DAXX MITOTIC FUNCTION PLAYS A PIVOTAL ROLE IN CHEMOTHERAPY RESISTANCE AND CANCER PROGRESSION

By

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In loving memory of my mother, Maria Rosaria Migliaccio

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LIST OF ABBREVIATIONS

APC	Anaphase Promoting Complex
BRCA1	BReast CAncer type 1 susceptibility protein
Bub1	Budding Uninhibited by Benzimidazoles 1
Bub3	Budding Uninhibited by Benzimidazoles 3
BubR1	Bub1-Related kinase
Cdc20	Cell-Division Cycle protein 20
Cdc27	Cell-Division Cycle protein 27
CHFR	CHeckpoint with Forkhead and Ring finger
Daxx	Death domain associated protein
DHB	Daxx Helical Bundle domain
DMSO	Dimethyl Sulfoxide
DUB	De-Ubiquitylating enzyme
EDTA	Ethylenediaminetetraacetic Acid
FACS	Fluorescence Activated Cell Sorting
HEp2	Cell line derived from human larynx carcinoma
H1299	NSCLC cell line, p53 null
HCT116	Colorectal carcinoma cell line
HDAC2	Histone Deacethylase-2
IHC	Immunohistochemistry
IP	Immunoprecipitation
JNK	Jun N-terminal kinase
MCF10A	Not transformed breast epithelial cell line
Mad2	Mitotic Arrest Deficient-2
MCC	Mitotic Checkpoint Complex

MDM2	Murine Double Minute-2
MN	Micronuclei
NMR	Nuclear Magnetic Resonance
NSCLC	Non Small Cell Lung Carcinoma
p53	Tumor protein 53
PTEN	Phosphatase and TENsin homolog
SAC	Spindle Assembly Checkpoint
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAP	Tandem Affinity Purification
Ub	Uquitin
USP7	Ubiquitin Specific processing Protease-7
USP7i	USP7 Inhibitor

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Breast cancer accounts yearly for 200,000 newly diagnosed American women and loss of 40.000 of them, representing the number one killer in middle-aged women. Taxanes are considered among the most active chemotherapeutic agents for breast cancer treatment however intrinsic and acquired resistances to taxanes, limit the successful therapeutic outcomes of breast cancer patients. Thus it is crucial to identify mechanisms of resistance and predictive markers that would allow differentiation of patients in taxane responders or non-responders to improve patients overall and cancer-free survival.

While seeking for additional markers of taxane response, we identified the novel role of protein Daxx in taxane sensitivity in experimental models and, importantly, in breast cancer patients (Chapter 3). Daxx, a ubiquitously expressed nuclear protein with relevant roles in transcription and cancer progression, acts as a mitotic checkpoint protein that ensures cell death upon exposure to taxanes. We have demonstrated that this function is mediated by Daxx mitotic partner Ubiquitin Specific processing Protease-7 (USP7). We found that USP7 has a crucial role in mitotic progression, regulating stability of mitotic checkpoint proteins CHFR, Aurora A kinase and Bub3. Results

described in this dissertation show that decreased USP7 expression impairs response to taxanes in cancer cell lines (Chapter 4).

In addition, here we show that Daxx-USP7 complex is fundamental for faithful chromosomes segregation in daughter cells. Decreased expression of USP7 leads to accumulation of several mitotic abnormalities and ultimately to aneuploidy, as we previously observed for Daxx (Chapter 5).

In conclusion, this work identified a new biological role of Daxx and USP7 in mitosis where loss of these proteins can contribute to cancer initiation/progression and development of chemotherapy resistance.

Thus, the study presented in this dissertation proposes Daxx and USP7 as predictive markers for taxane chemotherapy response to allow proper patient's stratification. In addition, by explaining one of the mechanisms of taxane resistance, this study allows rational choice of alternative therapeutic strategies to defeat breast cancer.

CHAPTER 1
INTRODUCTION

Breast Cancer

"Breast cancer" refers to an abnormal and malignant growth within the breast tissue. It is the oldest described tumor in humans, as cases of breast cancer were documented in an ancient Egyptian document. The "Edwin Smith Papyrus", which dates *circa* 1650 BC, is part of a medical treatise that among 48 cases of trauma surgery [1], describes 8 cases of breast tumors, referred as ulcers, treated by cauterization. In the manuscript also appears the comment that "there is no cure" for this disease.

Indeed the belief that breast cancer was incurable dominated among physicians until the 19th century until when advances in surgical practices - concomitantly to introduction of clinical microscopy and chemotherapy - allowed the increase of the survival of breast cancer patients [1]. However, in spite of the enormous progress achieved in the last 20 years in treatment and detection, breast cancer still represents the number one killer of middle-aged women worldwide (American Cancer Society).

Most commonly, breast cancer originates from epithelial cells localized in the breast ducts, tubular structures that connect the lobules to the nipple to secrete milk (ductal carcinoma), or less frequently from cells lining the lobules or milk glands (lobular carcinoma).

At the early stages, breast cancer is asymptomatic. Therefore, the diagnosis of breast cancer starts with detection of anomalies, generally a lump, during either clinical or a self breast examination, which is then confirmed by radiological exam (mammogram) and breast biopsy. At the later stages symptoms appear such as changes in the size, shape, or feel of the breast or nipple. In advanced breast cancer,

skin ulcers, leak of fluids from the nipple and pain or discomfort in the breast or armpit area may also appear.

Epidemiology and Risk Factors

About 1 in 8 U.S. women (just under 12%) will develop invasive breast cancer over the course of her lifetime. Every year, breast cancer accounts for about 200.000 newly diagnosed American women and a loss of more than 40.000 of them, representing the second most deadly cancer (American cancer society). According to the disease state, the 5 year survival is very different: for a non-invasive (or in-situ) tumor the survival rates are the highest, about 97%; they are decreased to 78% for a local invasive tumor and are down to 22% for an invasive/metastatic cancer. These numbers highlight the importance amongst middle aged women of conducting routine screening.

The incidence of breast cancer is 100 times higher among women than men. This is mainly due to hormonal susceptibility of the female breast tissue and the changes in breast mass due to menarche, pregnancies and menopause. Indeed the odds to being diagnosed with breast cancer are highly connected to 'reproductive' risk factors such as early menarche, late menopause, use of contraceptives, first pregnancy after age 30 and breastfeeding history.

Beside sex, there are other genetic factors that may increase the odds of being diagnosed with breast cancer. About 10% of all breast cancer cases have an inherited component [2]. The first cause of familial breast cancer (about the 3% of hereditary breast cancer [2]) is attributable to germline mutations in breast cancer susceptibility protein type 1 and 2 (BRCA1 and BRCA2) genes normally involved in DNA damage response and double strand brake repair [3]. Women that are diagnosed with mutations in BRCA1 gene have a 51% risk of breast cancer and 25% risk of ovarian cancer by age 50, while

mutations in BRCA2 gene confer a 63% risk of breast cancer by age 70 [4]. Other genetic conditions associated with higher risk of breast cancer include the Li-Fraumeni syndrome-1, in which patients harbor germline mutation of the *TP53* gene [5, 6]. A number of other factors increase the risk of breast cancer such as mutation of genes ataxia telangiectasia mutated (*ATM*) [7] and phosphatase and tensin homolog (*PTEN* which causes the Cowden disease) [8]. Loss of these genes usually correlates with poor tumor prognosis.

Also 'environmental' risk factors are associated with breast cancer, such as obesity, poor diet, smoking, alcohol consumption, exposure to chemicals (pesticides, antibiotics or hormones) and ionizing radiations.

Prognostic and Predictive Factors

For the successful treatment of breast cancer patients, it is fundamental to have available established prognostic and predictive factors to help clinicians select an optimal treatment option for a given patient. While prognostic factors may be able to predict the outcome and recovery from the disease, predictive factors instead are indicative of response to the therapy.

The major prognostic factor for early breast cancer is the lymph node involvement [9]. A patient with negative nodes has the highest 5 year survival rate but it progressively decreases as the number of positive nodes increases [10]. Of high clinical relevance Her2/neu and hormone receptor statuses (estrogen or progesterone receptors –ER or PR) have predictive and prognostic features [9]. The presence of ER or PR (about 45% of patients are ER and PR positive and about 30% are ER and PR negative) is indicative of higher 5 year survival rates; however it is also an indicator of higher chances of cancer recurrence. An ER or PR positive tumor will be also responsive to hormonal

therapy (eg. tamoxifen). Similarly, the Human Epithelial growth factor Receptor-2 status (Her2), which is over-expressed in 20-25% of invasive breast cancer, is usually an indicator of tumor aggressiveness and poor outcome. Patients with tumors positive for Her2 are predicted to be responsive to DNA damaging agents. Her2-dependent tumors can also be treated with a humanized monoclonal antibody (Trastuzumab or Herceptin®) against Her2 which inhibits the proliferation and survival of tumor cells.

In spite of the success of these prognostic and predictive markers, it is still difficult to identify patients that are responsive to chemotherapy. Particularly, there is a need to predict response to therapy among women with triple negative disease (ER, PR and Her2 negative).

Breast Cancer Treatment

After breast cancer diagnosis, the treatment chosen for each patient will be assessed according to tumor stage and the patient's genetic background and it will include surgery (if tumor is operable), chemotherapy and/or radiotherapy.

For patients with *in situ* breast cancer a lumpectomy, single or even double mastectomies (for patients with familial breast cancer) are among the surgical options. Radiation therapy may also be recommended after lumpectomy or mastectomy.

Chemotherapy represents the only therapeutic approach for inoperable breast cancer and it is the preferred treatment option for operable breast cancer patients either before or after surgical removal of the tumor.

Taxane Chemotherapy

A number of agents with established cytotoxic activity are used in therapy; taxanes (paclitaxel (or Taxol®) and docetaxel (Taxotere®) are considered among the most active [11].

Taxol® was first isolated from the Pacific yew, *Taxus brevifolia*, in 1962. Its antitumor activity was immediately recognized, but due to the extremely low yield of drug obtained from the tree and the poor solubility, the interest on the efficacy of this molecule was slow to rise [12]. As the xenograft mouse model was introduced, the efficacy of Taxol® in killing tumors prompted the introduction of this drug in clinical trials in 1983 [13]. The successful entry of paclitaxel into clinical trials boosted an interest in understanding the mechanism of taxane-induced cell death. Work done from Dr. Horwitz uncovered that paclitaxel had a unique mechanism of action as it was found to act on microtubules [14, 15]. However, later on, it was recognized that proteins targeted by this treatment, went beyond tubulin, acknowledging that downstream targets of taxanes include mitotic checkpoint proteins [16]. After the semi-synthesis of Taxol® was achieved, the use of this drug was approved for treatment of refractory ovarian cancer. From 1994 Taxol® has been employed as chemotherapic agent for breast ovarian, colon and lung malignancies.

In 2003 the American Chemical Society established that the discovery of Taxol® was a National Historic Chemical Landmark as life-saving compound in oncology [13].

Taxanes Activity

Although taxanes are successful in the selective killing of tumor cells in clinical settings, current understanding of how this happens is controversial and incomplete. For a long time, apoptosis had been considered the main mechanism of cell death in response to taxane treatment. Currently, several distinct models of therapy response are recognized [17-19], wherein different modes of tumor cell death are likely determined by drug concentration [20] and genetic background of the cells within a tumor [21].

Taxanes bind to microtubles and inhibit their depolymerization and functions in both mitosis and interphase [14, 15, 22]. At pharmacological concentrations, taxanes reversibly bind to a subunit of the tubulin heterodimers which forms microtubules; this accelerates polymerization and inhibits the depolymerization of tubulin, disrupting microtubule dynamics. This event, in turn, activates a mitotic checkpoint which invokes mitotic arrest [23]. This mitotic arrest does not persist indefinitely. After some period of time, cells usually undergo an aberrant exit from mitosis, characterized by the lack of metaphase, anaphase and cytokinesis. The nuclear envelope is re-formed around individual chromosomes or groups of chromosomes producing large nonviable cells with multiple micronuclei, which are morphologically distinguishable from apoptotic cells. This type of cell death, known as mitotic catastrophe, is activated during mitosis as a result of deranged spindle formation coupled with blocks of different checkpoint mechanisms thus causing aberrant chromosome segregation and nuclear fragmentation [24-28].

Taxanes and Mitotic Checkpoints

Taxanes inhibit microtubules dynamics. But since the formation of the mitotic spindle, the attachment of kinetochores and correct chromosome partitioning all rely on microtubule dynamics, taxanes are also known as mitotic spindle poisons [29].

The mitotic block observed upon taxane exposure derives from activation of the Spindle Assembly Checkpoint (SAC) [19] which controls metaphase-anaphase transition by ensuring that all kinetochores are correctly attached to spindles before anaphase [23, 30, 31]. When cells are exposed to conditions of prolonged mitotic stress in the presence of these toxins, the SAC is eventually inactivated and cells exit mitosis [32] as micro-nucleated or tetraploid [33]. This abnormal mitotic exit is referred as “mitotic slippage”, or

"mitotic catastrophe" [34, 35] and is dependent upon the ubiquitination and proteolysis of cyclin B and Securin.

Several mitotic checkpoint proteins, including MPS1, Survivin, CHFR, and members of Mad and Bub protein families (Mad1, Mad2, BubR1, Bub1 and Bub3), sense improper tension between kinetochores and microtubules of the mitotic spindle and transmit a signal to inhibit mitotic progression. Inactivation of these checkpoint proteins has been associated with either increases in sensitivity or resistance to taxane treatment [36-40] and for Mad2 and BubR1 findings are controversial [36, 37, 41].

The factors that determine prolongation of mitotic block and, thus, resistance to treatment by taxanes, remain incompletely characterized. Inactivation of CHFR, mitotic-associated E3 ubiquitin ligase [42-44]) which degrades the mitotic kinase Aurora A [45] leads to decreased sensitivity to mitotic spindle poisons [46]. Down-regulation of breast cancer susceptibility gene 1 (BRCA1) by siRNA leads to increased taxane resistance in breast cancer cell line MCF-7 [47]. Another report describes the microtubule depolymerizing drug nocadozole to induce delay in mitotic exit upon depletion of p31comet in HeLa cells. p31comet acts in mitosis by counteracting spindle checkpoint function of Mad2 [48]. Thus, recent efforts have started to link sensitivity of tumor cells to taxane treatment with genetic defects in the cell cycle checkpoints in association with cancer chemotherapy. It has been suggested that inactivation of mitotic checkpoint proteins can contribute to the selective response of taxane treatment in vivo [49]. However, mutations in known checkpoint proteins occur rather rarely [50, 51]; thus broader studies are necessary to search for novel molecular targets of taxanes therapy.

Predictive Markers for Taxane Response

Many cancer patients are resistant or become resistant to Taxol® during drug administration [52-54]. Over the last 30 years, basic research has provided invaluable support in developing therapeutic strategies, but unfortunately it has failed so far to understand how almost half of the patients develop therapy resistance [52, 53].

Predicting and overcoming resistance or incomplete response to these agents would represent a major improvement in the clinical management of breast cancer [55-57]. Factors contributing to taxane resistance include: alteration in expression of tubulin or microtubules-associated proteins [58-60], multidrug-resistance [61, 62], deregulations in numerous cellular pathways [63] such as cell cycle control [64], cell proliferation [65, 66], apoptosis [67] and nuclear-cytoplasmic transport for both proteins and RNA [68, 69]. Numerous studies support the evidence that SAC plays a pivotal role in taxane resistance. Spindle checkpoint defects have been described in increased sensitivity and resistance to paclitaxel and oftentimes results were controversial [36, 40, 41].

Down-regulation of checkpoint proteins CHFR, survivin and Bub1 were shown to increase paclitaxel sensitivity [36, 38, 70]. Conversely , inactivation of mitotic checkpoint proteins which lead to taxane resistance include BRCA1 [47], Mad2 antagonist protein p31[comet, 48] and Bub3 [71]. Among checkpoint proteins, Aurora A kinase has been linked to taxane resistance [72-74]; it is amplified in breast cancer [75-77] and is over-expressed in several tumors [78-83] with poor prognosis [84-86].

Protein Daxx: a Novel Player in Paclitaxel Resistance

Daxx is 120 KDa highly conserved and ubiquitously expressed protein [87].

We have found that sensitivity to paclitaxel treatment in breast cancer cell lines and mouse cells correlates with the level of Daxx, a ubiquitously expressed nuclear

protein. Upon paclitaxel exposure, cells with high levels of Daxx induce a transient mitotic block followed by mitotic catastrophe, while cells with low Daxx are blocked in prometaphase and continue proliferation after drug removal. While screening for Daxx expression in breast malignancy, we documented extensive heterogeneity of Daxx in primary breast cancer specimens.

Studies published from our laboratory showed correlation between Daxx and cellular response to paclitaxel [88]. Cell lines with extreme level of Daxx: T47D (low level of Daxx, Daxx/actin = 1.0) and MDA MB 468 (high levels of Daxx, Daxx/actin = 14.0) behaved very differently when exposed to paclitaxel. Results from colony formation assay showed a high survival rate of T47D cells (low level of Daxx) and low survival rate of MDA MB 468 cells (high level of Daxx) at all time-points tested. Thus, these data demonstrated that low levels of Daxx correlate with increased resistance to paxlitaxel treatment in breast cancer cell lines. To further confirm this correlation, Daxx was depleted in MDA MB 468 breast cancer cells and HEp2 human epithelial carcinoma cells by stable expression of anti-Daxx shRNA. Depletion of Daxx by shRNAs reproduced the original finding that level of Daxx is critical for paclitaxel response [88].

To this end it is essential to examine the function of Daxx as a novel mitotic checkpoint protein that determines sensitivity to paclitaxel and can be used as a predictive marker in selection of breast cancer patients to receive taxane therapy. In addition, it will be important to determine the mechanism of this sensitivity by elucidating the role of Daxx in mitotic progression. Identification of Daxx as a novel mitotic checkpoint release protein which determines resistance to taxanes, will aid in proper selection of patients to receive this therapy and contribute to our understanding of

mechanisms that connect cell division, genome instability and breast cancer progression.

Daxx: an Enigmatic and Controversial Protein

The first description of protein Daxx dates back in 1997 when Yang *et al.* identified Daxx as a Fas "death-domain associating protein" [89] in a yeast two-hybrid screen. The interaction between Daxx and the cytosolic portion of Fas was reported to trigger a FADD-independent activation of the Jun N-terminal kinase pathway, which in turn activated apoptosis. This report challenged the apoptosis field that not all the signals from the Fas receptor were mediated and translated in the cell by FADD. However reports which followed were unable to reproduce the same results [90]. Data collected in mouse embryonic fibroblasts which were deficient in FADD or caspase-8 could not activate apoptosis thus suggesting that Daxx did not elicit a FADD independent pathway [91, 92]. Most importantly other groups failed to reproduce Daxx and Fas interaction reporting also that the apoptotic activation prompted by Daxx over-expression was not mediated by JNK [93]. Contradictory evidence on the pro-apoptotic function of Daxx were also supported by the *in vivo* work done by the Leder group on Daxx knockout mouse. Loss of Daxx caused embryonic lethality by day 8.5-9, but quite unexpectedly it was not marked by proliferation abnormalities as lack of a pro-apoptotic protein would. Conversely, Daxx knockout was characterized by extensive apoptosis, supporting instead an anti-apoptotic function of Daxx [94, 95].

In addition, to challenge the initial findings, accumulating evidence, given by biochemical fractionations and immunofluorescence experiments, described Daxx as a nuclear protein [93, 96] with obvious ND10/PML body association in interphase cells [96].

Daxx binds to sumoylated PML via its two independent SUMO interaction motifs (SIMs) that results in Daxx accumulation in the ND10/PML nuclear bodies [97, 98].

In addition Daxx was found to co-localize at heterochromatin loci with ATRX a chromatin remodeling enzyme in a cell cycle dependent manner [99]. Thus, these results raised the question on how a nuclear confined protein such as Daxx could trigger apoptosis by interacting with Fas, a membrane embedded protein.

Indeed, Daxx localization has been object of a second diatribe. Using cellular biochemical fractionation of NIH-3T3 fibroblasts, the Lalioti group showed that the majority of endogenous Daxx accumulated in the nuclear compartment while a minor part was detected in low-density microsomes [100]. They also confirmed these finding by immunofluorescence staining, thus assuming that two distinct intracellular Daxx pools may exist: one in the nucleus and another in the cytosol within low density microsomes.

Intracellular localization of Daxx has also been attributed to shuttling between cellular compartments in response to stresses [101-105] mainly to guarantee cell survival.

Daxx was reported to re-locate from the nucleus to the cytoplasm during glucose deprivation [105, 106], oxidative stress [105] and chemical hypoxia [102]. These re-localization studies however were mostly done with over-expressed protein and were not supported by following studies. The first conflicting report showed *via* biochemical separation that Daxx remains in the nucleus after exposure to hydrogen peroxide or UV treatment [107]. The second, that we undertook, was done to analyze endogenous Daxx cellular localization using several cell lines, including those that were reported to display cytoplasmic Daxx accumulation. None of the cell line, challenged with same or harsher stresses than those published, showed any redistribution of Daxx from the nuclear to

cytosolic compartment by any means tested (immunofluorescence, time-lapse microscopy and biochemical fractionation) [108]. Thus, while there is some tantalizing evidence to suggest existence and function of Daxx in the cytoplasm, more extensive studies of endogenous protein trafficking are required.

Daxx spatiotemporal relocalization appears to occur within the nucleus in a cell cycle dependent manner [99] and in response to some stresses[109]. At the end of S-phase Daxx is relocalized from ND10 nuclear bodies to condensed heterochromatin by phosphorylated ATRX, a chromatin remodeling protein [99]. The biological role of this redistribution is still under investigation. It has been show that Daxx, along with other ND10-associated proteins can be released from ND10 into the nucleoplasm during heat shock and heavy metal exposure [109].

Daxx has also been known to interact with the CENtromeric Protein-C (CENP-C) and relocalize to centromeres [110, 111]. The function of this interaction has been recently uncovered in mouse cells [112] and will be discussed below.

In summary, Daxx localization within the nuclear compartment has been confirmed by a number of reports [113]. Through its two independent SUMO interaction motifs (SIMs) Daxx binds to sumoylated PML in the ND10/PML nuclear bodies [97, 98].

Daxx Structure

Since its discovery, it was apparent that Daxx plays important roles in regulating a wide array of functions even if amidst some controversy. To orchestrate these cellular processes, spanning from transcriptional regulation to antiviral response which will be discussed in the next paragraphs, Daxx interacts with a long list of proteins (more than 50). However, the molecular mechanisms on how Daxx contributes to these disparate cellular functions, is unknown, mainly because the structural characterization of this

protein is poor. hDaxx is a 740-amino acid protein that was described to contain, a coiled coil region [111], a predicted "Pair of Amphiphatic Helices" (PAH) [114] and two SUMO interacting motifs (SIM) [97, 98]. These poorly characterized domains have been used to set boundaries to design deletion constructs for Daxx to probe interaction with over 50 putative partner proteins [113]. Future work was undoubtedly necessary for detailed and proper characterization of Daxx protein structure.

DHB Domain Characterization of Daxx

In collaboration with Dr. McIntosh, University of Vancouver Using NMR spectroscopy, we have demonstrated that the C-terminal half of Daxx is intrinsically disordered, whereas a folded domain is present at its N-terminus. This domain forms a left-handed four-helix bundle with topology which differs from the Sin3 PAH domains. This Daxx Helical Bundle (DHB) domain interacts with the N-terminal residues of the tumor suppressor Ras-association domain family 1C (Rassf1C) which in turn folds into an amphipathic a-helix upon binding the Daxx domain. Indeed Daxx was reported to be able to recruit/sequester Rassf1C [115] in ND10 upon Rassf1C over-expression and the two proteins were shown to interact and regulate mitosis [116].

Based on a proposed Daxx recognition motif as hydrophobic residues preceded by negatively-charged groups, we found that peptide models of p53 and MDM2 also bound the DHB domain [117]. This provides a structural foundation for understanding Daxx functions and molecular interactions.

Daxx a Multifunctional Protein

As described above, Daxx was identified as a proapoptotic Fas-interacting protein [89] and later demonstrated to have anti-apoptotic activity [94, 95, 118], is a ubiquitously expressed and highly conserved nuclear protein that also possesses intrinsic

transcription repression activity [119]. Indeed the best characterized Daxx function is that of transcription co-repressor [120]. Daxx does not bind directly to DNA but only indirectly [113]. In several reports Daxx was described to be recruited through its SUMO interaction motifs, with sumoylated transcription factors [121-123] or chromatin modifiers such as HDAC2 [124] and DNA methyltransferases [125]. Some of these examples will be described in the next paragraphs.

Daxx regulates also stability of p53 E3-ligase MDM2 through binding of Deubiquitinating Enzyme USP7 (also known as HAUSP) and hence can regulate p53-mediated apoptosis. According to the work initially published by Tang *et al.* [126], Daxx ultimately prevents MDM2 self-ubiquitination, enabling this E3 ligase to target p53 for proteolytic degradation [126, 127]. Upon DNA damage Daxx-USP7-MDM2 complex dissociates so that MDM2 is destabilized by self-ubiquitination and proteolitic degradation. This leads to stabilization and activation of p53.

Daxx has also been described to interact with the CENtromeric Protein-C (CENP-C) and localize to centromeres [110, 111]. The functional significance of Daxx association with CEN/periCEN was dim until recent findings which identified Daxx-containing complex as a novel chaperone for histone H3.3 [128, 129]. This transcription-associated variant of histone H3 was recently shown to be enriched in repetitive regions of genome including telomeric and pericentromeric loci [129]. Several studies have shown that Daxx-mediated incorporation of H3.3 into MaSat in mouse cells correlates with transcription elevation from this region of genome [128] suggesting Daxx role in maintenance of heterochromatin structure at these genomic loci [128].

Daxx Role in Mitosis

Despite extensive characterization of Daxx intranuclear localization during interphase [90, 99] little is known about the localization of this protein in mitosis. It has been previously reported that the major Daxx housing domain in interphase, ND10/PML bodies, undergoes dramatic changes during mitosis with number of domains decreased [130-132] and protein composition of domains changed. ND10/PML bodies become depleted of Daxx, Sp100 and SUMO-1 concomitantly with a reported hyperphosphorylation of the PML protein [110, 133]. As cells leave mitosis and re-enter G1, ND10 reform their interphase protein composition with Daxx reappearing at this domain [110]. To analyze the localization of Daxx during mitosis, mouse cells were stained with Daxx antibodies, and DNA counterstained with Hoechst 33258. Localization of Daxx was evaluated and documented relative to stages of mitosis as determined by the degree of chromatin condensation and chromosome localization. In prophase Daxx is accumulated in a dot-like pattern characteristic of ND10-the major site of Daxx localization during interphase. The main transition of Daxx localization occurs when cells progress into prometaphase. Instead of a dot-like localization, Daxx is visible in a pattern characteristic of mitotic spindles, where it remains during the end of prometaphase and part of metaphase (characterized by aligned chromosomes). Once cells proceed into anaphase (chromosome separation pattern), Daxx disappears from the mitotic spindle and remains diffuse during the end of mitosis [134].

Another indication that Daxx may participate in mitosis is the above mentioned interaction between Daxx and the intrinsic kinetochore component CENP-C [111]. Depletion of Ams2, a Daxx-like motif-containing GATA factor in S. pombe results in chromosome missegregation [135].

We found that Daxx$^{-/-}$ embryos are developmentally retarded by day 8 and completely disintegrated by day 11.5 [99]. DNA staining of day 9.5 Daxx$^{-/-}$ embryos revealed accumulation of mitotic cells in prometaphase and high level of apoptosis, while a dismal level of apoptotic cells and all mitotic stages were observed in Daxx$^{+/+}$ embryos. Daxx$^{+/+}$, Daxx $^{+/-}$ and Daxx$^{-/-}$ cell lines were collected from day 9.5 embryos; karyotyping of Daxx$^{-/-}$ cells revealed high aneuploidy and genomic instability. All three tested Daxx$^{-/-}$ cell lines were tri/tetraploid, with high heterogeneity in chromosome number between five metaphases analyzed for each of these cell lines. In general, a high level of genomic instability and aneuploidy is often observed upon depletion of mitotic checkpoint proteins [23, 136], confirming a potential function of Daxx in mitosis progression indicating prometaphase as a potential stage of Daxx activity.

Daxx Role in Carcinogenesis

Several reports show a connection with Daxx expression and carcinogenesis. Daxx over-expression has been documented within the stroma of prostate cancer samples [137]. The role of Daxx in prostate cancer may be explained by Daxx functions as a co-repressor of the androgen receptor (AR) a hormone receptor essential for the homeostasis of prostate tissue [138]. It was shown that Daxx can also negatively influence AR transcription activity in colon cancer cells [139] in a SUMO-dependent manner.

Daxx decreased expression was found in tumor specimens of adenocarcinoma. The authors reported that this phenomenon can be explained by the loss of Daxx regulation of proliferation and differentiation in colon cells [140].

Another example of Daxx involvement in tumorigenesis, involves the proto-oncogene c-met, in which over-expression is documented in tumors, including breast cancer, and elevates metastatic potential. Our laboratory demonstrated that Daxx is a

repressor of c-met transcription via HDAC2 recruitment/stabilization on c-met promoter. Inverse correlation between Daxx and c-Met in metastatic breast cancer specimens suggests potential function of Daxx as a c-met repressor during cancer progression [120]. Thus, in breast cancer pathogenesis, reduced Daxx not only elevates taxane resistance [88], but also accelerates metastatic progression via c-met up-regulation.

A recent report also documented somatic mutations of Daxx gene in 25% of patients affected by pancreatic neuroendocrine tumors (PanNETs) [141]. Among PanNETs the authors found collectively 43% of tumor with mutated Daxx or its partner ATRX. Due to the reported activity of Daxx/ATRX complex in chromatin remodeling at repetitive G-rich sequences, like telomeres [112, 142], subsequent work was done to analyze telomere length in PanNETs [143]. This study found that Daxx and ATRX mutations positively correlate with long telomeres, which were extended by alternative lengthening of telomeres (ALT), a mechanism independent by the enzyme telomerase [143, 144].

Taken together, these studies highlight Daxx role as tumor suppressor; further studies are needed to better characterize Daxx function in the process of carcinogenesis.

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CHAPTER 2
MATERIAL AND METHODS

Cell Culture

HEp2, H1299 and HCT116 parental or p53-/- cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco BRL, Carlsbad, CA) and grown in a humidified 5% CO2 incubator. MCF10A were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were cultured under recommended conditions. Taxol® (Paclitaxel; Sigma, St Louis, MO, USA; 100 mM in dimethyl sulfoxide) was used at a final concentration of 10 nM. Thymidine (Sigma) was dissolved in 1N NaOH for 1M stock and used at a final concentration of 2 mM. MLN8054 (Millennium Pharmaceuticals Inc., Cambridge, MA) was dissolved in DMSO at 5 mg/ml and used at 4 µM final concentration.

Immuno-precipitation and Mass Spectrometry Analysis

Cells synchronized by DTB and blocked in 10nM Taxol® were lysed 30 minutes at RT in lysis buffer consisting of 50 mM Tris-HCL (pH 7.45), 150 mM NaCl, 1mM EDTA, 1% Triton X-100, in presence of 10 mM N-ethylmaleimide (Sigma), 5 mM iodoactetamide (Sigma), 1 mM phenylmethylsulfonylfluoride (Calbiochem, EMD Chemicals, Gibbstown, NJ, USA), 1 mg/mL aprotinin (Sigma), 1 mM leupeptin (Sigma), 1 mM pepstatin (Sigma). Lysate was then pre-cleared by centrifugation at 1800g for 10' at RT and filtration trough 0.45 micron filter (Corning). Pre-cleared lysates (Input) were incubated with preconditioned FLAG magnetic beads for 3 hours at RT on end-over-end rotator. Beads were than washed four times with lysis buffer without protease inhibitors

and eluted by thrombin cleavage (1 enzyme unit, New England Labs) for 30' in lysis buffer.

Protein samples were then analyzed for efficiency of pull-down and co-IP by western blot analysis as described below. For Mass spectrometry analysis samples were loaded on Protean II precast gels 8-16% Tris-HCl (#161-1457, BioRad, Hercules, CA). Bands were then revealed with Novex colloidal blue staining kit (#LC6025, Invitrogen, Carlsbad, CA, USA).

Immunofluoresence

Immunofluoresence analysis was completed as previously described [116]. Cells were fixed, permeabilized and then stained with the following primary antibodies: Daxx 5.14 monoclonal [99], PML 14 rabbit [96], USP7 (Bethyl labs, Montgomery, TX), Aurora A (Cell Signaling Technologies, Danvers, MA) and alpha-Tubulin (Sigma). Then labeling was done using appropriate FITC- or Texas Red-conjugated secondary antibodies (Invitrogen) and HOECHST (Sigma). Images were analyzed using Leica TCS SP5 confocal microscope.

Western Blotting

Protein samples were separated by 4-20% SDS-PAGE (Biorad), transferred to nitrocellulose membranes (Whatman, Dassel, Germany) and blocked with 3% non-fat milk/PBS, 0.1% Tween (PBST). Primary antibodies to Daxx 677 rabbit (in house), USP7 rabbit (Bethyl labs), Cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CHFR (Abcam, Cambridge, MA, USA), Aurora A (Cell signaling), Bub3 (BD Transduction Laboratories, Franklin Lakes, NJ, USA), Actin (Sigma) or were diluted in 3% milk/PBST and incubated overnight at 4°C. Membranes were then washed 3X with PBST for 1 hr at RT with appropriate secondary antibody (Millipore, Billerica, MA, USA;

all 1:2,500). Membranes were then washed with PBST and exposed using ECL reagent (Amersham, GE Healthcare, Pittsburg, PA, USA). Densitometry analysis of cyclin B or CHFR and actin western blots was performed using the Quantity One software from Bio-Rad.

Transient and Stable Depletions

For transient siRNA transfections smart pools were purchased from Dharmacon and employed according manufacturer instructions.

For stable depletion of HEp2, H1299 and MCF10A cells a lentiviral expression system kindly provided by Peter M. Chumakov (Lerner Research Institute, Cleveland [145] was used as previously described [116]. shRNAs and Daxx [116], for control and USP7 were designed according to the Dharmacon si*DESIGN* algorithm. control shRNA was directed against base pairs 1262-1284 of SETDB1 (TCCTCTTTCTTATCCTCGTATGT).

Time Lapse Microscopy

Time lapse imaging of cells was performed according to [146]. Briefly, control and Daxx-depleted HEp2 cells were stably transfected with GFP-histone H2B (gift of Dr. Duane Compton, Dartmouth) and analyzed by Leica TCS SP5 confocal microscope equipped with environmental chamber; images were taken every 2 min. Mitotic stages were determined by three hallmark events including 1) first indication of chromatin condensation marked as late G2/prophase transition (T=0); 2) invagination of the nucleus marking the prophase/pro-metaphase transition; and 3) beginning of chromosome segregation marking the metaphase/anaphase transition. Three experiments were completed for each shRNA group with an average of 20-30 cells per experiment.

APC Assay

Cellular pellets were resuspended in lysis buffer (20mM Tris-HCl, pH 7.2, 2mM DTT, 0.25mM EDTA, 5mM KCl, 5mM $MgCl_2$) on ice and subjected to 1,500psi N_2 in a nitrogen disruption chamber. The lysate was spun for 15min at 15,000g. Supernatants were divided into single use aliquots and flash frozen in N_2. For assays, extracts, on ice, were supplemented with an energy regenerating system (30U/mL rabbit creatine phosphokinase type I, 7.5mM creatine phosphate, 1mM ATP, 1mM $MgCl_2$, 0.1mM EGTA), non-destructible cyclin B, and cycloheximide. Proteins were then added in a final volume of 14mL. ^{35}S-labeled substrate (1mL) was added; aliquots were made and shifted to 30°C. Samples were quenched at the indicated times by the addition of sample buffer, resolved by SDS-PAGE and imaged using a Typhoon phosphorimager (GE Healthcare).

Mitotic Stages Assessment

Cells were transiently depleted by control or USP7 siRNAs (Dharmacon, Thermo Fisher Scientific, Waltham, MA, USA). 72h post-transfection cells were fixed and than DNA stained with hoechst 33342. Mitotic stages were determined by microscopy and were categorized as prophase-prometaphase (P), metaphase (M) or anaphase (A) or telophase (T) according DNA morphology. Mitotic stages assessment for each sample was conducted counting at least 100 mitotic events *per* experiment.

Micronuclei Scoring

Cells stably depleted by control or USP7 shRNAs were grown on coverslips than fixed and stained for DNA according to immunofluorescence procedure. The criteria adopted for of micronuclei (MN) scoring were previously described [147, 148]. MN score represent the number of MN *per* cell. For each sample at least 300 cells were counted.

Colony Formation Assay

Cells exposed to control or 10 nM Taxol® were originally plated on 3.5 cm dishes (Corning) for treatment. Following exposure, cells were trypsinized and re-plated (in triplicates) at 1:1,000 dilution on six well plates (Corning, Lowell, MA) for colony formation analysis. Five to seven days afterwards, colonies were stained with crystal violet and counted.

Immunohistochemistry

For this study, twenty two women were identified with locally advanced HER-2 non-amplified breast cancer that were treated with standard taxane/anthracycline based neoadjuvant chemotherapy at H. Lee Moffitt Cancer Center. Initial core biopsy was performed for the diagnosis and all patients underwent neoadjuvant chemotherapy followed by definitive surgery with either lumpectomy or mastectomy and axillary lymph node dissection. Two patients had invasive lobular carcinoma, one patient papillary carcinoma, while the remainder had invasive ductal carcinoma. Four tumors were hormone receptor negative and eighteen tumors were hormone receptor positive. Tissue blocks were obtained from initial biopsy (prior to neoadjuvant chemotherapy) to perform immunohistochemical staining for Daxx. Slides were de-paraffinized with xylene and re-hydrated through decreasing concentrations of ethanol to water, including an intermediary step to quench endogenous peroxidase activity (3% hydrogen peroxide in methanol). For heat-induced antigen retrieval, sections were heated in a water bath at 95°C while submerged in Trilogy buffer (Cell Marque, Hot Springs, AR) for 25 minutes and afterwards incubated with a universal protein blocker Sniper (Biocare Medical, Walnut Creek, CA) for 15 min, RT. Monoclonal mouse anti-Daxx 5.14 was added o/n at RT. Mach 2 goat anti-mouse-horse radish peroxidase-conjugated (Biocare Medical,

Walnut Creek, CA) was then added for 30 min, RT. Detection of Daxx was achieved by incubating slides in 3'3' diaminobenzidine (Biocare Medical, Walnut Creek, CA) for 15 min, RT. Slides were counterstained with hematoxylin (Vector Laboratories Inc., Burlingame, CA) for 10 sec and mounted with Cytoseal XYL (Richard-Allen Scientific, Kalamazoo, MI). Slides were analyzed using Leica DM2000 microscope and pictures were taken using Leica DFC480 CCD camera with Leica FireCam 1.7.1 software. For each specimen, at least one thousand cells were examined for Daxx expression, and the number of cells with an evident signal were recorded and categorized by the intensity of staining (0 for undetectable, 5 for highest) of Daxx multiplied by the percent of staining cells (Daxx score).

Mouse Xenografts

HEp2 xenografts were generated in Nu/Nu mice by subcutaneous injection of 5 x 10^6 HEp2 cells containing a 1:1 mixture of matrigel (BD Bioscience)/DMEM suspension. Tumors were monitored daily and grown to a volume of approximately 150 mm^3 (day 7-9 after cell injection) before drug treatment. Vehicle (1 part of 1:1 solution of 50% EtOH/Cremaphor EL (Sigma) to 10 parts of PBS) or 20 mg/kg Taxol® (LC Laboratories, Woburn, MA; Paclitaxel stock = 25 mg/mL dissolved in EtOH/Cremaphor EL) was injected intraperitoneally (IP) every second day for a total of five injections. Up to fifteen animals were used for each experimental group. Tumor volume was measured by calipers and calculated on a daily basis using the formula $V= (1/6) \pi a(b)^2$ where (a) and (b) are the measured length and width (millimeters) of the tumor, respectively. Increases or reductions in tumor size were determined according to the relative initial tumor size beginning on day one of injection. Experiment was terminated at day 15 of first drug injection or when tumor volume reaches 1000 mm^3.

Metaphase Spreads and Karyotyping

HEp2 and H1299 cells stably expressing control or USP7 shRNAs were treated with 50 ng/mL colcemid (Invitrogen, Carlsbad, CA) for 2 hours. Similarly, MCF10A cells were treated with 50 ng/mL colcemid for 16 hours before proceeding with metaphases preparations. Cells were collected and resuspended in a hypotonic solution of 2% KCl and 2% $Na_3C_6H_5O_7$ for 7 minutes at 37°C. Metaphase spreads were then prepared and stained with Giemsa-trypsin (G-band) procedure. Analysis was carried out using the OLYMPUS BX41 microscope equipped with a BASLER scA1400-17gmASI digital camera. Images were analyzed using the Applied Spectral Imaging (ASI) software V7.0.6.8860. Unless stated differently, for each experiment at least one-hundred metaphases for each sample were counted.

CHAPTER 3
CELLULAR LEVELS OF DAXX CORRELATE WITH TAXANE RESISTANCE

Introductory Remarks

Taxanes, a group of cytotoxic drugs which includes paclitaxel (Taxol®) and docetaxel (Taxotere®), are among the most successful anticancer agents for breast cancer chemotherapy [11, 18, 149]. Taxanes affect microtubule stability, but mutations or alterations in tubulin occur very rarely in cancers [150]. Taxane activity affects mainly mitotic checkpoints [16, 151] and leads to a prolonged arrest of cells in mitosis, that will eventually trigger cell death [21] by a mechanism still largely unknown [17, 152-154]. Currently, many mitosis-related proteins have been scrutinized for their ability to affect taxane sensitivity, but like tubulin, many of these proteins are unaltered in cancer and cannot explain the majority of drug resistance seen in patients exposed to these compounds.

Many breast cancer patients are resistant or become resistant to taxanes during drug administration [52-54]. A number of studies have been carried out to determine a genomic profile that could be predictive for taxane treatment [63, 155-158]. Despite the extensive efforts toward identification of predictive markers for taxane treatment in breast cancer, the clinical applications of results have been limited. This was partly due to lack of reproducibility of methods among several groups [159] but also to the fact that the loss of checkpoint genes involved in mitotis and taxane response rarely occurs in cancer [50, 51]. In addition, these approaches do not account for the fact that regulation of mitosis occurs largely at the post-translational level [160].

Giovinazzi S, Lindsay CR, Morozov VM, Escobar-Cabrera E, Summers MK, Han HS, McIntosh LP, Ishov AM. Regulation of mitosis and taxane response by Daxx and Rassf1. Oncogene. 2012 Jan 5;31(1):13-26. doi: 10.1038/onc.2011.211.

Thus, predicting and overcoming resistance or incomplete response to these agents would represent a major improvement in the clinical management of breast cancer [55-57].

Functional screens have been directed at finding novel targets affecting sensitivity to Taxol® and other compounds but it remains unclear whether these targets play a direct role in Taxol® sensitization or offer prognostic value to clinicians [161]. The existence of a mitotic stress checkpoint(s), separate in function from the SAC has been proposed in regard to cells that have been exposed to spindle toxins like Taxol® [46]. Thus, it is essential to identify these mitotic guardian proteins or pathways involved in Taxol® sensitization/resistance because foreknowledge of these targets may prove useful for proper selection of patients for taxane-based chemotherapy. To this end, we sought to further understand the phenomenon of Daxx-dependent Taxol® resistance related to mitosis and to determine its importance in tumors subjected to this type of chemotherapy.

Intrinsic and acquired resistances to taxanes represent the most limiting factors to the successful treatment of breast cancer patients.

Exploring the mechanism of Daxx-based resistance, we observed that, upon Taxol® treatment, cells with reduced level of Daxx remain in a prolonged mitotic arrest and complete cell division after Taxol® removal, while cells with high levels of Daxx exit from Taxol®-induced mitotic block as micro-nucleated cells incapable for proliferation in cell culture settings [88, 116].

Daxx is a highly conserved and developmentally essential nuclear protein [94, 99, 108]. Daxx is involved in numerous cellular processes such as transcriptional regulation [113],

anti-viral immunity [162], apoptosis [90, 95] and carcinogenesis [163, 164]. Exploring the mechanism of Daxx-based resistance, we observed that, upon Taxol® treatment, cells with reduced level of Daxx remain in a prolonged mitotic arrest and complete cell division after Taxol® removal, while cells with high levels Daxx exit from Taxol®-induced mitotic block as micro-nucleated cells incapable for proliferation in cell culture and xenograft settings [88, 116].

Results

Duration of Mitotic Stages is Affected in the Absence of Daxx

Resistance to Taxol® was observed in human breast cancer (MBA-MD-468) and human larynx carcinoma (HEp2) cells with experimentally reduced Daxx [88]. To understand the function of Daxx in Taxol® response, we utilized HEp2 cells expressing control or anti-Daxx short hairpin RNAs (shRNAs) (Figure 3-1a). We used this model cell line as taxane-based therapy is one of treatment options in head and neck cancer [165] and given the ability of HEp2 cells to recapitulate the Daxx-dependent Taxol® response observed in breast cancer cell lines as shown previously [88]. HEp2 cells were synchronized using a double thymidine block and released for cyclin B protein level analysis to monitor G2/M/G1 progression. Although control shRNA cells showed destruction of cyclin B by 9 h post-thymidine release, Daxx-depleted cells showed prolonged stabilization of cyclin B at 9.5–11 h post-release, suggesting that Daxx is required for normal mitosis (Figure 3-1b). Next, we studied mitotic progression by time-lapse microscopy in control- and Daxx-depleted cells stably transfected with histone H2B-GFP. The occurrence of chromatin condensation in Daxx-depleted cells was more rapid, indicating faster progression of prophase compared with control cells (Daxx shRNA cells had average 7.5 min and control shRNA average 10.2 min). Contrarily, the

average prometaphase/metaphase timing of Daxx depleted cells (37.6 min) was longer than in control depleted cells (average 31.2 min). No differences in mitotic progression were observed in control shRNA compared with parental HEp2 cells (data not shown). The combination of these data suggests that depletion of Daxx in human cells results in perturbation of normal mitosis, implying that Daxx is necessary for proper mitotic progression.

Cyclin B is Stabilized in Daxx-Depleted Cells Treated with Taxol®

Cells with reduced Daxx display increased resistance to Taxol® treatment because the majority of cells arrest in mitosis for longer period of time (and thus are able to complete normal division upon Taxol® wash-out), while control cells exit mitosis towards micro-nucleated cells (and stop proliferation). A similar effect was previously observed upon cell exposure to low or high Taxol® concentrations [88]. We sought to understand the Taxol® resistance phenomenon more in depth by analyzing the cyclin B levels in synchronized control, Daxx- depleted cells upon 10 nM Taxol® treatment. Whereas cyclin B protein levels decreased by 13 hrs post-release in control cells, it was stabilized in Daxx- depleted cells (Figure 3-2). Stabilized cyclin B upon Daxx depletion is a biochemical indication of cells arrested in mitosis, while control cells exit mitosis by micronucleation, as confirmed morphologically for Daxx-depleted cells [88].

Daxx is not Required for Activation of the Anaphase Promotion Complex (APC) *in vitro* for Degradation of Mitotic Cyclins

To determine whether Daxx was required for the activity of mitotic E3 ubiquitine ligase APC/C or release from the spindle checkpoint, we utilized an *in vitro* system using mitotic extracts, which recapitulates both of these activities [166]. APC activity was determined by monitoring the destruction of radio-labeled Securin which remained

stable throughout all extracts derived from cells (control- or Daxx -depleted) and confirmed an active spindle checkpoint (Figure 3-3a). During the incubation, extracts undergo a slow spontaneous release from spindle checkpoint-mediated inhibition. The loss of Daxx did not delay the kinetics of this release, suggesting that it is not required for direct activation of the APC upon checkpoint silencing. We tested this idea directly, by asking whether the Mad2 antagonist, p31Comet, was able to induce APC activity toward Securin in these extracts. Addition of p31Comet to control as well as Daxx deficient mitotic extracts (produced from mitotic cells in either nocodazole or Taxol® block) resulted in equal activation of the APC and subsequent destruction of Securin (Figure 3-3b) while addition of recombinant Daxx protein did not induce activation of the APC (data not shown). Taken together, these results imply that the prolonged mitotic arrest observed upon Daxx depletion is not due to an inability to activate the APC, at least *in vitro*. However, as the mechanism(s) of spindle checkpoint silencing/release are poorly understood, we cannot exclude that Daxx may participate in an upstream event that is not recapitulated in our *in vitro* settings.

Analysis of Mitosis Related Proteins upon Daxx Depletion

Daxx exhibits transcription repression activity [113]; thus it could potentially regulate mitotic progression and Taxol® sensitivity repressing mitotic checkpoint proteins. In this regard, Daxx depletion does not change accumulation of several mitosis-related proteins including Cdc27, Cdc20, and Mad2 (Figure 3-4a). No cell cycle specific changes of Daxx protein level were observed either (Figure 3-4b). The combination of these data may suggest that Daxx-mediated Taxol® sensitivity is independent of the previously reported transcription repression activity of Daxx, at least for tested SAC-related proteins.

Daxx Dependent Tumor Response to Taxol®

Next, we sought to understand the importance of Daxx in tumor response to Taxol® using neoplasm generated by a xenograft system. Taxane-based therapy is one of treatment options in head and neck cancer [165]; therefore, we assessed the anti-neoplastic activity of Taxol® exerted on tumors generated from control- or Daxx-depleted HEp2 larynx carcinoma cells. By comparing the daily changes in tumor volume (beginning of treatment at approximately 150 mm^3 of tumor) between Taxol® and vehicle treated groups, we could determine regressions in tumor growth. Response to Taxol®of control neoplasms compared to vehicle was markedly sharper due to the sudden drop in volume even after the first injection of drug. The regression trend was observed further after the 2^{nd}-5^{th} drug administration from day 4-11 (Figure 3-5a). This drug response, in contrast, was reduced in Daxx-depleted xenografts as the sizes of Taxol®-treated tumors in these groups closely followed that of vehicle-administered tumors. The residual tumor size (calculated as a ratio of sizes between Taxol®-injected to vehicle-injected tumors at the end of experiment) of Daxx-depleted tumors after five administrations of Taxol® averaged to be 0.65 while the residual size of control-depleted tumors was much smaller at 0.27 (Figure 3-5a). We concluded that tumors generated from Daxx-depleted cells had reduced response rate to Taxol® administration compared to control-depleted tumors.

Taxane-induced Mitotic Catastrophe in Xenografts

We reasoned that the differential Taxol® response of these tumor groups may, in part, be linked to the cellular outcomes as documented previously [88]: Taxol®-resistant cells arrest in a prolonged mitotic state with sustained cyclin B protein levels and continue cell division upon drug decay, while non-resistant cells exit mitosis forming

micronucleated cells incapable of entering next cycle. To address this possibility, we analyzed cellular morphology at tumor xenografts sections. Based on DNA staining, cells were categorized as 1) interphase, 2) mitosis, 3) apoptosis, and 4) micronuclei [88]. In control shRNA xenografts, we observed an increased number of micronucleated cells (indication of Taxol® response) after 2nd and 5th injections of Taxol®, elevating to 50% at the end of treatment. In contrast, the number of interphase and mitotic cells (indication of Taxol® resistance) in Daxx-depleted xenografts remained high, while micronuclei were low (less than 10% at the end of treatment), suggesting that cells keep cycling (Figure 3-5 c). Occurrence of apoptotic cells was similar across all xenografts and increased marginally upon Taxol® exposure. Thus, in current experimental settings, depletion of Daxx elevated resistance of experimental tumors to Taxol® treatment, with majority of cells continuously cycling – while tumors derived from control-depleted cells formed micronuclei and thus stop proliferation.

In Breast Cancer Patients, Daxx is a Predictive Factor for Paclitaxel Response

To address the clinical ramifications of Daxx regulation of taxane sensitivity, twenty-two women with locally advanced HER-2 non-amplified breast cancer who were treated with standard taxane and anthracycline based neoadjuvant chemotherapy at H. Lee Moffitt Cancer Center were identified for this study. Patients were classified as either responders or non responders based on the clinical response measured with the longest diameter by physical examination performed by the treating physician at the time of encounter, with responders experiencing >75% reduction. Daxx score was calculated based on the Daxx IHC staining intensity multiplied by the percent of staining cells. Based on the above definition of response, 10 patients were classified as responders and twelve as non-responders. Comparison of pretreatment samples

between the responders and non-responders was performed using an independent sample t test. Responders to therapy had a higher mean Daxx score compared to non-responders (Figure 3-6; p=0.06). This data suggested that Daxx score could predict the response to neoadjuvant taxane and anthracycline based chemotherapy. The small sample size and the retrospective nature of the study are the main limitation of this finding that will be further validated in a future prospective study.

Summary of Results

In this chapeter, we presented several evidences that levels of Daxx determine taxane resistance in 1) cell lines (see text), 2) mouse xenografts (Figure 3-5) and 3) breast cancer patients (Figure 3-6). Moreover, we showed that this resistance is determined by Daxx-dependent regulation of mitotic progression, as documented by 1) elevated stability of cyclin B (Figures 3-1 and 3-2); 2) prolongation of prometa/metaphase stages of mitosis (see text). We also found that Daxx does not affect directly APC, at least in *in vitro* setting (Figure 3-3), necessitating in vivo study to pin Daxx function in mitosis and taxol resistance. Studies presented in this chapter have established new roles for Daxx in cell cycle progression—the importance of which may be intensified because of its function as a trigger for Taxol® sensitization—which adds to our understanding of mechanisms linking cell division, chemotherapy response and cancer progression.

Discussion

Taxane chemotherapy is considered among the most responsive treatment options for many cancer patients, either alone or as adjuvant in combination with anthracyclins [11]. Nevertheless, large numbers of patients are resistant or become resistant to taxane therapy during treatment. The response rate of docetaxel is ~50%

even after the first-line chemotherapy administration and decreases to 20-30% by second- or third-line administration [52, 53]. Thus, development of new genomic prognosis factors and in-depth understanding of drug activity on both a cellular and organism levels are needed for optimization of adjuvant therapy and proper patient stratification. Numerous studies have been carried out to determine a genomic profile that could be predictive to taxane treatment [155-159] while alternative approaches have sought to understand selective resistance to taxanes to decipher mechanisms which regulate responses [150, 167, 168]. Inactivation of mitotic proteins can contribute to the selective response of taxane treatment in vivo [49]. Divergent response to Taxol® exposure is usually seen in cells deficient of mitotic checkpoint proteins or other regulators of cell division. To date, loss of function of the majority of mitotic proteins, including Mad2, Bub1 and BubR1, and response to paclitaxel is controversial. Hence, identification of factors, that increase drug resistance upon inactivation, is largely incomplete or uncharacterized.

To this end, Daxx was verified as a novel regulator of Taxol® response in cell culture conditions, animal models and primary human tumor specimens. Human breast cancer cells and larynx carcinoma HEp2 cells with experimentally modified levels of Daxx, show reduced responses to Taxol® as previously described [88]. A mouse xenograft system also recapitulates our initial findings, becoming the first indication that Daxx could be important for the fate of tumors exposed to taxane based chemotherapy (Figure 3-5, panels A and B). We also found that control groups displayed an increased amount of micronucleation upon Taxol® treatment, which may account for the rapid loss of xenografts tumor volume observed in these regimental settings (Figure 3-5, panel C).

In contrast, Daxx-depleted tumors displayed increased mitotic and interphase index (Figure 3-5, panel C), indicating that cells were capable to maintain mitotic block via elevated cyclin B stability (Figure 3-2) and continue proliferation after Taxol® decay that happens fast in nude mice [169]. Thus, mitotic cells from Daxx-depleted tumors can potentially reenter G1 after drug decay, providing a working model for how cells or tumors devoid of either of these protein targets can survive chemotherapy treatment and proliferate (Model in Figure 3-7).

The nature of Daxx function in cells is largely attributed to regulation of apoptosis or transcription. While this functionality is debatable in many circumstances, the prevailing idea of the role of Daxx is that of a modulator or adapter of many cellular functions which are critical to cell vitality [113]. Indeed, Daxx has been found critical and necessary for embryonic development in mice as Daxx-/- embryos exhibit extensive apoptosis and lethality by E11.5 [99, 170].

In addition to function in Taxol® response, we also report an unexpected role of Daxx in the regulation of mitosis. In the absence of Daxx, the duration of prophase and the prometaphase/metaphase transition is altered. Indeed, the stability of cyclin B protein is changed in the absence of Daxx as well (Figure 3-1). This may suggest that the activity of E3 ubiquitin ligase APC is altered in the absence of Daxx. We attempted to study the role of Daxx as direct regulator of APC using an in vitro assay, but the results were undistinguishable (Figure 3-3), suggesting that cellular in vivo mitotic environment is necessary for proper execution of Daxx function in mitosis. Differential degradation of cyclin B in Daxx-depleted cells could also be due to mis-regulation of cyclin B on a different operational level, namely, the alteration in stability of the APC

activator Cdc20 [171]. However, we found no differences in and accumulation of Cdc20 as well as Cdc27 and Mad2 (Figure 3-4). In addition our results indicate that upon Daxx depletion and taxane exposure both dynamics of degradation and/or accumulation of cyclin B levels are affected.

Evidences presented in this chapter suggest that Daxx represents a trigger for cellular Taxol® sensitivity.These findings in addition to recently reported functions of Daxx repression of c-met oncogene expression [120] and Daxx mutation in tumors[141, 143], may further uncover Daxx role in tumor progression. In the future, Daxx may serve as useful molecular marker for proper selection of cancer patients for taxane chemotherapy. In order to achieve this goal, clinical studies additional to presented here (Figure 3-6) will be required examining the status of Daxx expression in tumors before and after taxane treatment as well as studies in patients with an established history of taxane resistance. Daxx expression vary in breast cancer cell lines [88], but the mechanism of Daxx down-regulation has largely been unstudied.

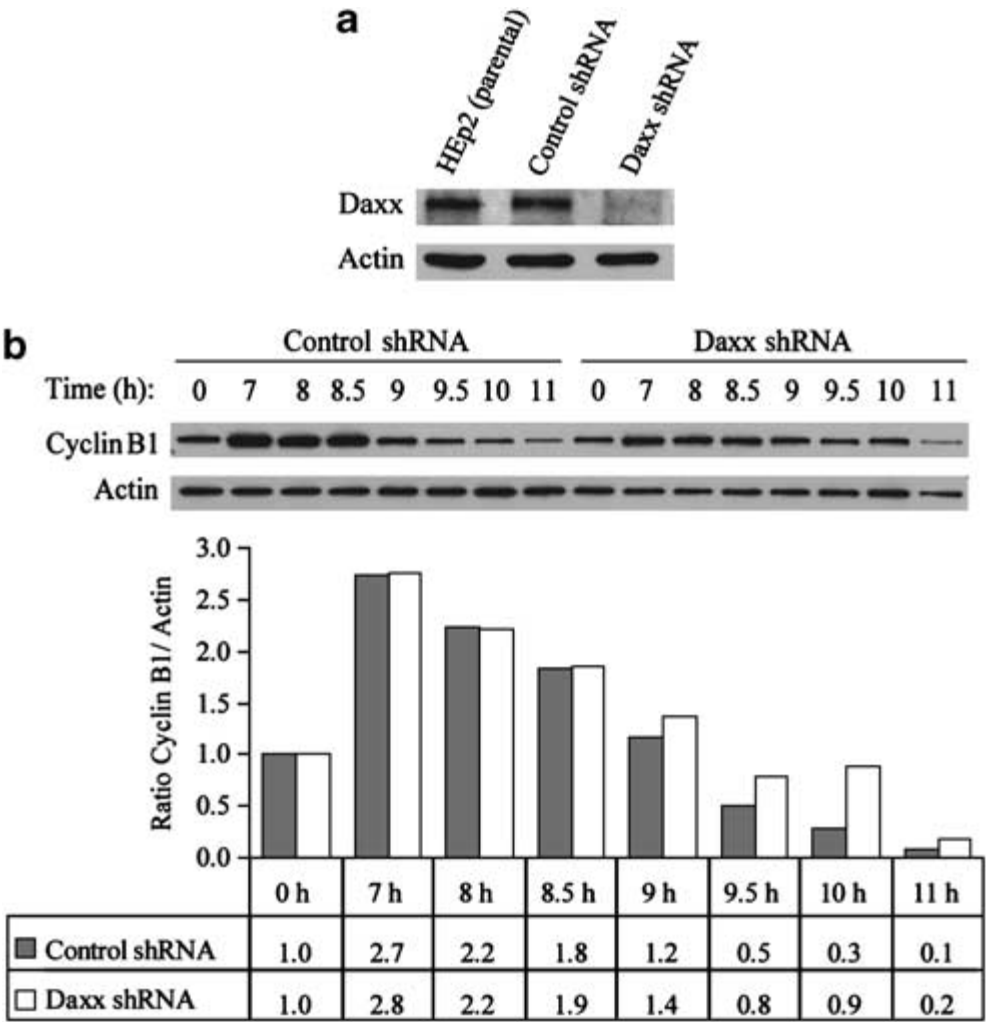


Figure 3-1. Daxx-dependent stability of cyclin B. (a) Western blot analysis of Daxx depletion in HEp2 cells. (b) Control- and Daxx depleted HEp2 cells were synchronized by a double thymidine block (0 h) and then released into normal media for progression through mitosis (6–11 h). Top: Western blot analysis of cyclin B protein stability. Bottom: Relative quantization of cyclin B protein levels (normalized to actin). Cyclin B protein is stabilized longer in Daxx-depleted cells (at 9.5–11 h, post-thymidine release), indicating that Daxx-depleted cells are delayed in mitosis. Data show a representative experiment out of four.

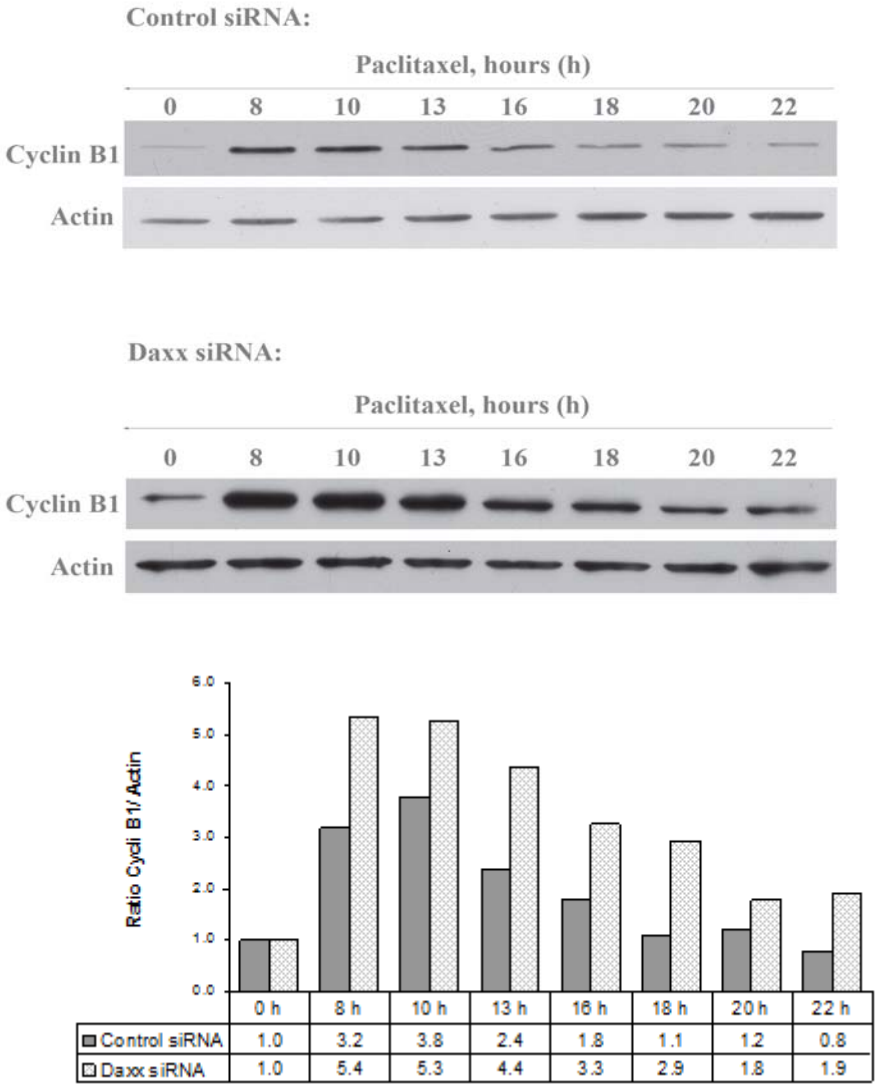


Figure 3-2. Depletion of Daxx prolongs cyclin B stability. Western blot analysis of cyclin B protein stability in HEp2 control- or Daxx-siRNA cells treated with Taxol® for the indicated amount of time (6–22 h). Cells were synchronized using DTB and released (0 h) into normal media containing 10 nM Taxol®. The bottom panel: densitometry analysis of cyclin B normalized by actin; for each cell line, the cyclin B/actin ratio at 0 h set as 1.0. Whereas cyclin B protein levels rapidly decrease by 13 h post-thymidine release in control shRNA cells, cyclin B protein levels were stabilized longer in Daxx- and Rassf1A-depleted cells (through 22 h, post-thymidine release), indicating that Daxx and Rassf1A depletion prolongs exit from mitosis in response to Taxol® exposure. Data show a representative experiment out of three.

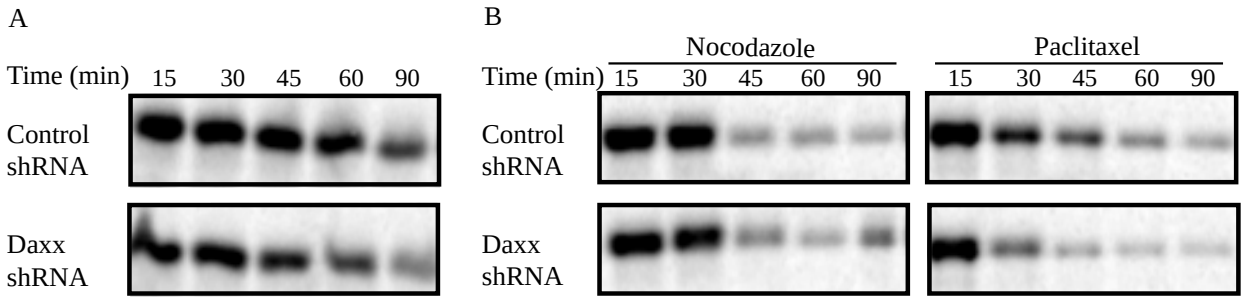


Figure 3-3. Daxx is not required for in vitro activation of the APC. A) Extracts were generated from nocodazole-arrested cells expressing control- or Daxx-shRNAs and the stability of the APC substrate Securin, labeled with 35S, was monitored by autoradiography. B) Extracts were generated as in (A) from cells treated with 10 μM nocodazole or 10 nM Taxol® (paclitaxel). The requirement for Daxx to activate the APC was tested by addition of the Mad2 antagonist p31 Comet.

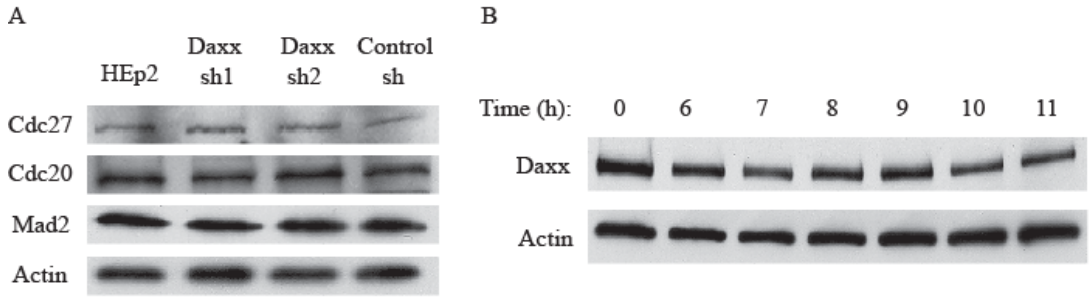


Figure 3-4. Analysis of mitosis-related proteins. A) Analysis of mitotic checkpoint proteins in control- and Daxx-depleted cell lines. Parental HEp2 cells and cells stably expressing control or two independent anti-Daxx shRNAs were analyzed by Western blot for levels of Cdc27, Cdc20, and Mad2; proteins levels are unchanged. B) Cell cycle-dependent expression of Daxx. HEp2 cells were synchronized using a double thymidine block to arrest cells in the G1/S-boundary and then released and allowed to progress through S, G2, M phase (0-11hr post thymidine release, compare with cyclin B dynamics on Figure 3-1). Daxx protein level showed no significant changes during cell cycle.

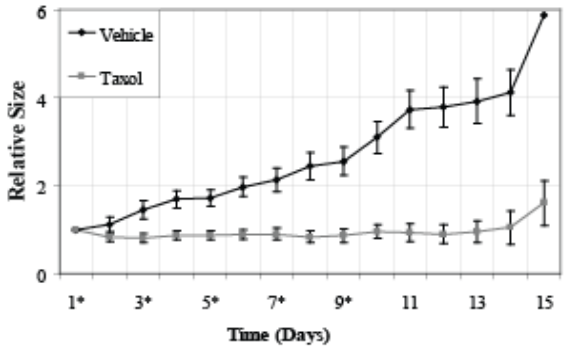
Figure 3-5. Depletion of Daxx increases resistance of experimental tumors to Taxol®. Control- or Daxx- depleted tumors derived from injection of HEp2 cells into Nu/Nu mice were treated with vehicle or 20mg/kg Taxol®. A) Table summarizing key statistical data generated from tumors exposed to vehicle or Taxol® regimens. Relative tumor sizes for vehicle (A) or Taxol®-treated tumors (B) were recorded at the end of treatment and the relative residual tumor volumes were calculated by dividing the values from (B) over (A). B) Graphs charting the changes in relative tumor size of control-or Daxx-depleted xenografts exposed to vehicle or Taxol®. Asterisks at day number (x axis) denote time of injection of vehicle or Taxol® (days 1, 3, 5, 7, 9). Note discrepancy in tumor growth in vehicle and Taxol® treated tumors in control shRNA groups, while the rate of growth in Daxx-shRNA group exposed to Taxol® have reduced response. C) Treatment response at cellular level. Tumor xenografts were extracted 24 hours following the 2nd or 5th Taxol® injection or at the end of experiment (day 15) and cellular response was analyzed based on appearance of chromatin (stained for DNA) by light microscopy and characterized as 1) interphase, 2) mitotic, 3) micronucleated, and 4) apoptotic as described previously[88]. Control xenografts exhibited an increased number of micronucleated cells (indication of Taxol® response), while Daxx-depleted xenografts show increased numbers of mitotic and interphase cells (indication of Taxol® resistance and continuous proliferation) and correspondingly less micronuclei; number apoptotic cells is similar among groups.

A

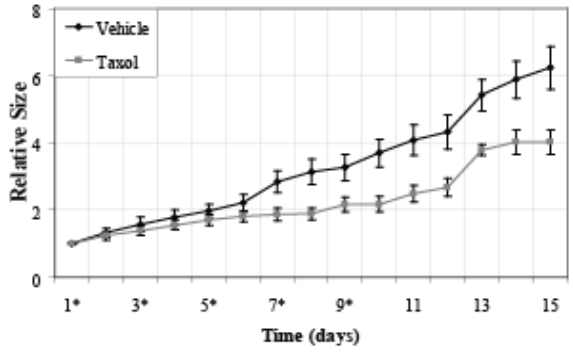
Xenograft	End Tumor Size: Vehicle (A)	End Tumor Size: Paclitaxel (B)	Residual Tumor Size (B/A)	N (Animals) Control/ Paclitaxel (Total)
Control shRNA	5.87	1.61	0.27	8/14 (22)
Daxx shRNA	6.23	4.02	0.65	10/15 (25)

B

Control shRNA:

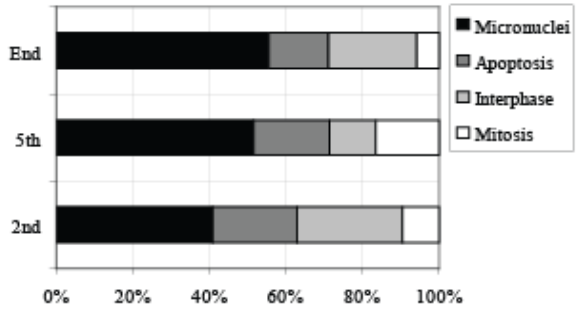


Daxx shRNA:

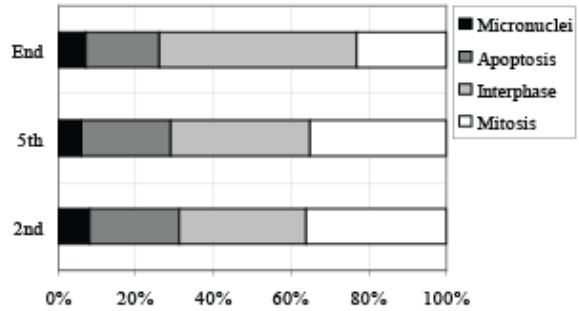


C

Control shRNA:



Daxx shRNA:



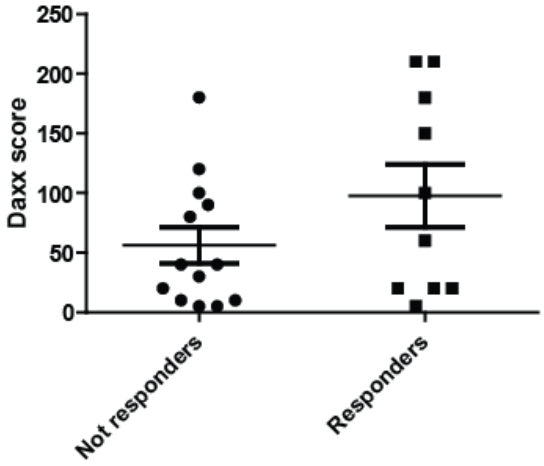


Figure 3-6. Daxx levels have reverse correlation with taxane chemotherapy response in breast cancer patients. Twenty-two women with breast cancer who were treated with standard taxane and anthracycline based neoadjuvant chemotherapy were classified as either responders (up to 75% reduction of tumor size; 10 patients) or non-responders (less than 75% reduction of tumor size; 12 patients). Daxx score was calculated based on the Daxx IHC staining intensity multiplied by the percent of staining cells. Comparison of pretreatment samples between the responders and non-responders was performed using an independent sample t test. Responders to therapy had a higher mean Daxx score compared to non-responders ($p=0.06$).

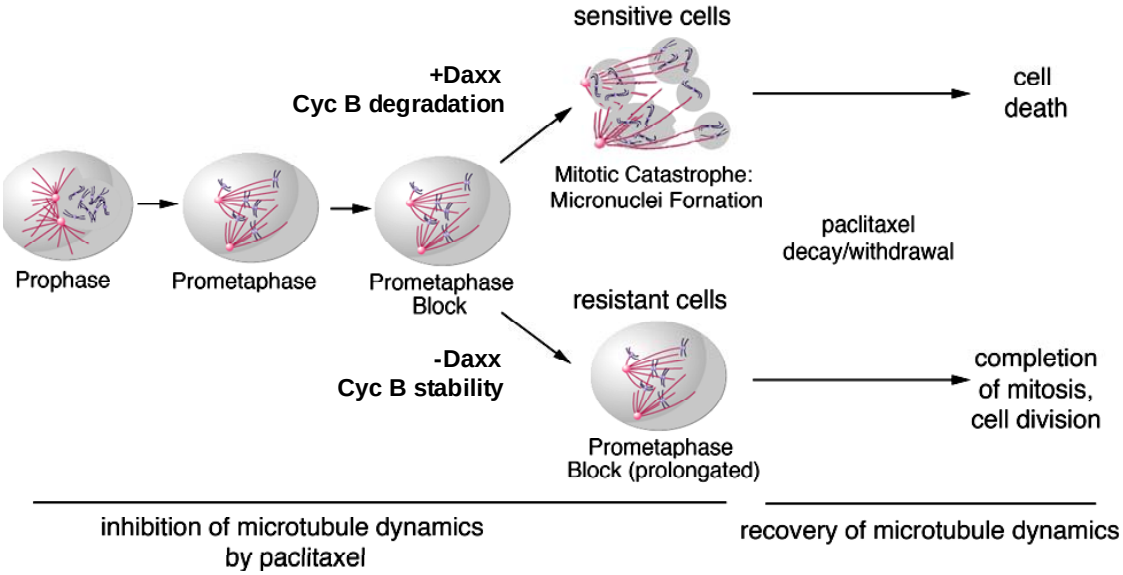


Figure 3-7. Model of Daxx-dependent Taxol® response. At pharmacological concentrations, Taxol® reversibly inhibits microtubule dynamics blocking cells in prometaphase. Cells that are sensitive to Taxol® activate mitotic block only transiently, followed by cyclin B proteolysis, micronuclei formation, block of proliferation and cell death, while Daxx negative cells have a more prolonged prometaphase block due to elevated cyclin B stability; they continue proliferation after drug decay/withdrawal and microtubule dynamics restoration--thus surviving chemotherapy.

CHAPTER 4
DAXX INTERACTS WITH UBIQUITIN SPECIFIC PROTEASE-7, USP7, TO REGULATE MITOSIS AND CELLULAR TAXOL® RESPONCE

Introductory Remarks

Taxanes (paclitaxel and docetaxel) are powerful drugs for breast cancer treatment; however, a large number of patients are resistant to this therapy for unknown reasons. Therefore it will be essential to develop prognostic tools and predictive markers to differentiate the patient population for appropriate chemotherapy selection.

According to our model (Figure 3-7), Daxx deficient cells cannot resolve prometaphase/metaphase transition as efficiently as control cells. This would explain the observation that, upon taxanes treatment, cells with a low Daxx expression are protected from mitotic catastrophe and are able to complete mitosis and survive after taxane decay. Given high level of taxane resistance in clinical conditions, we attempted to understand the role of Daxx in such resistance and cell cycle regulation (Chapter 3). We demonstrated that low levels of Daxx induce stabilization of cyclin B which allows cells exposed to taxanes to stay in a prolonged mitotic arrest and escape action of chemotherapy.

Growing evidence describes mitosis as a dynamic process controlled by multi-protein complexes [172, 173]. Considering the ability of Daxx to interact and recruit a wide variety of partners [113], it is highly probable that additional proteins may participate in the Daxx-dependent checkpoint regulation as well as contribute to taxane chemotherapy resistance.

To this end, we used a functional proteomic approach to isolate Daxx-containing multiprotein complex upon paclitaxel treatment. The hypothesis was that the identification of a Daxx interacting mitotic network could speed up detection of new

markers of taxane resistance and lead to the development of new approaches to combat this resistance.

Our proteomic analysis identified Ubiquitin Specific processing Protease-7 (USP7) as the most abundant protein in Daxx mitotic complex.

USP7 is also known as Herpes-virus Associated Ubiquitin Specific Protease, or HAUSP, because it was first identified playing a role in antiviral response by interacting and regulating stability of a herpes virus E3-ubiquitin ligase [174-176].

USP7 is a deubiquitylating enzyme or DUB [177], which, by catalyzing the removal of ubiquitin chains from substrate proteins, rescue them from degradation. Due to the roles of its substrates, USP7 is involved in disparate cellular processes, in normal or stressed conditions [127, 178-180].

USP7 partakes in transcriptional regulation by stabilizing the transcription factor REST essential for the maintenance of neuronal stem/progenitor cells [181, 182]. USP7 participates in DNA damage response by stabilizing Claspin [183], an adaptor protein that controls duration of stress checkpoint response [184].

Deubiquitylating activity of USP7 has been shown to regulate mono-ubiquitylation of tumor suppressor PTEN [179] and transcription factor FOXO4 [185], thus enabling nucleoplasm-cytosol shuttling of these proteins.

USP7 has been described also in epigenetic regulation due to its role in stabilization of PRC1 complex subunits (MEL18 and BMI1), and regulation of monoubiquitylation of the histone H2B [186-188]. A recent study reported that USP7 can interact and increase the activity of DNMT1 *in vitro* though binding, suggesting that USP7 can play an important role inDMNT1-mediated epigenetic maintenance [189].

The interest on USP7 biological roles spiked when it was shown that USP7 regulates the fate of the tumor suppressor p53 [190, 191]. The Gu lab reported that USP7 directly interacts and stabilizes p53. However subsequent genetic studies demonstrated that this USP7 mediated regulation of p53 is not the predominant one in cells. Contrarily to what was expected, genetic inactivation of USP7 in mice resulted in p53 stabilization, data not compatible with the notion of p53 as major USP7 substrate [192, 193].

Indeed, later on, it was demonstrated that USP7, in association with Daxx, regulates p53 [127, 191, 192], *via* MDM2 [126], the E3 ubiquitin ligase that antagonize p53. In interphase, Daxx forms a bridge between USP7 and p53-specific E3 ligase MDM2 [126, 127, 191, 192]. In normal conditions, this ternary complex allows USP7 to remove the ubiquitin moieties from self-ubiquitylated MDM2. Stabilized MDM2 in turn can ubiquitylate p53 and maintain the levels of this protein low throughout the cell cycle. In response to genotoxic stress Daxx-USP7-MDM2 ternary complex promptly dissociates, inducing therefore MDM2 self-destruction and stabilization of p53. These results explain the early lethality observed in USP7 knockout mice (Embryonic day 3.5) where cells isolated from these embryos are characterized by loss of MDM2, abnormal accumulation of p53 with consequent cell proliferation arrest and apoptosis [194]. However, deletion of p53 gene in USP7 knockout mice was not able to rescue USP7 induced embryonic lethality (contrarily to what was shown for MDM2 knockout mice [195]), providing the genetic proof of the importance a p53-independent function of USP7 [196, 197].

Regarding the involvement in cancer progression, USP7 has been described as both tumor suppressor and oncogene [179, 185, 190, 191]. Due to potential oncogenic properties, mostly attributed to p53 regulation, USP7 represented a promising anti-

neoplastic target. Indeed several specific USP7 inhibitors (USP7i) have been developed so far [198, 199] and are currently tested at the pre-clinical level. These drugs have shown to be potent anti-proliferative agents [199, 200] in several cancer cell lines but the therapeutical use of these USP7 inhibitors can be limited by frequent inactivation of p53 gene in tumorigenesis and limited knowledge regarding alternative pathways controlled by USP7.

In the current and the following chapters (Chapter 4 and 5) we will demonstrate that USP7 and Daxx play a pivotal role in mitosis and their silencing contribute to genomic instability and chemotherapy response. Here we show that this newly identified Daxx and USP7 mitotic interaction occurs and functions independently from p53.

So far, participation of USP7 in mitosis has only been speculated. USP7 was shown to interact and stabilize checkpoint protein CHFR [178], but its direct involvement in mitotic regulation has not yet been demonstrated.

Results

Daxx Mitotic Complex Isolation

We have previously shown that protein Daxx participates in mitosis regulation thus affecting taxane resistance [88, 134]. In order to gain insights of Daxx function in this tightly controlled cell cycle stage, we isolated Daxx mitotic complex using the pOZ-FH-N expression vector for Tandem Affinity Purification (TAP) [201]. The plasmid pOZ-FH-N coding for FLAG (F) and HA (H) tags was modified to harbor a thrombin cleavage site (TCS) between the tags. For mitotic complex isolation, stable HEp2 cell lines expressing pOZ-F-TCS-H or pOZ-F- TCS-H-Daxx were synchronized by double thymidine block (TDB) and released in Taxol® to arrest cells in mitosis (95% by FACS analysis) to mimic conditions of Taxol® treatment. Cell lysates were subjected to FLAG

immuno-precipitation (IP) followed by thrombin cleavage. The eluted fractions were resolved by SDS-PAGE, colloidal Comassie stained (Figure 4-1, panel A) and identical gel areas from Daxx or control IPs were sequenced by mass spectrometry.

Daxx Interacts with USP7 in Mitosis

The most abundant protein identified exclusively in Daxx mitotic complex was the deubiquitylating enzyme USP7 [127, 178, 179] (Ubiquitin-specific-processing protease 7, also known as HAUSP [202]) (see Table 1). The association of Daxx and USP7 was previously published [126, 180]; yet, neither mitotic-specific binding between Daxx and USP7, or USP7 function in mitosis, were previously reported.

This novel mitotic interaction is reproducible as confirmed by co-immunoprecipitation experiments (Figure 4-1). Mass spectrometry data was validated by FLAG IP in cellular system and protocol used for the complex isolation (Figure 4-1, panel B) where USP7 was pulled-down specifically in FLAG- Daxx IP but not FLAG only IP. These results were further validated by immunoprecipitation of endogenous Daxx in nocodazole arrested HEp2 cells (Figure 4-1, panel C). We were also able to prove reciprocal binding of USP7 and Daxx by immunoprecipitating endogenous USP7 in cells arrested in mitosis with both nocodazole (Figure 4-1, panel D, left) and Taxol® (Figure 4-1, panel D, right). Our results therefore strongly demonstrate that Daxx and USP7 interact in mitosis. Interestingly this association occurs in cells arrested in prometaphase by Taxol®, which keeps the spindle assembly check-point (SAC) active, or nocodazole which instead leaves the SAC inactivated. Thus Daxx and USP7 interaction occurs in prometaphase independently from the SAC activation status.

Depletion of USP7 Causes Delay of Early Mitotic Events

We previously demonstrated that Daxx depletion causes stabilization of cyclin B1, blocking transiently cells in mitosis [116] (Figures 3-1 and 3-2). To test whether Daxx and USP7 cooperate to maintain this block, USP7 was depleted by siRNA in HEp2 cells (Figure 4-2, panel A). Cells were then synchronized using a double thymidine block and released to monitor cyclin B protein levels during progression through G2/M/G1 stages. While cells transfected with control siRNA showed degradation of cyclin B by 9 hrs post-thymidine release, USP7 depleted cells showed stabilization of cyclin B at 9-11 hrs post-release. Thus, as was shown for Daxx [134] in Chapter 3, depletion of USP7 also led to stabilization of cyclin B in mitosis (Figure 4-2, panel A).

Since Daxx and USP7 were previously reported to be involved in the regulation of tumor suppressor p53 stability [127, 191, 192], via MDM2 de-ubiquitylation and stabilization [126], we tested whether p53 may be involved in Daxx and USP7 mitotic function. To this end USP7 was depleted in non-small lung carcinoma cell line H1299, which is p53 null [203]. This cell line has similar levels of Daxx and USP7 compared to HEp2 cells (Figure 4-2). H1299 cells were then transfected with control or USP7 siRNAs and synchronized to monitor cyclin B stability, as described above, in cells entering (7 hrs post DT release), progressing (9 hrs post DT release) and exiting mitosis (11 hrs post DT release). Cyclin B was stabilized in H1299 cells upon USP7 depletion (Figure 4-2, panel B). Therefore we concluded that down-modulation of either Daxx or USP7 causes cyclin B accumulation. In addition, as previously observed for Daxx [88], we now show that silencing of USP7 affects cyclin B stability independently from p53.

We had previously shown that Daxx depletion causes impaired mitotic progression and a transient mitotic block [134]. Therefore we next sought to investigate how USP7

may affect cellular advancement though mitotic stages. Consistently to what was observed for Daxx, USP7 depletion does not increase mitotic index *per se* (data not shown). However, USP7 depletion resulted in an increase of prometa- and metaphase stages (P/M) of mitosis. An increase in P/M index was observed in both HEp2 cells (Figure 4-3, panel A) and H1299 cells (Figure 4-3, panel B). Therefore we concluded that USP7 silencing causes a p53-independent mitotic delay, in agreement with stabilization of cyclin B (Figure 4-2) and the effects documented for Daxx depletion [116] (Chapter 3).

USP7 Depletion Destabilizes CHFR Protein

USP7 has been reported to remove ubiquitin chain and increase stability of a mitotic checkpoint protein, the E3 ligase Checkpoint with Forkhead and Ring finger (CHFR) [178].

Loss of CHFR expression was documented in up to 50% of tumor specimens from a variety of tissues including breast cancer [70, 204, 205]. CHFR knockout mice are viable with high rates of spontaneous tumors due to the chromosomal instability and mitotic defects linked to accumulation of Aurora A [45], the key mitotic kinase that promotes bipolar spindle assembly [83, 206-208]; it also controls mitosis by regulating cyclin B localization and stability [207, 209].

To test whether mitotic function of USP7 is mediated by CHFR, we monitored this protein stability in HEp2 cells stably expressing control or USP7 shRNAs. Cells depleted by USP7 showed decrease in CHFR protein levels (0 hours) and a reduced stability of this protein upon protein synthesis block with cycloheximide (CHX, Figure 4-4, panel A).

Similarly, H1299 cells transiently depleted of USP7 had reduced stability and decreased CHFR protein levels upon CHX treatment, compared to control depleted cells (Figure 4-4, panel B).

Thus, USP7 depletion destabilizes mitotic E3 ligase CHFR independently from p53 cellular status.

Loss of USP7 Leads to Accumulation of Aurora A and Multipolar Spindles

CHFR is required for the maintenance of an early mitotic checkpoint essential to blocking mitotic progression through the metaphase stage while the cell is withstanding stresses [46, 210]. Being a RING domain E3 ligase, CHFR binds and ubiquitylates one of the pivotal mitotic kinases, Aurora A [45, 204, 211] targeting this kinase for proteasomal degradation. We tested whether we could observe changes in accumulation of Aurora A upon depletion of USP7. An increase in Aurora A protein levels was observed in both HEp2 and H1299 cell lines upon depletion of USP7 (Figure 4-5, panel A). Upon depletion of USP7 in H1299 p53 null cells, the increment of Aurora A is noticeably higher that in p53 wild type HEp2 cells as shown by the quantification of Aurora A protein levels (Ratio Aurora A/Actin, Figure 4-5 A). This difference may be explained by the G1-S block and apoptosis triggered by p53 accumulation upon USP7 depletion in HEp2 cells, while H1299 cells can progress through G1/S checkpoint, thus continuously accumulating Aurora A.

Since Aurora A kinase governs faithful replication, separation/ localization of centrosomes and bipolar spindle assembly [212], we asked whether we could observe mitotic abnormalities in USP7 depleted cells.

In agreement to what was reported for Aurora A amplification or accumulation [208, 213-215] we observed a significant increase of multipolar mitoses in cells with either stably

(Figure 4-5, panel B) or transiently (Figure 4-6) depleted USP7 as shown by immuno-fluoresce staining for Aurora A and α-tubulin. Although localization of Aurora A was preserved in cells lacking USP7, an increase in multipolar mitoses was observed in both HEp2 and H1299 cells. Transient depletion of Daxx revealed that multipolarity can be mediated by absence of either Daxx or USP7 with the highest effect observed with decreased levels of the latter (Figure 4-6). This result is in agreement with our hypothesis that Daxx may trigger USP7 DUB activity in mitosis.

We also observed an increase in overnumerary poles (>4 poles) in USP7 shRNA cell lines (Figure 4-5, panel C) potentially due to failed cytokynesis and centrosome over-replication in cells going through multiple cell divisions upon USP7 depletion (and, therefore, with elevated Aurora A).

In order to confirm this data in a non-tumorigenic cell line, we counted multipolar mitoses of human mammary epithelial cells, MCF10A, upon transient depletion with cell-permeable non-targeting control or USP7 siRNAs (Accell technology, Dharmacon). Analysis of up to four-hundred mitoses in MCF10A control or USP7 depleted cells confirmed data obtained in HEp2 and H1299 cancer cell lines (not shown).

USP7 Depletion Elevates Taxane Resistance that Can Be Attenuated with Aurora A inhibitor MLN8054

In order to investigate the existence of a correlation between USP7 and cellular response to paclitaxel, USP7 mRNA expression was analyzed across the NCI-60 cell-line screen [216, 217]. This expression profile was then correlated with sensitivity to paclitaxel. Analysis done by our collaborator Dr. Reinhold at the NHI-NCI, revealed that USP7 expression significantly correlates with paclitaxel response ($P < 0.05$) in the NCI-60 cell lines.

To investigate the role of USP7 in taxane response, HEp2 cells stably expressing USP7 or control shRNAs were tested for paclitaxel induced cell death measured by colony formation assay. Increased survival of USP7 depleted cells was observed in comparison to cells expressing the control shRNA (Figure 4-7). After 18 hours of taxol treatment only 7% of colonies in control cells survived, while USP7 depleted cells tolerated the same condition to a much higher extent showing a 25% survival rate. Therefore, experimental down-regulation of USP7 reduced sensitivity to paclitaxel treatment in HEp2 cells as it was previously shown for Daxx.

Since Aurora A over-expression correlates with poor patient's outcomes [84-86] and resistance to taxanes [72-74], selective Aurora A inhibitors, such as MLN8054 [218-220], have been developed. Use of these drugs in cells with overexpressed Aurora A has been shown to sensitize tumor cells to chemotherapeutic agents including taxanes [221-223], even when spindle checkpoint proteins are absent [224]. Thus, the potential of abrogating taxol resistance in combinatorial treatments with Aurora A kinase inhibitors are promising for clinical applications.

Currently, MLN8054, as selective inhibitor of Aurora A [218, 220], is in phase-I clinical trials in patients with advanced malignancies and solid tumors including breast cancer. Thus we chose this drug as an inhibitor of Aurora A to test whether it could override USP7 induced paclitaxel resistance. MLN8054 was tested alone or in combination with Taxol® in cells stably expressing control or USP7 shRNAs in colony formation assay (Figure 4-7). Cells expressing control or USP7 depleted cells did not show significantly different response to MLN8054 alone; however a different outcome was observed when this drug was used in combination with Taxol®. The drug combination did not synergize

in the killing of control depleted cells (8% survival in MLN8054+Taxol® treatment *vs* 7% with Taxol® alone), but it was effective in diminishing taxol resistance in USP7 shRNA cells (17% survival in MLN8054+Taxol® treatment *vs* 29% with Taxol® alone, P=0.01). The combination of the two drugs was also more effective than MLN8054 alone in killing USP7 depleted cells (17% survival in MLN8054+Taxol® treatment *vs* 25% with MLN8054 alone, P=0.05).

Our results indicate that in the absence of USP7, functional inactivation of the mitotic spindle checkpoint using Aurora A kinase inhibitor, MLN8054, can elevate cellular taxol response and significantly decrease USP7-induced taxane resistance.

Summary of Results

In this chapter, we presented evidence that Daxx and USP7 interact in mitosis (Figure 4-1) and that this association determines mitotic progression and taxane resistance. For the first time we have shown that DUB enzyme USP7 is involved in mitotic regulation as cells depleted by USP7 display 1) elevated stability of cyclin B (Figure 4-2); 2) prolongation of prometa/metaphase stages of mitosis (Figure 4-3); 3) decreased stability of mitotic E3 Ub ligase CHFR (Figure 4-4) with consequent accumulation of CHFR substrate Aurora A and multipolar mitotic spindles (Figure 4-5 and 4-6). We also found that taxol response in 60 cancer cell lines correlates with levels of USP7 and that taxol resistance is elevated upon experimental depletion of USP7 (Figure 4-7), as it was shown in Chapter 3 and [88, 116] for Daxx. Moreover, we demonstrated and that this resistance can be at least partially attenuated by inhibition of Aurora A (Figure 4-7). Thus, studies presented in this chapter have established new roles for Daxx and USP7 in cell cycle progression where absence of these molecules impairs cytotoxic activity of taxane administration (Model in Figure 4-8).

Discussion

Daxx is a multifunctional protein that plays a pivotal role in both physiological and pathological cellular processes. We previously demonstrated that cells with low levels of Daxx have reduced sensitivity to taxanes (Chapter 3 and [88, 116]), powerful chemotherapeutic agents, by persisting in a pro-metaphase block that allows cells to escape taxane-induced cell death. In this chapter we dissected the mechanisms of Daxx-dependent taxanes resistance that also proves function of this protein in mitotic progression. We have shown that Daxx interacts (Figure 4-1) and cooperates with Ubiquitin Specific processing Protease-7 (USP7) to regulate mitosis (Figures 4-2 and 4-3). We have demonstrated that depletion of USP7 delays early mitotic events as shown by accumulation of prometa/metaphases (Figure 4-3), biochemically confirmed by stabilization of cyclin B (Figure 4-2), as it was previously observed for Daxx [88, 116] (Chapter 3). Therefore our results strongly indicate that Daxx and USP7 interact and cooperate in mitosis to ensure accurate mitotic progression.

Tang *et al*. demonstrated that in interphase cells, Daxx creates a bridge between USP7 and MDM2; moreover, upon Daxx binding, USP7 DUB activity toward MDM2 increases [126]. But how Daxx activates USP7 is still unknown, mainly due to lack of biochemical characterization of USP7-Daxx binding. Recent developments in USP7 field showed that USP7 activity can be enhanced upon binding of co-factors. The metabolic enzyme (GMPS), a known USP7 associating enzyme, increases deubiquitylating activity up to 100-fold through binding of a "switching" loop in close proximity to USP7 catalytic domain [225]. Thus we can speculate that Daxx may bind USP7 in a similar fashion to enhance its activity in mitosis. It is also plausible that Daxx contribution to this interaction is to recruit USP7 substrates. Future studies are needed

to dissect Daxx and USP7 association in mitosis *versus* interphase

In order to understand how Daxx and USP7 regulate mitotic progression we asked whether CHFR, a USP7 substrate, is involved. CHFR is a mitotic E3 ligase that targets checkpoint proteins for destruction during mitosis. Our results demonstrate that upon USP7 depletion stability of CHFR is reduced in both HEp2 and H1299 cells, indicating that this regulation occurs independently from p53 (Figure 4-4).

Genetic studies [45] demonstrated that the mitotic defects observed in CHFR depleted cells are mostly mediated by increased levels of one of its substrates, Aurora A kinase. In normal cells Aurora A kinase activity is required for centrosomes maturation and separation, thus bipolar spindle formation. The phenotype of cells with high levels of Aurora A protein is mainly characterized by multipolar mitoses. Hence, the next question we asked was whether Aurora A had accumulated in cells depleted by USP7 and if we could observe the associated phenotypes. Indeed, depletion of USP7 correlated with higher Aurora A protein levels compared to control depleted cells, in both p53 WT (HEp2) and p53 null (H1299) cell lines. H1299 cells accumulated Aurora A protein to an higher extent than HEp2 cells; this effect can be explained by absence of p53-mediated G1/S block that allows cell with over-amplified centrosomes to progress through cell cycle [226]. Study of multipolar events, as result of Aurora A accumulation, revealed that USP7 depletion leads to a significant increase of over-numerary spindle poles (Figure 4-5) in comparison to control depleted cells.

We next sought to understand if a similar effect could be seen in Daxx depleted cells. Therefore we depleted HEp2 cells by siRNA for non-targeting control, Daxx and USP7 and evaluated the multipolar phenotype. An increase of multipolar mitoses was

observed in both HEp2 and H1299 cell lines depleted by Daxx or USP7, compared to control siRNA treated cells (Figure 4-6). Yet, the highest extent of multipolar phenotype was associated with USP7 silencing. These results can be partly due to the fact USP7 is known to stabilize Daxx in normal condition [227]. Hence, USP7 depletion accounts for cumulative effects due to reduction in both proteins. In addition, loss of the complex component with enzymatic property (USP7) is more likely to produce the strongest multipolar phenotype.

Results obtained from colony formation assay with experimentally reduced levels of USP7 and *in silico* correlative analysis in the NCI-60 platform, show that USP7 expression influences response to taxanes in cancer cell lines. Thus, these results indicate that USP7, as shown in Chapter 3 for Daxx, can be used as a predictive marker for taxane response in cancer patients. Therefore, Daxx and USP7 can be used for proper stratification of breast cancer patients to receive taxane-based treatment.

We collected evidence that USP7, as well as Daxx [88, 134], is a regulator of taxane response. Previously, USP7 and Daxx were identified as regulators of p53 stability and as such proposed to regulate G1/S cell cycle arrest and apoptosis. In this chapter we demonstrated that another stage of cell cycle is regulated by Daxx and USP7 interaction. As shown in our model in Figure 4-7, we propose that during normal mitotic progression Daxx activates USP7 for de-ubiquitination of mitotic E3 ligase CHFR, rescuing this protein from degradation. In turn, stabilized CHFR ubiquitylates and targets Aurora A for degradation, thus 'fine tuning' the levels of this protein on the entrance of mitosis. Proper levels of Aurora A ensure bipolar spindle formation and normal mitotic progression guaranteed by timely degradation of cyclin B on the

metaphase/anaphase onset. Deregulation of USP7 and/or Daxx reduces stability of CHFR, thus Aurora A accumulates. Stabilization of Aurora A leads to cyclin B accumulation, potentially by two independent mechanisms. The first one is controlled by the SAC which halts mitotic progression due to the inability to resolve multipolar mitoses. The second one relies on the ability of Aurora A to directly bind and stabilize cyclin B as part of checkpoint response [209]. Finally, stabilization of cyclin B holds cells in mitosis. This allows cancerous cells exposed to taxanes to acquire a resistant trait by having a prolonged mitotic block and continue proliferation after drug decay and microtubule dynamics restoration, thus surviving chemotherapy (see model in Figure 3-7).

In addition, we demonstrated that a selective small molecule inhibitor of Aurora A, MLN8054, can attenuate USP7-mediated taxane resistance, most likely through accelerated mitotic slippage toward micronucleation [33]. Previously it was shown that knockdown of Aurora A by RNA interference enhanced the chemosensitivity of paclitaxel in pancreatic cancer cells [74]. This evidence, together with our results, suggests that combinatorial drug regimens of taxanes with MLN8054 may improve the outcome of chemotherapy response in cancer patients. We also demonstrated that cytotoxic effects of combination of Taxol and MLN8054 or Taxol alone are similar in control depleted cell lines. However, in USP7 depleted cells, the combinatorial treatment represents the most efficacious means to kill chemoresistant cells. The results presented here demonstrate a role for USP7 in mediating mitotic progression and taxane response mainly through regulation of Aurora A.

Table 4-1. List of USP7 peptides identified by mass spectrometry

Position (aa)	Sequence
847-854	DGPGNPLR
480-487	FDDDVVSR
941-948	LLEIVSYK
302-310	VLLDNVENK
875-882	KLYYQQLK
1024-1033	IQSLLDIQEK
746-755	IQDYDVSLDK
409-420	FMYDPQTDQNIK
756-784	ALDELMDGDIIVFQKDDPENDNSELPTAK
Protein coverage by amino acid count: 102/1102 = 9.3%	

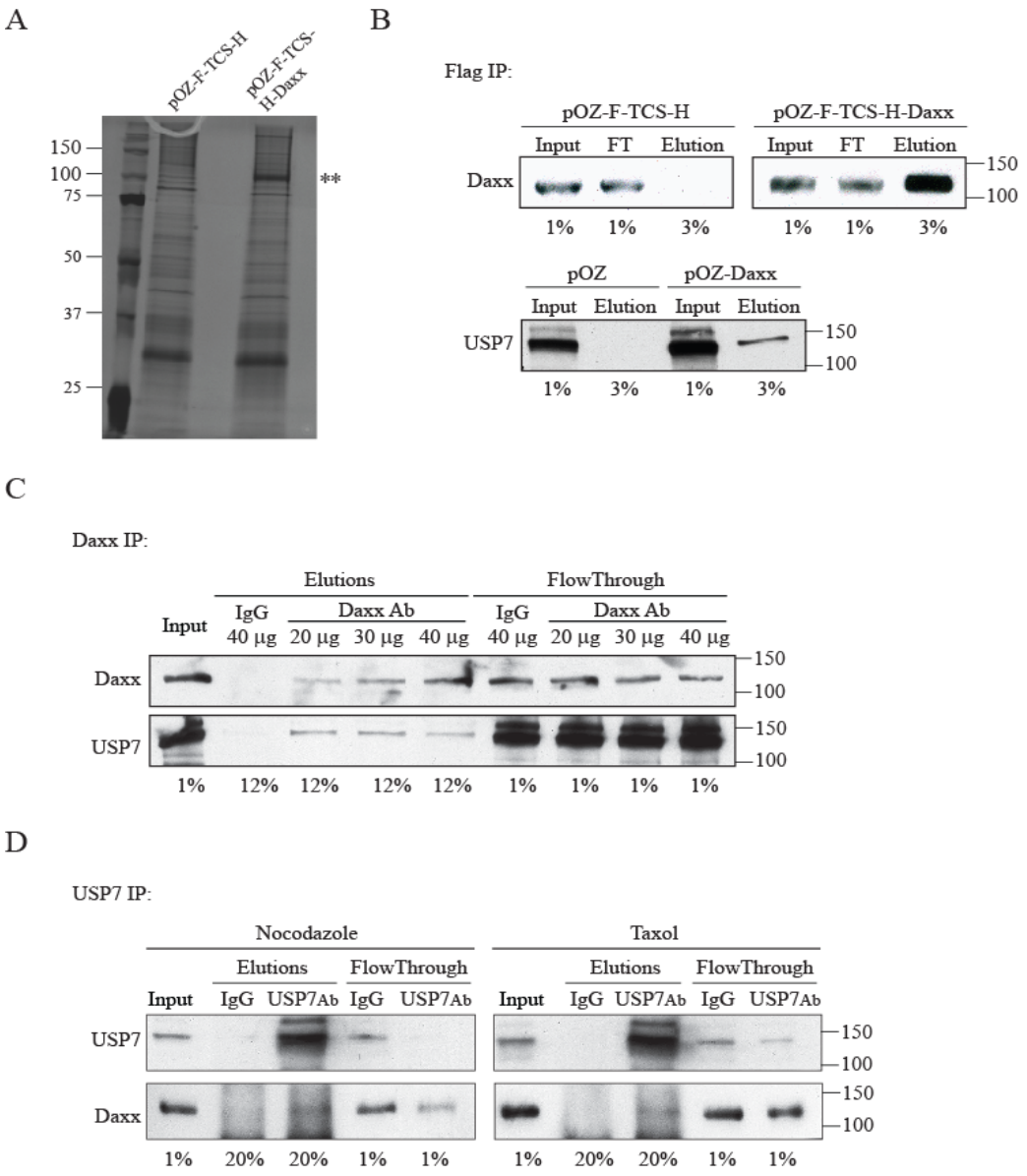


Figure 4-1. Daxx interacts with USP7 in mitosis. (A) HEp2 cells stably expressing pOZ_F-TCS-H or pOZ_F-TCS-H-Daxx were synchronized in mitosis as described in materials and methods. Cell lysates (Inputs) were immuno-precipitated with anti-FLAG-M2 magnetic beads. After extensive washes, complexes were eluted and resolved on SDS-PAGE and colloidal comassie stained. (B) Western blot analysis of immunoprecipitation experiment conducted as in (A). Cell lysates labeled as inputs (I), flow through (FT) and eluted samples (Elution) recovered by thrombin cleavage were immunoblotted with Daxx or USP7 antibodies. Mitotic interaction between Daxx and USP7 is reproducible. Representative experiment out of three. (C) Co-IP of USP7 with Daxx endogenous immunoprecipitation in HEp2 cells synchronized in mitosis by nocodazole exposure. (D) Co-IP of Daxx with USP7 endogenous immunoprecipitation in HEp2 cells synchronized in mitosis by nocodazole or Taxol® exposure. Numbers below the blots represent the sample percentiles.

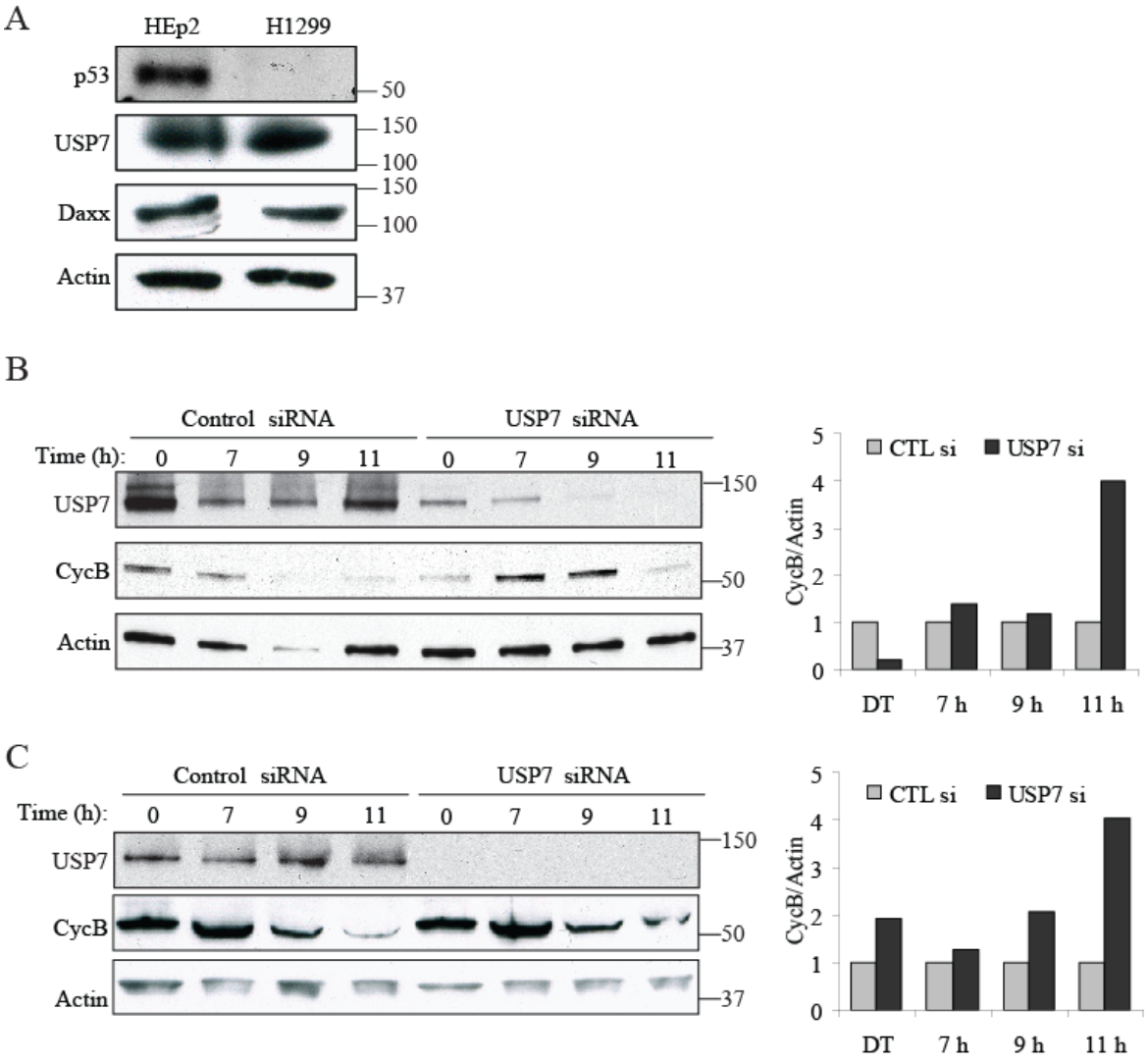


Figure 4-2. USP7 depletion results in stabilization of cyclin B1 (cycB) in a p53 independent manner. (A) Western blot analysis in HEp2 and H1299 cells to compare expression of p53, Daxx and USP7. HEp2 cells express wild-type p53 while H1299 are p53 null but similar levels of Daxx and USP7 are expressed in both cell lines. B and C, left panels: Western blot analysis of HEp2 (B) or H1299 (C) cells synchronized by DTB and simultaneously transfected with either control or USP7 siRNAs. Samples were taken 72h post- transfection at 0, 7, 9 and 11 hours after DTB release to allow cells to progress through mitosis. Right panels: Relative quantization of cycB protein levels using actin as internal control for each timepoint. Data are normalized using control siRNA transfected cells. CycB is stabilized in USP7-depleted cells in a p53 independent manner. Data shows representative experiments out of three.

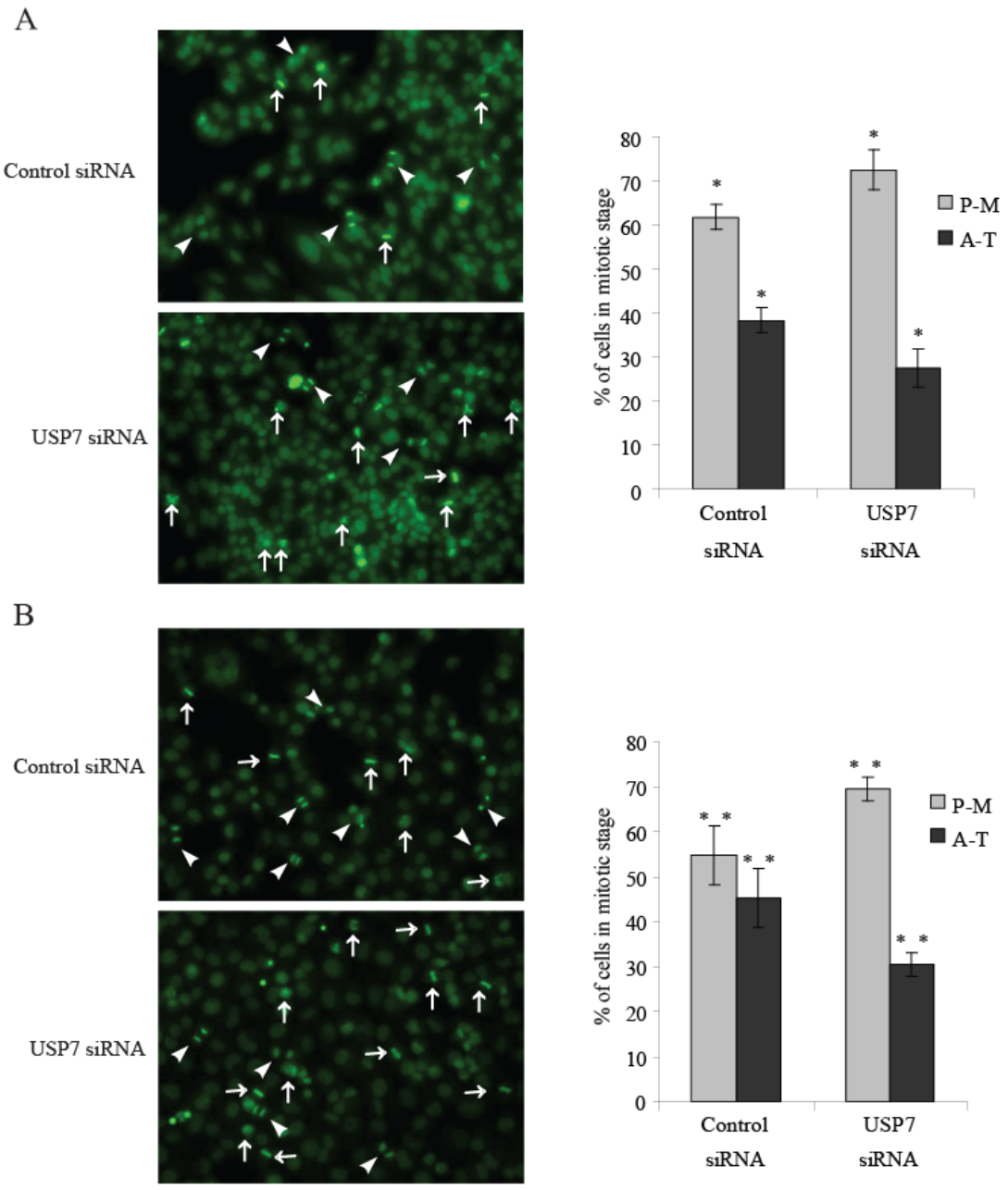


Figure 4-3. USP7 depletion causes accumulation of mitotic cells in prometa-metaphase. HEp2 (A) and H1299 (B) cells were transfected with control or USP7 siRNAs. 72h post-transfection DNA was stained with hoechst 33342. Mitotic stages were distinguished according DNA morphology. The left panels represent the frequencies of mitotic stages divided into two groups: from prometaphase to metaphase (P-M, indicated by arrows) or from anaphase to cytodieresis (A-C, arrowheads). Upon USP7 depletion a dramatic increase of cells in P-M was observed. For each experiment counted there were at least one hundred mitotic events (± SD, n=3). *P-Value<0.01; ** P-Value<0.02.

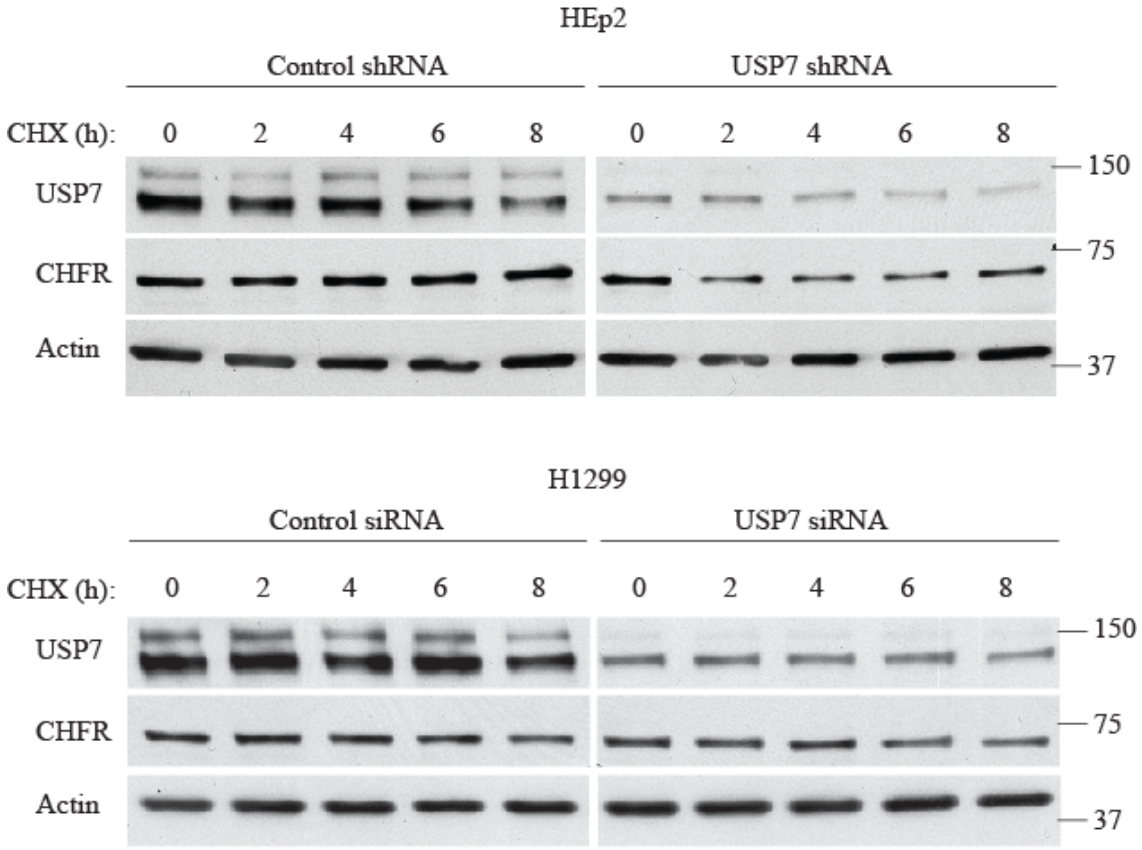
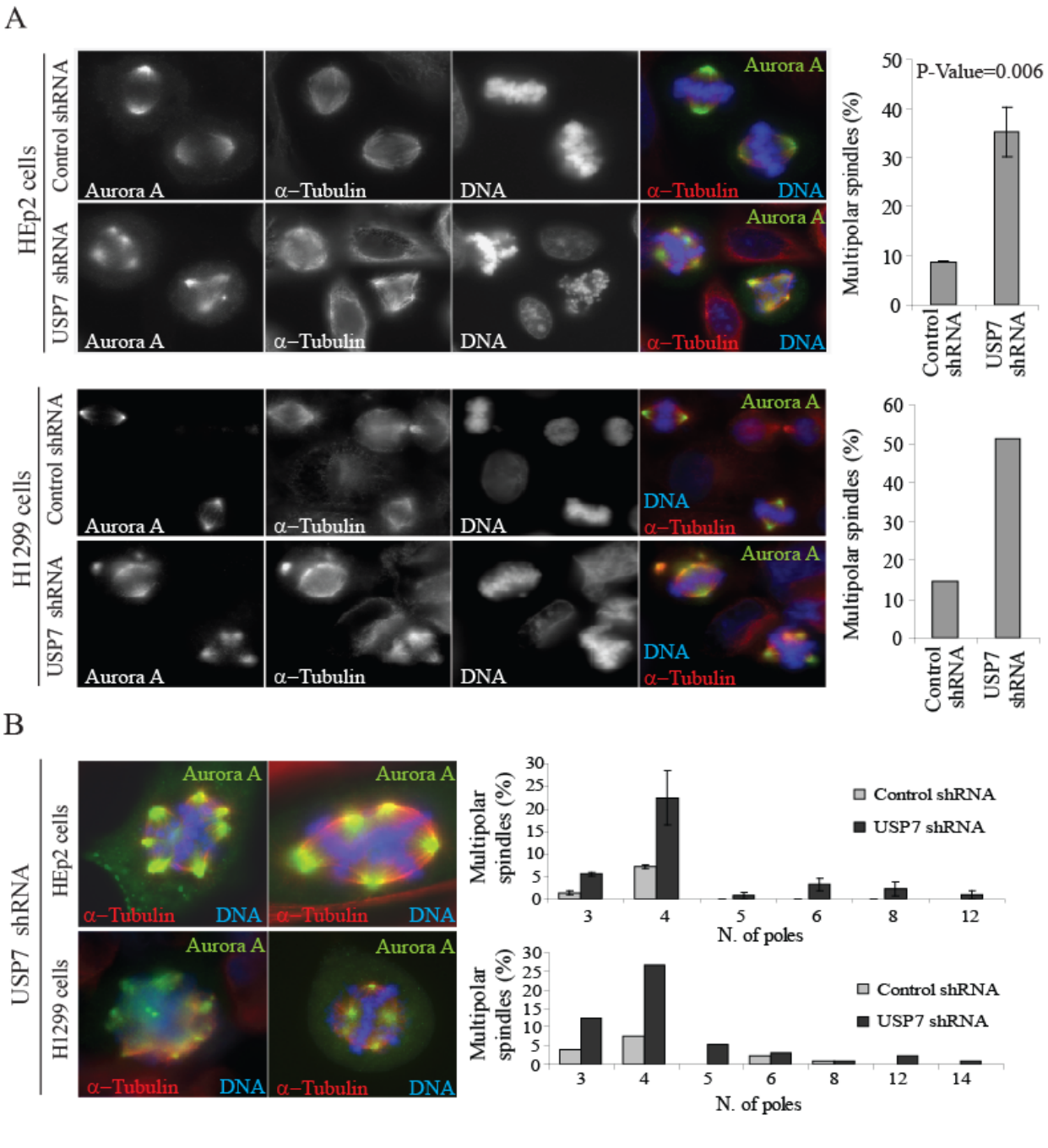


Figure 4-4. USP7 depletion destabilizes CHFR protein. Western blot analysis of Hep2 cells (top) stably expressing control or USP7 shRNA and H1299 cells (bottom) transiently transfected with control or USP7 siRNAs exposed to 1 μg/mL of cyclohexamide (CHX) for 2, 4, 6 or 8 hours. Depletion of USP7 causes the reduction of CHFR protein levels in both cell lines.

Figure 4-5. USP7 depletion causes accumulation of multipolar cell divisions which are mediated by accumulation of Aurora A kinase, a CHFR substrate. (A) Immunofluoresce staining of HEp2 and H1299 stable cell lines expressing shRNAs using Aurora A kinase and α-tubulin to label spindle poles. Hoechst was used to stain DNA. The panels on the right represent the quantification of multipolar events over the total number of mitosis (± SD, n=3). Analysis was conducted counting at least 100 mitotic events for each experiment. (B) Immunofluoresce staining of Aurora A kinase, α-tubulin and DNA in HEp2 and H1299 stably expressing USP7 shRNA. In USP7 depleted cells are observed multipolar divisions with overnumerary poles (n>4). Quantification of these events is presented on the right panel (± SD, n=3). (C) Representative Western blot analysis showing accumulation of Aurora A kinase in HEp2 and H1299 cells depleted by USP7. Cells were synchronized by DTB and releases in growth media for 11 hours to progress through mitosis. Numbers represent quantification of Aurora A protein over internal control (actin).


A
HEp2 cells
Control shRNA
USP7 shRNA
Aurora A
α–Tubulin
DNA
P-Value=0.006
Multipolar spindles (%)
Control shRNA
USP7 shRNA
H1299 cells
B
USP7 shRNA
HEp2 cells
H1299 cells
Multipolar spindles (%)
N. of poles
Control shRNA
USP7 shRNA
C
HEp2
H1299
CTL sh
USP7 sh
CTL si
USP7 si
Aurora A
Actin
Ratio Aurora A/Actin:
1
1.9
1
24

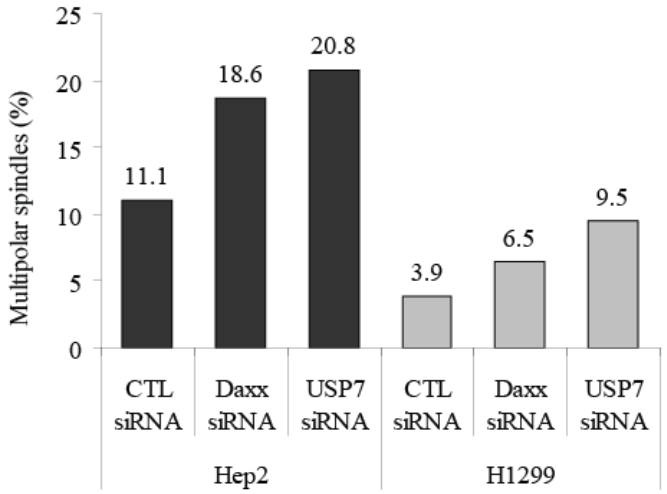


Figure 4-6. Transient depletion of USP7 and Daxx leads to the accumulation of multipolar mitoses. Graph represents the quantification of multipolar events over the total number of mitosis in control, Daxx and USP7 siRNAs, in HEp2 and H1299 cells. For each experiment staining of Aurora A, α-tubulin and DNA was performed to label spindle poles and assess chromosomal segregation. A minimum of one hundred mitotic events were counted *per* experiment. Transient depletion of USP7 and Daxx in HEp2 and H1299 cells causes increase of multipolar spindles.

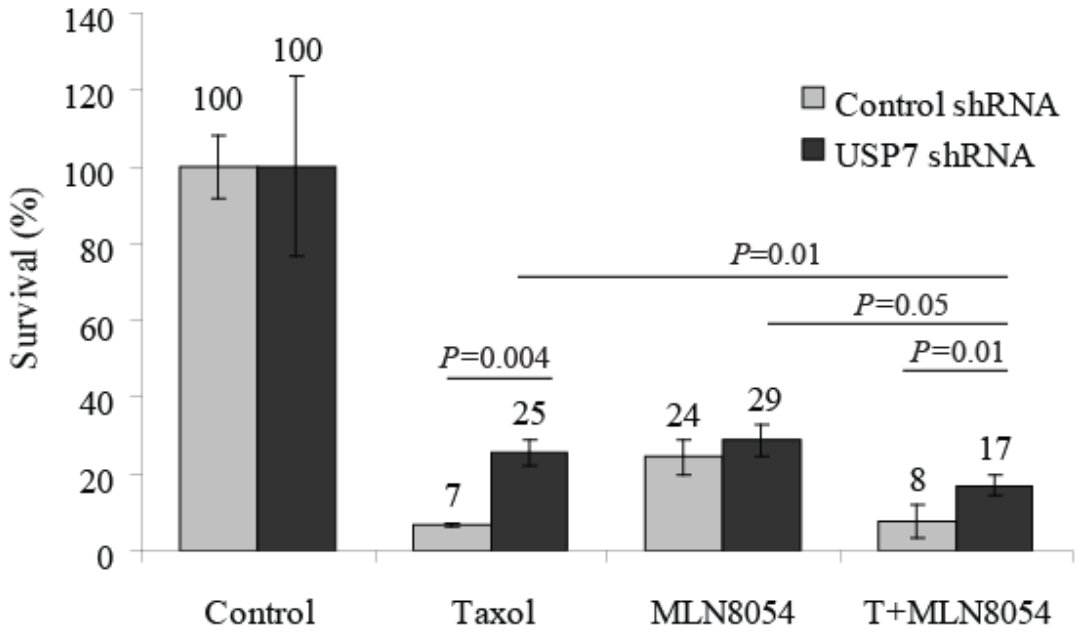


Figure 4-7. Depletion of USP7 Desensitizes Cells to Paclitaxel which can be rescued by Aurora A inhibition. Percentage of colonies formed from control- or USP7-depleted HEp2 cells which were synchronized using a double thymidine block and then released and exposed to control, 10nM taxol, 4uM MLN8054 and both drugs for 18 hours. After treatment, cells were replated for colony formation assay (±SD, n=3).

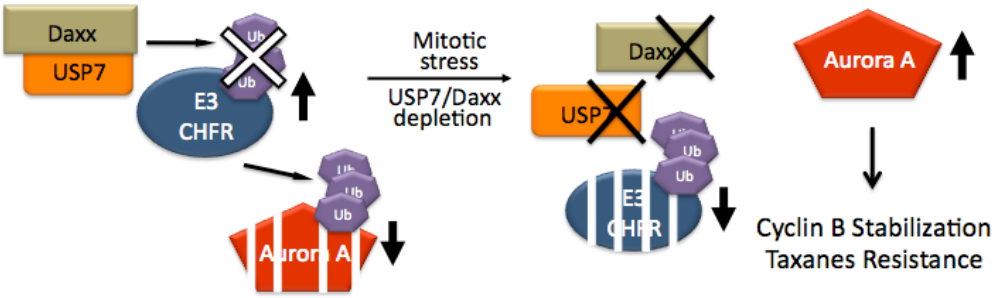


Figure 4-8. USP7/Daxx regulation of mitosis and taxane resistance. Daxx activates USP7 for de-ubiquitination of mitotic E3 ligase CHFR; stabilization of CHFR elevates Aurora A ubiquitination and degradation. Deregulation of USP7 or Daxx reduces stability of CHFR, thus accumulating Aurora A. It turns into stabilization of Cyclin B that holds cells in mitosis thus elevating survival upon taxanes treatment. Ub: ubiquitin.

CHAPTER 5
DAXX AND USP7 ARE GUARDIANS OF GENOMIC STABILITY

Introductory Remarks

As it was first postulated by the German biologist Theodor Boveri in 1902, the precise partitioning of duplicated chromosomes to daughter cells is essential for the development and survival of all organisms. Defects in segregation lead to aneuploidy, the state where entire chromosomes are gained or lost. Aneuploidy is a hallmark of the majority of tumor cells and several lines of evidence indicate that it contributes to the evolution of cancer [228-232]. Aneuploidy is also the leading cause of spontaneous miscarriages and hereditary birth defects in humans [233, 234].

In order to segregate correctly the genetic material during mitosis, a sophisticated and well conserved system among all eukaryotic cells tightly regulates cell division. During this process the dividing cell ensures that kinetochores of each chromatid are correctly attached to the bipolar spindle. During the initial formation of the mitotic plate, at the prometa/metaphase transition, many sister chromatids do not achieve the necessary biorientation. Some sister chromatids may be attached to microtubules irradiating from the same spindle pole (known as syntelic attachments) or one chromatid may become attached at the same time with microtubules from the two opposite poles (known as merotelic attachment) [235]. These abnormal attachments must be corrected so that until all chromosomes are under proper tension and bi-orientation, the metaphase-anaphase transition is delayed [236, 237]. In eukaryotic cells this delicate mission is assigned to the Spindle Assembly Checkpoint (SAC) [30].

To the SAC belongs a long list of proteins with checkpoint function identified by genetic screenings [238]. These proteins can be categorized as ‘sensors’ (Aurora B,

Mad1, Bub1 and Mps1) [239, 240] or as 'signal transducers' (Cdc20 plus the Mitotic Checkpoint Complex or MCC, formed by Mad2, Bub3 and BubR1 proteins) [241] or as 'effectors' (anaphase promotion complex/cyclosome - APC/C) [234, 242, 243]. In short, these molecules can sense lack or faulty tension, even on a single kinetochore, to produce a 'wait' signal, preventing Cdc20 to associate and activate the APC/C. This system avoids segregational errors by inhibiting APC/C^{Cdc20} mediated destruction of cyclin B and securin, thereby avoiding entrance in anaphase.

Once all the kinetochore-microtubules attachments satisfy the SAC, Cdc20 can then bind and activate APC/C to ubiquitinate cyclin B and securin for proteolytic degradation [30, 146]. Drops in securin levels lift the inhibition on separase. This enzyme then cleaves the cohesin rings which hold sister chromatids together to finally progress from metaphase to anaphase [244].

Thus, maintenance of a functional SAC is fundamental for the genomic stability of cells. Genetic studies conducted with hypomorphic or heterozygous mouse models for the components of the SAC, demonstrated that these mice are characterized by aneuploidy [245-247] and increased rates of spontaneous (Mad2+/- [247], CENP-E+/- [248, 249], Bub1 hypomorphic [250, 251]) or carcinogen-induced (Bub3 [245] and BubR1 [246]) tumors.

Surveillance, detection and correction of failed or improper microtubule/kinetochore attachment are tightly regulated where recruitment of SAC components is hierarchical and sequential [252]. The protein Bub3 (budding uninhibited by benzimidazoles 3 homolog) [253] is a key SAC component that is immediately localized to unattached kinetochores [254]. Here it recruits other components of the MCC and the SAC sensor Bub1 to generate two inhibitory complexes that suppress the function of APC/C

by either sequestering or inhibiting Cdc20 respectively [255, 256]. Another fundamental checkpoint function governed by Bub3 is to establish the formation of correct microtubules-kinetochore attachments coordinating microtubule binding proteins [257, 258].

Cells with reduced levels of Bub3 compromise localization of the other components of the SAC, Bub1 and BubR1 [258], resulting in failed SAC activation [257]. Due to inability to correctly attach and sense improperly bound kinetochores-microtubules, reduced expression of Bub3 displays chromosome segregation errors in the form of micronuclei [259] misaligned and lagging chromosomes [258].

During the characterization of USP7 depleted cell lines we noticed a peculiar phenotype. Cells lacking USP7 showed signs of chromosomal instability. These results were reminiscent of the aneuploidy we previously observed in cells isolated from Daxx knock out mouse embryos where extensive aneuploidy was observed in $Daxx^{-/-}$ MEFs, compared to Daxx+/- or +/+ MEFs which displayed normal karyotype. This condition was observed in three independent $Daxx^{-/-}$ MEF cell lines, suggesting Daxx may be important for accurate chromosomal separation.

In this chapter we present evidence that USP7, as previously demonstrated for Daxx, causes genomic instability characterized by abnormal chromosomes segregation, accumulation of micronuclei and increased aneuploidy. We demonstrated that these mitotic abnormalities may be mediated by reduction of checkpoint protein Bub3 in USP7 depleted cells. Our results indicate that USP7 interacts with Bub3 in mitosis. This binding favors Bub3 stabilization as cells depleted by USP7 have reduced levels of Bub3. These effects are occurring in similar extents in cell lines harboring wild type or mutated p53.

Thus, results presented in this chapter provide the first evidence that loss of USP7 leads to genomic instability by destabilizing a key mitotic checkpoint component in a p53 independent manner.

Results

USP7 Depletion Causes Genomic Instability

While generating USP7 shRNA cells, we noticed several interphase nuclei abnormalities that are usually recognized as derived from mitotic segregational defects [260]. Cells with reduced levels of USP7 have high levels of nuclear blebs and micronuclei (MN) compared to control-depleted cells (Figure 5-1). In USP7 shRNA cells, a small sub-population of cells that is still positive for USP7 does not have nuclei abnormalities.

Loss of USP7 leads to increase in micronuclei formation

To study the significance of this observed nuclear abnormalities we decided to quantify the extent of micronuclei (MN) accumulation. MN are the manifestation of chromosomal instability and are generated as a consequence of cellular exposure to genotoxic stresses or as result of mitotic abnormalities [261]. MN originate from chromosomal fragments or whole chromosomes that are not segregated during mitosis because they are not properly attached to spindle microtubules [262]. At the end of mitosis nuclear envelope reforms around the 'lost' genetic material in the cytoplasm, where MN appear as tiny nuclei by DNA staining [263]. In mammalian cells, MN form during anaphase [264] from lagging chromosome fragments –in case of failed repair of DNA double strand breaks [261]– or whole chromosomes –in case of defects in assembly of kinetochore proteins [265, 266], abnormal spindles [267, 268] or non-functional SAC [238, 260, 269, 270].

To statistically assess the accumulation of MN upon USP7 depletion compared to the control depleted cells, we adopted the previously established rules for MN scoring [148]. The MN score represents the percentage of MN *per* cell, where as MN was considered each rounded DNA material in the cytosol, non- overlapping with the nuclear material and not exceeding 1/3 of the volume of the nucleus. Only occasional MN were detected in HEp2 and H1299 control depleted cell lines (Figure 5-2, first panel in enlarged box); a dramatic increase of MN score was observed in USP7 depleted cell lines (Figure 5-2). In addition, the MN population was heterogeneous across the USP7 shRNA cells in regards of both size and number *per* cell, as shown by the pictures in three distinct fields (Figure 5-2). We could clearly distinguish two different MN sub-populations. The first one was characterized by multiple and larger MN (Figure 5-2, arrows). This type of MN usually arises from multipolar mitoses due to the inability of the cell to correctly partition groups of chromosomes. The increased incidence of multipolar events upon USP7 depletion and the underlying mechanism were presented in Chapter 4 (Figure 4-5). The second one was represented by a small MN, often located between two interphase nuclei (Figure 5-2, arrowheads). Since this type of MN derives from lagging chromosome at the anaphase onset, it is likely that other mitotic segregation problems (besides Aurora A induced multipolarity) may be encountered by cells with depleted USP7.

Loss of USP7 causes increase of lagging chromosomes

One of the mechanisms responsible for the MN formation is the presence of unattached or lagging chromosomes in anaphase [264]. To better understand the nature of MN formation, immunofluorescence staining was done in control or USP7 depleted HEp2 cells. Cells were labeled with centromeric protein CENP-A and peri-centromeric

protein CENP-B to detect centromeres/kinetochores. Anaphases with lagging chromosomes were detected in almost all mitotic events in HEp2 cells stably depleted of USP7 (80% of lagging chromosomes *versus* 10% of control shRNA cells, p=0.00002; Figure 5-3). This data, together with the MN scoring, strongly indicates that USP7 depletion causes significant chromosome mis-segregation that may be driven 1) by multipolarity due to accumulation of Aurora A, and 2) from lagging chromosomes in anaphase.

Loss of USP7 promotes abnormal karyotype

To understand in detail the role of USP7 in genomic instability HEp2 and H1299 cells stably expressing control or USP7 shRNAs were karyotyped. Our expectation was to observe deviation from the cell line chromosomal modal numbers due to the high extent of micronucleation and multipolarity in cells with silenced USP7.

Karyotypes of the analyzed cell lines were nearly triploid for HEp2 cells with chromosomal modal number of 72 ± 7.8, and nearly tetraploid for H1299 cells with chromosomal modal number of 94.2 ± 12.5. However, upon USP7 depletion, a deviation from these numbers was observed. In USP7 depleted HEp2 cell line, the calculated chromosomal modal number was 73.1 ± 15.2. The doubling of the standard deviation indicates an increased genomic instability in these cells. More dramatic were the effects in H1299 USP7 shRNA cell line, in which the modal number was reduced from 94.2 of the control to 85.1 chromosomes *per* cell. As well, the standard deviation of chromosomes modal number was increased almost three-fold. Thus, consistently with the morphologic manifestation of genomic instability (MN and lagging chromosomes- Figures 5-2 and 5-3), the number of cells with gain or loss of chromosomes was increased in USP7 shRNA cells in both p53 positive and negative cells (Figure 5-4).

In order to confirm this data in a non-tumorigenic cell line, we analyzed karyotypes of human mammary epithelial cells, MCF10A, upon transient depletion with cell-permeable non-targeting control or USP7 siRNAs (Accell technology, Dharmacon). Analysis of up to one thousand metaphases in MCF10A control or USP7 depleted cells confirmed data obtained in HEp2 and H1299 cancer cell lines: USP7 silencing causes almost a two-fold increase in aneuploidy.

This data is in agreement with evidence previously collected from karyotyping of Daxx-/- MEFs which were characterized by severe aneuploidy compared to normal kariotype displayed by Daxx +/+ MEFs. Hence, our data indicates a common role of Daxx and USP7 in mitotic regulation and maintenance of genomic stability.

USP7 Interacts and Controls Stability of SAC Protein Bub3

USP7 loss induces high rates of unattached chromosome that may result from a change in stability of mitotic checkpoint proteins. While we were screening for mitotic proteins which would have differential stability upon USP7 depletion, the 'interactome' landscape of human DUBs was published [271]. This report indicated that USP7, among other proteins, interacts with checkpoint protein Bub3 in HeLa cells. Bub3 protein levels were consistently low upon USP7 depletion, in both HEp2 and H1299 cells (Figure 5-5, panel A). These results are consistent with the increase of lagging chromosomes and MN (Figures 5-2 and 5-3) and the data previously published for Bub3 [238]. Indeed many reports indicate that Bub3 localizes to unattached kinetochores to recruit and interact with other members of the MCC to maintain Cdc20 inactive until all kinetochores are correctly attached and aligned to the mitotic plate [254, 257-259]. Haplo-insufficiency of Bub3 leads to chromosomal mis-aggregation and chromosomal instability [241, 259].

To test USP7/Bub3 interaction, immuno-precipitation experiments of endogenous USP7 were done in HEp2 cells synchronized in mitosis by either nocodazole or Taxol® exposure. Cell lysates (Input) collected from synchronized cells were used in IP experiments with control IgG or USP7 antibodies conjugated to protein G magnetic beads (Dynabeads, Invitrogen). Bub3 was pulled-down only with USP7 antibodies indicating that endogenous proteins, Bub3 and USP7, interact *in vivo* in cells arrested in mitosis. In addition the results suggest that this association occurs independently from the activation status of the SAC, since Bub3 is co-immuno-precipitated with USP7 in both nocodazole (SAC off) and Taxol® (SAC on) (Figure 5-5, panel B).

Summary of Results

Results presented in this chapter uncover a novel and pivotal function of USP7 in the maintenance of genomic stability. Upon USP7 depletion we observed mitotic abnormalities including 1) micronuclei accumulation, 2) mis-shaped nuclei, 3) lagging chromosomes in anaphase and 4) karyotype instability. Our results suggest that these abnormalities are mediated by decreased levels of SAC component Bub3, interaction partner and potential substrate of USP7 in mitosis. Thus our results show that USP7 regulates mitosis by affecting stability of multiple checkpoint proteins: Bub3, CHFR and Aurora A, as described in Chapters 4 and 5.

Discussion

The work presented in this chapter indicates that, as previously demonstrated for Daxx, USP7 is also essential in maintaining genomic stability in mammalian cells, independently from the cell tissue of origin. Indeed analysis of interphase cells with reduced expression of USP7 presented several manifestations of mitotic abnormalities

such as ill shaped nuclei, massive micronucleation, nuclear buds and nucleoplasmic bridges (Figures 5-1 and 5-3).

These events are generally considered as markers of genotoxic stress and are hallmarks of tumor cells due to the genomic instability of the cancerous growth.

Closer analysis of mitotic figures revealed that loss of USP7 causes a wide array of mitotic abnormalities such as multipolar spindles (as shown in Chapter 4), increase of MN (Figure 5-2) and accumulation of lagging chromosomes (Figure 5-3). Collectively this data indicates that USP7 depletion causes genomic instability.

Karyotype analysis of HEp2 and H1299 cells stably expressing control or USP7 shRNAs revealed that USP7 depleted cells were more aneuploid than the control depleted cells (Figure 5-4); moreover, we recapitulated the same results upon transient depletion of USP7 in non-tumorigenic MCF 10A breast epithelial cells (not shown).

Next, we hypothesized that the loss of USP7 would cause genomic instability by deregulating proteins belonging to the SAC. To test this hypothesis we analyzed by western blot stability of several components of the SAC (Mad2, Cdc20 and Cdh1, data not shown). Only Bub3 showed marked reduction upon USP7 depletion in both HEp2 and H1299 cells. Prior studies RNAi and genetic demonstrated that decreased Bub3 causes failure in activating the spindle checkpoint machinery thus ablating the ability to correct or establish proper microtubules-kinetochores attachments. These findings may explain the observed mitotic abnormalities upon USP7 silencing. Hence, we can conclude that cells lacking USP7 are impaired in the spindle checkpoint assembly and signaling, dictated by decreased Bub3 protein. These cells enter anaphase with sister chromatids improperly attached to the spindle poles. This explains the appearance of

lagging chromosomes, the increase in micronuclei and the aneuploidy, as reported in this chapter.

Interestingly Bub3 and USP7 endogenous proteins interact in mitosis as shown in Figure 5-5, suggesting that Bub3 could be a direct target of deubiquitinating activity of USP7. Thus the reduced levels of the checkpoint protein Bub3 observed in USP7 depleted cells suggest that Bub3 may be a novel USP7 substrate.

In conclusion, we identified a new biological role of Daxx and USP7 in G2/M progression, which is independent from p53 cellular status. In this chapter, we presented novel evidence for a pivotal role for Daxx and USP7 in genomic stability thus indicating tumor suppressors function for these proteins. Recent findings of Daxx mutations in neuroendocrine pancreatic tumor and pediatric glioblastoma further confirm this notion.

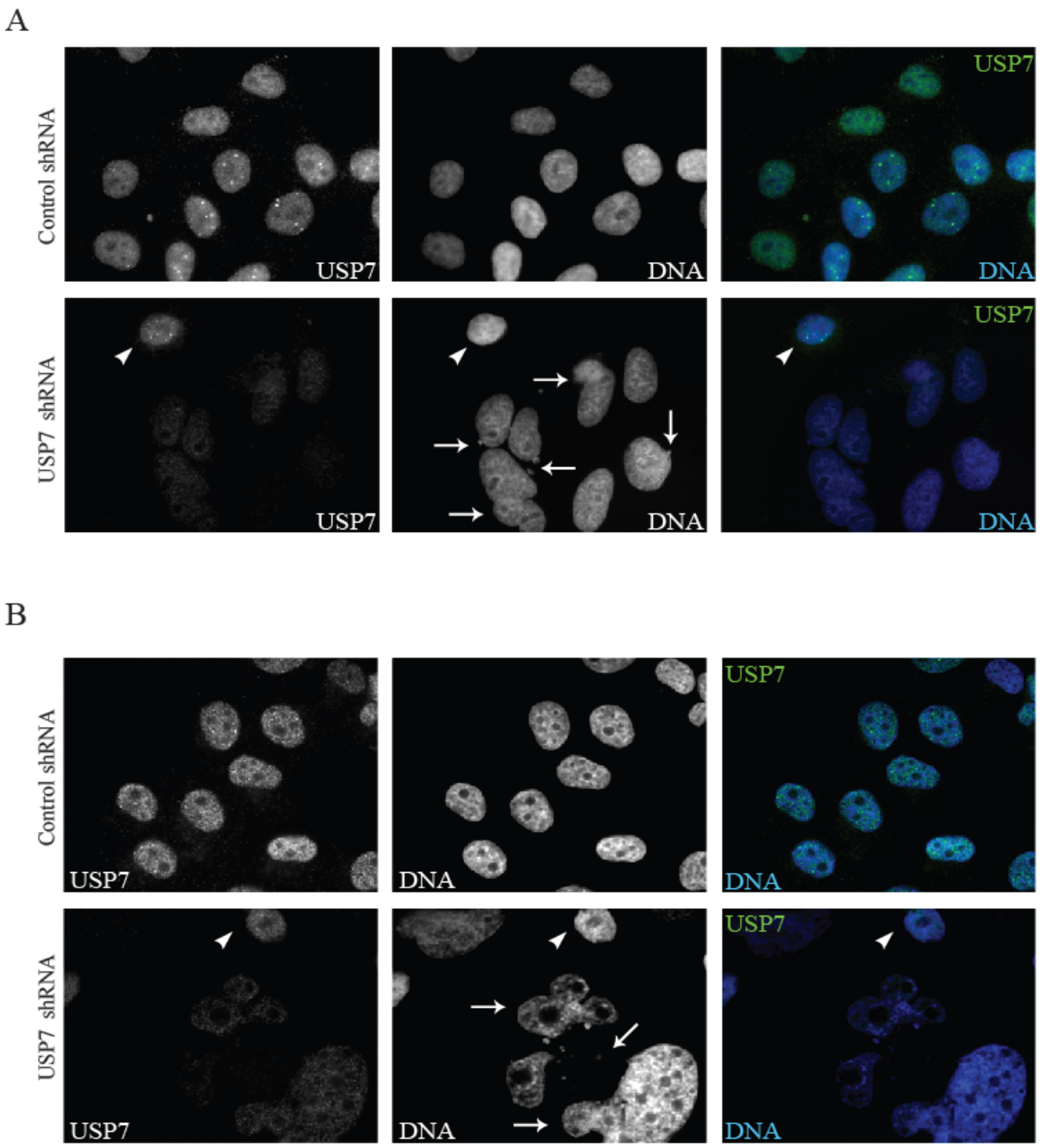


Figure 5-1. USP7 depletion causes genomic instability. Analysis for USP7 (green) and DNA (blue) in HEp2 (A) and H1299 (B) cell lines stably expressing control or USP7 shRNAs. The majority of USP7 depleted cells show genomic instability (micronucleation and irregular nuclear shape, indicated by arrows) while USP7 positive cells (white arrowheads) do not.

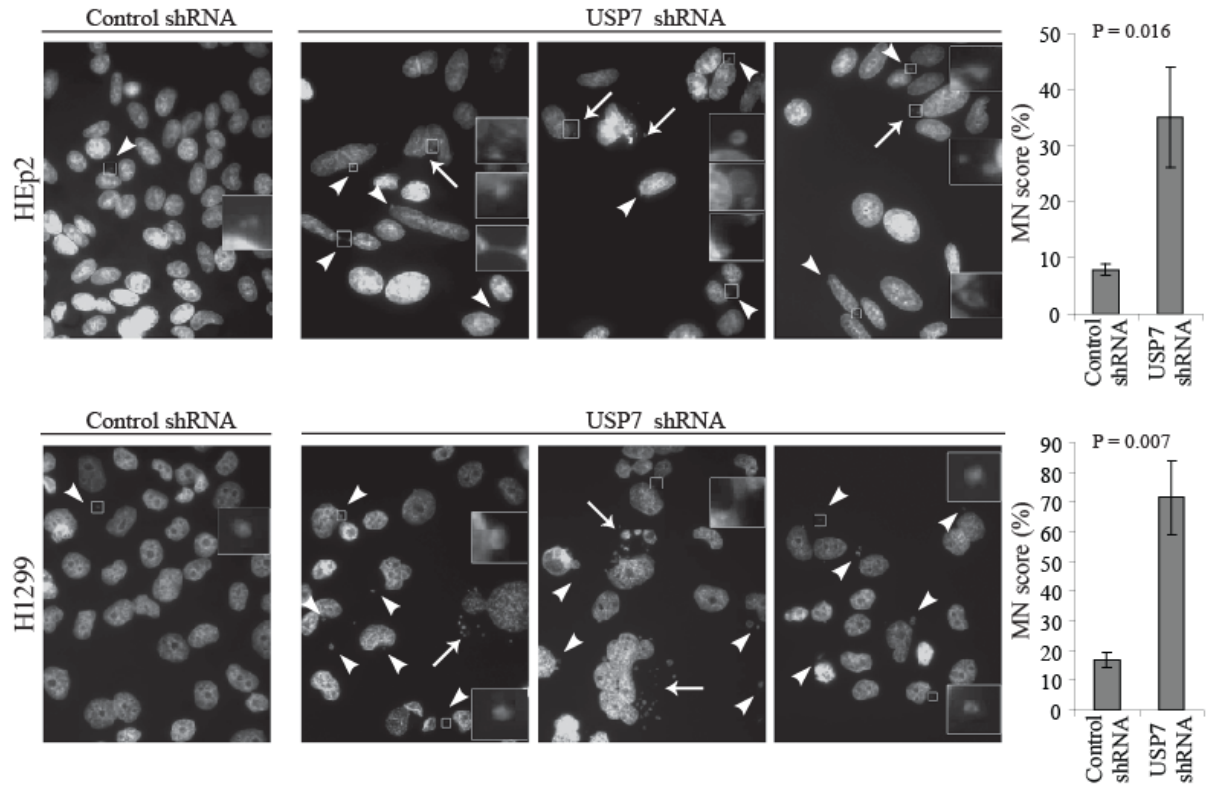


Figure 5-2. Loss of USP7 causes accumulation of micronuclei. Depletion of USP7 elevates micronuclei (MN) score in both p53 WT (HEp2) and null cell lines (H1299). Three representative fields show heterogeneity of MN size and number of MN *per* cell in USP7 shRNA cell lines. White arrowheads indicate single MN while white arrows point to groups of MN. Right: quantifications of MN accumulation over the total number of cells (MN score). For each experiment a minimum of 300 cells were counted (± SD, n=3) and for the scoring of micronuclei the criteria previously described were adopted [148].

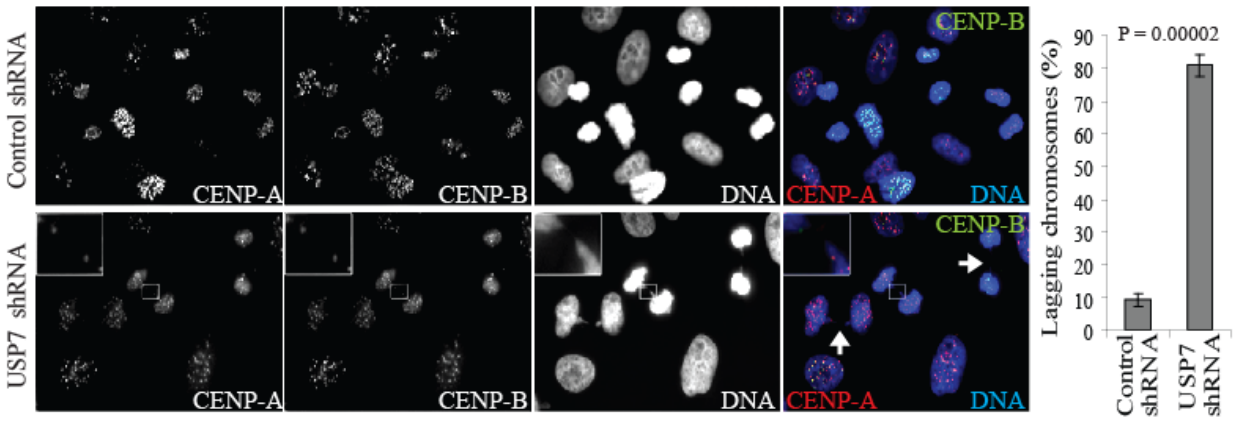


Figure 5-3. USP7 depletion causes mitotic abnormalities. USP7 depleted cells accumulate mitotic bridges and lagging chromosomes. Left: immunofluorescence staining of HEp2 cells labeled with centromeric marker CENP-A (red), CENP-B (green) and DNA (blue). Right: quantifications of lagging chromosomes over the total number of anaphases (lagging chromosomes %). For each experiment a minimum of 300 anaphases were counted (± SD, n=3)

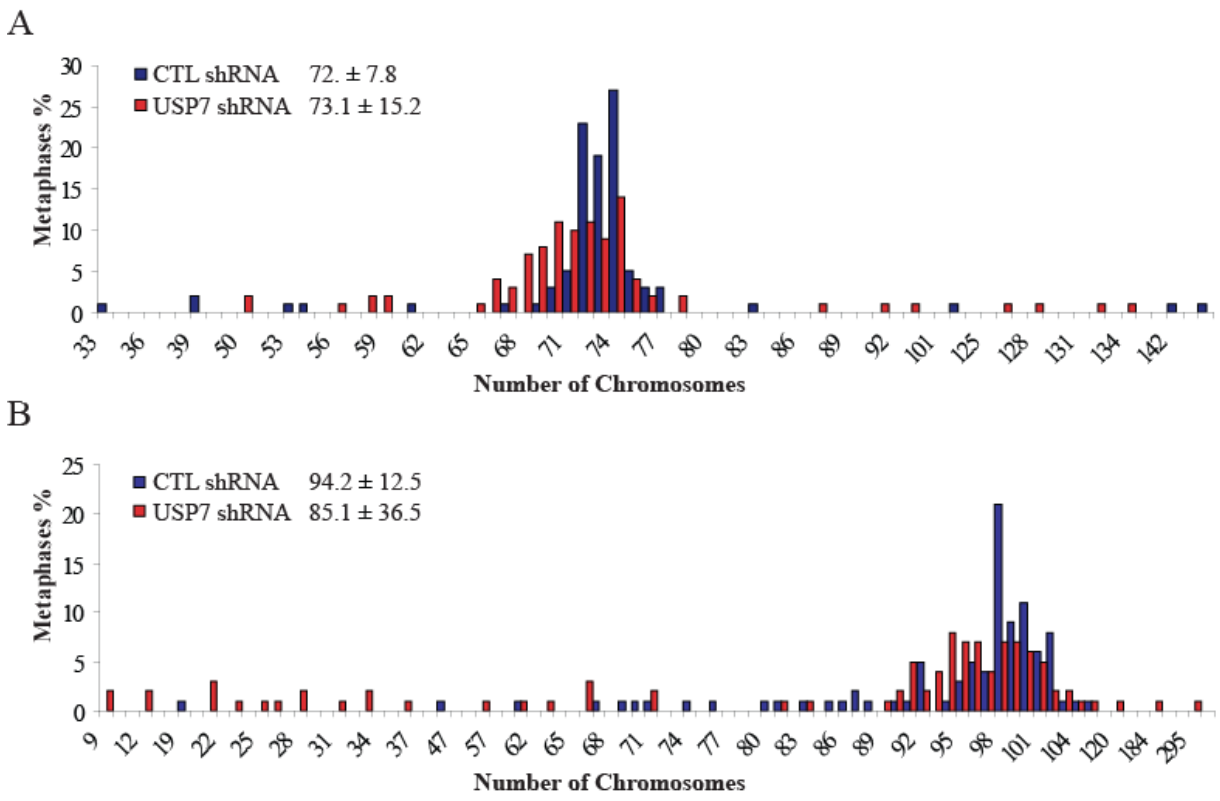


Figure 5-4. Depletion of USP7 leads to increased aneuploidy. Metaphase chromosomal analysis of HEp2 (panel A) or H1299 (panel B) cells stably deplete by control or USP7 shRNAs. Results were generated from 100 metaphases. Numbers represent sample's weighted average ± weighted s.d.

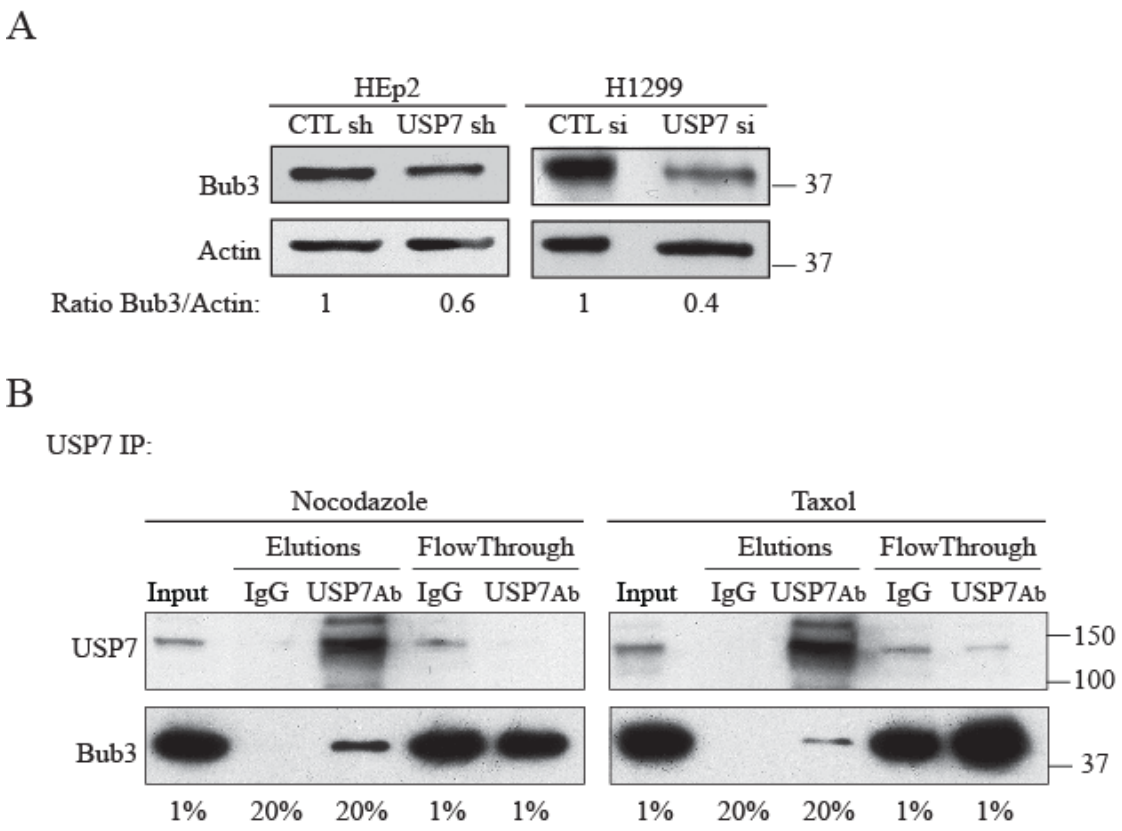


Figure 5-5. USP7 regulates stability and interacts with Bub3. A) Protein levels of checkpoint protein Bub3 are decreased upon depletion of USP7 in HEp2 and H1299 cells. Numbers below the blot represent relative quantification of Bub3 signal over actin chosen as loading control. Representative western blots of three independent experiments. B) Immunoprecipitation experiments of endogenous USP7 in HEp2 cells synchronized in mitosis by nocodazole or Taxol® exposure. Bub3 is pulled down only with USP7 antibody but not with IgG control. Numbers below the blots represent the sample percentile loaded.

CHAPTER 6
SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

Summary and Conclusions

Breast cancer is the most common cancer among middle aged women and ranks second in cancer-related mortality. With development of adjuvant therapies in breast cancer over the past 40 years, we are getting closer to a cure for breast cancer and efforts are continuing to find better treatments for metastatic breast cancer, which unfortunately remains incurable. The focus in research to prevent further metastatic incurable disease by selective and more effective therapy is needed to decrease mortality in breast cancer. Taxanes, a group of cytotoxic drugs which includes paclitaxel (Taxol®) and docetaxel (Taxotere), are among the most successful anticancer agents for breast cancer chemotherapy [11, 18, 149]. Taxane activity affects cell cycle at several key points, including mitotic checkpoint [16, 151] and leads to mitotic arrest that will eventually trigger cell death [21] by a mechanism still largely unknown [17, 152-154].

Many breast cancer patients are resistant or become resistant to Taxol® during drug administration [52, 53]. Indeed, intrinsic and acquired resistances to taxanes represent the most limiting factors to the successful treatment of breast cancer patients. Thus, predicting which patients will respond to taxane therapy and which will not benefit but only experience the side effects of the chemotherapy, is critical.

The scientific work presented in this dissertation was designed and executed for the characterization of taxane resistance machinery and to identify novel predictive markers that would allow differentiation of breast cancer patients for most suitable therapies and improve overall and cancer-free survival.

Seeking markers of taxane response, we identified a novel role of nuclear protein Daxx in taxane sensitivity. During previous studies published by our laboratory, Daxx was identified as a novel regulator of paclitaxel response in cell culture conditions [88]. Breast cancer cell lines with low levels of Daxx expression were resistant to drug application and importantly this data was recapitulated upon Daxx depletion in primary mouse and human cells.

In order to better understand this phenomenon we wanted to assess whether Daxx mediated taxane resistance could be reproduced also in breast cancer patients.

To address the clinical ramifications of Daxx regulation of taxane sensitivity, twenty-two women treated with standard taxane and anthracycline based neoadjuvant chemotherapy for locally advanced breast cancer were classified as either responders or non responders based on the clinical outcome. Responders to therapy had a higher mean Daxx score (calculated based on IHC staining intensity multiplied by the percent of staining cells) compared to non-responders, suggesting that Daxx could predict the response to taxane-based chemotherapy. Thus, the characterization of Daxx as novel biomarker predicting taxane responsiveness would be a crucial addition to the available predictive markers for patient populations with early and metastatic breast cancer.

In addition, we found that Daxx depletion affects normal mitotic progression, accumulating cells in prometaphase/metaphase *via* reduced degradation of mitotic cyclin B (Chapter 3 and reference [116]). In the absence of Daxx, cells remain in a prolonged mitotic block, while wild type cells undergo a transient arrest in mitosis. The prolonged mitotic arrest allows cells with down-modulated Daxx to escape taxane action (model in figure 3-7). To understand the mechanism of how Daxx affects mitosis

progression and taxane resistance, we isolated Daxx mitotic complex and identified Ubiquitin Specific processing Protease-7 (USP7), a deubiquitylating enzyme (DUB), as Daxx interacting protein in mitosis. Deubiquitinating enzyme USP7 has been described as both a tumor suppressor and an oncogene [179, 185, 190, 191]. Due to potential oncogenic properties, mostly attributed to p53 regulation, USP7 represented a promising anti-neoplastic target [198, 199, 272].

Earlier reports described that, in interphase, Daxx bridges USP7 and p53-specific E3 ubiquitin ligase MDM2, to maintain low levels of tumor suppressor p53 in normal cells[126, 127, 191, 192]. Thus, USP7/Daxx interaction was associated to p53-dependent G1/S checkpoint regulation. However, in this dissertation we presented evidence that Daxx and USP7 control another stage of cell cycle, mitosis, in a p53 independent manner. We found that USP7 depletion affects mitotic progression and taxane resistance similarly to Daxx inactivation, suggesting that both proteins are cooperating and functioning as new mitotic regulators assuring proper response to taxane treatment (Chapters 4). Their action regulates stability of several checkpoint proteins, such as E3 ubiquitin ligase CHFR and it substrate Aurora A (Chapter 4) and mitotic checkpoint protein Bub3 (Chapter 5). We have demonstrated that cells with decrease expression of USP7 have reduced levels of Bub3 and CHFR but accumulate Aurora A. All these effectors are regulated directly or indirectly by USP7 and are involved in taxanes response. Cells or tumors over-expressing Aurora A (as we have shown in Chapter 4 uopn USP7 down-modulation) are resistant to paclitaxel administration [72, 74]. As well, cells deficient in SAC protein Bub3 have been shown unresponsive to paclitaxel [71]. Contrarily, cells that lack CHFR are more sensitive to taxanes [46, 273] that may contradict our model. Therefore, to

assess the contribution of CHFR in USP7 mediated mitotic anomalies, we tested the ability of USP7 depletion to accumulate Aurora A in CHFR null HCT116 colon carcinoma cell lines. Aurora A and cyclin B were both stabilized in p53 isogenic HCT-116 cell lines (data not shown), thus indicating that CHFR contributes only partially to USP7-mediates stabilization of Aurora A in HEp2 and H1299 cells. Furthermore this data highlights the p53 and tissue independency of USP7 mitotic regulation. Thus, Daxx/USP7-mediated regulation of mitosis and sensitivity to taxane occurs through fine-tuning protein levels of checkpoint proteins Aurora A and Bub3 in agreement with previous reports.

We previously reported that Daxx interacts in mitosis with protein Rassf1. The major isoform of this gene, Rassf1A, has been implicated as a mitotic regulator and has been shown to interact with several key mitotic-related proteins, including Aurora A [274] and Cdc20 [275], though the latter is debatable [276]. However our results clearly show that the Daxx and USP7 effects are reproduced in several cell lines including H1299, which besides being defective for p53 expression, they are also Rassf1A null. Thus, although the mitotic interaction between Daxx and Rassf1A may regulate mitosis by some mechanism not yet identified, Daxx regulation of mitosis and taxanes response is most likely achieved via interaction with USP7.

The role of chromosomal instability in affecting taxane sensitivity was also proposed [277]. Indeed, clinical trials have been devoted to determine the relationship of an unstable karyotype to taxanes response [278]. Clinical evidence gathered from breast and ovarian cancer specimens strongly indicated that tumors which display severe chromosomal instability are less sensitive to paclitaxel. Moreover karyotypes of residual

tumors with paclitaxel resistant phenotype were greatly more aneuploid than the responsive ones, indicating that a selective pressure for this trait occurs [278].

Current study provides evidence that the loss of these two proteins leads to reduced response to taxanes and provides dual mechanistic explanation for this phenomenon. Results presented in Chapters 3 and 4 provide strong evidence of a new USP7/Daxx function in mitosis and response to the anti-mitotic drugs taxanes. Data presented in Chapter 5 indicates that the loss of Daxx and USP7 are linked to genomic instability a determining factor in efficacy of taxane cytotoxicity as well as a hallmark of cancer progression (Model in Figure 6-1).

According to our current understanding, Daxx and USP7 control and safeguard mitosis. Any dysfunction in this checkpoint contributes to both cancer progression and taxane resistance in breast cancer (Model in Figure 6-1). This novel view on USP7/Daxx function warrants the use of these genes as novel markers of therapeutic resistance and highlights novel targets for clinical intervention, such as Aurora kinase. This would allow proper breast cancer patients stratification and rational design of new clinical trials which may include combinatorial use of taxanes and Aurora A inhibitors.

In addition, this study offers insights on USP7 as a therapeutic target. Small molecule inhibitors of USP7 DUB activity were developed to activate G1/S block that supposes to trigger p53 mediated cell death [198, 200]. Efficacy and employment of these drugs is limited by mutations or inactivation in p53 gene, occurring in >50% of human cancers, including breast cancer [279, 280]. Our study demonstrated that silencing of USP7 correlates with taxane resistance, thus it warns for future clinical use of USP7 inhibitors in combinatorial regimens with taxanes.

In conclusion, our study provides new insights for therapeutic approaches and predictive markers which will help in reducing breast cancer mortality and will increase the likelihood of a cure.

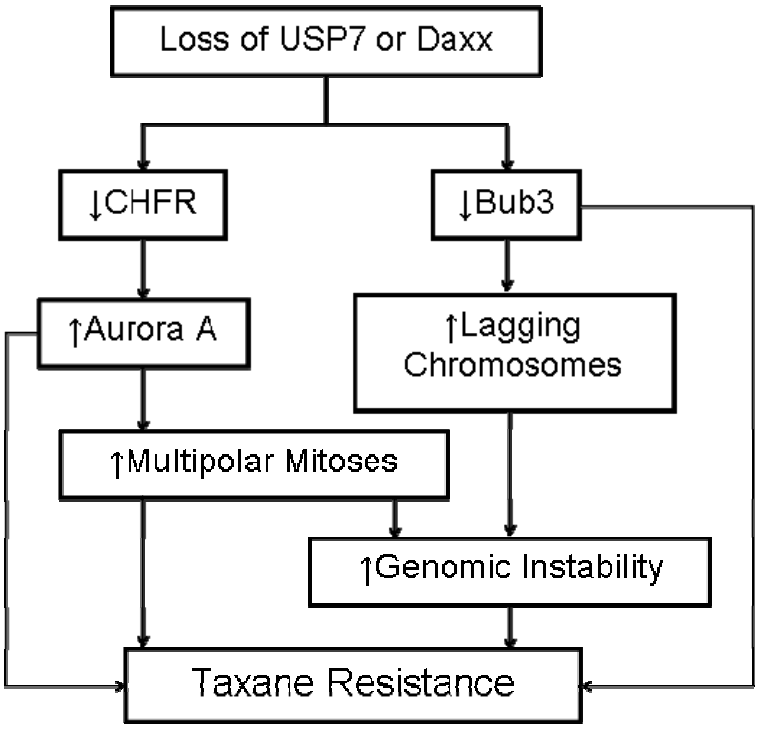


Figure 6-1. Proposed model for Daxx and USP7 regulation of taxane response and genomic instability. Decreased expression of USP7 and/or Daxx leads to down-modulation of two checkpoint proteins: CHFR and Bub3. On one hand, reduced CHFR allows Aurora A accumulation which in turn causes multipolar mitoses (Chapter 4) and genomic instability. Aurora A accumulation explains the observed taxanes resistance upon loss of Daxx and/or USP7 which can be reversed by administration of Aurora A inhibitors (Chapter 4). On the other hand, reduced levels of Bub3 lead to increase of unproperly attached and lagging chromosomes. This causes additional genomic instability thus further contributing to Daxx/USP7 mediated taxanes resistance (Chapter 5). Ultimately loss of Daxx and or USP7 can contribute to cancer progression therapy unresponsiveness in breast cancer patients.

Future Directions

Research presented in this study dissected USP7/Daxx-dependent mechanism of mitotic progression which affects response to microtubule poisonous drug administration, such as taxanes. This newly identified mechanism adds to our basic knowledge of cell cycle regulation machinery with the hope of offering rational and more effective treatments to defeat breast cancer. This contribution will be significant as it will rationally explore novel targeted and combinatorial regimens for treatment and will also aid in proper selection of breast cancer patients to receive taxane-based therapy.

Examine the Mechanism of Daxx Repression in Resistant Cells

In Chapter 3, we demonstrated that reduction of Daxx expression can dictate paclitaxel resistance, at least in a subpopulation of patients, suggesting Daxx as both biomarker and target in taxane sensitivity. Levels of Daxx proteins vary among breast cancer cell lines and patient specimens, where high levels of Daxx are associated with taxane sensitive phenotypes and that low levels of Daxx are associated with resistance to taxanes opening questions of Daxx regulation. Recently published screening for Daxx mutation identified that Daxx gene is mutated in about 25% of pancreatic neuroendocrine tumors, 7.1% of adult glioblastoma, 7.7% of oligodendroglioma and 1% of medulloblastomas, however no mutations were detected in a cohort of 96 breast cancer patients [143], suggesting that variability of Daxx accumulation between patients is not due to Daxx gene mutations. Thus, future work must be done to identify how repression of Daxx is achieved in breast tumors. Understanding the mechanism which leads to this gene inactivation may indicate clinical approaches to counteract Daxx repression.

Analysis of human Daxx promoter revealed three CpG reach islands, suggesting DNA methylation and epigenetic regulation as a possible mechanism of Daxx repression. If this proves true, we predict that the combination of paclitaxel with drugs that could elevate Daxx expression (demethylating agent 5-azacitidine/Vidaza or HDAC inhibitor SAHA-Vorinostat) may represent a successful combinatory treatment of therapy-resistant breast cancer patients. We postulate that de-repression of Daxx promoter should increase Daxx accumulation and, in turn, restore taxane responsiveness – both in cell lines and in patient settings. Therefore, an improved understanding of the mechanism of Daxx down-regulation may identify new combinatorial regimens to overcome taxane resistance.

Preliminary data in MDA-MD-468 and T-47D cell lines suggest that Daxx is regulated at the transcriptional level. This may be proven true at least in a sub-population of breast cancers, but it is possible that Daxx might be regulated at the protein level as well. Indeed, genetic inactivation of USP7 was proven to destabilize Daxx protein, suggesting Daxx as a USP7 substrate. Hence, it is plausible that USP7 negative tumors are affected also by reduced Daxx protein levels.

Validate Predictive Role of Protein USP7 as a Marker for Sensitivity to Taxane-based Chemotherapy in Breast Cancer Patients

In Chapter 4, we determined that Daxx interacts with USP7 in mitosis. We demonstrated that USP7 controls mitosis and taxane resistance similarly to what was observed for Daxx (Chapter 3) in cell lines. Thus, we will next determine how USP7 contributes to the paclitaxel treatment in mouse xenograft models. Then, it will be essential to determine whether USP7 and its mitotic substrates can be used as

predictive factors for taxane response by screening both pre- and post-treatment breast cancer specimens from patients with known outcomes to taxane-based treatment.

Earlier reports described USP7 as under-expressed in breast cancer tumor specimens [272]. Thus, similarly to what is planned for Daxx, the mechanism of USP7 repression in resistant cells and breast cancer patients will also be examined.

Explore Use of Aurora A Inhibitors to Reverse Taxane Resistance

Our working model presented in Chapter 4 (Figure 4-7), indicates that USP7/Daxx regulation of mitotic progression occurs through Aurora A. Aurora A is a putative oncogene [281, 282]; its amplification in breast cancer [75-77], or over-expression in tumors [78-83] correlates with poor patient outcomes [84-86] and resistance to taxanes [72-74]. Silencing or inhibition of Aurora A kinase activity sensitizes tumor cells to chemotherapeutic agents including taxanes [221-223], suggesting that this gene can be targeted in new therapeutic approaches. Thus, this essential mitotic kinase is a potential target to overcome taxane resistance. Our future work will test whether inhibition of Aurora A with inhibitors such as MLN8054 [218, 220] and the newly developed MLN8237 [283], both currently in phase I clinical trials, can override taxane resistance determined by USP7/Daxx. We already tested this hypothesis at the cellular level (Figure 4-6) for MLN8054 and we will do the same for MLN8237. Then we will test these molecules in xenograft models to evaluate which drug can fully override Daxx/USP7 mediated resistance in combinatorial settings with paclitaxel.

Biochemical Characterization of Daxx and USP7 Mitotic Interaction

We plan to continue characterization of USP7/Daxx mitotic interaction; this work will be useful in understanding Daxx modulatory function of USP7. To this end we will map by *in vitro* and *in vivo* pull-down assay Daxx and USP7 interacting domains. Next,

we will perform NMR characterization of Daxx/USP7 interaction to observe potential conformational changes of USP7 upon Daxx binding. Experiments will be done in collaboration with Dr. McIntosh, University of Vancouver as we did previously for Daxx Helical Bundle (DHB) domain [117]. These experiments will also be fundamental in determining Daxx function as modulator of USP7 enzymatic activity. Finally, we will characterize the role of Daxx post-translational modifications in regulation of USP7 interaction. To this end, we already mapped mitotic-specific phosphorylation residues of Daxx that, based on amino acid signature, may be modified by one of mitotic kinases. Studies in this direction may open another avenue in the modulation of Daxx/USP7-dependent taxane response.

Examine the Role of Daxx and USP7 in Genomic Instability

In Chapter 4, we showed that reduced levels of Daxx and USP7 lead to an increase in multipolar mitoses mediated by Aurora A accumulation. Cells that exit and survive such abnormal mitosis are affected by numerical chromosomal aberration. In Chapter 5, we demonstrated that cells silenced of USP7 are indeed characterized by genomic instability and that these karyotype abnormalities are induced by reduced levels of Bub3 and consequent lagging chromosomes in anaphase.

Thus, it will be interesting to address with future work whether these two events that lead to genomic instability (accumulation of Aurora A and reduced levels of Bub3) are related or separate and compounding events. Moreover, we will determine Daxx contribution to regulation of Bub3 stability.

Determine whether USP7 Regulates stability and Interacts with Components of the SAC and Kinetochore

Proper segregation of genetic material and cell death upon mitotic stresses both rely on competent mitotic checkpoint. We found that USP7 silencing reduces stability the SAC component Bub3, causing genetic instability and lack of response to microtubule poisonous drugs as taxanes. Thus, our study indicates that USP7 modulates the role of checkpoint proteins at kinetochore. In the future, assessing whether USP7 is mediating stability of other components of the SAC or affects their localization and functionality will be essential in fully understanding the biological and pathological roles of this enzyme. It will be important to determine if USP7 regulates the kinetochore structure and function. Detailed microscopy study of inter-kinetochore distances and defects in kinetochore- microtubule attachments will be undertaken.

In conclusion, the study presented in this dissertation provides future directions that may prove useful in the identification of novel therapeutic markers and approaches that will help in treating breast cancer patients.

LIST OF REFERENCES

1. Morrison, W.B. Cancer chemotherapy: an annotated history. *J Vet Intern Med* **24**, 1249-1262 (2010).

2. Lynch, H.T., Silva, E., Snyder, C. & Lynch, J.F. Hereditary breast cancer: part I. Diagnosing hereditary breast cancer syndromes. *Breast J* **14**, 3-13 (2008).

3. Boulton, S.J. Cellular functions of the BRCA tumour-suppressor proteins. *Biochem Soc Trans* **34**, 633-645 (2006).

4. Ford, D. & Easton, D.F. The genetics of breast and ovarian cancer. *Br J Cancer* **72**, 805-812 (1995).

5. Li, F.P., Fraumeni, J.F., Jr., Mulvihill, J.J., Blattner, W.A. *et al.* A cancer family syndrome in twenty-four kindreds. *Cancer Res* **48**, 5358-5362 (1988).

6. Varley, J.M. Germline TP53 mutations and Li-Fraumeni syndrome. *Hum Mutat* **21**, 313-320 (2003).

7. Zhao, L., Gu, A., Ji, G., Zou, P. *et al.* The Association between ATM IVS 22-77 T>C and Cancer Risk: A Meta-Analysis. *PLoS One* **7**, e29479 (2012).

8. Hollander, M.C., Blumenthal, G.M. & Dennis, P.A. PTEN loss in the continuum of common cancers, rare syndromes and mouse models. *Nat Rev Cancer* **11**, 289-301 (2011).

9. Cianfrocca, M. & Goldstein, L.J. Prognostic and predictive factors in early-stage breast cancer. *Oncologist* **9**, 606-616 (2004).

10. Fisher, B., Bauer, M., Wickerham, D.L., Redmond, C.K. *et al.* Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer. An NSABP update. *Cancer* **52**, 1551-1557 (1983).

11. O'Shaughnessy, J. Extending survival with chemotherapy in metastatic breast cancer. *Oncologist* **10 Suppl 3**, 20-29 (2005).

12. Wall, M.E. Camptothecin and taxol: discovery to clinic. *Med Res Rev* **18**, 299-314 (1998).

13. Oberlies, N.H. & Kroll, D.J. Camptothecin and taxol: historic achievements in natural products research. *J Nat Prod* **67**, 129-135 (2004).

14. Schiff, P.B., Fant, J. & Horwitz, S.B. Promotion of microtubule assembly in vitro by taxol. *Nature* **277**, 665-667 (1979).

15. Schiff, P.B. & Horwitz, S.B. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc Natl Acad Sci U S A* **77**, 1561-1565 (1980).

16. Wood, K.W., Cornwell, W.D. & Jackson, J.R. Past and future of the mitotic spindle as an oncology target. *Curr Opin Pharmacol* **1**, 370-377 (2001).

17. Gascoigne, K.E. & Taylor, S.S. How do anti-mitotic drugs kill cancer cells? *J Cell Sci* **122**, 2579-2585 (2009).

18. Higa, G.M. The microtubule as a breast cancer target. *Breast Cancer* **18**, 103-119.

19. Matson, D.R. & Stukenberg, P.T. Spindle poisons and cell fate: a tale of two pathways. *Mol Interv* **11**, 141-150.

20. Liebmann, J.E., Cook, J.A., Lipschultz, C., Teague, D. *et al.* Cytotoxic studies of paclitaxel (Taxol) in human tumour cell lines. *Br J Cancer* **68**, 1104-1109 (1993).

21. Morse, D.L., Gray, H., Payne, C.M. & Gillies, R.J. Docetaxel induces cell death through mitotic catastrophe in human breast cancer cells. *Mol Cancer Ther* **4**, 1495-1504 (2005).

22. Higa, G.M. The microtubule as a breast cancer target. *Breast Cancer* **18**, 103-119 (2011).

23. Cleveland, D.W., Mao, Y. & Sullivan, K.F. Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. *Cell* **112**, 407-421 (2003).

24. Kroemer, G., El-Deiry, W.S., Golstein, P., Peter, M.E. *et al.* Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ* **12 Suppl 2**, 1463-1467 (2005).

25. Chang, B.D., Xuan, Y., Broude, E.V., Zhu, H. *et al.* Role of p53 and p21waf1/cip1 in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs. *Oncogene* **18**, 4808-4818 (1999).

26. Roninson, I.B., Broude, E.V. & Chang, B.D. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updat* **4**, 303-313 (2001).

27. Castedo, M., Perfettini, J.L., Roumier, T., Andreau, K. *et al.* Cell death by mitotic catastrophe: a molecular definition. *Oncogene* **23**, 2825-2837 (2004).

28. Castedo, M., Perfettini, J.L., Roumier, T., Valent, A. *et al.* Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy. *Oncogene* **23**, 4362-4370 (2004).

29. Abal, M., Andreu, J.M. & Barasoain, I. Taxanes: microtubule and centrosome targets, and cell cycle dependent mechanisms of action. *Curr Cancer Drug Targets* **3**, 193-203 (2003).

30. Musacchio, A. & Salmon, E.D. The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol* **8**, 379-393 (2007).

31. Rieder, C.L. Mitosis in vertebrates: the G2/M and M/A transitions and their associated checkpoints. *Chromosome Res* **19**, 291-306.

32. Mantel, C., Guo, Y., Lee, M.R., Han, M.K. *et al.* Cells enter a unique intermediate 4N stage, not 4N-G1, after aborted mitosis. *Cell Cycle* **7**, 484-492 (2008).

33. Wysong, D.R., Chakravarty, A., Hoar, K. & Ecsedy, J.A. The inhibition of Aurora A abrogates the mitotic delay induced by microtubule perturbing agents. *Cell Cycle* **8**, 876-888 (2009).

34. Brito, D.A. & Rieder, C.L. Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. *Curr Biol* **16**, 1194-1200 (2006).

35. Brito, D.A., Yang, Z. & Rieder, C.L. Microtubules do not promote mitotic slippage when the spindle assembly checkpoint cannot be satisfied. *J Cell Biol* **182**, 623-629 (2008).

36. Niikura, Y., Dixit, A., Scott, R., Perkins, G. *et al.* BUB1 mediation of caspase-independent mitotic death determines cell fate. *J Cell Biol* **178**, 283-296 (2007).

37. Sudo, T., Nitta, M., Saya, H. & Ueno, N.T. Dependence of paclitaxel sensitivity on a functional spindle assembly checkpoint. *Cancer Res* **64**, 2502-2508 (2004).

38. Carvalho, A., Carmena, M., Sambade, C., Earnshaw, W.C. *et al.* Survivin is required for stable checkpoint activation in taxol-treated HeLa cells. *J Cell Sci* **116**, 2987-2998 (2003).

39. Lens, S.M., Wolthuis, R.M., Klompmaker, R., Kauw, J. *et al.* Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. *Embo J* **22**, 2934-2947 (2003).

40. Lee, E.A., Keutmann, M.K., Dowling, M.L., Harris, E. *et al.* Inactivation of the mitotic checkpoint as a determinant of the efficacy of microtubule-targeted drugs in killing human cancer cells. *Mol Cancer Ther* **3**, 661-669 (2004).

41. Kienitz, A., Vogel, C., Morales, I., Muller, R. *et al.* Partial downregulation of MAD1 causes spindle checkpoint inactivation and aneuploidy, but does not confer resistance towards taxol. *Oncogene* **24**, 4301-4310 (2005).

42. Bothos, J., Summers, M.K., Venere, M., Scolnick, D.M. *et al.* The Chfr mitotic checkpoint protein functions with Ubc13-Mms2 to form Lys63-linked polyubiquitin chains. *Oncogene* **22**, 7101-7107 (2003).

43. Kang, D., Chen, J., Wong, J. & Fang, G. The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition. *J Cell Biol* **156**, 249-259 (2002).

44. Chaturvedi, P., Sudakin, V., Bobiak, M.L., Fisher, P.W. *et al.* Chfr regulates a mitotic stress pathway through its RING-finger domain with ubiquitin ligase activity. *Cancer Res* **62**, 1797-1801 (2002).

45. Yu, X., Minter-Dykhouse, K., Malureanu, L., Zhao, W.M. *et al.* Chfr is required for tumor suppression and Aurora A regulation. *Nat Genet* **37**, 401-406 (2005).

46. Scolnick, D.M. & Halazonetis, T.D. Chfr defines a mitotic stress checkpoint that delays entry into metaphase. *Nature* **406**, 430-435 (2000).

47. Chabalier, C., Lamare, C., Racca, C., Privat, M. *et al.* BRCA1 downregulation leads to premature inactivation of spindle checkpoint and confers paclitaxel resistance. *Cell Cycle* **5**, 1001-1007 (2006).

48. Xia, G., Luo, X., Habu, T., Rizo, J. *et al.* Conformation-specific binding of p31(comet) antagonizes the function of Mad2 in the spindle checkpoint. *Embo J* **23**, 3133-3143 (2004).

49. Wassmann, K. & Benezra, R. Mitotic checkpoints: from yeast to cancer. *Curr Opin Genet Dev* **11**, 83-90 (2001).

50. Cahill, D.P., Lengauer, C., Yu, J., Riggins, G.J. *et al.* Mutations of mitotic checkpoint genes in human cancers. *Nature* **392**, 300-303 (1998).

51. Haruki, N., Saito, H., Harano, T., Nomoto, S. *et al.* Molecular analysis of the mitotic checkpoint genes BUB1, BUBR1 and BUB3 in human lung cancers. *Cancer Lett* **162**, 201-205 (2001).

52. Crown, J., O'Leary, M. & Ooi, W.S. Docetaxel and paclitaxel in the treatment of breast cancer: a review of clinical experience. *Oncologist* **9 Suppl 2**, 24-32 (2004).

53. Bonneterre, J., Spielman, M., Guastalla, J.P., Marty, M. *et al.* Efficacy and safety of docetaxel (Taxotere) in heavily pretreated advanced breast cancer patients: the French compassionate use programme experience. *Eur J Cancer* **35**, 1431-1439 (1999).

54. Ravdin, P., Erban, J. & Overmoyer, B. Phase III comparison of docetaxel and paclitaxel in patients with metastatic breast cancer. *Eur J Cancer* **suppl1:32** (2003).

55. Henderson, I.C., Berry, D.A., Demetri, G.D., Cirrincione, C.T. *et al.* Improved outcomes from adding sequential Paclitaxel but not from escalating Doxorubicin dose in an adjuvant chemotherapy regimen for patients with node-positive primary breast cancer. *J Clin Oncol* **21**, 976-983 (2003).

56. Aapro, M.S. Neoadjuvant therapy in breast cancer: can we define its role? *Oncologist* **6 Suppl 3**, 36-39 (2001).

57. Zhou, J. & Giannakakou, P. Targeting microtubules for cancer chemotherapy. *Curr Med Chem Anticancer Agents* **5**, 65-71 (2005).

58. Berrieman, H.K., Lind, M.J. & Cawkwell, L. Do beta-tubulin mutations have a role in resistance to chemotherapy? *Lancet Oncol* **5**, 158-164 (2004).

59. Goncalves, A., Braguer, D., Kamath, K., Martello, L. *et al.* Resistance to Taxol in lung cancer cells associated with increased microtubule dynamics. *Proc Natl Acad Sci U S A* **98**, 11737-11742 (2001).

60. Orr, G.A., Verdier-Pinard, P., McDaid, H. & Horwitz, S.B. Mechanisms of Taxol resistance related to microtubules. *Oncogene* **22**, 7280-7295 (2003).

61. Brooks, T.A., Minderman, H., O'Loughlin, K.L., Pera, P. *et al.* Taxane-based reversal agents modulate drug resistance mediated by P-glycoprotein, multidrug resistance protein, and breast cancer resistance protein. *Mol Cancer Ther* **2**, 1195-1205 (2003).

62. Chien, A.J. & Moasser, M.M. Cellular mechanisms of resistance to anthracyclines and taxanes in cancer: intrinsic and acquired. *Semin Oncol* **35**, S1-S14; quiz S39 (2008).

63. Villeneuve, D.J., Hembruff, S.L., Veitch, Z., Cecchetto, M. *et al.* cDNA microarray analysis of isogenic paclitaxel- and doxorubicin-resistant breast tumor cell lines reveals distinct drug-specific genetic signatures of resistance. *Breast Cancer Res Treat* **96**, 17-39 (2006).

64. Quinn, J.E., Kennedy, R.D., Mullan, P.B., Gilmore, P.M. *et al.* BRCA1 functions as a differential modulator of chemotherapy-induced apoptosis. *Cancer Res* **63**, 6221-6228 (2003).

65. Yu, D., Jing, T., Liu, B., Yao, J. *et al.* Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. *Mol Cell* **2**, 581-591 (1998).

66. Yu, D., Liu, B., Jing, T., Sun, D. *et al.* Overexpression of both p185c-erbB2 and p170mdr-1 renders breast cancer cells highly resistant to taxol. *Oncogene* **16**, 2087-2094 (1998).

67. Wang, T.H., Wang, H.S. & Soong, Y.K. Paclitaxel-induced cell death: where the cell cycle and apoptosis come together. *Cancer* **88**, 2619-2628 (2000).

68. Darshan, M.S., Loftus, M.S., Thadani-Mulero, M., Levy, B.P. *et al.* Taxane-induced blockade to nuclear accumulation of the androgen receptor predicts clinical responses in metastatic prostate cancer. *Cancer Res* **71**, 6019-6029 (2011).

69. Carbonaro, M., O'Brate, A. & Giannakakou, P. Microtubule disruption targets HIF-1alpha mRNA to cytoplasmic P-bodies for translational repression. *J Cell Biol* **192**, 83-99 (2011).

70. Satoh, A., Toyota, M., Itoh, F., Sasaki, Y. *et al.* Epigenetic inactivation of CHFR and sensitivity to microtubule inhibitors in gastric cancer. *Cancer Res* **63**, 8606-8613 (2003).

71. Xu, M., Takanashi, M., Oikawa, K., Nishi, H. *et al.* Identification of a novel role of Septin 10 in Paclitaxel-resistance in cancers through a functional genomics screen. *Cancer Sci* (2012).

72. Anand, S., Penrhyn-Lowe, S. & Venkitaraman, A.R. AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. *Cancer Cell* **3**, 51-62 (2003).

73. McGrogan, B.T., Gilmartin, B., Carney, D.N. & McCann, A. Taxanes, microtubules and chemoresistant breast cancer. *Biochim Biophys Acta* **1785**, 96-132 (2008).

74. Hata, T., Furukawa, T., Sunamura, M., Egawa, S. *et al.* RNA interference targeting aurora kinase a suppresses tumor growth and enhances the taxane chemosensitivity in human pancreatic cancer cells. *Cancer Res* **65**, 2899-2905 (2005).

75. Miyoshi, Y., Iwao, K., Egawa, C. & Noguchi, S. Association of centrosomal kinase STK15/BTAK mRNA expression with chromosomal instability in human breast cancers. *Int J Cancer* **92**, 370-373 (2001).

76. Staff, S., Isola, J., Jumppanen, M. & Tanner, M. Aurora-A gene is frequently amplified in basal-like breast cancer. *Oncol Rep* **23**, 307-312.

77. Barr, A.R. & Gergely, F. Aurora-A: the maker and breaker of spindle poles. *J Cell Sci* **120**, 2987-2996 (2007).

78. Gritsko, T.M., Coppola, D., Paciga, J.E., Yang, L. *et al.* Activation and overexpression of centrosome kinase BTAK/Aurora-A in human ovarian cancer. *Clin Cancer Res* **9**, 1420-1426 (2003).

79. Katayama, H., Brinkley, W.R. & Sen, S. The Aurora kinases: role in cell transformation and tumorigenesis. *Cancer Metastasis Rev* **22**, 451-464 (2003).

80. Li, D., Zhu, J., Firozi, P.F., Abbruzzese, J.L. *et al.* Overexpression of oncogenic STK15/BTAK/Aurora A kinase in human pancreatic cancer. *Clin Cancer Res* **9**, 991-997 (2003).

81. Sakakura, C., Hagiwara, A., Yasuoka, R., Fujita, Y. *et al.* Tumour-amplified kinase BTAK is amplified and overexpressed in gastric cancers with possible involvement in aneuploid formation. *Br J Cancer* **84**, 824-831 (2001).

82. Sen, S., Zhou, H., Zhang, R.D., Yoon, D.S. *et al.* Amplification/overexpression of a mitotic kinase gene in human bladder cancer. *J Natl Cancer Inst* **94**, 1320-1329 (2002).

83. Marumoto, T., Zhang, D. & Saya, H. Aurora-A - a guardian of poles. *Nat Rev Cancer* **5**, 42-50 (2005).

84. Das, K., Lorena, P.D., Ng, L.K., Shen, L. *et al.* Aurora-A expression, hormone receptor status and clinical outcome in hormone related cancers. *Pathology* **42**, 540-546.

85. Jeng, Y.M., Peng, S.Y., Lin, C.Y. & Hsu, H.C. Overexpression and amplification of Aurora-A in hepatocellular carcinoma. *Clin Cancer Res* **10**, 2065-2071 (2004).

86. Lassus, H., Staff, S., Leminen, A., Isola, J. *et al.* Aurora-A overexpression and aneuploidy predict poor outcome in serous ovarian carcinoma. *Gynecol Oncol* **120**, 11-17.

87. Kiriakidou, M., Driscoll, D.A., Lopez-Guisa, J.M. & Strauss, J.F., 3rd Cloning and expression of primate Daxx cDNAs and mapping of the human gene to chromosome 6p21.3 in the MHC region. *DNA Cell Biol* **16**, 1289-1298 (1997).

88. Lindsay, C.R., Scholz, A., Morozov, V.M. & Ishov, A.M. Daxx shortens mitotic arrest caused by paclitaxel. *Cell Cycle* **6**, 1200-1204 (2007).

89. Yang, X., Khosravi-Far, R., Chang, H.Y. & Baltimore, D. Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell* **89**, 1067-1076 (1997).

90. Salomoni, P. & Khelifi, A.F. Daxx: death or survival protein? *Trends Cell Biol* **16**, 97-104 (2006).

91. Juo, P., Kuo, C.J., Yuan, J. & Blenis, J. Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. *Curr Biol* **8**, 1001-1008 (1998).

92. Zhang, J., Cado, D., Chen, A., Kabra, N.H. *et al.* Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. *Nature* **392**, 296-300 (1998).

93. Torii, S., Egan, D.A., Evans, R.A. & Reed, J.C. Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs). *EMBO J* **18**, 6037-6049 (1999).

94. Michaelson, J.S., Bader, D., Kuo, F., Kozak, C. *et al.* Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. *Genes Dev* **13**, 1918-1923 (1999).

95. Michaelson, J.S. The Daxx enigma. *Apoptosis* **5**, 217-220 (2000).

96. Ishov, A.M., Sotnikov, A.G., Negorev, D., Vladimirova, O.V. *et al.* PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. *J Cell Biol* **147**, 221-234 (1999).

97. Santiago, A., Godsey, A.C., Hossain, J., Zhao, L.Y. *et al.* Identification of two independent SUMO-interacting motifs in Daxx: evolutionary conservation from Drosophila to humans and their biochemical functions. *Cell Cycle* **8**, 76-87 (2009).

98. Lin, D.Y., Huang, Y.S., Jeng, J.C., Kuo, H.Y. *et al.* Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Mol Cell* **24**, 341-354 (2006).

99. Ishov, A.M., Vladimirova, O.V. & Maul, G.G. Heterochromatin and ND10 are cell-cycle regulated and phosphorylation-dependent alternate nuclear sites of the transcription repressor Daxx and SWI/SNF protein ATRX. *J Cell Sci* **117**, 3807-3820 (2004).

100. Lalioti, V.S., Vergarajauregui, S., Pulido, D. & Sandoval, I.V. The insulin-sensitive glucose transporter, GLUT4, interacts physically with Daxx. Two proteins with capacity to bind Ubc9 and conjugated to SUMO1. *J Biol Chem* **277**, 19783-19791 (2002).

101. Jung, Y.S., Kim, H.Y., Lee, Y.J. & Kim, E. Subcellular localization of Daxx determines its opposing functions in ischemic cell death. *FEBS Lett* **581**, 843-852 (2007).

102. Jung, Y.S., Kim, H.Y., Kim, J., Lee, M.G. *et al.* Physical interactions and functional coupling between Daxx and sodium hydrogen exchanger 1 in ischemic cell death. *J Biol Chem* **283**, 1018-1025 (2008).

103. Junn, E., Taniguchi, H., Jeong, B.S., Zhao, X. *et al.* Interaction of DJ-1 with Daxx inhibits apoptosis signal-regulating kinase 1 activity and cell death. *Proc Natl Acad Sci U S A* **102**, 9691-9696 (2005).

104. Karunakaran, S., Diwakar, L., Saeed, U., Agarwal, V. *et al.* Activation of apoptosis signal regulating kinase 1 (ASK1) and translocation of death-associated protein, Daxx, in substantia nigra pars compacta in a mouse model of Parkinson's disease: protection by alpha-lipoic acid. *FASEB J* **21**, 2226-2236 (2007).

105. Song, J.J. & Lee, Y.J. Role of the ASK1-SEK1-JNK1-HIPK1 signal in Daxx trafficking and ASK1 oligomerization. *J Biol Chem* **278**, 47245-47252 (2003).

106. Song, J.J. & Lee, Y.J. Tryptophan 621 and serine 667 residues of Daxx regulate its nuclear export during glucose deprivation. *J Biol Chem* **279**, 30573-30578 (2004).

107. Khelifi, A.F., D'Alcontres, M.S. & Salomoni, P. Daxx is required for stress-induced cell death and JNK activation. *Cell Death Differ* **12**, 724-733 (2005).

108. Lindsay, C.R., Giovinazzi, S. & Ishov, A.M. Daxx is a predominately nuclear protein that does not translocate to the cytoplasm in response to cell stress. *Cell Cycle* **8** (2009).

109. Nefkens, I., Negorev, D.G., Ishov, A.M., Michaelson, J.S. *et al.* Heat shock and Cd2+ exposure regulate PML and Daxx release from ND10 by independent mechanisms that modify the induction of heat-shock proteins 70 and 25 differently. *J Cell Sci* **116**, 513-524 (2003).

110. Everett, R.D., Earnshaw, W.C., Pluta, A.F., Sternsdorf, T. *et al.* A dynamic connection between centromeres and ND10 proteins. *J Cell Sci* **112 (Pt 20)**, 3443-3454 (1999).

111. Pluta, A.F., Earnshaw, W.C. & Goldberg, I.G. Interphase-specific association of intrinsic centromere protein CENP-C with HDaxx, a death domain-binding protein implicated in Fas-mediated cell death. *J Cell Sci* **111 (Pt 14)**, 2029-2041 (1998).

112. Elsaesser, S.J. & Allis, C.D. HIRA and Daxx Constitute Two Independent Histone H3.3-Containing Predeposition Complexes. *Cold Spring Harb Symp Quant Biol* (2010).

113. Lindsay, C.R., Morozov, V.M. & Ishov, A.M. PML NBs (ND10) and Daxx: from nuclear structure to protein function. *Front Biosci* **13**, 7132-7142 (2008).

114. Hollenbach, A.D., Sublett, J.E., McPherson, C.J. & Grosveld, G. The Pax3-FKHR oncoprotein is unresponsive to the Pax3-associated repressor hDaxx. *EMBO J* **18**, 3702-3711 (1999).

115. Kitagawa, D., Kajiho, H., Negishi, T., Ura, S. *et al.* Release of RASSF1C from the nucleus by Daxx degradation links DNA damage and SAPK/JNK activation. *EMBO J* **25**, 3286-3297 (2006).

116. Giovinazzi, S., Lindsay, C.R., Morozov, V.M., Escobar-Cabrera, E. *et al.* Regulation of mitosis and taxane response by Daxx and Rassf1. *Oncogene* (2011).

117. Escobar-Cabrera, E., Lau, D.K., Giovinazzi, S., Ishov, A.M. *et al.* Structural Characterization of the DAXX N-Terminal Helical Bundle Domain and Its Complex with Rassf1C. *Structure* **18**, 1642-1653 (2010).

118. Michaelson, J.S. & Leder, P. RNAi reveals anti-apoptotic and transcriptionally repressive activities of DAXX. *J Cell Sci* **116**, 345-352 (2003).

119. Salomoni, P. & Pandolfi, P.P. The role of PML in tumor suppression. *Cell* **108**, 165-170 (2002).

120. Morozov, V.M., Massoll, N.A., Vladimirova, O.V., Maul, G.G. *et al.* Regulation of c-met expression by transcription repressor Daxx. *Oncogene* **27**, 2177-2186 (2008).

121. Chang, C.C., Lin, D.Y., Fang, H.I., Chen, R.H. *et al.* Daxx mediates the small ubiquitin-like modifier-dependent transcriptional repression of Smad4. *J Biol Chem* **280**, 10164-10173 (2005).

122. Kuo, H.Y., Chang, C.C., Jeng, J.C., Hu, H.M. *et al.* SUMO modification negatively modulates the transcriptional activity of CREB-binding protein via the recruitment of Daxx. *Proc Natl Acad Sci U S A* **102**, 16973-16978 (2005).

123. Shih, H.M., Chang, C.C., Kuo, H.Y. & Lin, D.Y. Daxx mediates SUMO-dependent transcriptional control and subnuclear compartmentalization. *Biochem Soc Trans* **35**, 1397-1400 (2007).

124. Hollenbach, A.D., McPherson, C.J., Mientjes, E.J., Iyengar, R. *et al.* Daxx and histone deacetylase II associate with chromatin through an interaction with core histones and the chromatin-associated protein Dek. *J Cell Sci* **115**, 3319-3330 (2002).

125. Puto, L.A. & Reed, J.C. Daxx represses RelB target promoters via DNA methyltransferase recruitment and DNA hypermethylation. *Genes Dev* **22**, 998-1010 (2008).

126. Tang, J., Qu, L.K., Zhang, J., Wang, W. *et al.* Critical role for Daxx in regulating Mdm2. *Nat Cell Biol* **8**, 855-862 (2006).

127. Song, M.S., Song, S.J., Kim, S.Y., Oh, H.J. *et al.* The tumour suppressor RASSF1A promotes MDM2 self-ubiquitination by disrupting the MDM2-DAXX-HAUSP complex. *EMBO J* **27**, 1863-1874 (2008).

128. Drane, P., Ouararhni, K., Depaux, A., Shuaib, M. *et al.* The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3. *Genes Dev* **24**, 1253-1265 (2010).

129. Goldberg, A.D., Banaszynski, L.A., Noh, K.M., Lewis, P.W. *et al.* Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell* **140**, 678-691 (2010).

130. Koken, M.H., Linares-Cruz, G., Quignon, F., Viron, A. *et al.* The PML growth-suppressor has an altered expression in human oncogenesis. *Oncogene* **10**, 1315-1324 (1995).

131. Stuurman, N., de Graaf, A., Floore, A., Josso, A. *et al.* A monoclonal antibody recognizing nuclear matrix-associated nuclear bodies. *J Cell Sci* **101 (Pt 4)**, 773-784 (1992).

132. Terris, B., Baldin, V., Dubois, S., Degott, C. *et al.* PML nuclear bodies are general targets for inflammation and cell proliferation. *Cancer Res* **55**, 1590-1597 (1995).

133. Sternsdorf, T., Jensen, K. & Will, H. Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1. *J Cell Biol* **139**, 1621-1634 (1997).

134. Giovinazzi, S., Lindsay, C.R., Morozov, V.M., Escobar-Cabrera, E. *et al.* Regulation of mitosis and taxane response by Daxx and Rassf1. *Oncogene*.

135. Chen, E.S., Saitoh, S., Yanagida, M. & Takahashi, K. A cell cycle-regulated GATA factor promotes centromeric localization of CENP-A in fission yeast. *Mol Cell* **11**, 175-187 (2003).

136. Chan, G.K. & Yen, T.J. The mitotic checkpoint: a signaling pathway that allows a single unattached kinetochore to inhibit mitotic exit. *Prog Cell Cycle Res* **5**, 431-439 (2003).

137. Waghray, A., Schober, M., Feroze, F., Yao, F. *et al.* Identification of differentially expressed genes by serial analysis of gene expression in human prostate cancer. *Cancer Res* **61**, 4283-4286 (2001).

138. Mizuta, H. & Kuroda, Y. Cloning and functional characterization of a rat Daxx that functions as a corepressor for the androgen receptor. *Cell Biol Int* **28**, 609-614 (2004).

139. Lin, D.Y., Fang, H.I., Ma, A.H., Huang, Y.S. *et al.* Negative modulation of androgen receptor transcriptional activity by Daxx. *Mol Cell Biol* **24**, 10529-10541 (2004).

140. Tzeng, S.L., Cheng, Y.W., Li, C.H., Lin, Y.S. *et al.* Physiological and functional interactions between Tcf4 and Daxx in colon cancer cells. *J Biol Chem* **281**, 15405-15411 (2006).

141. Jiao, Y., Shi, C., Edil, B.H., de Wilde, R.F. *et al.* DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. *Science* **331**, 1199-1203 (2011).

142. Law, M.J., Lower, K.M., Voon, H.P., Hughes, J.R. *et al.* ATR-X syndrome protein targets tandem repeats and influences allele-specific expression in a size-dependent manner. *Cell* **143**, 367-378 (2010).

143. Heaphy, C.M., de Wilde, R.F., Jiao, Y., Klein, A.P. *et al.* Altered telomeres in tumors with ATRX and DAXX mutations. *Science* **333**, 425 (2011).

144. Yeager, T.R., Neumann, A.A., Englezou, A., Huschtscha, L.I. *et al.* Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. *Cancer Res* **59**, 4175-4179 (1999).

145. Sablina, A.A., Budanov, A.V., Ilyinskaya, G.V., Agapova, L.S. *et al.* The antioxidant function of the p53 tumor suppressor. *Nat Med* **11**, 1306-1313 (2005).

146. Meraldi, P., Draviam, V.M. & Sorger, P.K. Timing and checkpoints in the regulation of mitotic progression. *Dev Cell* **7**, 45-60 (2004).

147. Countryman, P.I. & Heddle, J.A. The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. *Mutat Res* **41**, 321-332 (1976).

148. Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N. *et al.* HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat Res* **534**, 65-75 (2003).

149. Ring, A.E. & Ellis, P.A. Taxanes in the treatment of early breast cancer. *Cancer Treat Rev* **31**, 618-627 (2005).

150. Hari, M., Wang, Y., Veeraraghavan, S. & Cabral, F. Mutations in alpha- and beta-tubulin that stabilize microtubules and confer resistance to colcemid and vinblastine. *Mol Cancer Ther* **2**, 597-605 (2003).

151. Rieder, C.L. & Maiato, H. Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint. *Dev Cell* **7**, 637-651 (2004).

152. Chen, J.G. & Horwitz, S.B. Differential mitotic responses to microtubule-stabilizing and -destabilizing drugs. *Cancer Res* **62**, 1935-1938 (2002).

153. Holland, A.J. & Cleveland, D.W. Beyond genetics: surprising determinants of cell fate in antitumor drugs. *Cancer Cell* **14**, 103-105 (2008).

154. Gascoigne, K.E. & Taylor, S.S. Cancer cells display profound intra- and interline variation following prolonged exposure to antimitotic drugs. *Cancer Cell* **14**, 111-122 (2008).

155. Chang, J.C., Wooten, E.C., Tsimelzon, A., Hilsenbeck, S.G. *et al.* Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. *Lancet* **362**, 362-369 (2003).

156. Chang, J.C., Wooten, E.C., Tsimelzon, A., Hilsenbeck, S.G. *et al.* Patterns of resistance and incomplete response to docetaxel by gene expression profiling in breast cancer patients. *J Clin Oncol* **23**, 1169-1177 (2005).

157. Iwao-Koizumi, K., Matoba, R., Ueno, N., Kim, S.J. *et al.* Prediction of docetaxel response in human breast cancer by gene expression profiling. *J Clin Oncol* **23**, 422-431 (2005).

158. Miyoshi, Y., Kim, S.J., Akazawa, K., Kamigaki, S. *et al.* Down-regulation of intratumoral aromatase messenger RNA levels by docetaxel in human breast cancers. *Clin Cancer Res* **10**, 8163-8169 (2004).

159. Mauriac, L., Debled, M. & MacGrogan, G. When will more useful predictive factors be ready for use? *Breast* **14**, 617-623 (2005).

160. Nigg, E.A. Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol* **2**, 21-32 (2001).

161. Swanton, C., Marani, M., Pardo, O., Warne, P.H. *et al.* Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. *Cancer Cell* **11**, 498-512 (2007).

162. Saffert, R.T. & Kalejta, R.F. Promyelocytic leukemia-nuclear body proteins: herpesvirus enemies, accomplices, or both? *Future Virol* **3**, 265-277 (2008).

163. Heaphy, C.M., de Wilde, R.F., Jiao, Y., Klein, A.P. *et al.* Altered telomeres in tumors with ATRX and DAXX mutations. *Science* **333**, 425.

164. Jiao, Y., Shi, C., Edil, B.H., de Wilde, R.F. *et al.* DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. *Science* **331**, 1199-1203.

165. De Mulder, P.H. The chemotherapy of head and neck cancer. *Acta Otorhinolaryngol Belg* **53**, 247-252 (1999).

166. Summers, M.K., Pan, B., Mukhyala, K. & Jackson, P.K. The unique N terminus of the UbcH10 E2 enzyme controls the threshold for APC activation and enhances checkpoint regulation of the APC. *Mol Cell* **31**, 544-556 (2008).

167. Wang, Y. & Cabral, F. Paclitaxel resistance in cells with reduced beta-tubulin. *Biochim Biophys Acta* **1744**, 245-255 (2005).

168. Hari, M., Yang, H., Zeng, C., Canizales, M. *et al.* Expression of class III beta-tubulin reduces microtubule assembly and confers resistance to paclitaxel. *Cell Motil Cytoskeleton* **56**, 45-56 (2003).

169. Kubota, T., Matsuzaki, S.W., Hoshiya, Y., Watanabe, M. *et al.* Antitumor activity of paclitaxel against human breast carcinoma xenografts serially transplanted into nude mice. *J Surg Oncol* **64**, 115-121 (1997).

170. Michaelson, J.S., Bader, D., Kuo, F., Kozak, C. *et al.* Loss of daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development [In Process Citation]. *Genes Dev* **13**, 1918-1923 (1999).

171. Nilsson, J., Yekezare, M., Minshull, J. & Pines, J. The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction. *Nat Cell Biol* **10**, 1411-1420 (2008).

172. Herzog, F., Primorac, I., Dube, P., Lenart, P. *et al.* Structure of the anaphase-promoting complex/cyclosome interacting with a mitotic checkpoint complex. *Science* **323**, 1477-1481 (2009).

173. Eytan, E., Braunstein, I., Ganoth, D., Teichner, A. *et al.* Two different mitotic checkpoint inhibitors of the anaphase-promoting complex/cyclosome antagonize the action of the activator Cdc20. *Proc Natl Acad Sci U S A* **105**, 9181-9185 (2008).

174. Everett, R.D., Meredith, M., Orr, A., Cross, A. *et al.* A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. *EMBO J* **16**, 1519-1530 (1997).

175. Boutell, C., Canning, M., Orr, A. & Everett, R.D. Reciprocal activities between herpes simplex virus type 1 regulatory protein ICP0, a ubiquitin E3 ligase, and ubiquitin-specific protease USP7. *J Virol* **79**, 12342-12354 (2005).

176. Canning, M., Boutell, C., Parkinson, J. & Everett, R.D. A RING finger ubiquitin ligase is protected from autocatalyzed ubiquitination and degradation by binding to ubiquitin-specific protease USP7. *J Biol Chem* **279**, 38160-38168 (2004).

177. Wilkinson, K.D. Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *FASEB J* **11**, 1245-1256 (1997).

178. Oh, Y.M., Yoo, S.J. & Seol, J.H. Deubiquitination of Chfr, a checkpoint protein, by USP7/HAUSP regulates its stability and activity. *Biochem Biophys Res Commun* **357**, 615-619 (2007).

179. Song, M.S., Salmena, L., Carracedo, A., Egia, A. *et al.* The deubiquitinylation and localization of PTEN are regulated by a HAUSP-PML network. *Nature* **455**, 813-817 (2008).

180. Tang, J., Qu, L., Pang, M. & Yang, X. Daxx is reciprocally regulated by Mdm2 and Hausp. *Biochem Biophys Res Commun* **393**, 542-545.

181. Huang, Z., Wu, Q., Guryanova, O.A., Cheng, L. *et al.* Deubiquitylase HAUSP stabilizes REST and promotes maintenance of neural progenitor cells. *Nat Cell Biol* **13**, 142-152 (2011).

182. Huang, Z., Zhou, W. & Bao, S. Role of deubiquitylase HAUSP in stem cell maintenance. *Cell Cycle* **10**, 1182-1183 (2011).

183. Faustrup, H., Bekker-Jensen, S., Bartek, J., Lukas, J. *et al.* USP7 counteracts SCFbetaTrCP- but not APCCdh1-mediated proteolysis of Claspin. *J Cell Biol* **184**, 13-19 (2009).

184. Mailand, N., Bekker-Jensen, S., Bartek, J. & Lukas, J. Destruction of Claspin by SCFbetaTrCP restrains Chk1 activation and facilitates recovery from genotoxic stress. *Mol Cell* **23**, 307-318 (2006).

185. van der Horst, A., de Vries-Smits, A.M., Brenkman, A.B., van Triest, M.H. *et al.* FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. *Nat Cell Biol* **8**, 1064-1073 (2006).

186. Sarkari, F., Sanchez-Alcaraz, T., Wang, S., Holowaty, M.N. *et al.* EBNA1-mediated recruitment of a histone H2B deubiquitylating complex to the Epstein-Barr virus latent origin of DNA replication. *PLoS Pathog* **5**, e1000624 (2009).

187. van der Knaap, J.A., Kozhevnikova, E., Langenberg, K., Moshkin, Y.M. *et al.* Biosynthetic enzyme GMP synthetase cooperates with ubiquitin-specific protease 7 in transcriptional regulation of ecdysteroid target genes. *Mol Cell Biol* **30**, 736-744 (2010).

188. van der Knaap, J.A., Kumar, B.R., Moshkin, Y.M., Langenberg, K. *et al.* GMP synthetase stimulates histone H2B deubiquitylation by the epigenetic silencer USP7. *Mol Cell* **17**, 695-707 (2005).

189. Felle, M., Joppien, S., Nemeth, A., Diermeier, S. *et al.* The USP7/Dnmt1 complex stimulates the DNA methylation activity of Dnmt1 and regulates the stability of UHRF1. *Nucleic Acids Res* **39**, 8355-8365.

190. Li, M., Chen, D., Shiloh, A., Luo, J. *et al.* Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* **416**, 648-653 (2002).

191. Cummins, J.M. & Vogelstein, B. HAUSP is required for p53 destabilization. *Cell Cycle* **3**, 689-692 (2004).

192. Cummins, J.M., Rago, C., Kohli, M., Kinzler, K.W. *et al.* Tumour suppression: disruption of HAUSP gene stabilizes p53. *Nature* **428**, 1 p following 486 (2004).

193. Li, M., Brooks, C.L., Kon, N. & Gu, W. A dynamic role of HAUSP in the p53-Mdm2 pathway. *Mol Cell* **13**, 879-886 (2004).

194. Kon, N., Kobayashi, Y., Li, M., Brooks, C.L. *et al.* Inactivation of HAUSP in vivo modulates p53 function. *Oncogene* **29**, 1270-1279 (2009).

195. Montes de Oca Luna, R., Wagner, D.S. & Lozano, G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* **378**, 203-206 (1995).

196. Kon, N., Zhong, J., Kobayashi, Y., Li, M. *et al.* Roles of HAUSP-mediated p53 regulation in central nervous system development. *Cell Death Differ* **18**, 1366-1375.

197. Kon, N., Kobayashi, Y., Li, M., Brooks, C.L. *et al.* Inactivation of HAUSP in vivo modulates p53 function. *Oncogene* **29**, 1270-1279.

198. Colland, F. The therapeutic potential of deubiquitinating enzyme inhibitors. *Biochem Soc Trans* **38**, 137-143 (2010).

199. Colland, F., Formstecher, E., Jacq, X., Reverdy, C. *et al.* Small-molecule inhibitor of USP7/HAUSP ubiquitin protease stabilizes and activates p53 in cells. *Mol Cancer Ther* **8**, 2286-2295 (2009).

200. Nicholson, B. & Suresh Kumar, K.G. The multifaceted roles of USP7: new therapeutic opportunities. *Cell Biochem Biophys* **60**, 61-68 (2011).

201. Nakatani, Y. & Ogryzko, V. Immunoaffinity purification of mammalian protein complexes. *Methods Enzymol* **370**, 430-444 (2003).

202. Robinson, P.A., Lomonte, P., Leek, Markham, A.F. *et al.* Assignment1 of herpesvirus-associated ubiquitin-specific protease gene HAUSP to human chromosome band 16p13.3 by in situ hybridization. *Cytogenet Cell Genet* **83**, 100 (1998).

203. Maxwell, S.A. Two-dimensional gel analysis of p53-mediated changes in protein expression. *Anticancer Res* **14**, 2549-2556 (1994).

204. Privette, L.M., Weier, J.F., Nguyen, H.N., Yu, X. *et al.* Loss of CHFR in human mammary epithelial cells causes genomic instability by disrupting the mitotic spindle assembly checkpoint. *Neoplasia* **10**, 643-652 (2008).

205. Toyota, M., Sasaki, Y., Satoh, A., Ogi, K. *et al.* Epigenetic inactivation of CHFR in human tumors. *Proc Natl Acad Sci U S A* **100**, 7818-7823 (2003).

206. Marumoto, T., Hirota, T., Morisaki, T., Kunitoku, N. *et al.* Roles of aurora-A kinase in mitotic entry and G2 checkpoint in mammalian cells. *Genes Cells* **7**, 1173-1182 (2002).

207. Hirota, T., Kunitoku, N., Sasayama, T., Marumoto, T. *et al.* Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells. *Cell* **114**, 585-598 (2003).

208. Lukasiewicz, K.B., Greenwood, T.M., Negron, V.C., Bruzek, A.K. *et al.* Control of centrin stability by Aurora A. *PLoS One* **6**, e21291.

209. Qin, L., Tong, T., Song, Y., Xue, L. *et al.* Aurora-A interacts with Cyclin B1 and enhances its stability. *Cancer Lett* **275**, 77-85 (2009).

210. Matsusaka, T. & Pines, J. Chfr acts with the p38 stress kinases to block entry to mitosis in mammalian cells. *J Cell Biol* **166**, 507-516 (2004).

211. Privette, L.M. & Petty, E.M. CHFR: A Novel Mitotic Checkpoint Protein and Regulator of Tumorigenesis. *Transl Oncol* **1**, 57-64 (2008).

212. Marumoto, T., Honda, S., Hara, T., Nitta, M. *et al.* Aurora-A kinase maintains the fidelity of early and late mitotic events in HeLa cells. *J Biol Chem* **278**, 51786-51795 (2003).

213. D'Assoro, A.B., Lingle, W.L. & Salisbury, J.L. Centrosome amplification and the development of cancer. *Oncogene* **21**, 6146-6153 (2002).

214. Lingle, W.L., Lutz, W.H., Ingle, J.N., Maihle, N.J. *et al.* Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity. *Proc Natl Acad Sci U S A* **95**, 2950-2955 (1998).

215. Salisbury, J.L., D'Assoro, A.B. & Lingle, W.L. Centrosome amplification and the origin of chromosomal instability in breast cancer. *J Mammary Gland Biol Neoplasia* **9**, 275-283 (2004).

216. Gmeiner, W.H., Reinhold, W.C. & Pommier, Y. Genome-wide mRNA and microRNA profiling of the NCI 60 cell-line screen and comparison of FdUMP[10] with fluorouracil, floxuridine, and topoisomerase 1 poisons. *Mol Cancer Ther* **9**, 3105-3114.

217. Liu, H., D'Andrade, P., Fulmer-Smentek, S., Lorenzi, P. *et al.* mRNA and microRNA expression profiles of the NCI-60 integrated with drug activities. *Mol Cancer Ther* **9**, 1080-1091.

218. Hoar, K., Chakravarty, A., Rabino, C., Wysong, D. *et al.* MLN8054, a small-molecule inhibitor of Aurora A, causes spindle pole and chromosome congression defects leading to aneuploidy. *Mol Cell Biol* **27**, 4513-4525 (2007).

219. Huck, J.J., Zhang, M., McDonald, A., Bowman, D. *et al.* MLN8054, an inhibitor of Aurora A kinase, induces senescence in human tumor cells both in vitro and in vivo. *Mol Cancer Res* **8**, 373-384 (2010).

220. Manfredi, M.G., Ecsedy, J.A., Meetze, K.A., Balani, S.K. *et al.* Antitumor activity of MLN8054, an orally active small-molecule inhibitor of Aurora A kinase. *Proc Natl Acad Sci U S A* **104**, 4106-4111 (2007).

221. Cammareri, P., Scopelliti, A., Todaro, M., Eterno, V. *et al.* Aurora-a is essential for the tumorigenic capacity and chemoresistance of colorectal cancer stem cells. *Cancer Res* **70**, 4655-4665.

222. Sun, C., Chan, F., Briassouli, P. & Linardopoulos, S. Aurora kinase inhibition downregulates NF-kappaB and sensitises tumour cells to chemotherapeutic agents. *Biochem Biophys Res Commun* **352**, 220-225 (2007).

223. Scharer, C.D., Laycock, N., Osunkoya, A.O., Logani, S. *et al.* Aurora kinase inhibitors synergize with paclitaxel to induce apoptosis in ovarian cancer cells. *J Transl Med* **6**, 79 (2008).

224. Morrow, C.J., Tighe, A., Johnson, V.L., Scott, M.I. *et al.* Bub1 and aurora B cooperate to maintain BubR1-mediated inhibition of APC/CCdc20. *J Cell Sci* **118**, 3639-3652 (2005).

225. Faesen, A.C., Dirac, A.M., Shanmugham, A., Ovaa, H. *et al.* Mechanism of USP7/HAUSP Activation by Its C-Terminal Ubiquitin-like Domain and Allosteric Regulation by GMP-Synthetase. *Mol Cell* **44**, 147-159.

226. Meraldi, P., Honda, R. & Nigg, E.A. Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53-/- cells. *EMBO J* **21**, 483-492 (2002).

227. Tang, J., Qu, L., Pang, M. & Yang, X. Daxx is reciprocally regulated by Mdm2 and Hausp. *Biochem Biophys Res Commun* **393**, 542-545 (2010).

228. Rajagopalan, H. & Lengauer, C. Aneuploidy and cancer. *Nature* **432**, 338-341 (2004).

229. Kolodner, R.D., Cleveland, D.W. & Putnam, C.D. Cancer. Aneuploidy drives a mutator phenotype in cancer. *Science* **333**, 942-943 (2011).

230. Kops, G.J., Weaver, B.A. & Cleveland, D.W. On the road to cancer: aneuploidy and the mitotic checkpoint. *Nat Rev Cancer* **5**, 773-785 (2005).

231. Weaver, B.A. & Cleveland, D.W. Does aneuploidy cause cancer? *Curr Opin Cell Biol* **18**, 658-667 (2006).

232. Weaver, B.A. & Cleveland, D.W. The role of aneuploidy in promoting and suppressing tumors. *J Cell Biol* **185**, 935-937 (2009).

233. Hassold, T. & Hunt, P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet* **2**, 280-291 (2001).

234. Wei, Y., Multi, S., Yang, C.R., Ma, J. *et al.* Spindle assembly checkpoint regulates mitotic cell cycle progression during preimplantation embryo development. *PLoS One* **6**, e21557 (2011).

235. Pinsky, B.A. & Biggins, S. The spindle checkpoint: tension versus attachment. *Trends Cell Biol* **15**, 486-493 (2005).

236. Carmena, M. & Earnshaw, W.C. The cellular geography of aurora kinases. *Nat Rev Mol Cell Biol* **4**, 842-854 (2003).

237. Maresca, T.J. & Salmon, E.D. Welcome to a new kind of tension: translating kinetochore mechanics into a wait-anaphase signal. *J Cell Sci* **123**, 825-835 (2010).

238. Rao, C.V., Yamada, H.Y., Yao, Y. & Dai, W. Enhanced genomic instabilities caused by deregulated microtubule dynamics and chromosome segregation: a perspective from genetic studies in mice. *Carcinogenesis* **30**, 1469-1474 (2009).

239. Elowe, S. Bub1 and BubR1: at the interface between chromosome attachment and the spindle checkpoint. *Mol Cell Biol* **31**, 3085-3093 (2011).

240. Kelly, A.E. & Funabiki, H. Correcting aberrant kinetochore microtubule attachments: an Aurora B-centric view. *Curr Opin Cell Biol* **21**, 51-58 (2009).

241. Sudakin, V., Chan, G.K. & Yen, T.J. Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J Cell Biol* **154**, 925-936 (2001).

242. Peters, J.M. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat Rev Mol Cell Biol* **7**, 644-656 (2006).

243. Farr, K.A. & Cohen-Fix, O. The metaphase to anaphase transition: a case of productive destruction. *Eur J Biochem* **263**, 14-19 (1999).

244. Losada, A., Hirano, M. & Hirano, T. Cohesin release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. *Genes Dev* **16**, 3004-3016 (2002).

245. Babu, J.R., Jeganathan, K.B., Baker, D.J., Wu, X. *et al.* Rae1 is an essential mitotic checkpoint regulator that cooperates with Bub3 to prevent chromosome missegregation. *J Cell Biol* **160**, 341-353 (2003).

246. Dai, W., Wang, Q., Liu, T., Swamy, M. *et al.* Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency. *Cancer Res* **64**, 440-445 (2004).

247. Michel, L.S., Liberal, V., Chatterjee, A., Kirchwegger, R. *et al.* MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. *Nature* **409**, 355-359 (2001).

248. Weaver, B.A. & Cleveland, D.W. Aneuploidy: instigator and inhibitor of tumorigenesis. *Cancer Res* **67**, 10103-10105 (2007).

249. Weaver, B.A., Silk, A.D., Montagna, C., Verdier-Pinard, P. *et al.* Aneuploidy acts both oncogenically and as a tumor suppressor. *Cancer Cell* **11**, 25-36 (2007).

250. Baker, D.J., Jin, F., Jeganathan, K.B. & van Deursen, J.M. Whole chromosome instability caused by Bub1 insufficiency drives tumorigenesis through tumor suppressor gene loss of heterozygosity. *Cancer Cell* **16**, 475-486 (2009).

251. Jeganathan, K., Malureanu, L., Baker, D.J., Abraham, S.C. *et al.* Bub1 mediates cell death in response to chromosome missegregation and acts to suppress spontaneous tumorigenesis. *J Cell Biol* **179**, 255-267 (2007).

252. Chan, G.K., Liu, S.T. & Yen, T.J. Kinetochore structure and function. *Trends Cell Biol* **15**, 589-598 (2005).

253. Hoyt, M.A., Totis, L. & Roberts, B.T. S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. *Cell* **66**, 507-517 (1991).

254. Martinez-Exposito, M.J., Kaplan, K.B., Copeland, J. & Sorger, P.K. Retention of the BUB3 checkpoint protein on lagging chromosomes. *Proc Natl Acad Sci U S A* **96**, 8493-8498 (1999).

255. Braunstein, I., Miniowitz, S., Moshe, Y. & Hershko, A. Inhibitory factors associated with anaphase-promoting complex/cylosome in mitotic checkpoint. *Proc Natl Acad Sci U S A* **104**, 4870-4875 (2007).

256. Tang, Z., Shu, H., Oncel, D., Chen, S. *et al.* Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint. *Mol Cell* **16**, 387-397 (2004).

257. Logarinho, E. & Bousbaa, H. Kinetochore-microtubule interactions "in check" by Bub1, Bub3 and BubR1: The dual task of attaching and signalling. *Cell Cycle* **7**, 1763-1768 (2008).

258. Logarinho, E., Resende, T., Torres, C. & Bousbaa, H. The human spindle assembly checkpoint protein Bub3 is required for the establishment of efficient kinetochore-microtubule attachments. *Mol Biol Cell* **19**, 1798-1813 (2008).

259. Kalitsis, P., Earle, E., Fowler, K.J. & Choo, K.H. Bub3 gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis. *Genes Dev* **14**, 2277-2282 (2000).

260. Gisselsson, D. Classification of chromosome segregation errors in cancer. *Chromosoma* **117**, 511-519 (2008).

261. Fenech, M., Kirsch-Volders, M., Natarajan, A.T., Surralles, J. *et al.* Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis* **26**, 125-132 (2010).

262. Cimini, D., Fioravanti, D., Salmon, E.D. & Degrassi, F. Merotelic kinetochore orientation versus chromosome mono-orientation in the origin of lagging chromosomes in human primary cells. *J Cell Sci* **115**, 507-515 (2002).

263. Norppa, H. & Falck, G.C. What do human micronuclei contain? *Mutagenesis* **18**, 221-233 (2003).

264. Hoffelder, D.R., Luo, L., Burke, N.A., Watkins, S.C. *et al.* Resolution of anaphase bridges in cancer cells. *Chromosoma* **112**, 389-397 (2004).

265. Bakhoum, S.F., Genovese, G. & Compton, D.A. Deviant kinetochore microtubule dynamics underlie chromosomal instability. *Curr Biol* **19**, 1937-1942 (2009).

266. Bakhoum, S.F., Thompson, S.L., Manning, A.L. & Compton, D.A. Genome stability is ensured by temporal control of kinetochore-microtubule dynamics. *Nat Cell Biol* **11**, 27-35 (2009).

267. Bakhoum, S.F. & Compton, D.A. Cancer: CINful centrosomes. *Curr Biol* **19**, R642-645 (2009).

268. Zyss, D. & Gergely, F. Centrosome function in cancer: guilty or innocent? *Trends Cell Biol* **19**, 334-346 (2009).

269. Gisselsson, D., Hakanson, U., Stoller, P., Marti, D. *et al.* When the genome plays dice: circumvention of the spindle assembly checkpoint and near-random chromosome segregation in multipolar cancer cell mitoses. *PLoS One* **3**, e1871 (2008).

270. To-Ho, K.W., Cheung, H.W., Ling, M.T., Wong, Y.C. *et al.* MAD2DeltaC induces aneuploidy and promotes anchorage-independent growth in human prostate epithelial cells. *Oncogene* **27**, 347-357 (2008).

271. Sowa, M.E., Bennett, E.J., Gygi, S.P. & Harper, J.W. Defining the human deubiquitinating enzyme interaction landscape. *Cell* **138**, 389-403 (2009).

272. Hussain, S., Zhang, Y. & Galardy, P.J. DUBs and cancer: the role of deubiquitinating enzymes as oncogenes, non-oncogenes and tumor suppressors. *Cell Cycle* **8**, 1688-1697 (2009).

273. Banno, K., Yanokura, M., Kawaguchi, M., Kuwabara, Y. *et al.* Epigenetic inactivation of the CHFR gene in cervical cancer contributes to sensitivity to taxanes. *Int J Oncol* **31**, 713-720 (2007).

274. Rong, R., Jiang, L.Y., Sheikh, M.S. & Huang, Y. Mitotic kinase Aurora-A phosphorylates RASSF1A and modulates RASSF1A-mediated microtubule interaction and M-phase cell cycle regulation. *Oncogene* **26**, 7700-7708 (2007).

275. Song, M.S., Song, S.J., Ayad, N.G., Chang, J.S. *et al.* The tumour suppressor RASSF1A regulates mitosis by inhibiting the APC-Cdc20 complex. *Nat Cell Biol* **6**, 129-137 (2004).

276. Liu, L., Baier, K., Dammann, R. & Pfeifer, G.P. The tumor suppressor RASSF1A does not interact with Cdc20, an activator of the anaphase-promoting complex. *Cell Cycle* **6**, 1663-1665 (2007).

277. Swanton, C., Tomlinson, I. & Downward, J. Chromosomal instability, colorectal cancer and taxane resistance. *Cell Cycle* **5**, 818-823 (2006).

278. Swanton, C., Nicke, B., Schuett, M., Eklund, A.C. *et al.* Chromosomal instability determines taxane response. *Proc Natl Acad Sci U S A* **106**, 8671-8676 (2009).

279. Hollstein, M., Sidransky, D., Vogelstein, B. & Harris, C.C. p53 mutations in human cancers. *Science* **253**, 49-53 (1991).

280. Levine, A.J., Momand, J. & Finlay, C.A. The p53 tumour suppressor gene. *Nature* **351**, 453-456 (1991).

281. Zhou, H., Kuang, J., Zhong, L., Kuo, W.L. *et al.* Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. *Nat Genet* **20**, 189-193 (1998).

282. Giet, R. & Prigent, C. Aurora/Ipl1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases. *J Cell Sci* **112 (Pt 21)**, 3591-3601 (1999).

283. Manfredi, M.G., Ecsedy, J.A., Chakravarty, A., Silverman, L. *et al.* Characterization of Alisertib (MLN8237), an investigational small-molecule inhibitor of aurora A kinase using novel in vivo pharmacodynamic assays. *Clin Cancer Res* **17**, 7614-7624.

BIOGRAPHICAL SKETCH

Serena Giovinazzi was born in 1980 in Taranto, Italy, second daughter of high-school teachers Giuseppe Giovinazzi and Maria Rosaria Migliaccio.

Serena attended scientific high school with biology specialization and graduated in 1998. Then she graduated with honors from the University of Bologna, Italy, in 2004 with a M.S. in pharmaceutical biotechnology. During the last year of college she worked on her research project for the master degree thesis at the Biochemistry & Molecular Biology Department of the Chiron/Novartis Vaccines Research Centre in Siena, Italy.

After college graduation, from 2004 to 2007 Serena worked on several research projects as microbiologist at the Biochemistry & Molecular Biology Department of the Chiron/Novartis Vaccines Research Centre in Siena, Italy, under the supervision of Dr. Renata Grifantini and Dr. Guido Grandi. In February 2007 she obtained a research fellowship at the Istituto Superiore di Sanità (ISS) in Rome, Italy, which is the leading technical and scientific public body of the Italian National Health Service, where she studied the role of a human micro-RNA cluster in cancer.

Serena joined the University of Florida's IDP graduate school program in 2008 and pursed her Ph.D work in the laboratory of Dr. Alexander Ishov studying Daxx function in cellular taxol response and mitosis. She has presented her work at several national and international conferences and published her research.

Since 2005 Serena has been involved in training new lab members and serving as teaching assistant for several courses in biological sciences.

In 2004 Serena was awarded of the *summa cum laude* degree from University of Bologna for the achievements during her academic studies. She also received travel awards (in 2009, 2010 and 2011) from the graduate school, outstanding international

student awards (in 2009 and 2011) from the college of medicine at UFL, and the Medical Guild research incentive award in 2010 from University of Florida.