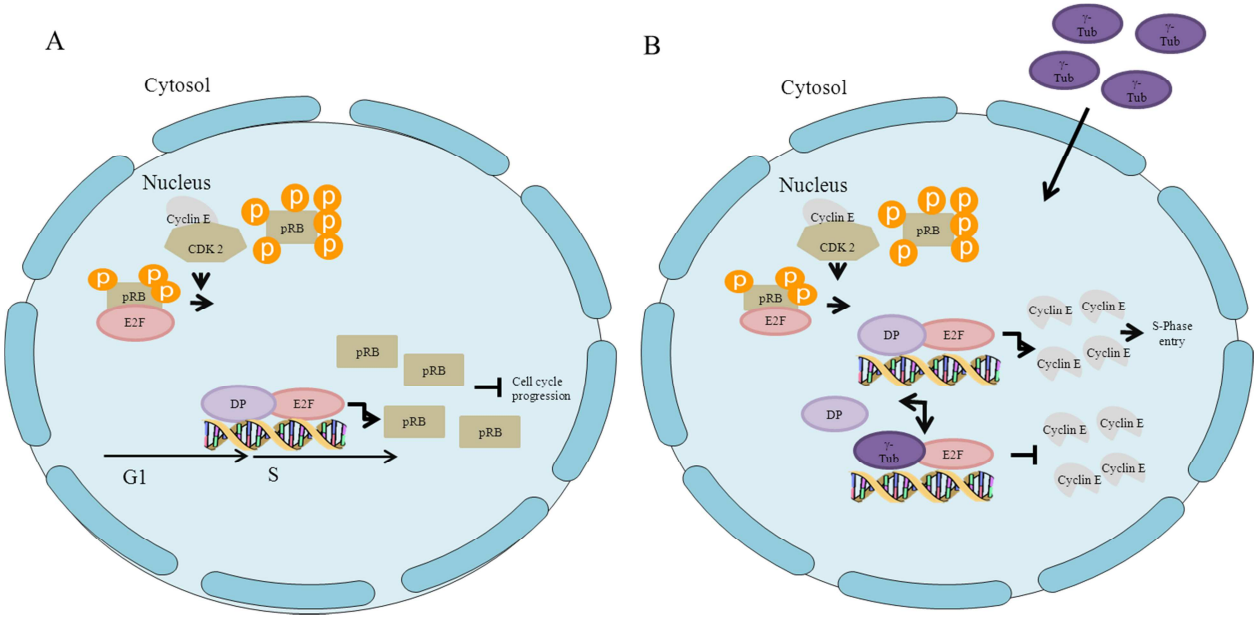

Figure 4. γ-Tubulin protein levels regulate E2F transcriptional activity. *A*) In the absence of γ-tubulin during G1/S-phase transition, the released E2F-Dp1 complex transcribes RB. ***B*)** In the presence of γ-tubulin during G1/S transition, the released E2F-Dp1 complex transcribes gene products necessary for S-phase entry, such as cyclin E. As nuclear γ-tubulin levels increase, the E2F–γ-tubulin complex forms and inhibits the transcriptional activity of E2Fs.

9.2.2 Paper II

γ-Tubulin is a member of the tubulin family, which plays key roles in microtubule nucleation and cell cycle regulation. Microtubule nucleation requires the γ-tubulin ring complex, and during the M phase (mitosis), this complex accumulates at the centrosome to support mitotic spindle formation. The ubiquitination of γ-tubulin by BRCA1/BARD1 was shown to be critical for regulating microtubule nucleation and centrosome duplication, and blocking this pathway causes centrosome amplification. In this study, we identified BAP1 as a deubiquitination enzyme for γ-tubulin. BAP1 was downregulated in metastatic adenocarcinoma breast cell lines compared to non-cancerous human breast epithelial cells. Furthermore, we could show that low expression of BAP1 is associated with reduced overall survival of breast cancer patients. Reduced expression of BAP1 in breast cancer cell lines was associated with mitotic abnormalities. Importantly, rescue experiments involving the expression of a full-length but non-catalytic mutant of BAP1 reduced ubiquitination of γ-tubulin and prevented mitotic defects. The results from our study uncovered a new mechanism for BAP1 in the deubiquitination of γ-tubulin, which was required to prevent the formation of abnormal mitotic spindle and genome instability in breast carcinoma cells (Figure 5).

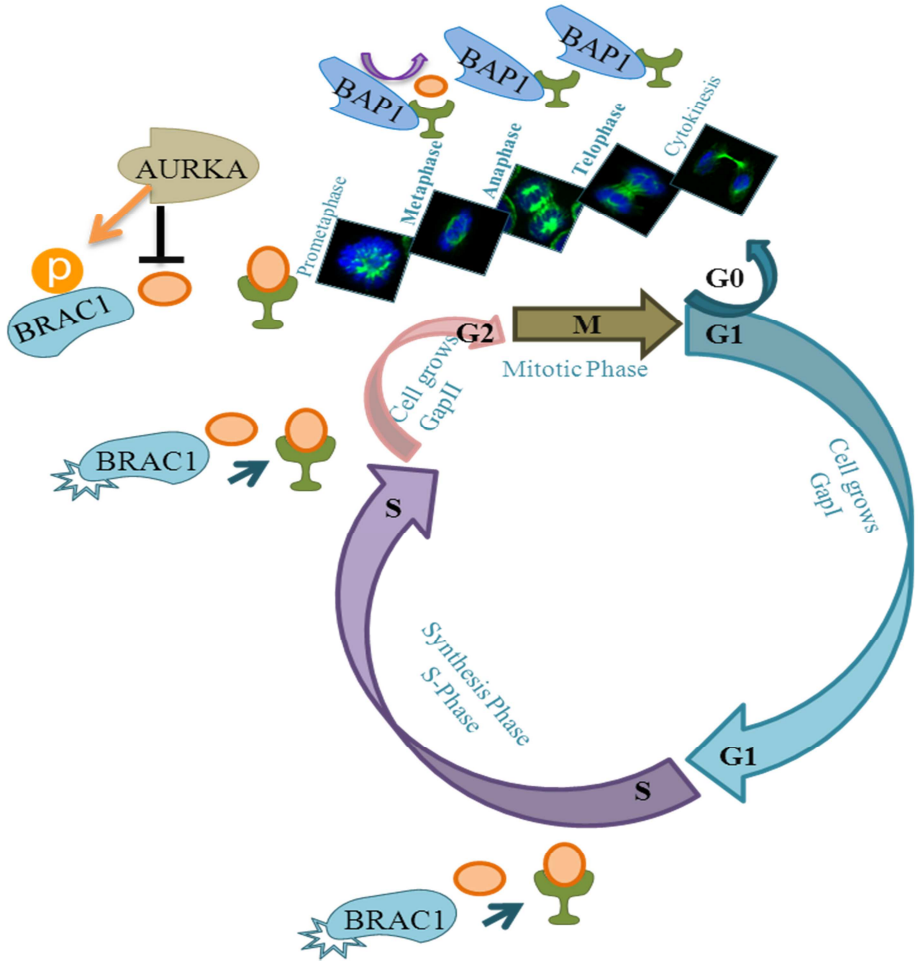

Figure 5. BAP1 expression rescues chromosome segregation defects associated with abnormal spindles in breast carcinoma. The model shows a dynamic balance of γ-tubulin ubiquitination by the BRCA1/BARD1 complex and deubiquitination by BAP1, which is required to prevent the formation of an abnormal mitotic spindle and genome instability in breast carcinoma.

9.2.3 Paper III

Cancer in children is rare, and neuroblastoma accounts for less than 15% of all childhood cancers, but it is the most common solid tumour in children younger than 1 year of age. In children of all ages, it is the most common solid tumour that arises from the sympathetic nervous system, and it is composed of undifferentiated and poorly differentiated neuroblasts arising from the different stages of the sympathoadrenal lineage of neural crest origin.

In the present study, we found that BAP1 expression in neuroblastoma has a prognostic implication in non-NMYC amplified neuroblastoma. NMYC is a transcription factor, and high levels of NMYC promote proliferation of neuroblastoma. High BAP1 expression was associated with better overall survival in neuroblastoma patients. Furthermore, we could show that rescue experiments involving expression of BAP1 inhibited cell growth by arresting the cells in the S phase of the cell cycle. In addition, synchronisation of the BAP1-expressing cells showed that upon prolonged S-phase arrest, the majority of the cells are in the G0 phase. This suggests that since BAP1-expressing cells are unable to pass the S phase, they are further directed toward cell death. Together, our findings may have important implications for BAP1, which can play a key role in the regulation of the cell cycle and cell death.

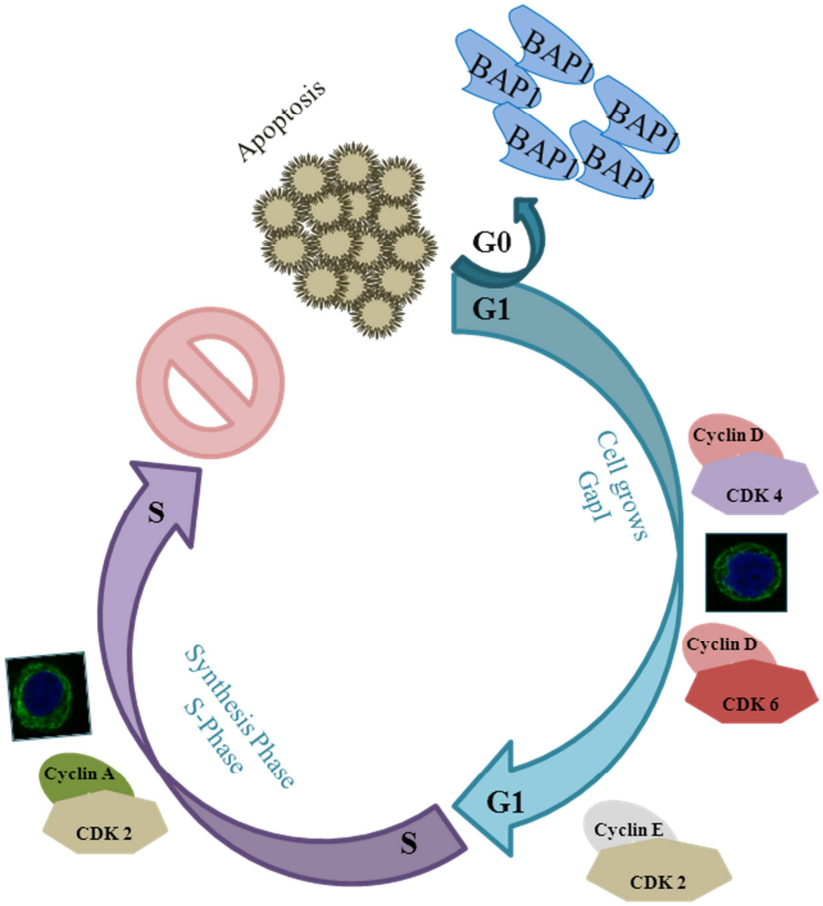

Figure 6. BAP1 protein levels regulate cell cycle progression. The tumour suppressor function of BAP1 in neuroblastoma was mediated through arrest of the cells in S phase and by promoting cell death.

10. Conclusions

- The expression of E2F transcriptional target genes was negatively regulated by γ-tubulin.
- γ-Tubulin levels and localisation determine optimal cell cycle progression.
- Deubiquitination of γ-tubulin by BAP1 is an important factor for preventing the formation of an abnormal mitotic spindle and genome instability.
- High expression levels of BAP1 are associated with a significantly prolonged survival in breast cancer patients.
- BAP1 plays a key role in the regulation of S-phase progression and cell death in neuroblastoma.
- High expression levels of BAP1 are associated with better overall survival in neuroblastoma patients.

11. Popular summary

The progression of the cell cycle is tightly coordinated; any kind of premature entry of a cell into the next phase leads to a significant propensity toward genomic alterations. A major cause of tumour formation is genomic instability, which can be minimised by high-fidelity DNA replication in the S phase, proper chromosome segregation, and error-free repair of sporadic DNA damage during cell cycle progression. Alterations in these processes can cause cellular senescence, apoptosis or tumour initiation. In this thesis, we investigated the mechanism that causes cell cycle dysregulation and genomic instability in non-transformed and cancer cells.

In the first project, we investigated the role of nuclear γ-tubulin in cell cycle progression. γ-Tubulin is a member of the tubulin family, which plays key roles in microtubule nucleation and cell cycle regulation. We observed translocation of γ-tubulin into the nucleus during the late G1 to early S phase of the cell cycle. Nuclear γ-tubulin interacted with the transcription factor E2F1 and reduced its activation. In the absence of γ-tubulin, increased transcriptional activity of E2F elevates E2F-mediated expression of Retinoblastoma protein, which is a key regulator of entry into cell division. It leads to a delay in S-phase entry. Our conclusion from this study is that a transient transcription of genes necessary for S-phase entry is regulated by the E2F–γ-tubulin complex during the G1/S transition.

Chromosomal instability and aneuploidy are associated with spindle defects in several types of cancer, including breast carcinoma. Microtubule nucleation requires the γ-tubulin ring complex, and during the M phase (mitosis), this complex accumulates at the centrosome to support mitotic

spindle formation. The post-translational modification of γ-tubulin through ubiquitination is vital for regulating microtubule nucleation and centrosome duplication. In our second study, we identified BRCA1-associated protein-1 (BAP1) as a deubiquitination enzyme for γ-tubulin. We found a low expression level of BAP1, which is a tumour suppressor protein, in metastatic adenocarcinoma breast cell lines compared to non-cancerous human breast epithelial cells. Mitotic abnormalities that usually happen as a result of an error in cell division were also observed in breast cancer cell lines with low expression of BAP1. Furthermore, we could show that low expression of BAP1 is associated with reduced overall survival of breast cancer patients. In summary, we found that BAP1 prevents mitotic abnormalities by removing ubiquitin from γ-tubulin, which is required to regulate mitotic spindle organisation.

In our third study, we aimed to identify the role of BAP1 in neuroblastoma, which is a childhood cancer. Since we found reduced expression of BAP1 in neuroblastoma cell lines, we decided to determine the consequences of rescuing its expression. BAP1 expression in neuroblastoma cell lines arrested the cells in S phase and promoted cell death in neuroblastoma cell lines. By analysing neuroblastoma patient samples, it was found that high BAP1 expression was associated with better overall survival. This finding suggests that BAP1 acts as a cell cycle regulator, and this may be one of the mechanisms through which BAP1 carries out its tumour suppressor function.

12. Acknowledgements

This work was performed at the Department of Laboratory Medicine, Center for Molecular Pathology (Malmö) and Translational Cancer Research, Medicon Village (Lund), Department of laboratory medicine, Lund University, Lund, Sweden.

I wish to express my sincere gratitude to

Ramin Massoumi, my supervisor. Thanks for supporting me, listening to my ideas, giving me the freedom to act on them and for undoubting trust in me from day one. You are a warm hearted person and I will always be very grateful that I was able to join your group.

Håkan, Karin, Noori and Maite, my Co-supervisors. Thanks for the support and the kind positive words.

A big thanks to past and present members of the Massoumi's group: Kristofer, Raji, Tamae, Hengning, Kasia, Karna and Alissa for creating a relaxed working atmosphere and for the helpful discussion and advice during our group meetings.

Co-authors. Thank you for all your comments and input on the manuscript and thank you for sharing knowledge and data.

The past and present members of CMP and TCR. You made the years as a PhD student a pleasant time. Also, I got a lot of help from many of you with a lot of techniques used in this thesis.

Sofia, Marica, Sophie, Caro B and Susann. Thank you for all the laughters and nice scientific / non scientific conversations throughout the years.

Kristin and Elisabeth. Thanks for your administrative help.

Stephan Bamgurtner. Special thanks for believing in me and giving me the possibility to do my master thesis in your lab. I will be always thankful to you for introducing me to research field.

My friends in Sweden, especially Sayeh, Siker and Omid. Thanks for your friendship, fun and for all the good memories.

Lastly a big thanks to my family for genuine support throughout this time.

Mitra, Goli and Masoud. You are my family in Sweden, thanks for friendship, encouragement and support all these years.

Iraj.You are the one who started me out on this path, and I have thoroughly enjoyed every step of it.

Anoosh. Thanks for your support, love, encouragement and so on. I have learned many positive things from you, specially the hard work and determination.

Afsheed. Special thanks for your truthful laugh, support, care, love and special food. You are such a nice and happiest aunt I have ever seen, thank you for everything.

It is difficult for me to put into words how much I love and cherish my amazing grandmother Hamideh. Your strength, grace and kindness inspires me and so many others. I do not know where I would be without your guidance and care. Thank you for everything.

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