AURORA KINASES IN SOLELY ESTROGEN-INDUCED ONCOGENESIS: RELATION TO CENTROSOME AMPLIFICATION AND CHROMOSOMAL INSTABILITY

By

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACI: August Copenhagen Irish

ADH: atypical ductal hyperplasia

AF: activation function

ANOVA: Analysis of Variance

APC: anaphase promoting complex

ATP: adenosine 5'-triphosphate

Aur: Aurora kinase

BC: breast cancer

BRCA: breast cancer gene

CA: centrosome amplification

Cdk: cyclin dependant kinase

CIN: chromosomal instability

COC: combination oral contraceptive

DCIS: ductal carcinoma *in situ*

DES: diethylstilbestrol

E_2: 17β-estradiol

ER: estrogen receptor

ERE: estrogen response element

ERT: estrogen replacement therapy

G1, 2: gap phases of the cell cycle

H&E: hematoxylin and eosin

HRT: hormone replacement therapy

IDBC: invasive ductal breast cancer

M: mitosis

MDM2: mouse double minute 2

MIN: microsatellite instability

NEK: nima related kinase

PCR: polymerase chain reaction

PP1: protein phosphatase 1

RITA: Reactivation of $p53^{wt}$ and Induction of Tumor cell Apoptosis

S: DNA synthesis phase of the cell cycle

SERM: selective estrogen receptor modulator

SNP: single nucleotide polymorphism

TPX2: targeting protein for Xklp2

Tx: tamoxifen citrate

Abstract

Persistent Aurora (Aur) A and B over-expression, centrosome amplification (CA), chromosomal instability (CIN) and aneuploidy invariably occur together in a vast majority of human neoplasms. These molecular changes are frequently found (>80%) in human sporadic ductal carcinoma *in-situ* (DCIS) and in primary invasive ductal breast cancers (IDBC). In solely 17β-estradiol (E_2)-induced mammary tumors in ACI rats, Li et al. have previously shown AurA protein over-expression, CA, CIN and aneuploidy (Li et al., 2004). AurA and B, mitotic kinases involved in controlling proper cell division, are modulated by cell cycle progression. Their over-expression is implicated in the deregulation of chromosomal duplication/segregation, and cytokinesis leading to CA, CIN and aneuploidy. To determine whether the over-expression of Aur kinases is a common feature of E_2-induced oncogenesis, AurA and B expression were examined in the Syrian hamster tumors of the kidney. Western blot analysis of the E_2-induced Syrian hamster tumors of the kidney revealed that both AurA and B protein expression was markedly increased when compared to cholesterol-treated controls. Moreover, immunohistochemistry revealed that this increase in AurA and B expression was specifically localized to E_2-induced tumor cells. Using an *in vitro* kinase assay, a significant increase in AurA kinase activity was detected in these tumors of the kidney and a significant increase in AurA mRNA levels

was detected as measured by real-time PCR. The over-expression of both kinases was markedly reduced in E_2-induced tumor-bearing hamsters subjected to either a 10-day E_2-withdrawal period or treated for a similar period with Tamoxifen citrate (Tx) plus E_2, when compared to tumors of the kidney from hamsters treated with E_2 alone. These results indicate that both AurA and B are under estrogen control mediated by estrogen receptor α (ERα). Additionally, examination of centrin and γ-tubulin expression in Syrian hamster tumors of the kidney indicates an increase in centrosome number and size, a characteristic of CA. The AurA expression was co-localized to isolated amplified tumor centrosomes, identified by their high levels of centrin and γ-tubulin expression. CIN and aneuploidy in Syrian hamster tumors of the kidney has previously been demonstrated (Papa et al., 2003). In order to assess, during E_2-induced oncogenesis, whether the over-expression of AurA and B contributes to the deregulation of the centrosome cycle via their specific protein substrates, we analyzed the protein expression of centrin, histone H3, PP1 and TPX2, all of which were significantly over-expressed in Syrian hamster tumors of the kidney and in ACI rat mammary gland tumors. Additionally, the expression of MDM2, which regulates the tumor suppressor gene p53, and p53 protein, an AurA substrate, were examined in Syrian hamster tumors of the kidney and in ACI rat mammary gland tumors. MDM2 expression was significantly increased in both Syrian hamster tumors of the kidney and in ACI rat mammary gland tumors at the protein and mRNA level,

and estrogen modulation studies showed that MDM2 protein expression is either directly or indirectly controlled by E_2. Binding studies show that MDM2 is bound to $p53^{wt}$. MDM2-$p53^{wt}$ binding was inhibited in Syrian hamster tumors of the kidney after treatment with the small molecule inhibitor RITA (Reactivation of p53 and Induction of Tumor cell Apoptosis). In E_2-induced Syrian hamster tumors of the kidney, Western blot analysis revealed that RITA treatment led to a significant increase in $p53^{wt}$ protein expression when compared to untreated tumor samples. In addition, RITA treatment led to the increased expression of Bax, a downstream target gene of p53, which promotes apoptosis. These data suggest that Aur kinase over-expression, induced by estrogens, may interfere with bipolar spindle formation and chromosome segregation, leading to CA, CIN and aneuploidy, thus supporting the concept that AurA over-expression and CA are causative events, induced by estrogen, that lead to tumor development. The over-expression of MDM2, in tandem with AurA, indicates a close relationship between these two entities in fostering CA, CIN, aneuploidy and tumor development, defining events of E_2-driven oncogenesis.

Chapter 1: Introduction and Background

1.1 The Relationship Between Breast Cancer and Estrogen

Breast cancer (BC) is the most commonly diagnosed female cancer worldwide with an estimated one million new cases diagnosed each year (Bray et al., 2004). Moreover, BC is the second leading cause of cancer related deaths in industrialized countries (Nkondjock and Ghadirian, 2005). Approximately 181,000 cases of BC are diagnosed in the United States each year with an estimated 41,000 dying from BC each year (2007). It has been well established that cancer has a genetic basis; however, only 5 – 10% of BC are familial (King et al., 1993). These familial cases largely result from inherited mutations in BC susceptibility genes such as BRCA1 and 2 (2005). In the case of sporadic BC, which accounts for about 90 – 95% of all BC cases, it has been well established that both endogenous, and to a lesser extent, exogenous estrogens play a crucial role in both the causation and development (Bilimoria and Morrow, 1995).

In addition to being female and post-menopausal (50+ years), the major risk factors of sporadic BC are associated with ovarian hormone exposure (Table 1), more specifically, the accumulation of a woman's lifetime exposure to estrogen (Bernstein, 2002). These well-established risk factors include: early age of menarche (<12 years), late onset of menopause (>55 years), nulliparity, late age of first full-term birth (>30 years) and fewer number of full-term births (Martin and Weber, 2000; 2005). All of these risk factors act

to directly increase a woman's exposure to circulating levels of estrogen through an increased number of uninterrupted ovulatory cycles.

Table 1. Breast Cancer risk factors

Early age of menarche	Post-menopausal obesity
Late onset of menopause	Alcohol consumption
Nulliparity	Physical inactivity
Late age of first full-term birth	COC / HRT
Fewer number of full-term births	Ovarian tumors

Other risk factors associated with BC may be attributed to individual diet and lifestyle choices which have been shown to indirectly increase endogenous levels of circulating estrogens. Obesity increases a woman's risk of post-menopausal BC (McPherson et al., 2000; Eliassen et al., 2006); however it does not increase the risk of pre-menopausal BC (Huang et al., 1997). In post-menopausal women, adipose tissue is the major source of circulating estrogen. Thus, obesity increases the level of estrogen and the likelihood of developing BC. Physical activity has been implicated as an important variable risk factor for BC (Colditz et al., 2003). An inverse association between physical activity and BC has been reported (Bernstein et al., 2005). In addition, moderate alcohol consumption (1-2 drinks per day) has been associated with a 30-50% increase in BC (Martin and Weber, 2000; Terry et al., 2006). It has been suggested that alcohol consumption within the

last five years is associated with an 82% increased risk in young women (age 20-49) relative to never drinkers (Berstad et al., 2007).

Other proposed BC risk factors are related to the exogenous administration of estrogens such as in combination oral contraceptives (COC) and hormone replacement therapy (HRT). While one may expect that the ingestion of these exogenous estrogens would increase a woman's risk of BC, a majority of studies report only a modest increase in BC risk in a subset of COC users under the age of 45 (Malone et al., 1993). A slight increase in the risk of developing BC has been reported in women taking COCs and 10 years after cessation (McPherson et al., 2000). However, recent reports suggest that, at most, COC use is a minor BC risk contributor (Nichols et al., 2007).

Estrogen replacement therapy (ERT) or combined HRT provide relief of menopausal symptoms and prevent osteoporosis in post-menopausal women, thus leading to their increased use in recent decades (Keating et al., 1999). Over the last 10 years, both therapies have come under fire for their suspected role in increasing BC risk. Several reports have shown data in favor of (Colditz et al., 1995; Schairer et al., 2000; Beral, 2003) and against (Dupont and Page, 1991) an increased BC risk after these post-menopausal hormone therapies. The most comprehensive of these studies, the Women's Health Initiative, a randomized, double-blind, placebo controlled disease prevention trial, reported no increase in BC risk in women taking ERT alone

(Anderson et al., 2004). However, women receiving combined HRT were 26% more likely to develop BC (Rossouw et al., 2002) and to be diagnosed at an advanced stage most likely due to an increase in breast density which delayed diagnosis (Chlebowski et al., 2003).

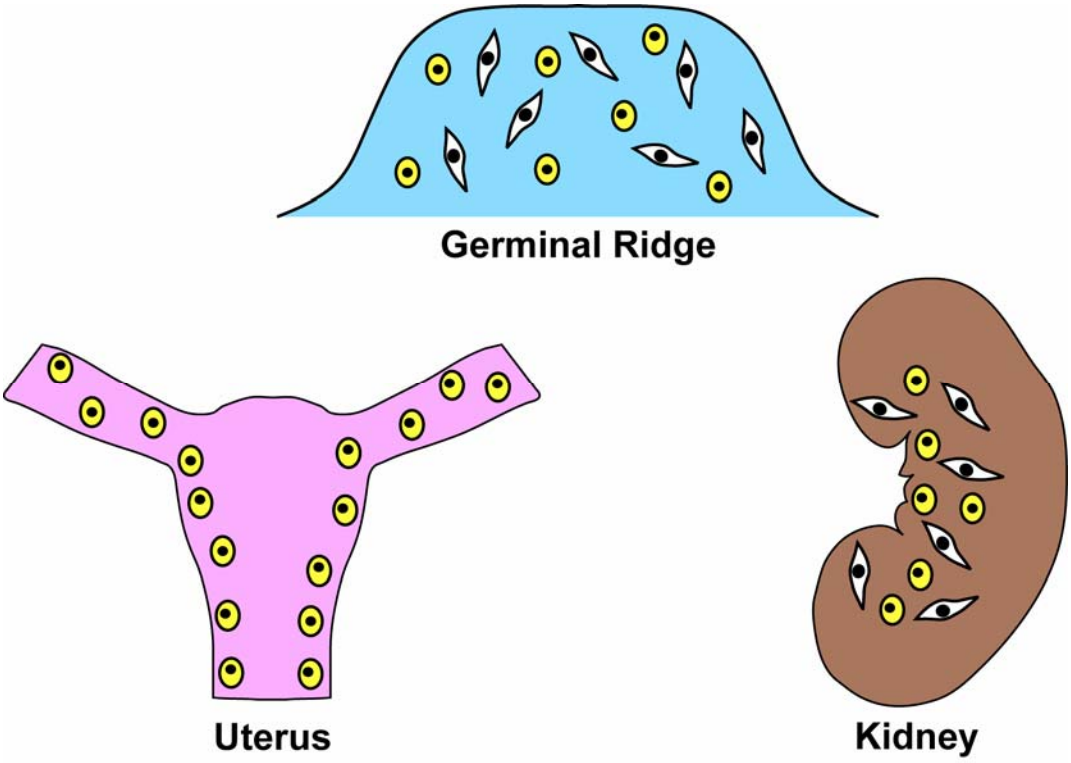
Additional strength for the link between BC and estrogen has been observed in some of the drugs used in its therapy. The two types of hormonal therapies used in the treatment of BC include selective estrogen receptor modulators (SERMs) and aromatase inhibitors. SERMs are used for treatment and prevention of BC in both pre- and post-menopausal women, whereas aromatase inhibitors are used for prevention only in post-menopausal women. SERMs, like tamoxifen, work by selectively inhibiting estrogen receptor α (ERα). The binding of tamoxifen to the ERα results in a conformational change which favors recruitment of co-repressors that inhibit transcription (Come et al., 2005). In at-risk populations, tamoxifen is effective in preventing cancer cell proliferation and reducing BC incidence by 48% (Johnston, 2005). Aromatase inhibitors have been shown to be effective in reducing serum estrogen levels by inhibiting the conversion of androgens to estrogens in peripheral tissues (Fentiman, 2004). While tamoxifen has been the standard of care for several decades, recent studies have shown that treatment with aromatase inhibitors may be more efficacious as a first–line treatment of advanced BC (Baum et al., 2002).

1.2 The Syrian Hamster Model in the Study of Human Breast Cancer

Estrogen-induced tumors of the Syrian hamster kidney were first described in 1959 by Kirkman (Kirkman, 1959). He showed that these tumors were not only estrogen-induced but also estrogen-dependent. When the estrogen source was removed, the tumors stopped growing and underwent a marked regression (Kirkman, 1959). The Syrian hamster kidney is an intensively studied model in estrogen oncogenesis because 100% tumor incidence is achieved using the natural hormone 17β-estradiol (E_2) in the absence of co-administration of any other carcinogen (Liehr, 1997). Moreover, no spontaneous tumors have been detected at this organ site (Kirkman, 1959; Pour et al., 1976).

In development, the hamster reproductive tract and urinary tract systems arise from the same germinal ridge of multipotential cells (Kirkman, 1959; Li et al., 2001) (Figure 1). Some of the reproductive germinal cells that are normally destined to reside in the uterus ectopically migrate to the urinary tract where they establish themselves in the corticomedullary region of the kidney. These misplaced interstitial cells, committed to an epithelial differentiation pathway, are referred to as ectopic uterine stem cells (Li et al., 2001). These cells remain dormant unless exposed to a sustained level of estrogen.

Figure 1. Schematic representation of the Syrian hamster germinal ridge (Adapted from (Li et al., 2001)



Bilateral tumors can be induced with E_2 and the synthetic estrogen diethylstilbestrol (DES) in both intact and castrated male hamsters. Similar tumors can also be induced in ovariectomized female hamsters, but because frequent estrogen exposure induces aplastic anemia in female hamsters resulting in a high mortality, they are not commonly used. Moreover, intact females do not develop kidney tumors when exposed to E_2/DES alone because of the inhibitory effects of ovarian progesterone (Kirkman, 1959). This response is in contrast to the role of this hormone in the mammary gland

where both estrogen and progesterone are mitogenic. Progestins, anti-estrogens, like tamoxifen, and androgens, when concomitantly administered with E_2 or DES completely block the formation of these tumors in the hamster kidney (Li and Li, 1978).

An advantage of this well-established animal model is that E_2-induced tumors occur in the absence of any intervening morphological stages, and the tumors develop via the continuous progression of a subset of interstitial stem cells (Li et al., 2003b). This is a marked contrast to human BC and other female rat E_2-induced mammary tumors from a variety of strains which progress through stages of hyperplasia, atypical ductal hyperplasia (ADH), dysplasia and ductal carcinoma *in-situ* (DCIS) in response to continuous estrogen exposure. However, the E_2-induced Syrian hamster kidney model and human BC share many crucial early molecular changes (Li and Li, 2003). These events include: over-expression and amplification of early response nuclear proto-oncogenes c-*myc*, c-*fos* and c-*jun* (Hou et al., 1996; Li et al., 1999), deregulation of cell-cycle entities including cyclins D and E, cyclin dependent kinase (cdk) 2 and 4, pRb and the cyclin dependent kinase inhibitor p27 (Liao et al., 2000), AurA/B over-expression (Hontz et al., 2007), CA, CIN and aneuploidy (Li et al., 2001).

1.3 The ACI Rat Model in the Study of Human Breast Cancer

Rodent models have been used in the study of human BC since the 1930s (Lacassagne, 1932; Lacassagne, 1933). Many studies have identified mouse and rat strains highly susceptible to estrogen-induced mammary carcinogenesis (Dunning et al., 1953; Nagasawa, 1979). However, these solely estrogen-induced mammary tumor models are limited by variability in tumor incidence depending on the strain, administration method, dose and tolerance (Dunning et al., 1953). Several of the rat tumor models become estrogen-dependent upon treatment with synthetic chemical carcinogens, including 7,12-dimethylbenz-(a)anthracene (DMBA) (Huggins et al., 1961) and methylnitrosourea (MNU) (Bots and Willighagen, 1975), or radiation to induce tumor development. While these chemically-induced rat mammary tumors are morphologically similar to human BCs (Russo and Russo, 2000; Singh et al., 2000), the cellular sequence is not the same, no ADH or DCIS, and they do not share the same molecular characteristics. For example, chemically-induced rat mammary tumors are highly diploid (>85%) (Aldaz et al., 1992; Haag et al., 1996), whereas human BCs are highly aneuploid, 89.6% (Arnerlov et al., 2001). The validity of these chemically-induced tumor models has also been questioned because their induction requires synthetic chemicals to which women are never exposed to in the environment.

The August Copenhagen Irish (ACI) rat, a cross between the August and the Copenhagen-Irish strains has emerged as a uniquely sensitive, solely

estrogen-induced mammary oncogenesis model (Dunning et al., 1953). Recently, the sensitivity of the ACI strain to estrogen-induced BC has been mapped to chromosomes 5 and 18 (Gould et al., 2004). These chromosomes are the location of estrogen-induced mammary cancer (Emca) 1 and 2 genes which act independently to increase tumor incidence and decrease latency. ACI rat mammary tumors are both estrogen-induced and -dependent as demonstrated by tumor regression when exogenous estrogen sources are removed or anti-estrogens are co-administered (Li et al., 2002b). Advantages of this model over other mouse and rat models include: **1.** Estrogen treatment of intact female ACI rats causes mammary tumors in 100% of the animals within 6 months (Li et al., 2002b); **2.** There is a very low incidence of spontaneous mammary gland tumor development – about 11% (Maekawa and Odashima, 1975); **3.** A rat mammary tumor virus has not been identified as is the case in mice with the mouse mammary tumor virus (MMTV); **4.** Estrogen concentrations used to induce tumor formation approach the physiological range of normal cycling rats (70-80 pg/mL) (Li et al., 2004); **5.** Concomitant treatment with SERMs, like tamoxifen, completely inhibits E_2-induced mammary tumor induction (Li and Li, 2003); **6.** Histopathologic and morphological changes including: hyperplasia, dysplasia, DCIS and invasive ductal breast cancer (IDBC) are similar to those reported in human BC; and **7.** Cellular and molecular features observed in E_2-induced ACI rat mammary tumors are similar to those observed in human ductal BC. For example, both

ACI rat mammary tumors and human BCs frequently show over-expression and amplification of early response nuclear proto-oncogenes c-*myc* (Li et al., 2002a), c-*fos* and c-*jun*, deregulation of cell-cycle entities including cyclins D1 and E1 (Weroha et al., 2006), cdk2 and 4, pRb and the cyclin dependent kinase inhibitor p27, AurA/B over-expression, CA, CIN and aneuploidy (Li et al., 2004).

1.4 Estrogen Action and Estrogen Receptors

Natural estrogens are steroid hormones synthesized from cholesterol, in the ovaries and adrenal gland. The synthesis and secretion of estrogens are controlled by follicle stimulating hormone produced by the pituitary gland. Estrogen is used to collectively describe the female hormones which include E_2, estrone and estriol. Of these, E_2 is the most abundant and potent. Estrogens influence growth, differentiation and functioning of target tissues including mammary gland, uterus and ovaries (Kuiper et al., 1997). Estrogens have also been shown to play a role outside the reproductive system. They play an important role in bone maintenance (Srivastava et al., 2001), brain function (McEwen, 1999) and the cardiovascular system where, depending on the E_2 levels, they may exert cardioprotective effects (Mendelsohn and Karas, 1999).

Estrogen displays its effects through at least four estrogen receptor (ER) pathways: classical ligand-dependent, ligand-independent, estrogen

response element (ERE)-independent and cell-surface (non-genomic) signaling (Hall et al., 2001). The classical mechanism of estrogen action, via ERs, is how most of its biological effects occur. Estrogens circulate in the body either un-bound or bound to albumin or sex hormone binding globulin (Kahn et al., 2002). Un-bound estrogen can freely diffuse in and out of cells; however, in target cells, estrogen is retained with high affinity and specificity by binding to ERs (Kuiper et al., 1997). This binding allows the ERs to dissociate from a heat shock protein and undergo a conformational change allowing dimerization to occur. The ER dimers can then bind to specific sequences of DNA referred to as EREs that act as transcription factors used to modulate transcription of target genes (Figure 2). The interaction with EREs can be direct or indirect via the action of co-activators which enhance transcription (McKenna et al., 1998) or co-repressors which down-regulate transcription (Dobrzycka et al., 2003).

ERs belong to the steroid/thyroid hormone superfamily of nuclear receptors. Two ERs have been identified, ERα by Jensen in 1958 (Jensen and DeSombre, 1973) and ERβ by Kuiper in 1996 (Kuiper et al., 1996). ERα and ERβ are separate gene products; ERα is localized to human chromosome 6q25.1 (Menasce et al., 1993) and ERβ to chromosome 14q22-24 (Enmark et al., 1997). The ERs can be subdivided into several functional domains, the highly conserved, 97 and 60% DNA- and ligand binding

domains, respectively, and the highly variable N-terminal domain (Hall et al., 2001).

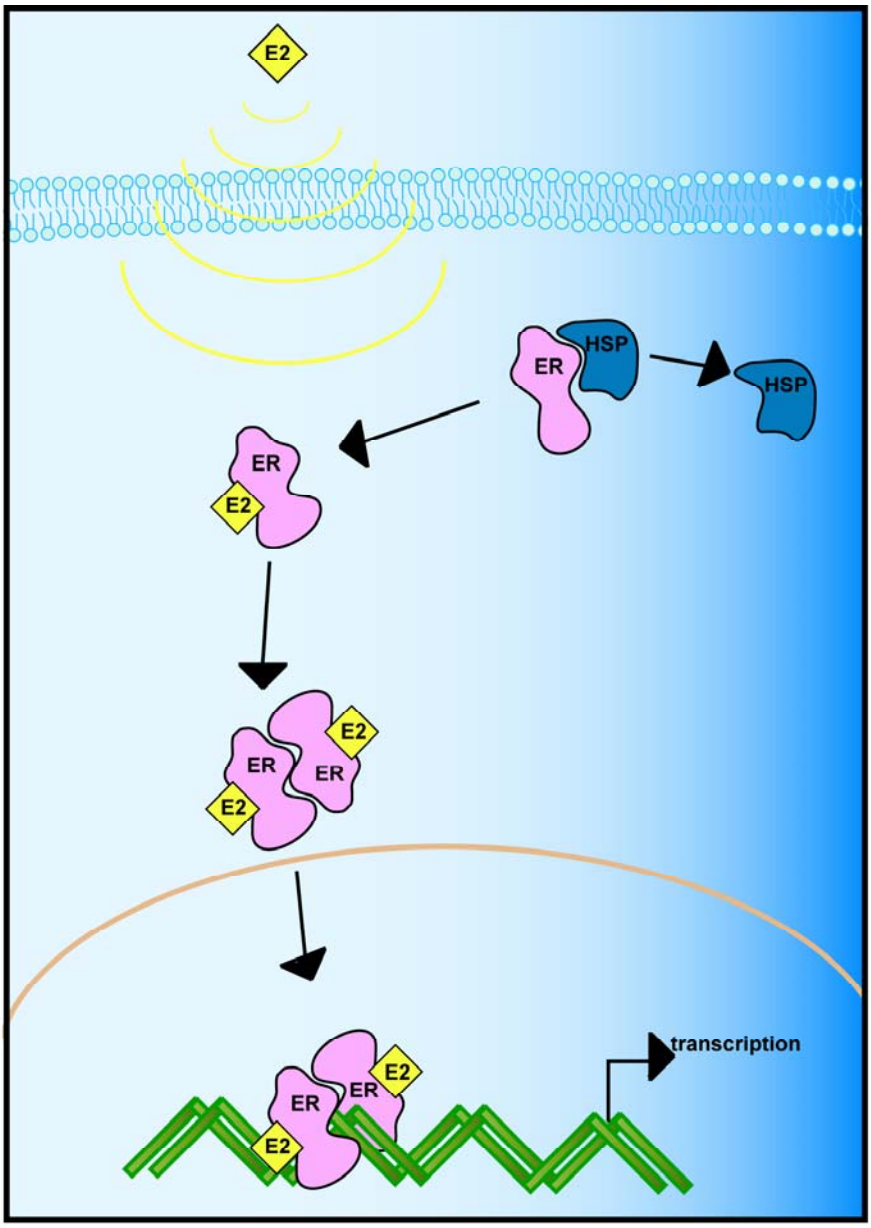
Both ERs are widely distributed throughout the body. ERα is expressed primarily in the pituitary, uterus, liver, heart and kidneys, whereas ERβ is expressed primarily in the lungs, ovary, prostate, gastrointestinal tract, bladder as well as hematopoietic and central nervous systems (Couse et al., 1997; Kuiper et al., 1997). ERα and ERβ are co-expressed in the mammary gland, epididymis, thyroid, adrenal, bone and certain regions of the brain (McEwen and Alves, 1999; Pettersson and Gustafsson, 2001). In many instances, when co-expressed, ERβ opposes the actions of ERα. The function of ERα is primarily related to cellular growth responses in target tissues, whereas the function of ERβ is primarily anti-proliferative and pro-differentiative (Imamov et al., 2005).

The existence of two ER subtypes allows estrogen to signal via three potential nuclear pathways: ERα homodimers, ERα/ERβ heterodimers and ERβ homodimers (Kuiper and Gustafsson, 1997). The transactivating functions of ERα and ERβ are mediated by transcription activation functions (AF-1 and AF-2) that allow the ERs to stimulate the transcription of estrogen-regulated genes. In addition to their role in transcription mediation, AFs mediate cell- and promoter-specificity (Matthews and Gustafsson, 2003). While both ERs are highly reactive to activation through the ligand-dependent AF-2, only ERα is highly reactive to ligand-independent activation through AF-

1. It has been suggested that this weaker activation of ERβ through AF-1 might be the origin of its repressive activity (Ogawa et al., 1998).

In addition to the nuclear ER, a G protein-coupled ER, GPR30, has been described (Carmeci et al., 1997). In response to estrogen, GPR30 can mediate rapid signaling events and modulate transcriptional activity. In a recent clinical study of human breast samples 42% and 62% of DCIS and IDBC were GRP30 positive, respectively (Filardo et al., 2006). While it is as yet unknown how ER and GPR30 interact and the role that this interaction may play in oncogenesis, GPR30 may represent an important mechanism to overcome ER loss in BC (Prossnitz et al., 2008).

Figure 2. Schematic representation of ER/estrogen mediated gene transcription. Estrogen (E2), estrogen receptor (ER), heat shock protein (HSP) (Adapted from (Michalides, Griekspoor et al. 2004)



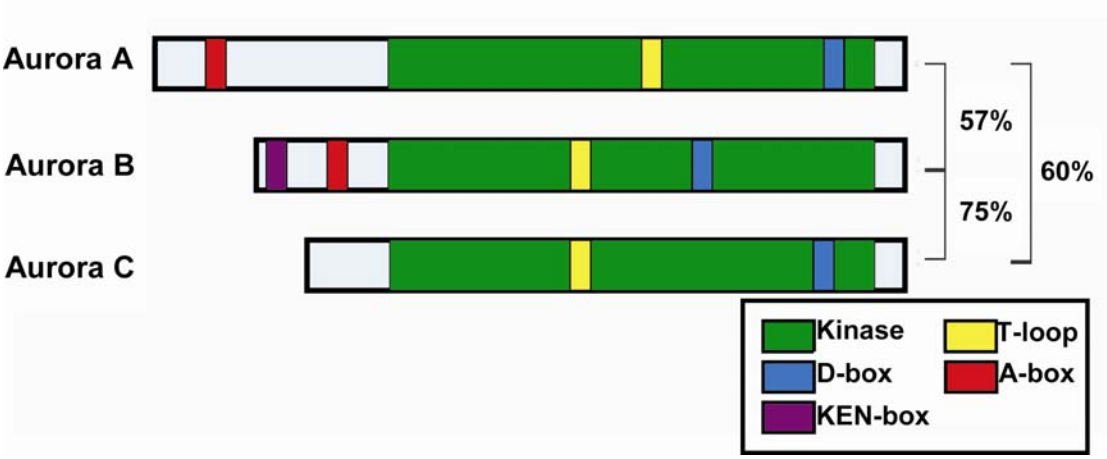
1.5 Overview of Aurora Kinases

Aurora kinases are mitotic serine/threonine kinases that phosphorylate a myriad of mitotic/centrosomal protein substrates required for proper chromosome duplication, segregation and cytokinesis. These mitotic events are important in maintaining genomic integrity. The serine/threonine family of mitotic kinases also includes cdk1, the polo-like kinases with four family members, the NEK kinases with 11 family members and the WARTS/LATS 1 related kinases (Marumoto et al., 2005).

Aurora kinases were first discovered in *Drosophila* (Glover et al., 1995); however, human tissue samples have been reported to express three Aurora kinases (Keen and Taylor, 2004) (Figure 3). Because numerous research groups discovered the various Aurora proteins independently, they are known by a plethora of names; however, nomenclature has agreed on Aurora A (AurA), B (AurB) and C (AurC) (Adams et al., 2001; Nigg, 2001). The catalytic domains of the Aurora kinases (Figure 3, shown in green) are highly conserved, but there is little homology between the NH_2-terminal extensions. Auto-phosphorylation of a threonine residue in the T-loop, (Figure 3, shown in yellow) is required for the activation of the protein's kinase activity. The destruction box (D-box), (Figure 3, shown in blue) is a short amino acid motif that is recognized by adaptors of the anaphase promoting complex (APC). These adaptors target the protein for degradation via the ubiquitin pathway. AurA degradation is dependent upon an intact A- and D-

box (Littlepage and Ruderman, 2002; Keen and Taylor, 2004). AurB is degraded by the proteasome via the Cdc27 subunit of the APC. Degradation of AurB does not depend on its D-box, but it does require intact KEN- and A-boxes (Nguyen et al., 2005). While AurA and B are expressed in most cell types, AurC is specifically expressed in the testis where it plays a role in spermatogenesis (Carmena and Earnshaw, 2003; Marumoto et al., 2005).

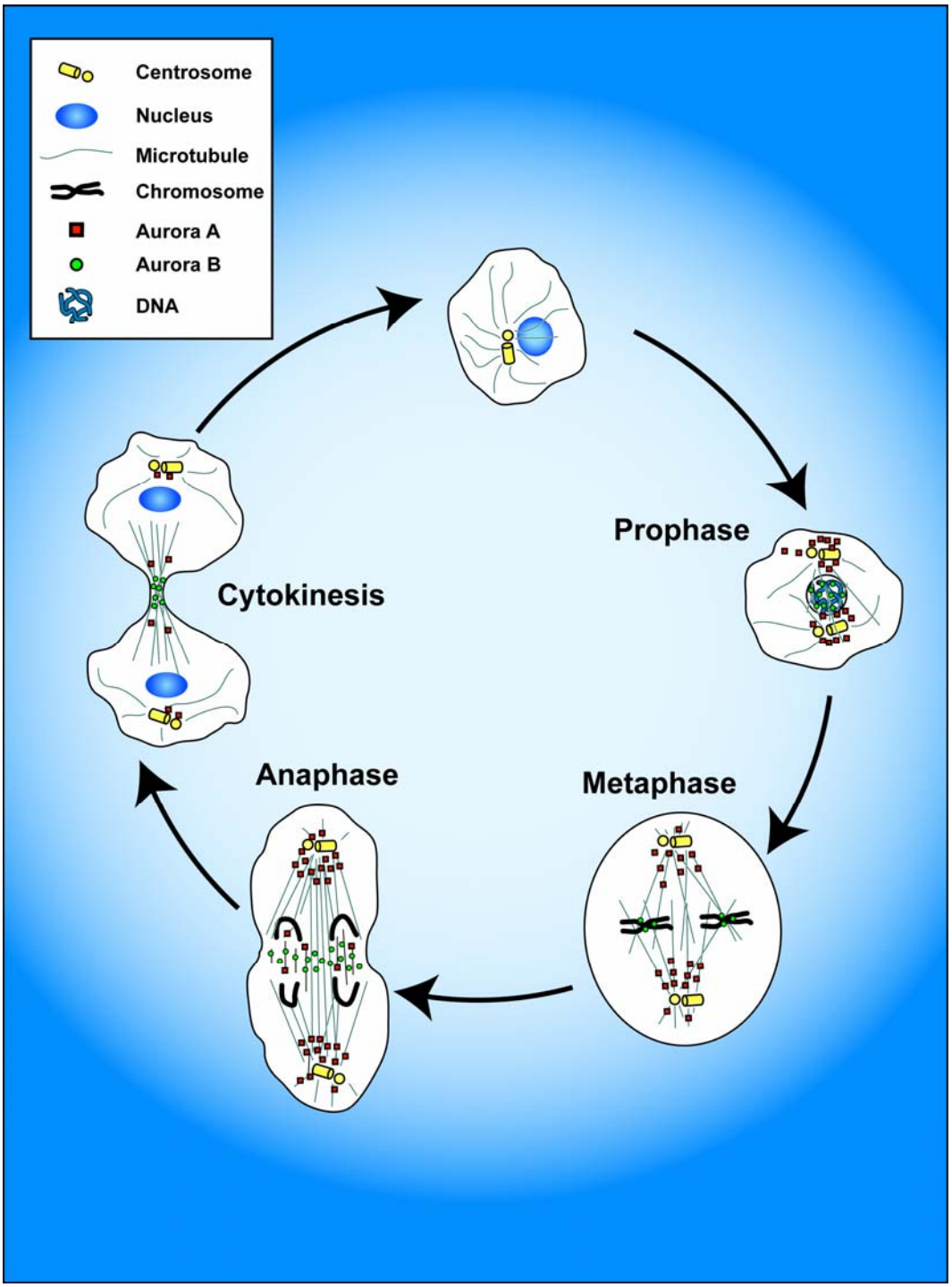
Figure 3. Schematic representation of the three human Aurora kinases. The numbers on the right indicate the proteins percentage sequence identities. (Adapted from ((Keen and Taylor, 2004; Nguyen et al., 2005))



Although the catalytic domains of the Aurora kinases are highly conserved, they have very distinct subcellular localizations (Carmena and Earnshaw, 2003). While AurA maintains an association with the centrosomes, AurB is a chromosomal passenger protein that migrates during mitosis

(Figure 4). In G1, the levels of both AurA and B are significantly reduced. By prophase, AurA is at the centrosomes whereas AurB is nuclear. In metaphase, AurA is on the microtubules near the spindle poles and AurB is on the centromeres. As the cells progress to anaphase, the majority of AurA is on the polar microtubules and AurB is at the spindle midzone. However, at the culmination of the cell cycle, both AurA and B are at the cell midbody where they participate in cytokinesis.

Figure 4. Localization of AurA and B kinase during mitosis (Adapted from (Carmena and Earnshaw, 2003)



AurA and B are essential for the proper execution of various mitotic events. The mitotic roles of AurA and B have been elucidated using techniques such as Aur-depletion, -inhibition and RNA interference (RNAi). AurA plays a role in centrosome duplication, maturation and separation, mitotic entry, bipolar spindle assembly and stability, chromosome alignment and cytokinesis, whereas AurB participates in phosphorylation of histone H3, chromosome alignment, chromosome segregation and cytokinesis. AurB binds three other chromosomal passenger proteins: inner centromere protein (INCENP), survivin and borealin (Keen and Taylor, 2004). Proper localization and function of any of these proteins is dependent on the other three. Substrates for both AurA (Mendez et al., 2000; Katayama et al., 2001; Du and Hannon, 2002; Giet et al., 2002; Gigoux et al., 2002; Kufer et al., 2002; Sakai et al., 2002; Castro et al., 2003; Hirota et al., 2003; Kunitoku et al., 2003; Dutertre et al., 2004; Katayama et al., 2004; Ouchi et al., 2004; Tien et al., 2004; Toji et al., 2004; Troiani et al., 2005) and AurB (Giet and Glover, 2001; Zeitlin et al., 2001; Bishop and Schumacher, 2002; Rogers et al., 2002; Goto et al., 2003; Kawajiri et al., 2003; Minoshima et al., 2003; Lan et al., 2004; Guse et al., 2005; Delacour-Larose et al., 2007) have been identified (Table 2), yet whether all of these substrates are actually phosphorylated *in vivo* remains to be determined. The expression of both AurA and B peaks at the G2/M transition and kinase activity is maximal during mitosis (Katayama et al., 2003).

Table 2. Known substrates of AurA and B. Common substrates to both are bolded.

AurA substrates

Ajuba	Eg5	p53
BRCA1	Hec 1	PP1
CDC25B	**Histone H3**	Ras GAP
Cdh-1	hsNuf2	TACC
CENP-A/B	Lats2	TPX2
Centrin 2	MBD3	**Survivin**
CPEB	NM23-H1	**Vimentin**

AurB substrates

CENP-A	INCENP	Myosin II
Desmin	MCAK	**Survivin**
GFAP	MgcRacGAP	Topoisomerase IIα
Histone H3	MKLP1	**Vimentin**

Presently, the most extensively studied Aurora kinase is AurA. Ectopic expression of AurA, active or not, leads to CA (Zhou et al., 1998; Meraldi et al., 2002). The over-expression of kinase active AurA has the ability to transform NIH3T3 cells and when implanted in nude mice induces tumor

formation, thus supporting the role of AurA as an oncogene (Bischoff et al., 1998; Zhou et al., 1998). While it appears that over-expression of AurA is sufficient to deregulate the centrosome cycle leading to centrosome abnormalities, its kinase activity is necessary to cause transformation leading to tumor formation.

AurA over-expression has been reported in hematological tumors and nearly all human solid tumors including: liver with a frequency or 61% (Jeng et al., 2004), esophageal with a frequency of 58% (Tong et al., 2004), lung (Haruki et al., 2001), gastric with a frequency of 50% (Sakakura et al., 2001), prostate (Bar-Shira et al., 2002), non-Hodgkins lymphoma (Yakushijin et al., 2004), bladder with a frequency of 75% (Sen et al., 2002), pancreatic with a frequency of 58% (Han et al., 2002; Li et al., 2003a), colorectal with a frequency of 50% (Bischoff et al., 1998), kidney with a frequency of 95% (Ehara et al., 2003), ovarian with a frequency of 57% (Gritsko et al., 2003), endometrial (Moreno-Bueno et al., 2003) and breast (Tanaka et al., 1999). Of note, reports indicate that AurA is over-expressed with a frequency of 94% in human DCISs and primary invasive mammary carcinomas (Tanaka et al., 1999) coming in second only behind kidney tumors (Ehara et al., 2003). Interestingly, AurA over-expression is not commonly correlated with gene amplification indicating that other mechanisms such as transcriptional activation and suppression of protein degradation must also play a role in AurA regulation (Marumoto et al., 2005). While significantly less is known

about AurB and its role in oncogenesis, it is known that AurA and B expression levels appear to rise or decline in parallel (Keen and Taylor, 2004). While no direct link has as yet been found between AurA over-expression and centrosome abnormalities, AurA overexpression, CA and aneuploidy invariably occur together.

The over-expression of Aurora kinases has been strongly linked to oncogenesis as well as tumor proliferation. This has led to the development of a new class of anti-cancer drugs that specifically targets the ATP-binding domain of the Aurora kinases. Currently, eight novel Aurora kinase inhibitors have been described. Of these, VX-680 (Harrington et al., 2004) has shown promising results both *in vitro* and *in vivo* and is the first Aurora kinase inhibitor to enter clinical trials. The high frequency of AurA over-expression, ~94%, in human BC (Tanaka et al., 1999) and in the ACI rat E_2-induced mammary tumors (Li et al., 2004), provides strong evidence that small molecule inhibitors like VX-680 may provide a new approach to BC therapy.

1.6 The Normal Centrosome Cycle

Centrosomes are cellular organelles located in the cytoplasm adjacent to the nucleus. They consist of a pair of barrel shaped centrioles composed of a radial array of nine microtubule triplets and a surrounding halo of protein dense pericentriolar matrix (Delattre and Gonczy, 2004). The pericentriolar matrix contains γ-tubulin ring complexes where microtubule nucleating activity

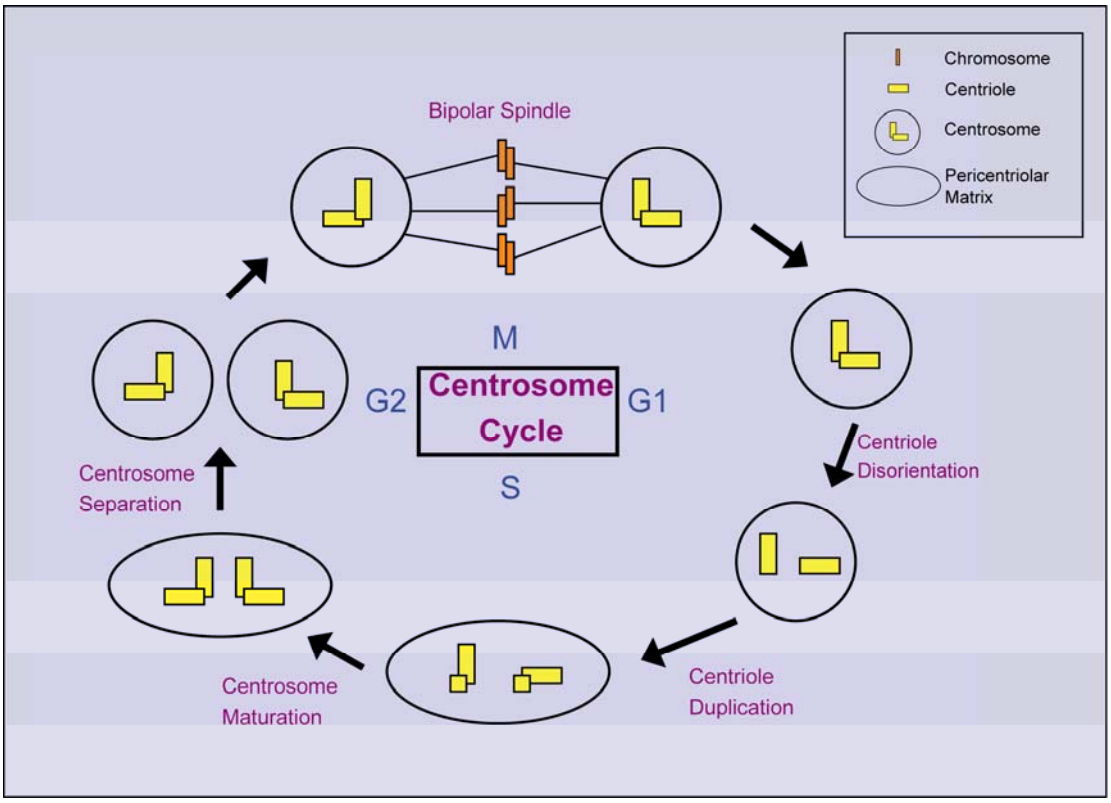
resides. In eukaryotic cells, the centrosome functions as the major microtubule organizing center (MTOC), the focal point from which microtubules emanate. Therefore, the centrosome influences all microtubule dependent processes including organelle transport, cell shape, polarity and motility (Meraldi and Nigg, 2002). Other important functions of the centrosomes occur during mitosis, when the centrosomes aid in establishing a bipolar spindle apparatus and cytokinesis (Wang et al., 2004).

In normal cells, centrosomes duplicate only once in each cell cycle to give rise to two mitotic spindle poles. The centrosome cycle has been divided into a series of discrete events commonly referred to as centriole disengagement, centriole duplication, centrosome maturation and centrosome separation (Meraldi and Nigg, 2002). Centrosome duplication is closely coordinated with the stages of the cell cycle (Figure 5). The interphase centrosome consists of a pair of centrioles surrounded by pericentriolar matrix. Duplication begins in S phase when the two centrioles lose their orthogonal arrangement in a process termed centriole disengagement that requires the protease separase (Tsou and Stearns, 2006). Centrin, a ubiquitous centrosome protein component, is required for centriole duplication (Salisbury et al., 2002) which begins with the formation of procentriole buds at the proximal end of each of the original centrioles. The procentrioles mature during S phase and G2. The separation of the two mature centrosomes occurs at the G2/M transition. The two mature centrosomes separate from

each other and move to opposite ends of the cell where they establish the bipolar mitotic spindle which helps ensure that chromosomes are equally segregated during mitosis.

While the mechanism by which the centrosome is duplicated during cell division is largely unknown, it remains an active area of research. A large body of evidence has accumulated suggesting that centrosome duplication is tightly linked to cell cycle progression through interacting regulatory pathways. These regulators include cdk2/cyclin A and E which control the cell, centrosome and DNA cycles and have been implicated in triggering centriole duplication (Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999; Tsou and Stearns, 2006). In addition, the mitotic kinases, Aurora, Polo and Nek families, have been implicated in centriole duplication (Meraldi and Nigg, 2001; Barr et al., 2004) as has the APC which ensures that centrioles duplicate only once per cell cycle (Tsou and Stearns, 2006).

Figure 5. The normal centrosome cycle



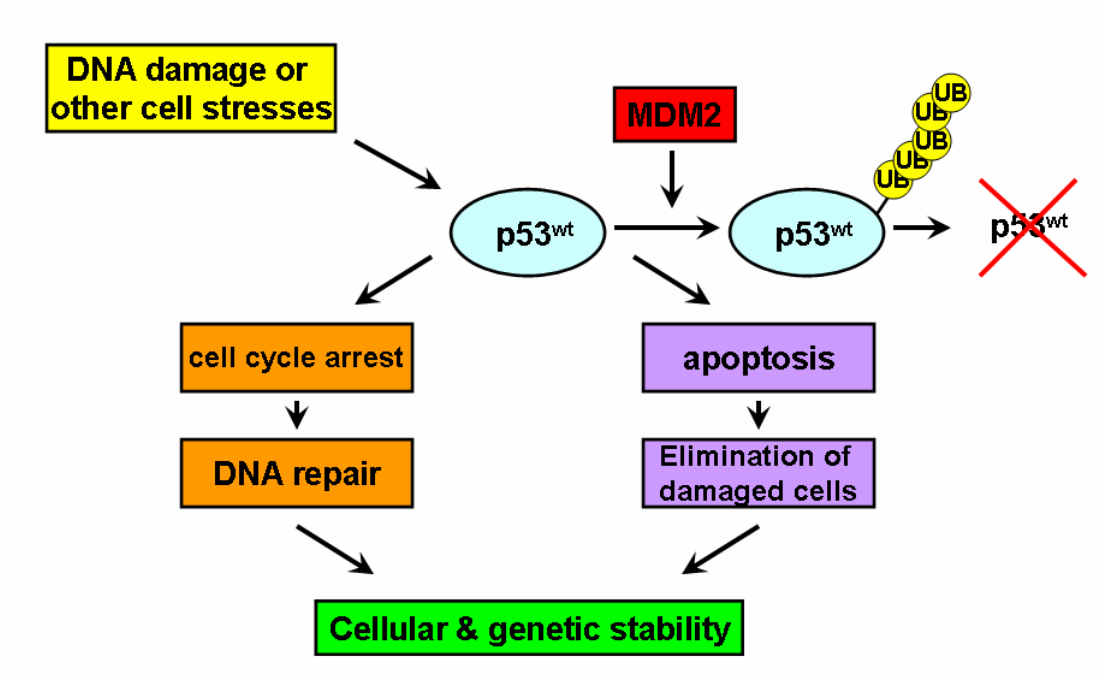
1.7 The p53-MDM2 Pathway

p53 is a tumor suppressor protein which plays an important role in protecting the genomic stability of the cell. In normal cells, p53 is kept at low levels by MDM2 to regulate cell growth under control (Barak et al., 1993; Wu et al., 1993). In response to DNA damage, or other cellular stresses, p53 is stabilized to modulate transcription of its effector genes (Vogelstein et al., 2000). This induces a variety of cellular responses. The most common responses are cell cycle arrest allowing the cell to repair damaged DNA, and

apoptosis leading to the elimination of damaged cells (Oren, 2003). These mechanisms ensure that damaged cells are either repaired or deleted from the genome, thus maintaining the genetic integrity of the cell. This is why p53 is called the guardian of the genome.

MDM2 (mouse double minute 2) is a nuclear proto-oncogene regulated by p53 through a p53 binding site present in the MDM2 gene (Wu et al., 1993). An important function of MDM2 is to regulate the expression of p53. MDM2 accomplishes this function in two ways: it may bind to and inhibit p53 transcriptional activity (Momand et al., 1992), or it may promote the rapid degradation of p53 through the ubiquitin-dependent proteosome pathway (Haupt et al., 1997).

Figure 6. The p53 pathway



When p53 was discovered in 1979, it was classified as an oncogene because of its increased expression in various human tumors. It was not until 10 years later, when it was discovered that the originally isolated p53 was a mutant, that p53 was correctly characterized as a tumor suppressor gene. Loss of $p53^{wt}$ function is common in many human malignancies (~50%) (Caron de Fromentel and Soussi, 1992; Greenblatt et al., 1994) thus leading to a loss of its tumor suppressor properties. Loss of $p53^{wt}$ function occurs either via mutation or through an inhibition of its transcriptional activation (Vogelstein et al., 2000). In human BC, p53 mutations are less common (~20%) (Pharoah et al., 1999; Gasco et al., 2002). In tumors that retain $p53^{wt}$,

like BC, p53 inactivation occurs through the deregulation and over-expression of MDM2 (Oliner et al., 1992; Finlay, 1993). When MDM2 is over-expressed, it binds to and ubiquitinates $p53^{wt}$ leading to its degradation by the proteosome. MDM2 possesses activity of an E3 ubiquitin ligase allowing it to target p53 for degradation. This inhibits $p53^{wt}$ function compromising the genetic stability of the cell. Loss of $p53^{wt}$ function induces a cascade of molecular changes that include CA, CIN/aneuploidy (Fukasawa et al., 1996; Fukasawa et al., 1997; Carroll et al., 1999) and oncogenic transformation.

In addition to $p53^{wt}$ regulation, the expression of ERα has been shown to induce MDM2 transcription (Phelps et al., 2003). MDM2 mRNA and protein levels are increased in ERα^{+} BC tumors (Marchetti et al., 1995; Bueso-Ramos et al., 1996). Recently, a single nucleotide polymorphism, SNP309 T/G, was identified in the MDM2 promoter region that also binds ERα (Kinyamu and Archer, 2003; Bond et al., 2004). This SNP has been associated with attenuation of the p53 pathway and accelerated tumor formation in a gender-specific and hormone-dependent manner, suggesting that hormones, like estrogen, may alter oncogenesis (Bond et al., 2006).

Interrupting the p53-MDM2 interaction may presumably restore endogenous p53 function and be an important anti-cancer approach. Two small molecule inhibitors that interrupt this interaction have been reported, nutlins (Vassilev et al., 2004) and Reactivation of p53 and Induction of Tumor cell Apoptosis (RITA) (Issaeva et al., 2004). Nutlins bind to the p53-binding

site of MDM2 blocking the p53-MDM2 interaction. In contrast, RITA binds to p53 inhibiting the binding of MDM2 to p53 and its inactivation. Both molecules are effective as anti-tumor agents *in vitro.* RITA has also shown promising anti-tumor effects *in vivo* without significant adverse effects.

1.8 Centrosome Amplification, Chromosomal Instability and Aneuploidy in Human Breast Cancer

Genomic instability is one of the driving forces in the development and progression of malignant tumors. Two forms of genomic instability have been identified in cancer, microsatellite instability (MIN) and CIN (Boland et al., 1998). MIN refers to gene mutations involved in DNA synthesis and repair, uncommon in most cancers, that essentially give rise to diploid tumors whereas CIN, a common characteristic of malignancy, results from the deregulation of the centrosome cycle leading to abnormal chromosomal separation and aneuploidy.

Aneuploidy, the state of an altered chromosome number, is a consistent feature observed in nearly all types of cancer (Lengauer et al., 1998). Almost a century ago, Boveri suggested that aneuploidy in cancer cells may occur through defects in chromosome segregation machinery (Boveri, 1914). Boveri's suggestion was based on his sea urchin embryo studies that exhibited abnormal chromosome segregation following fertilization of an egg with two sperm. Boveri noticed similarities between the chromosome

abnormalities in sea urchin embryos and those reported in cancer, these similarities led to his hypothesis that malignant tumors arise through centrosome defects that result in improper cell division. A growing body of evidence now supports his hypothesis.

CIN, the rate of change in chromosome number, is the result of the deregulation of the normal centrosome cycle leading to CA. CA is defined as an increase in centrosome volume and number within a single cell, accumulation of excess pericentriolar material, increased microtubule nucleation capacity and inappropriate phosphorylation of centrosomal proteins (Lingle et al., 1998). A consequence of CA is the formation of multipolar mitotic spindles (Wang et al., 2004) leading to abnormal segregation of chromosomes and aneuploidy (Salisbury et al., 1999). CA occurs at a very early stage of cell transformation, most often resulting in mitotic catastrophe and eventual apoptosis. However, selection drives survival and proliferation of some of these cells that have acquired a growth advantage (Salisbury et al., 1999).

Centrosome duplication and DNA replication are usually tightly linked and essential to ensure equal chromosomal segregation during cell division (Sluder and Hinchcliffe, 2000). In cancer, these two events may be uncoupled leading to CA, increased frequency of multipolar mitotic spindles and CIN (Lingle et al., 1998; Pihan et al., 2001; D'Assoro et al., 2002). The Aurora kinases play a major role in centrosome separation and maturation. All three

human Aurora kinases are over-expressed in many human cancers where CA has been observed. Because of this, the expression of the Aurora kinases, more specifically AurA, has become the focus as to one possible cause of CA (Zhou et al., 1998).

In a study of human breast tumors, significant centrosome alterations, including supernumerary centrioles, excess pericentriolar material, disrupted centriole barrel structure, unincorporated microtubule complexes, centrioles of unusual length, centrioles functioning as ciliary basal bodies and mispositioned centrosomes, were observed (Lingle and Salisbury, 1999). AurA over-expression, CA, CIN and aneuploidy are defining features of human BC, as well as in E_2-induced ACI rat mammary tumor and Syrian hamster kidney tumor models. These traits of human BC have been detected in 55-78% of human DCISs and 85-92% of human IDBCs (Makris et al., 1997; Arnerlov et al., 2001; Li et al., 2002a). Similarly, CA, CIN and aneuploidy have been reported in 84% and 91% of E_2-induced rat mammary DCISs and primary mammary tumors (Li et al., 2002a) respectively, and in 92% of E_2/DES-induced hamster kidney tumors (Li et al., 2001). There is growing evidence that these characteristics occur early and therefore, may be important in neoplastic transformation rather than a consequence of tumor progression.

The G1/S and G2/M cell cycle checkpoints, that normally ensure the orderly progression of cell cycle events, can also be inactivated in cancer

(Hartwell and Weinert, 1989). In normal cells, DNA damage triggers checkpoint activation through the tumor suppressor p53 and up-regulation of its downstream targets. However, in some malignant tumors a p53 mutation has been correlated with the occurrence of CA (Weber et al., 1998; Carroll et al., 1999; Ouyang et al., 2001; Jeng et al., 2004; Zhu et al., 2005). In BC, tumors that express $p53^{wt}$, as well as CA, also over-express MDM2 which inactivates $p53^{wt}$ by promoting its degradation (Carroll et al., 1999). In addition, the phosphorylation of $p53^{wt}$ by AurA at serine 315 makes it more susceptible to ubiquitination by MDM2 and proteolysis (Katayama et al., 2004). Recently, it has been shown that AurA also phosphorylates $p53^{wt}$ at serine 215 which abrogates its function. This phosphorylation inhibits $p53^{wt}$ DNA binding and transcriptional activation leading to an AurA override causing cell cycle progression, survival and transformation (Liu et al., 2004).

CA and CIN also develops independently of p53 mutations suggesting the presence of other mechanisms leading to the disruption of chromosome segregation. The oncogenes E6 and E7 (Duensing et al., 2000), associated with human papillomavirus (HPV), and mutations in BRCA1 and BRCA2 (Deng, 2002), tumor suppressor genes associated with the development of familial breast and ovarian cancers, have been suggested as possible culprits as have cyclin E and c-myc. Deregulation of cyclin E affects processes involved in duplication and segregation of chromosomes leading to CIN (Spruck et al., 1999), and c-myc over-expression has been implicated in

oncogenesis by allowing accelerated passage through the G1/S checkpoint resulting in genomic instability (Felsher and Bishop, 1999).

It is interesting that the over-expression of AurA and $p53^{wt}$ loss of function result in a similar cascade of CA, CIN and aneuploidy. Understanding these two converging pathways that lead to oncogenic transformation may prove to be an important step in the study of BC.

Chapter 2: Statement of Purpose

2.1 Significance

BC is the number one diagnosed cancer in women in the United States. About 90-95% of all BCs are sporadic. It is evident that endogenous and exogenous estrogens play a crucial role in both BC causation and development (Bilimoria and Morrow, 1995). The role of estrogens in breast oncogenesis is beginning to be unraveled. Recently, AurA has been shown to be over-expressed in human BC (Tanaka et al., 1999). Aur kinases are part of a family of mitotic kinases that when aberrantly expressed may cause errors in mitosis that may result in the transformation of normal cells. The over-expression of AurA has been correlated with CA, CIN and aneuploidy, all of which are characteristics of virtually all human solid tumors examined.

Over the years, Li and Li have shown that E_2-induced oncogenesis in the male Syrian hamster kidney and the female ACI rat mammary gland are unique animal models that mimic the molecular/cellular events reported in some human cancers (Li et al., 2001; Li et al., 2004). In particular, the morphological changes observed in the E_2-induced mammary tumors of the ACI rat are remarkably similar to those seen in human BC. Therefore, these animal models are excellent tools to study the mechanisms involved in E_2-induced oncogenesis.

2.2 Overall Hypothesis

The main objectives of these studies represent an attempt to understand the molecular and cellular events involved in solely E_2-induced oncogenesis, using the E_2-induced tumors of the kidney in the male Syrian hamster or the mammary gland in the female ACI rat. The main focus is to determine the role of the mitotic kinases AurA and B and the $p53^{wt}$-MDM2 interaction, likely contributors of CIN through CA, in estrogen oncogenesis. **We hypothesize that E_2 treatment in castrated, male Syrian hamsters induces oncogenesis in the kidney resulting from the over-expression and increased activity of AurA and B. We propose that the over-expression of the Aur kinases leads to increased expression and deregulated phosphorylation of some of their centrosomal protein substrates, thus eliciting CA and CIN. We further propose that the loss of $p53^{wt}$, a key tumor suppressor protein, is due to the over-expression of MDM2 and that by blocking the binding of MDM2 with the inhibitor, RITA, $p53^{wt}$ function will be retained.** These studies will provide evidence to determine whether or not the over-expression of the Aur kinases is a crucial and early event controlling the integrity of chromosomal segregation and cytokinesis at cell division during E_2-driven oncogenesis, and whether the over-expression of MDM2 leads to the loss of $p53^{wt}$ function during E_2-induced oncogenesis. The ultimate goal of these studies is to identify early molecular and cellular changes during E_2-induced oncogenesis that will

provide new novel targets for the prevention and therapeutic intervention of human sporadic BC.

2.3 Specific Aims Purpose

2.3.1 Specific Aim 1. To determine the tissue localization, expression, activity and regulation of the mitotic kinases, AurA and B, in E_2-induced tumors in the Syrian hamster kidney.

The Aur kinases are essential for proper mitosis and thus, essential in maintaining genomic integrity. Over-expression of active AurA kinase has been shown to transform NIH3T3 cells, and yield tumor formation when implanted in nude mice, thus supporting the role of AurA as an oncoprotein (Bischoff et al., 1998; Zhou et al., 1998). AurA over-expression has been reported in a number of human solid tumors and reports indicate that it is over-expressed in 94% of human DCISs and in 94% of primary invasive breast neoplasms (Tanaka et al., 1999). While significantly less is known about AurB and its role in oncogenesis, it has been shown that the expression levels of both kinases appear to rise/decline in parallel (Keen and Taylor, 2004).

Previous studies in the Li laboratory have determined the localization and expression of AurA in E_2-induced ACI rat mammary oncogenesis (Li et al., 2004). These studies showed an increase of AurA expression in both E_2-treated mammary glands and -induced tumors as compared to untreated

mammary glands at both the mRNA and protein levels. Parallel samples showed CA and CIN, suggesting a causal link among estrogen exposure, AurA over-expression, CA, CIN and aneuploidy eventually leading to mammary oncogenesis.

The localization, level of expression, kinetic activity and regulation of AurA and B were evaluated in hamster kidneys from age-matched control cholesterol-treated animals, E_2-treated animals and -induced tumors in the hamster kidney. Tissue localization was determined by immunohistochemistry, and the levels of protein and mRNA expression by Western blot analysis and real-time PCR, respectively. The kinetic activity was studied using an *in vitro* kinase assay and the estrogen regulation by an E_2 removal/inhibition study. The data obtained from these studies provides data on the over-expression of AurA and B which have shown to be important early events in E_2-induced oncogenesis leading to CA.

2.3.2 Specific Aim 2. To determine the differential expression/phosphorylation of selected Aur kinase substrates that contribute to the deregulation of the centrosome cycle thus causing CA and CIN during E_2-induced oncogenesis.

Evidence indicates that AurA and B over-expression elicits CA leading to CIN and aneuploidy, all of these events are defining characteristics of human sporadic BC. It is likely that the over-expressed AurA and B

inappropriately phosphorylates centrosomal protein targets leading to CA and abnormalities in chromosome segregation and cytokinesis. AurA and B kinases are known to bind/phosphorylate a myriad of protein substrates, some of which have yet to be identified. It is hypothesized that when AurA and B are over-expressed, they may be inappropriately acting on one or more of their protein substrates to precipitate development of CA.

AurA and B are known to play an important role in regulating crucial events during mitosis. Based on these known roles of AurA and B, we proposed to determine the expression level and phosphorylation of four known Aur substrates, centrin, histone H3, protein phosphatase 1 (PP1) and targeting protein for Xklp2 (TPX2). The rationale for the use of these substrates is as follows: **Centrin:** It is a ubiquitous component of the centrosome essential for proper centriole and centrosome duplication (Salisbury et al., 2002). Recently, it has been identified as an AurA substrate that may play a role in the ability of AurA over-expression to cause CA (Lukasiewicz, 2007). ***Histone H3:*** Phosphorylation of histone H3 is required for proper chromosome condensation, and has been suggested to play a role in the interaction of the N-terminus of histone H3 and DNA (Van Hooser et al., 1998). This role is important in facilitating CA by inappropriate or excessive phosphorylation, required for cell cycle progression, in the presence of AurA and B over-expression. **PP1:** It is a Ser/Thr phosphatase that can dephosphorylate AurA leading to its inactivation (Satinover et al., 2004). In

addition, active AurA can phosphorylate PP1 reducing its phosphatase activity (Katayama et al., 2001). ***TPX2:*** It interacts with AurA as both an activator and a substrate. TPX2 is a microtubule-associated protein that stimulates AurA auto-phosphorylation at Thr288 and induces a change in AurA conformation. This conformation change protects AurA from inactivation from PP1 dephosphorylation. TPX2 is phosphorylated by AurA and then recruits AurA to the spindle microtubules (Kufer et al., 2002).

Most of the information on the centrosomal protein substrates of AurA and B originated from studies employing *Xenopus, Drosophila*, yeast, bacteria and tumor cell lines. Using the male Syrian hamster kidney and the female ACI rat mammary gland models, the studies proposed in this aim applied a molecular approach to characterize known centrosomal proteins that specifically interact, and are phosphorylated by AurA and B. Tissue localization was determined by immunohistochemistry, and the levels of protein expression by Western blot analysis. The estrogen regulation of some of these substrates was analyzed in an E_2 pellet removal/inhibition study. The elucidation of the expression of these centrosomal proteins and phosphorylation by AurA and B was considered essential to assess whether these proteins play a crucial role in E_2-induced oncogenesis.

2.3.3 Specific Aim 3. To determine whether $p53^{wt}$ function is lost due to the enhanced degradation induced by the over-expression of MDM2 during E_2-induced oncogenesis, thus enhancing conditions for CA, CIN and oncogenic transformation.

Loss of $p53^{wt}$, an important tumor suppressor, is known to facilitate CA, CIN (Fukasawa et al., 1996; Fukasawa et al., 1997) and oncogenic transformation. Loss of $p53^{wt}$ function is common in many human malignancies. Mutations of $p53^{wt}$ account for about 50% of the loss observed (Caron de Fromentel and Soussi, 1992; Greenblatt et al., 1994). Mutations in $p53^{wt}$ are relatively uncommon in BC (15-20%) (Pharoah et al., 1999; Gasco et al., 2002) suggesting that loss of $p53^{wt}$ function is occurring by other means during breast oncogenesis. Two mechanisms have been proposed: 1. MDM2 binds to $p53^{wt}$ resulting in its inactivation. 2. The over-expression of AurA leads to the phosphorylation of $p53^{wt}$ making it more susceptible to MDM2 degradation (Katayama et al., 2004).

MDM2 is an oncoprotein that acts as an E3 ubiquitin ligase targeting $p53^{wt}$ for destruction by proteosomes. Over-expressed MDM2 decreases $p53^{wt}$ levels and inhibits the DNA damage checkpoint and DNA repair. MDM2 is over-expressed in 73% of human BCs at both the mRNA and protein levels (Bueso-Ramos et al., 1996).

Interrupting the $p53^{wt}$-MDM2 interaction may presumably restore endogenous $p53^{wt}$ function and be an important anti-cancer approach. A

small molecule inhibitor that binds to $p53^{wt}$, RITA (Issaeva et al., 2004) was used to inhibit the binding of MDM2 to $p53^{wt}$. RITA has shown promising anti-tumor effects *in vitro* and *in vivo* without significant adverse effects.

Protein and mRNA expression and regulation of MDM2 and $p53^{wt}$ were evaluated in hamster kidneys and mammary glands from age-matched control cholesterol-treated animals and E_2-induced tumors. The levels of protein and mRNA expression were determined by Western blot analysis and real-time PCR, respectively. The estrogen regulation of MDM2 and $p53^{wt}$ was studied by an E_2 pellet removal/inhibition study. In addition, RITAs ability to restore $p53^{wt}$ function was assessed in hamsters bearing E_2-induced tumors in the kidney. The data obtained from these studies provides important information on the $p53^{wt}$-MDM2 interaction during E_2-induced breast oncogenesis.

Chapter 3: Materials and Methods

3.1 Animals and treatment

Adult castrated male Syrian golden hamsters (LAK:LVG), outbred strain, weighing 90-100 g were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). They were housed in facilities certified by the American Association for the Accreditation of Laboratory Animal Care, acclimated for at least one week prior to use, maintained on a 12-hr light:12-hr dark cycle, fed certified rodent chow (Ralston-Purina 5002) and tap water *ad libitum*. The animal studies were carried out in adherence to the guidelines established in the "*Guide for the Care and Use of Laboratory Animals*," (US Department of Health and Human Resources, NIH, 1985). Hamsters in the treatment groups were sc implanted with 20 mg E_2 pellets as described previously (Li et al., 1980). Age-matched control animals were implanted with 20 mg pellets of cholesterol. To maintain constant levels of E_2, new pellets were implanted every 3.0 months. Their mean daily absorption was 96 ± 4 μg/day. The pellets were prepared by Hormone Pellet Press (Shawnee Mission, KS). Over a 6.0 month period of E_2 treatment, the average E_2 concentration in serum was 2.28 ± 0.43 ng/mL, and in the kidney was 4.57 ± 1.04 pg/mg protein (Li et al., 1994).

For the withdrawal studies, E_2-treated hamsters for 6.0 months and bearing tumors were divided into four groups: 1) Maintained on E_2 treatment. 2) Withdrawn of both E_2 pellets. 3) Withdrawn of the initial E_2 pellet, while the second one remained and additionally implanted with 2 pellets of 20 mg

Tamoxifen citrate (Tx). 4) Control age-matched cholesterol-treated. All animals were killed ten days after removal of the E_2 pellets and the Tx treatment, tumors were individually harvested from groups 1-3 and kidneys from group 4 for subsequent analysis. The withdrawal/treatment period was selected because it has been previously shown that after a 72 h E_2-withdraw period, all estrogens are completely cleared from the circulation of E_2-treated animals (Li et al., 1994).

For the RITA studies, 6.0 month, E_2-treated hamsters bearing tumors were divided into three groups. Each group was injected ip daily for 15 days with: 1) 0.1 mL/100 g BW vehicle. 2) 0.75 mg RITA/100 g BW. 3) 1.0 mg RITA/100 g BW. All animals were killed on day 16. Tumors were harvested for subsequent analysis. The treatment period/dose was based on a previous *in vivo* mouse study in which 1.0 mg RITA/100 g BW resulted in a decreased tumor volume and the inhibition of MDM2-$p53^{wt}$ complex formation (Issaeva et al., 2004).

Intact female ACI rats were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN) at 6-8 weeks of age. They were housed in facilities certified by the American Association for the Accreditation of Laboratory Animal Care, acclimated for at least one week prior to use, maintained on a 12-hr light:12-hr dark cycle, fed Teklad Rodent Diet 8604 and tap water *ad libitum*. The animal studies were carried out in adherence to the guidelines established in the "*Guide for the Care and Use of Laboratory Animals*," (US

Department of Health and Human Resources, NIH, 1985). Rats in the treatment groups were implanted sc with 20 mg pellets containing 3 mg of E_2 plus 17 mg of cholesterol as previously described (Li et al., 2004). Age-matched control animals were implanted with 20 mg pellets of cholesterol. The pellets were prepared by Hormone Pellet Press (Shawnee Mission, KS). Over the treatment period, the average E_2 serum levels were 123.5 ± 4.8 pg/mL (4.0 months) and 121.8 ± 3.0 pg/mL (6.0 months) (Li et al., 2002b).

For the withdrawal studies, 6.0 month E_2-treated rats bearing tumors, were divided into four groups: 1) Maintained on E_2 treatment. 2) E_2 pellet treatment removed. 3) Maintained on E_2-treatment plus additional implantation of 2 pellets of 20 mg Tx. 4) Control age-matched cholesterol-treated. All animals were killed 8 days after the removal of the E_2 pellet and the Tx treatment. Tumors were individually harvested from groups 1-3 and mammary glands from group 4 for subsequent analysis. The withdrawal/treatment period was selected based on tumor regression studies performed by DiAugustine (unpublished data).

3.2 Western blot

Kidneys, mammary glands and tumors were homogenized in lysis buffer containing 50 mM Tris-HCl pH7.4, 0.2 M NaCl, 2 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.5 mM Na_3VO_4, 20 mM Na-pyrophosphate, 1 mM PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 mM DTT. The supernatant

fractions were collected and their protein content determined with BCA reagents (Pierce, Rockford, IL). Proteins were separated by gel electrophoresis on PAGEr Gold Precast Gels (Cambrex Bio Science Rockland, Inc, Rockland ME) or Pierce Precise Protein Gels (Pierce) and transferred to a nitrocellulose membrane (PVDF membrane for Centrin). The following primary antibodies were used: AurA BL656 (Bethyl Labs, Montgomery, TX, 1:1000), AurB ab2254 (Abcam, Cambridge, MA, 1:1000), Centrin MCI (Salisbury, JL, Mayo Clinic, Rochester, MN, 1:25000), γ-tubulin MMR58 (Salisbury, JL, 1:10000), Bax 2772 (Cell Signaling Technology, Danvers, MA, 1:1000), histone H3 (FL-136) sc-10809 (1:1000), PP1 (E9) sc-7482 (1:5000), TPX2 (H-300) sc-32863 (1:1000), MDM2 (SMP14) sc-965 (1:200), p53 (Pab 240) sc-99 (1:200) and p21 (M-19) sc-471 (1:200), all from Santa Cruz Biotechnology, Santa Cruz, CA. The membranes were incubated overnight at 4^{o}C with primary antibody followed by incubation with appropriate secondary antibodies for 2 hrs. Protein expression was visualized with ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ). Densitometry was done using Alpha Imager 2000 (Alpha Innotech, San Leandro, CA) or Northern Light model B 95 (Imaging Research Inc., Ontario, Canada) and Scion Image software (Scion Corporation, Frederick, MD).

3.3 *In vitro* protein kinase assay

Proteins were extracted as described for Western blotting. Aliquots, 200 μg of total protein extracts, were incubated for 2 hrs at room temperature with 1 μg of AurA BL469 (Bethyl Labs) followed by a 2 hr incubation with 30 μL Protein A/G PLUS-agarose sc-2003 (Santa Cruz Biotechnology). Normal IgG was used as a negative control. Immunoprecipitated complexes were washed several times with PBS and immediately used for kinase assays (Upstate, Inc., Charlottesville, VA) following the manufacturers standard protocol with slight modifications. Immunoprecipitates were incubated for 30 mins at 30^{o}C with 5X reaction buffer (40 mM MOPS, pH 7.0, 1 mM EDTA), 10 μCi/μL of [^{32}P]γATP (Amersham Biosciences) diluted in Upstate Mg/ATP cocktail. Reactions were spotted onto P81 paper, subjected to a series of washes in 0.75% phosphoric acid and acetone and counts per min measured using a scintillation counter (Beckman Coulter, Fullerton, CA).

3.4 Reverse transcription PCR and quantitative real-time PCR

Total RNA was extracted using the protocol supplied by GibcoBRL for Trizol Reagent (Invitrogen Corp., Carlsbad, CA). RNA integrity was evaluated by agarose gel electrophoresis and the RNA concentration determined by spectrometry.

Reverse transcription for AurA was performed using 5 μg of total RNA/5 μl. First strand synthesis was performed using the manufacturer's

protocol for M-MLV reverse transcriptase (Invitrogen Corp.). The reverse transcription reaction was performed using an iCycler thermocycler (Bio-Rad Laboratories, Hercules, CA). The thermocycling protocol consisted of 1 hr incubation at 37^{o}C followed by 10 min incubation at 96^{o}C. The samples were stored at 4^{o}C until used for PCR analysis.

Reverse transcription for MDM2 and p53^{wt} was performed using 5 µg of total RNA/5 µl using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The reaction was performed using an iCycler thermocycler (Bio-Rad Laboratories). The thermocycling protocol consisted of incubations for 10 mins at 25^{o}C, 120 mins at 37^{o}C followed by 5 secs at 85^{o}C. The samples were stored at 4^{o}C until used for PCR analysis.

The cDNA synthesized during the reverse transcription reaction was used to perform the real-time PCR reaction. For AurA, cDNA was amplified using the Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen Corp.) and gene specific primers for β-actin (FWD 5'-CAG CCG AGA GGG AAA TTG TG-3'; REV 5'-TCG TTG CCA ATG GTG ATG AC-3'; 101 bp amplicon) and AurA (FWD 5'-TGG GTG TGT GCC TCG AAA-3'; REV 5'-GAT TGA AGG CCG GAT GCA-3'; 102 bp amplicon) in a 7300 Real Time PCR System (Applied Biosystems). The thermocycling protocol consisted of incubations for 2 mins at 50^{o}C followed by 10 mins at 95^{o}C. Then, the samples underwent 40 cycles of 45 secs each at 95^{o}C, 60^{o}C and 72^{o}C. The specificity of the amplicon was verified by running a dissociation step.

For MDM2 and $p53^{wt}$, cDNA was amplified using Power Sybr® Green PCR master mix (Applied Biosystems) and gene specific primers for β-actin (FWD 5'-CAG CCG AGA GGG AAA TTG TG-3'; REV 5'-TCG TTG CCA ATG GTG ATG AC-3'; 101 bp amplicon), MDM2 (Syrian hamster kidney - FWD 5'-ACA GAT GTT GGA CTC TGC GTG AGA-3'; REV 5'-ATC TAA GCC TTC TGC CTC CAG CTT -3'; 101 bp amplicon) (ACI rat mammary gland - FWD 5'-AGA TGT GCC TGA TGG CAA AA-3'; REV 5'-AAG TCG ACG GCT GGG AAT AG-3'; 128 bp amplicon) and $p53^{wt}$ (Syrian hamster kidney - FWD 5'-GAA GGA AAT ATG CAT GCC GAA T-3'; REV 5'-CTC ATA GGG CAC CAC CAC ACT-3'; 72 bp amplicon) (ACI rat mammary gland - FWD 5'-CGA AAT CCT ATC CGG TCA GT-3'; REV 5'-TGA GGG CCC AAG ATA GAA TC-3'; 91 bp amplicon) in a 7900HT Real Time PCR System (Applied Biosystems). The thermocycling protocol consisted of 2 min incubation at 50°C followed by 10 min incubation at 95°C. Then, the samples underwent 40 cycles of 45 secs each at 95°C, 56°C and 72°C. The specificity of the amplicon was verified by running a dissociation step.

3.5 Immunohistochemical analysis

Kidneys were excised, trimmed and fixed in 5% paraformaldehyde, followed by a rapid paraffin-embedding process. Tissue sections (6 μm) were prepared and dewaxed. Antigens were retrieved (Dako Target Retrieval Solution, Dako, Carpinteria, CA) by heating in a water bath set at 97°C for 20

mins or a digital decloaking chamber (Biocare Medical, Concord, CA) and treated with 3% H_2O_2 for 15 mins to block endogenous peroxidases. After blocking with 6% of the appropriate serum in 1% bovine serum albumin, the primary antibodies, AurA BL656 (Bethyl Labs, 1:1000), AurB ab2254 (Abcam, 1:50) and TPX2 (H-300) sc-32863 (Santa Cruz Biotechnology, 1:40), were applied to the sections overnight at 4^{o}C. Appropriate secondary antibodies were incubated for 1 hr at 25^{o}C followed by 1 hr with Vector Laboratories Elite ABC (Burlingame, CA). As negative controls, similar tissue sections were incubated replacing the primary antibodies with the appropriate normal serum. The slides were counterstained with hematoxylin, dehydrated in alcohol and mounted in Permount medium (1:1 Permount:Xylene) before being examined under the microscope.

3.6 Enrichment of amplified tumor centrosomes

The isolation of amplified E_2-induced tumor centrosomes was performed according to Moudjou and Bornens (Moudjou, 1994) with minor modifications. Primary tumors (8-10 g) from the kidney were minced and brought to 50 mL in culture medium containing 10 µl cytochalasin, 10 µl nocodazole and 50 µl protease inhibitor cocktail, all obtained from Sigma Aldrich (St. Louis, MO). The minced tumor was incubated for 30 mins at 4^{o}C, washed and then resuspended in 0.1X TBS/8% sucrose containing 2 µl cytochalasin, 2 µl nocodazole and 10 µl protease inhibitor cocktail and

pressed through a stainless steel sieve and filtered through a 105 micron nylon mesh monofilament cloth (Small Parts, Inc., Miami Lakes, FL). The resulting cell suspension was lysed in a buffer containing 1 mM Tris-HCl pH 8.0, 0.1% 2-mercaptoethanol, 0.5% Triton X-100 and 1X protease inhibitors, homogenized in a glass dounce and centrifuged at 2,500 x g for 10 mins. The lysate was placed in a 50 mL tube, under-laid with 60% sucrose and centrifuged at 10,000 x g for 30 mins. The upper 25 mL fraction was carefully aspirated, and the remaining sample was vortexed, over-laid onto a vegetable-dyed sucrose gradient (70%, 50% and 40% sucrose) and centrifuged at 40,000 x g for 1 hr. The gradient was fractionated [Fraction Collector and Econo Pump (Bio-Rad Laboratories) Fractionation System (Brandel, Gaithersburg, MD)] into 24 0.5 mL portions. The fraction volumes were brought to 1.5 mL with gradient buffer (10 mM PIPES pH 7.2, 0.1% Triton X-100 and 0.1% 2-mercaptoethanol), vortexed and centrifuged at 50,000 x g for 1 hr. The supernatant was discarded and the pelleted fractions were stored at -80^{o}C for further analysis by Western blotting.

3.7 Immunofluorescence analysis

Kidneys were excised and frozen in Tissue-Tek Cryo-OCT Compound (Andwin Scientific, Warner Cen, CA). Slides were fixed in absolute methanol for 10 mins at -20^{o}C, blocked in 5% normal goat serum, 1% glycerol, 0.1% BSA, 0.1% fish skin gelatin, 0.1% triton X-100 and 0.4% sodium azide (10%

stock) and incubated with primary antibodies. The following primary antibodies Centrin 20H5 (Salisbury, JL, 1:1000), γ-tubulin (Sigma Aldrich, 1:1000) and AurA (H-130) sc-25425 (Santz Cruz Biotechnology, 1:40) were applied to serial sections and incubated overnight at 25^{o}C. Appropriate secondary antibodies conjugated with Alexa fluor 488 or 568 (Molecular Probes, Eugene, OR) were incubated for 1 hr at 25^{o}C followed by a PBS wash. The slides were mounted with Pro Long Gold antifade reagent w/ DAPI (Invitrogen Corp.) and digital images recorded using a Zeiss Axiovert fluorescence microscope.

3.8 Co-Immunoprecipitation

Proteins were extracted as described for Western blotting. Aliquots, 500 μg total protein, were incubated with primary antibody, p53 (PAb 240) ab26 (Abcam Inc.) at 4^{o}C overnight. 50 μL of ImmunoPure Immobilized A/G beads (Pierce) were added to the proteins and incubated for an additional hr at 4^{o}C. Immune complexes were washed 3 times in immunoprecipitation buffer A containing 190 mM NaCl, 50 mM Tris-HCl pH7.4, 6 mM EDTA and 2.5% Triton X-100 and then 3 times in immunoprecipitation buffer B containing 150 mM NaCl, 10 mM Tris-HCl pH8.0, 5 mM EDTA and 0.1% Triton X-100. Proteins were released from immune complexes with 2-mercaptoethanol at 99^{o}C for 5 mins, separated by gel electrophoresis on Pierce Precise Protein Gels (Pierce) and transferred to nitrocellulose

membranes. Primary antibody against MDM2 (SMP14) sc-965 (Santa Cruz Biotechnology, 1:200) was incubated overnight at 4^{o}C. Appropriate secondary antibody was incubated for 2 hrs and protein expression was visualized with ECL chemiluminescence (Amersham Biosciences).

3.9 Statistical Analyses

One way analysis of variance (ANOVA) with Tukey post-hoc tests were used for statistical evaluation, with the exception of the AurA kinase activity that was analyzed using student *t* test. Values were expressed as the mean ± SE. Statistical significance was assumed when $p \leq 0.05$.

Chapter 4: Experimental Results

4.1 Over-expression of AurA and B and CA in early E_2-induced tumor foci of the Syrian hamster kidney

4.1.1. AurA and B expression in early tumorous foci

In order to assess the cellular location of AurA and B expression after various E_2-treatment intervals (3.5-6.0 months) in early tumor foci, serial kidney sections were examined by H&E staining (Figure 7A) and immunohistochemistry. Analysis of whole kidney serial sections of 3.5-month E_2-treated hamsters established that the expression of both AurA (Figure 7B) and B (Figure 7C) was confined essentially to cells present in early tumorous foci. AurA or B positive stained cells were not present in similar tumor foci sections in the absence of either primary antibody (Figure 7D). Positive stained AurA (Figure 7E) and B (Figure 7F) cells were also present in large well-established tumor foci derived from 6.0-month E_2-treated hamsters, but not in adjacent non-involved epithelial kidney cells.

4.1.2. Western blot analysis of Aur A and B in control and E_2-treated kidneys and E_2-induced tumors of the kidney

To determine the protein expression levels of AurA and B kinases, Western blot analysis of lysates from whole hamster kidney samples after 3.0-, 4.0-, 5.0- and 6.0-months and primary tumors after 6.0-months of E_2 treatment was performed (Figure 8A). Only modest increases in AurA protein

were seen in early E_2-treatment periods; however, a significant 8.7-fold rise was detected in primary tumors of the kidney (Figure 8B) when compared to control cholesterol-treated kidneys. A slower migrating AurA band, presumably the phosphorylated form of AurA, was observed only in E_2-induced tumors of the kidney. No detectable changes in AurB protein expression (Figure 8A) were observed after 3.0-5.0-months of E_2 treatment. A slight rise in AurB expression was detected in 6.0-month E_2-treated kidneys, and a significant 4.6-fold increase in primary E_2-induced tumors of the kidney compared to age-matched cholesterol-treated control kidneys (Figure 8B).

4.1.3. AurA mRNA expression in E_2-induced tumors

To determine whether the increased protein levels of AurA expression take place concurrently with similar increases in mRNA, real-time PCR analysis was performed in Syrian hamster E_2-induced tumors of the kidney and those of 3.0- and 5.0-month E_2-treated kidneys. A significant 6.0-fold increase in mRNA expression was observed in primary tumors of the kidney compared to age-matched cholesterol-treated control kidneys (Figure 9).

4.1.4. AurA activity in E_2-induced tumors of the kidney

To determine whether the over-expressed AurA protein in the E_2-induced tumors of the kidney was functionally active, a kinase assay was

performed using the synthetic construct, kemptide, as a substrate. The over-expression of AurA led to a concomitant 2.6-fold increase in its kinase activity in E_2-induced hamster tumors of the kidney relative to control cholesterol-treated kidneys (Figure 10).

4.1.5. Estrogen modulation of the expression of the Aur kinases in E_2-induced tumors of the kidney

In order to determine whether the protein expression of AurA and B was regulated by estrogen, Western blot analysis was performed in tumor samples from groups of 6.0-month tumor bearing animals continuously E_2-treated, after their E_2 pellets were withdrawn or concomitantly treated with Tx for 10 days. The withdrawal/treatment period was selected because it has been previously shown that after a 72 h E_2-withdraw period, all estrogens are completely cleared from the circulation of E_2-treated animals (Li et al., 1994). Compared to age-matched control cholesterol-treated kidneys, an 8.0-fold rise in AurA expression was detected in all tumors receiving sustained E_2 treatment (Figure 11B). When compared to tumor-bearing hamsters continuously treated with E_2, a 78% and a 79% reduction in AurA expression was observed after either a 10-day E_2-withdrawal period or after E_2 + Tx treatment, respectively. Likewise, E_2-elicited AurB over-expression markedly declined after either E_2-withdrawal (81%) or Tx (64%) co-administration. Cell proliferation, assessed by Ki-67 labeling, was not significantly altered in any

of the tumor groups (data not shown). The significant decline in AurA and B expression observed upon E_2 withdrawal and E_2 + Tx treatment, did not result in a concomitant reduction in the number/volume of amplified centrosomes.

4.1.6. Western blot analysis of centrosome proteins in control and E_2-treated kidneys and E_2-induced tumors of the kidney

Since centrin and γ-tubulin are ubiquitous centrosome protein components and markers associated with CA, their protein expression was determined in lysates of individual E_2-treated hamster kidneys after 3.0-, 4.0-, 5.0- and 6.0-months, and in primary tumors of the kidney after 6.0-months of treatment (Figure 12A). When compared to control cholesterol-treated hamster kidney samples, the centrin Western blot analysis of E_2-induced tumor samples shows a clear shift in the centrin band from ~16 to 20 kDa. The upper band (~20 kDa), largely present in the tumors, may represent the phosphorylated form of centrin. The level of centrin expression was significantly increased (5.7-fold) in the tumor samples when compared to that observed in control cholesterol-treated animals (Figure 12B). The expression of γ-tubulin was nearly absent in the kidneys of both control and E_2-treated animals, but showed a marked increase in expression, 70.0-fold, in tumor samples. A doublet was consistently observed in these samples (Figure 12B).

4.1.7. CA in E_2-induced tumorous foci of the Syrian hamster kidney

In view of the fact that centrin and γ-tubulin are markers of centrosome number and volume, their expression was monitored in E_2-induced tumor foci from 6.0-month E_2-treated hamsters. Initially, the foci were detected in kidney serial sections by H&E staining (Figure 13A), and later, the amplified centrosomes were visualized by their positive staining for centrin and γ-tubulin (Figure 13B-C). AurA was co-localized to the amplified centrosomes in the tumor focus (Figure 13C). The pattern of immunofluorescence staining of the centrosomal proteins in control cholesterol-treated kidneys (data not shown) and in 6.0-month E_2-treated adjacent uninvolved kidney cells (Figure 13D) was confined to the pair of centrioles apical to the nucleus.

4.1.8. Localization of AurA and other centrosome proteins in isolated amplified centrosomes from E_2-induced tumors of the kidney

To determine whether AurA expression occurs concomitantly with CA, enriched amplified centrosome preparations of E_2-induced tumors of the kidney were examined in 6.0- to 8.0-month E_2-treated hamsters employing a discontinuous sucrose gradient fractionated into 24 aliquots (Figure 14). Western blot analysis of the aliquots established that the centrosome proteins, centrin and γ-tubulin, were expressed at high levels in fractions 8-

10. Both proteins consistently exhibited their highest level of expression in fraction 9, while the intensity of their expression in other fractions was variable and for the most part significantly reduced. Notably, the expression of AurA was limited to fraction 9 confirming its distinctive association with the amplified tumor centrosome fraction. On the other hand, AurB expression was spread throughout fractions 6 to 10 (data not shown), expected of a mobile passenger protein.

4.2 Expression of selected Aur kinase substrates in E_2-induced tumor foci of the Syrian hamster kidney and the ACI rat mammary gland

4.2.1. Tissue localization of Aur substrates in early E_2-induced tumor foci of the kidney

Although over 25 Aur kinase substrates have been described (Meraldi et al., 2004; Li and Li, 2006; Lukasiewicz, 2007), only a few commercially available antibodies cross react with the hamster kidney or the rat mammary gland. Given such limitations, centrin, histone H3, PP1 and TPX2 were selected to determine whether their pattern of expression follows that of AurA/B. Their cellular localization was assessed in serial kidney sections containing early tumor foci from animals treated with E_2 at different time intervals. Sections were examined first by H&E (data not shown) and later by

immunohistochemistry (Figure 15A-D). Similarly to AurA, TPX2 expression was overwhelmingly confined to E_2-induced tumor foci cells. Its level of expression increased along with the size of the tumor foci (Figure 15A-C). However, cells contained in E_2-induced tumor foci, did not stain for centrin, histone H3 or PP1, irrespectively of the size of the foci or the length of E_2 treatment.

4.2.2. Western blot analysis of Aur substrates in control and E_2-induced tumors of the kidney

To determine whether the 8.7-fold increase in AurA protein expression observed in E_2-induced tumors of the kidney was in tandem with similar increases in the expression of some of its substrates, the protein level of expression of non-phosphorylated and phosphorylated centrin and histone H3, as well as, PP1 and TPX2, was analyzed by Western blot analysis from control cholesterol-treated kidneys and primary tumors after 6.0-months of E_2 treatment (Figure 16A). The expression of centrin, histone H3, PP1 and TPX2 was increased by 9.4, 11.5, 2.4 and 2.9-fold (Figure 16B), respectively, in primary E_2-induced tumors when compared to control cholesterol-treated kidneys, while phospho-centrin and -histone H3 expression did not.

4.2.3. Estrogen modulation of the expression of Aur substrates non- and phosphorylated in E_2-induced tumors of the kidney

To determine whether the protein expression of non-phosphorylated centrin and histone H3, and their phosphorylated isoforms were regulated by estrogen, Western blot analysis was performed in tumor samples from groups of 6.0-month tumor-bearing animals that were continuously treated with E_2, their E_2 pellets withdrawn or concomitantly treated with Tx for 10 days (Figure 17A). The expression of centrin significantly decreased 80% upon concomitant Tx + E_2 treatment (Figure 17B). No significant changes in the protein expression of histone H3 and the phosphorylated forms of centrin and histone H3 were observed after estrogen modulation.

4.2.4. Western blot analysis of Aur substrates in control and E_2-induced tumors of the mammary gland

Similarly, to determine the protein level of expression of non- and phosphorylated forms of centrin and histone H3, as well as, PP1 and TPX2, control cholesterol-treated mammary glands and primary tumors after 6.0-months of E_2 treatment were subjected to Western blot analysis (Figure 18A). Expression of centrin, histone H3, PP1 and TPX2 was significantly increased by 2.9, 15.3, 1.8 and 2.1-fold (Figure 18B), respectively, in E_2-induced mammary tumors when compared to control cholesterol-treated mammary

glands. No significant changes in the protein expression of phospho-centrin and -histone H3 were observed.

4.2.5. Estrogen modulation of the expression of Aur substrates non- and phosphorylated in E_2-induced tumors of the mammary gland

In order to determine whether the protein expression of non- and phosphorylated forms of centrin and histone H3 were regulated by estrogen, Western blot analysis was performed in tumor samples from groups of 6.0-month tumor-bearing animals continuously treated with E_2, as well as in animals in which their E_2 pellets were withdrawn or concomitantly treated with Tx for 8 days (Figure 19A). The expression of centrin significantly decreased upon E_2 withdrawal or concomitant Tx + E_2 treatment, 73% and 61%, respectively (Figure 19B). No significant changes in the protein expression of histone H3 and the phosphorylated forms of centrin and histone H3 were observed after estrogen modulation.

4.2.6. Localization of Aur substrates and other centrosome proteins in isolated amplified centrosomes from E_2-induced tumors of the kidney and mammary gland

To determine whether phospho-centrin and -histone H3 expression occurs in parallel to CA, enriched amplified centrosome preparations of E_2-

induced tumors of the kidney and mammary gland were examined. The enriched centrosome fractions were obtained from 6.0- to 8.0-month treated hamsters (Figure 14) and from rats (data not shown) as previously described in section 4.1.8. Western blot analysis of the 24 fractions demonstrated that the expression of the phosphorylated forms of centrin and histone H3 were not associated with the amplified tumor centrosome fraction in either the E_2-induced tumors of the kidney or those of the mammary gland.

4.3 MDM2 expression and regulation in E_2-induced tumor foci of the Syrian hamster kidney and the ACI rat mammary gland

4.3.1. Western blot analysis of MDM2 and p53 in control and E_2-induced tumors of the kidney

To determine the MDM2 and p53 protein level of expression, Western blot analysis of lysates from control cholesterol-treated kidneys and primary tumors after 6.0-months of E_2 treatment was performed (Figure 20A). A significant 8.8-fold increase in MDM2 expression was observed in primary E_2-induced tumors when compared to control cholesterol-treated kidneys (Figure 20B). Similarly, a small, significant, 2.9-fold increase in p53 expression was observed in primary tumors of the kidney compared to control cholesterol-treated kidneys (Figure 20B).

4.3.2. MDM2 and p53^{wt} mRNA expression in E_2-induced tumors of the kidney

To determine whether E_2-treatment affects the level of MDM2 and p53^{wt} mRNA expression of Syrian hamster kidneys treated with E_2 (3.0- and 5.0-months) and in E_2-induced tumors of the kidney, real-time PCR analysis was performed. No significant changes were observed in the levels of MDM2 and p53^{wt} mRNA expression in hamster kidneys treated with E_2, however, in primary E_2-induced tumors of the kidney, MDM2 mRNA expression exhibited a significant 3.5-fold increase, while p53^{wt} levels were unchanged when compared to age-matched cholesterol-treated control kidneys (Figure 21).

4.3.3. Estrogen modulation of MDM2 and p53 expression in E_2-induced tumors of the kidney

In order to determine whether the protein expression of MDM2 and p53 was regulated by estrogen, Western blot analysis was performed in tumor samples from groups of 6.0-month tumor bearing animals that were continuously E_2-treated, and in similar animals in which their E_2 pellets were withdrawn or concomitantly treated with Tx for 10 days (Figure 22A). Compared to age-matched control cholesterol-treated kidneys, a 13.3-fold rise in MDM2 expression was detected in all tumors receiving sustained E_2 treatment (Figure 22B). Upon a 10-day E_2-withdrawal period, MDM2

expression was significantly reduced by 48% compared to that of tumors continuously treated with E_2. Similarly, Tx co-administration resulted in a significant 47% decline in MDM2 expression in these tumors. In contrast, no change in p53 protein expression was observed after E_2 pellet withdrawal or concomitant Tx treatment.

4.3.4. Co-immunoprecipitation of MDM2 and $p53^{wt}$ in E_2-induced tumors of the kidney

To determine whether $p53^{wt}$ binds to MDM2 *in vivo*, a co-immunoprecipitation study was performed. $p53^{wt}$ was immunoprecipitated from control cholesterol-treated kidneys and primary E_2-induced kidney tumors and subjected to MDM2 Western blot analysis (Figure 23). A significant binding of $p53^{wt}$ to MDM2 was observed primarily in E_2-induced tumors.

4.3.5. RITA modulation of MDM2 and $p53^{wt}$ protein expression in E_2-treated kidneys and E_2-induced tumors of the kidney

In order to determine whether the MDM2-$p53^{wt}$ interaction was disrupted by RITA, Western blot analysis of MDM2 and $p53^{wt}$ was performed in control cholesterol-treated kidneys and in tumor samples from groups of 6.0-month tumor bearing animals that were continuously treated with E_2 or

concomitantly i.p. treated with 0.75 mg or 1.0 mg RITA for 15-days. (Figure 24A). Compared to age-matched control cholesterol-treated kidneys, an 11.7-fold increase in MDM2 expression was detected in all tumors receiving sustained E_2 treatment (Figure 24B). Upon concomitant RITA treatment, MDM2 expression was significantly increased by 13% compared to that of tumors continuously treated with E_2. No significant increase in $p53^{wt}$ expression was detected in tumors receiving sustained E_2 treatment. However, $p53^{wt}$ expression was significantly increased 64% after concomitant treatment with RITA compared to that of tumors continuously treated with E_2 alone.

4.3.6. RITA modulation of Bax and p21 protein expression in the kidney

In order to determine whether the protein expression of $p53^{wt}$ downstream targets was increased after the disruption of the MDM2-$p53^{wt}$ interaction by RITA, Western blot analysis of Bax and p21 was performed in control cholesterol-treated kidneys and in tumor samples from groups of 6.0-month tumor bearing animals that were continuously treated with E_2 or concomitantly treated with 0.75 mg or 1.0 mg RITA for 15-days (Figure 25A). Compared to age-matched control cholesterol-treated kidneys, a 3.4-fold increase in Bax expression was detected in all tumors receiving sustained E_2 treatment (Figure 25B). Upon concomitant RITA treatment, Bax expression

was significantly increased by 60% compared to that of tumors continuously treated with E_2. No significant increase in p21 expression was detected in tumors receiving sustained E_2 treatment or upon concomitant RITA treatment (Figure 25B).

4.3.7. Western blot analysis of MDM2 and p53 in control and E_2-induced tumors of the mammary gland

To determine the protein level of expression of MDM2 and p53, Western blot analysis of lysates from control cholesterol-treated mammary glands and primary tumors after 6.0-months of E_2 treatment was performed (Figure 26A). A significant 7.7-fold increase in MDM2 expression was observed in primary tumors compared to control cholesterol-treated mammary glands (Figure 26B), while no significant changes in p53 protein expression were observed.

4.3.8. MDM2 and $p53^{wt}$ mRNA expression in E_2-induced tumors of the mammary gland

Real-time PCR analysis was performed to determine the MDM2 and $p53^{wt}$ mRNA expression of ACI rat E_2-induced tumors of the mammary gland and mammary glands treated with E_2 for 3.0- and 5.0-months. MDM2 mRNA showed a significant 4.1-fold increase in primary tumors of the mammary

gland compared to age-matched cholesterol-treated control mammary glands (Figure 27). No significant changes in $p53^{wt}$ mRNA expression were observed in primary E_2-induced tumors of the mammary gland.

4.3.9. Estrogen modulation of the expression of MDM2 and p53 in E_2-induced tumors of the mammary gland

In order to determine whether the protein expression of MDM2 and p53 is regulated by estrogen, Western blot analysis was performed in tumor samples from groups of 6.0-month tumor bearing animals that were either continuously E_2-treated or had their E_2 pellet withdrawn or concomitantly treated with Tx for 8 days (Figure 28A). Compared to age-matched control cholesterol-treated mammary glands, a 6.8-fold rise in MDM2 expression was detected in all tumors receiving sustained E_2 treatment (Figure 28B). Upon an 8-day E_2-withdrawal period, MDM2 expression was significantly reduced by 66% compared to that of tumors continuously treated with E_2. Similarly, Tx co-administration resulted in a significant 46% decline in MDM2 expression in these tumors. In contrast, no change in p53 protein expression was observed after E_2 pellet withdrawal or concomitant Tx treatment.

4.3.10. Co-immunoprecipitation of MDM2 and $p53^{wt}$ in E_2-induced tumors of the mammary gland

A co-immunoprecipitation study was performed to determine whether $p53^{wt}$ binds to MDM2 *in vivo*. $p53^{wt}$ was immunoprecipitated from control cholesterol-treated mammary glands and primary E_2-induced mammary gland tumors and subjected to MDM2 Western blot analysis (Figure 29). As in the E_2-induced tumors of the kidney, $p53^{wt}$ binds to MDM2 in E_2-induced mammary tumors.

Figure 7. Immunostaining of AurA and B in Syrian hamster E_2-induced early tumor foci of the kidney

Representative kidney serial sections from 3.5-month treated hamster kidneys: A. H&E staining showing a small tumor focus. The cells present in the tumor foci were AurA (B) and B (C) positive. D. A negative control kidney serial section without primary antibodies. Representative kidney serial sections from 6-month treated hamster kidneys: AurA (E) and B (F) positive stained cells were confined to the E_2-induced tumor foci. Magnification: 40X.

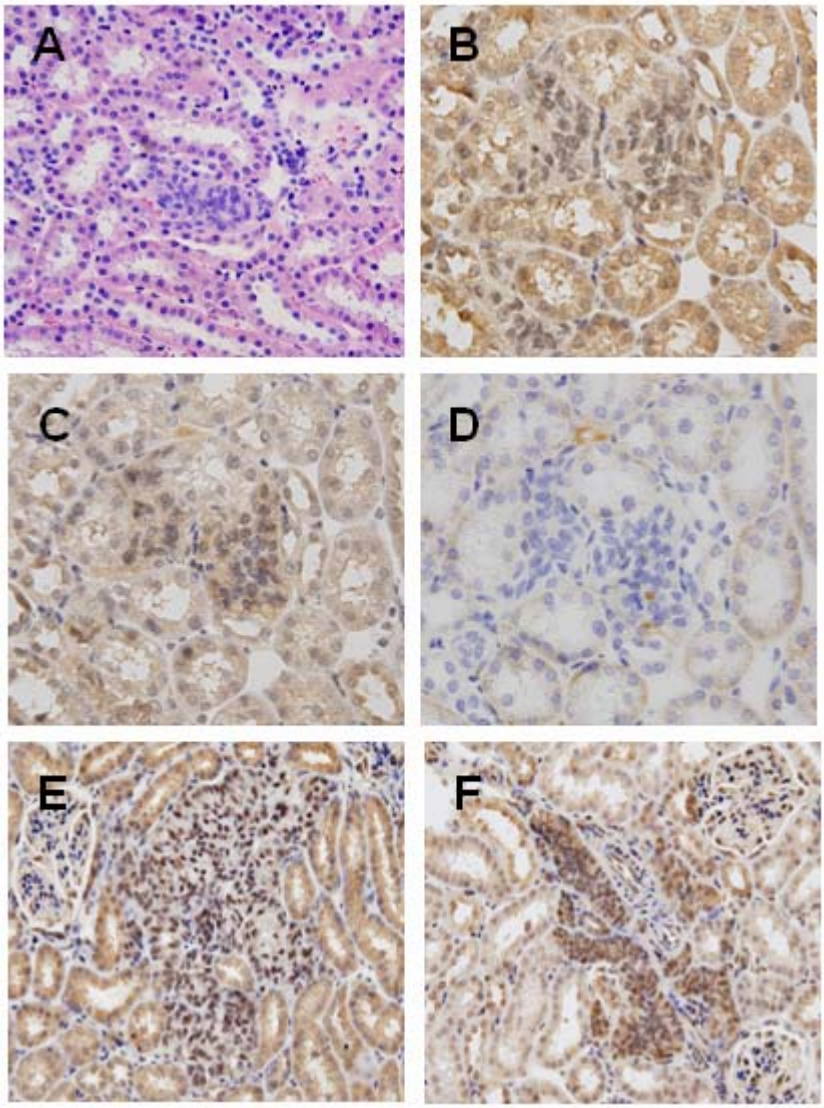

A
B
C
D
E
F

Figure 8. Effect of estrogen treatment on the protein expression of AurA and B in the Syrian hamster kidney

A. No significant changes in the expression of AurA were observed in either cholesterol-treated control (C) or in E_2-treated kidney samples after 3 (E3), 4 (E4) and 5-months (E5) E_2-treatment. After 6 months of E_2 treatment (E6), there was a slight increase in AurA expression followed by a marked rise in E_2-induced tumors (T). A doublet was present in the tumor samples which may represent the native and phosphorylated forms of AurA. AurB expression was detected only in T samples. GAPDH was used as a loading control. B. In T samples, the expression of AurA and B were significantly increased, 8.7- and 4.6-fold, respectively, as compared to C kidney samples. Data represent the mean ± SE, n=6. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, ** $p < 0.001$ vs C.

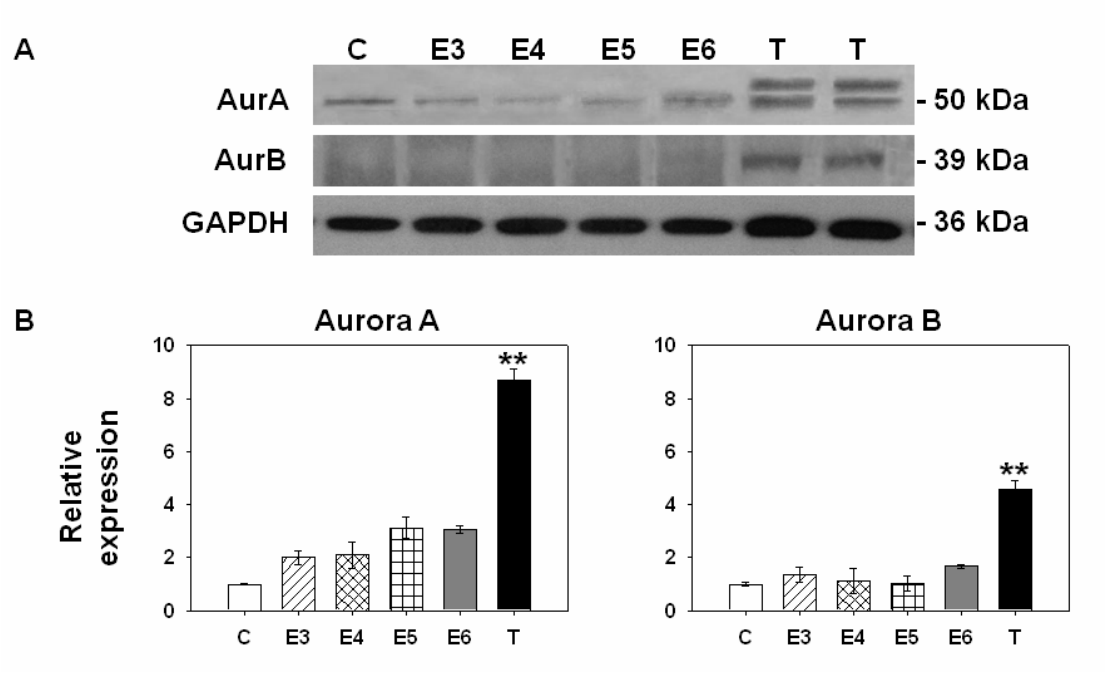

A
C
E3
E4
E5
E6
T
T
AurA
- 50 kDa
AurB
- 39 kDa
GAPDH
- 36 kDa
B
Aurora A
Aurora B
Relative expression
10
8
6
4
2
0
**
C
E3
E4
E5
E6
T

Figure 9. Effect of estrogen treatment in the mRNA expression of AurA by real-time PCR in the Syrian hamster kidney

AurA mRNA levels were analyzed by real-time PCR from Syrian hamster kidneys treated with cholesterol (C), E_2 for 3 (E3) or 5 (E5) months and E_2-induced kidney tumors (T). A significant 6.0-fold increase was detected in T samples compared to C samples. Data represent the mean ± SE, n=6. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, ** $p < 0.001$ vs C, E3 and E5.

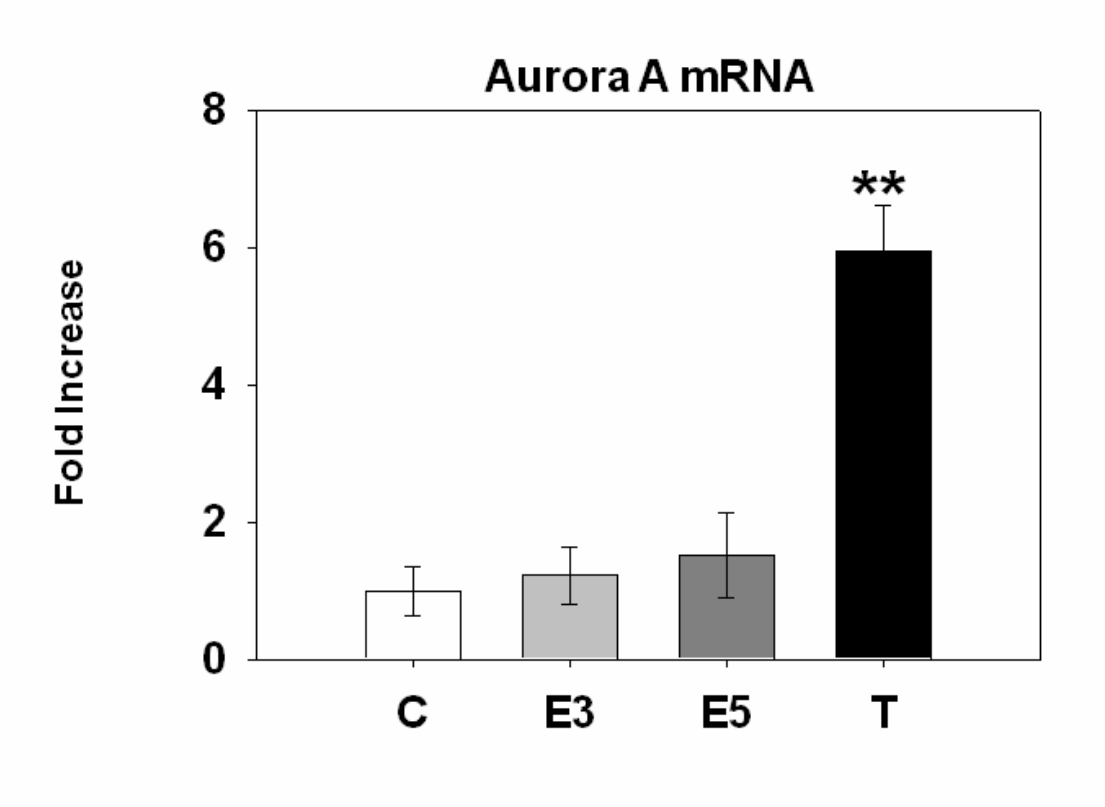

Aurora A mRNA
Fold Increase
8
6
4
2
0
**
C
E3
E5
T

Figure 10. Effect of estrogen treatment on AurA kinase activity in the Syrian hamster kidney and in E_2-induced tumors of the kidney

A significant 2.6-fold increase in AurA kinase activity was observed in E_2-induced hamster tumors of the kidney (T) when compared to cholesterol-treated control (C) hamster kidneys, t-test, * $p < 0.05$ vs C, n=6.

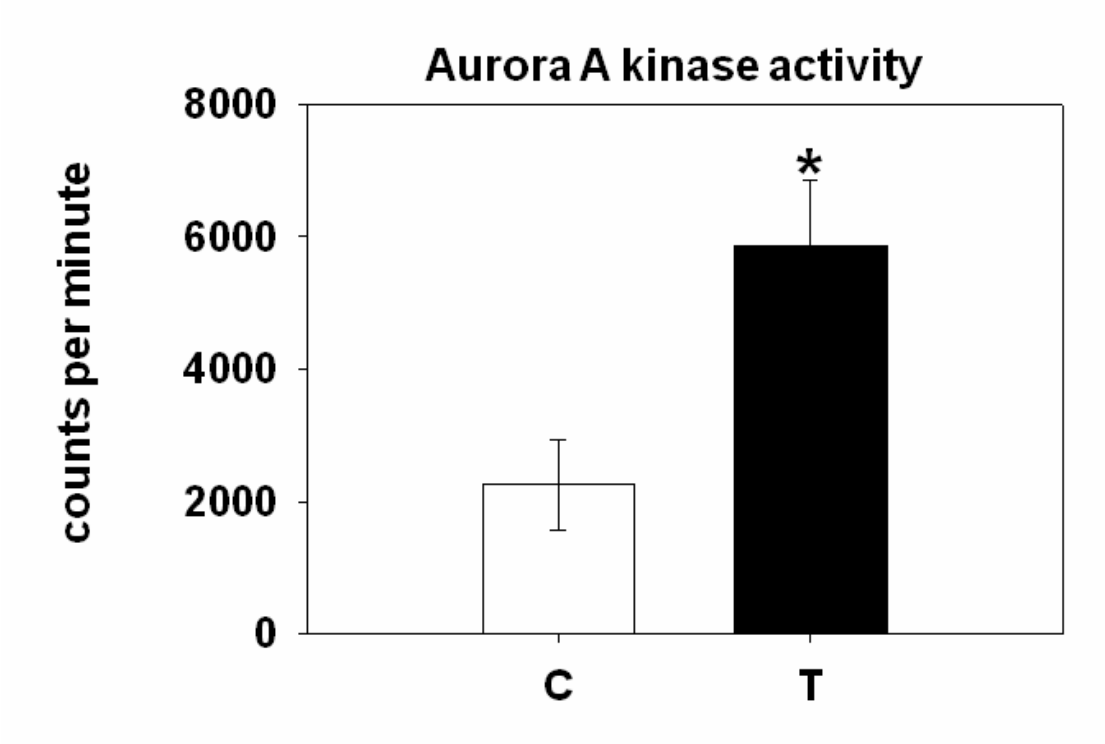

Aurora A kinase activity
counts per minute
8000
6000
4000
2000
0
*
C
T

Figure 11. Estrogen modulation of Aur kinase protein expression in the Syrian hamster E_2-induced tumors of the kidney

A. A significant decline in AurA and B expression was observed after the E_2 10-day withdrawal period (T-Eout), 78 and 81% respectively, and after concomitant treatment with Tx (T-tam), 79 and 64% respectively (B). GAPDH was used as a loading control. Data represent the mean ± SE, n=3/group. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, ** $p < 0.001$ vs C, ♠ $p < 0.05$ vs T.

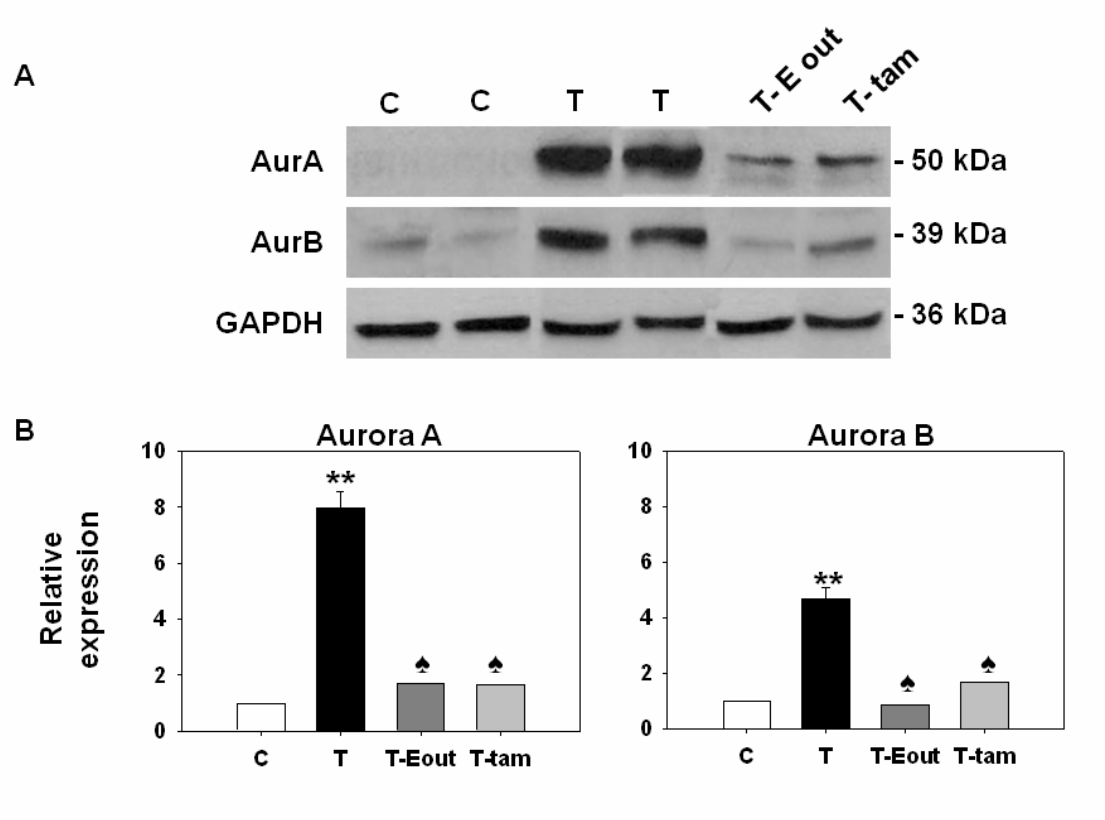

A
C
C
T
T
T-E out
T-tam
AurA
AurB
GAPDH
- 50 kDa
- 39 kDa
- 36 kDa
B
Aurora A
Aurora B
Relative expression
10
8
6
4
2
0
**
♠
C
T
T-Eout
T-tam

Figure 12. Effect of estrogen treatment in the protein expression of centrin and γ-tubulin in the Syrian hamster kidney and in E_2-induced tumors of the kidney

A. A clear shift in the centrin band was observed among control cholesterol-treated (C) and E_2-treated kidneys for 3 (E3), 4 (E4), 5 (E5) or 6 (E6) months and in E_2-induced tumor samples (T). The tumor upper band present may represent the phosphorylated form of centrin. γ-tubulin expression was very low in both C and in E_2-treated animals, but showed a marked increase in expression in the T samples. A doublet was consistently observed in these samples. GAPDH was used as a loading control. B. T samples showed a significant 5.7-fold increase in centrin and a 70.0-fold increase in γ-tubulin expression when compared to C samples. Data represent the mean ± SE, n=3/group. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, ** $p < 0.001$ vs C.

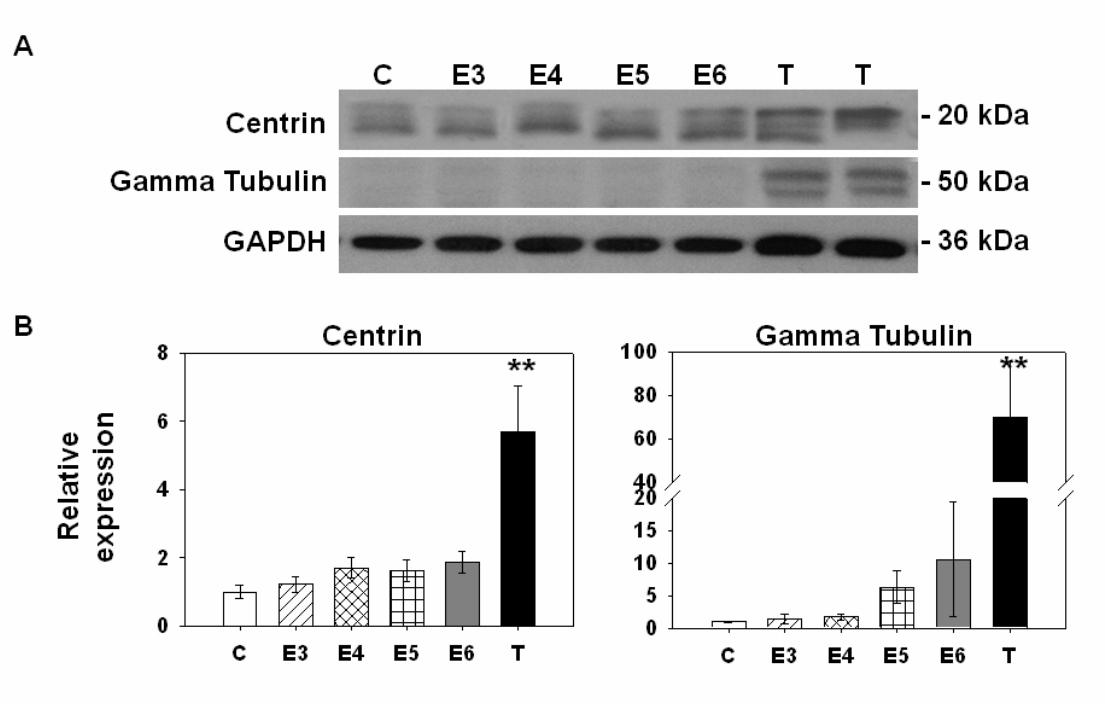

A
C
E3
E4
E5
E6
T
T
Centrin
- 20 kDa
Gamma Tubulin
- 50 kDa
GAPDH
- 36 kDa
B
Centrin
Gamma Tubulin
Relative expression
**
**
C
E3
E4
E5
E6
T

Figure 13. Effect of E_2 in centrosome number and size in Syrian hamster tumor foci of the kidney

Centrosomes and nuclei were observed by fluorescent microscopy of serial sections labeled with antibodies against centrin 20H5, γ-tubulin, AurA and DAPI (blue) (B-D) following a corresponding 6-month E_2-induced tumor foci area stained for H&E (A). In the tumor foci, centrosome amplification in size and number was evident (B). AurA co-localizes to the amplified centrosomes (C). Numerous centrosomes were much larger and often more numerous than those centrosomes in adjacent, uninvolved tissue (D).

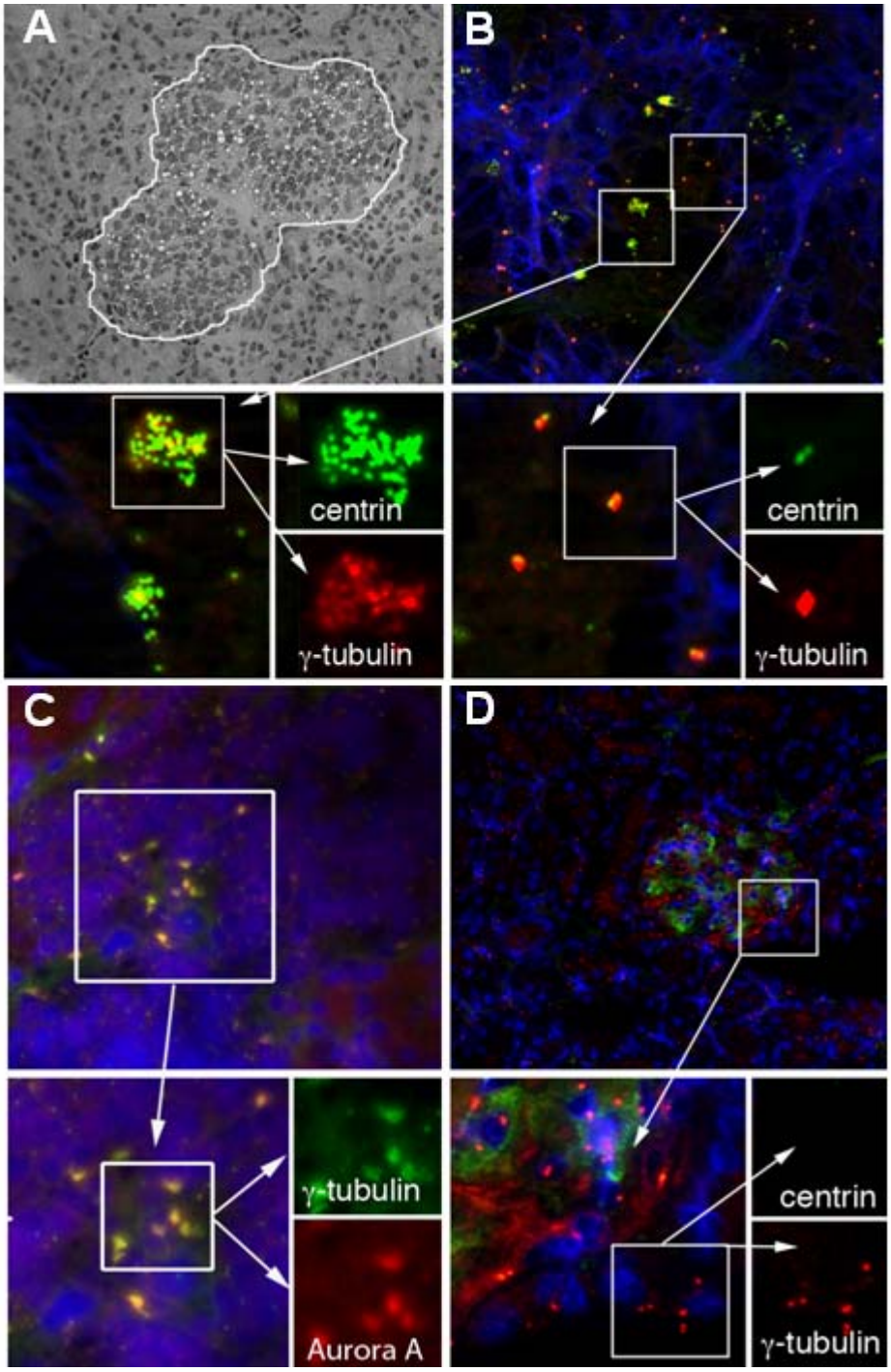

A
B
centrin
γ-tubulin
centrin
γ-tubulin
C
D
γ-tubulin
Aurora A
centrin
γ-tubulin

Figure 14. Localization of AurA and centrosome proteins, γ-tubulin and centrin, to isolated centrosome fractions of Syrian hamster E_2-induced tumors of the kidney

Centrosome isolation fractions from a combined sample of E_2-induced kidney tumors depicting the presence of AurA, γ-tubulin and centrin. Note that the AurA expression peak is located in fraction 9, where a major peak for γ-tubulin and centrin expression is also present.

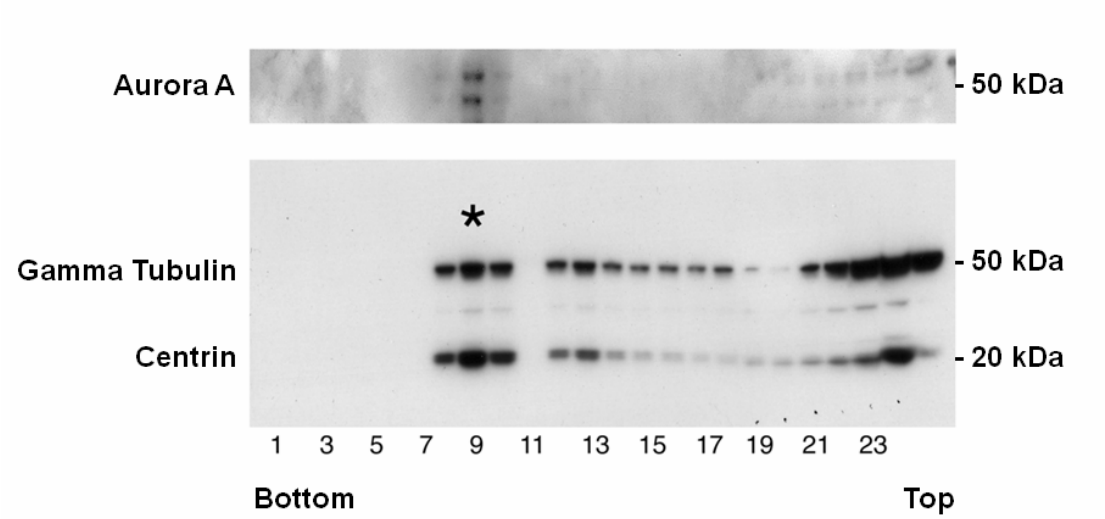

Aurora A
- 50 kDa
*
Gamma Tubulin
- 50 kDa
Centrin
- 20 kDa
1 3 5 7 9 11 13 15 17 19 21 23
Bottom
Top

Figure 15. Effect of estrogen treatment on the protein expression of TPX2 in Syrian hamster tumor foci of the kidney

The number of positive cells stained for TPX2 increased upon estrogen treatment. A. A small tumor focus from a 4.0-month E_2-treated kidney. B. An intermediate tumor focus from a 6.0-month E_2-treated kidney. C. A large tumor focus from a 6.0-month E_2-treated kidney. D. Kidney serial section without primary antibody.

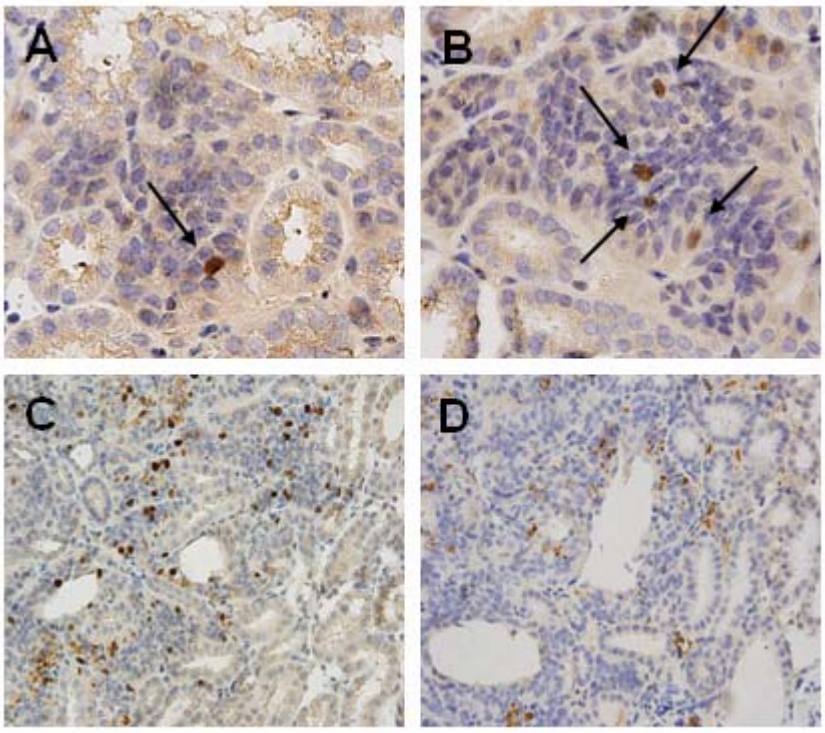

A
B
C
D

Figure 16. Protein expression of centrin, histone H3, PP1 and TPX2 in Syrian hamster E_2-induced tumors of the kidney

Western blot analysis (A) revealed that the expression of centrin, histone H3, PP1 and TPX2 expression was markedly increased (B), 9.4, 11.5, 2.4 and 2.9-fold, respectively, in E_2-induced tumors (T) when compared to control cholesterol-treated samples (C). GAPDH was used as a loading control. Data represent the mean ± SE, n=3. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, ** $p < 0.001$ vs C, * $p < 0.05$ vs C.

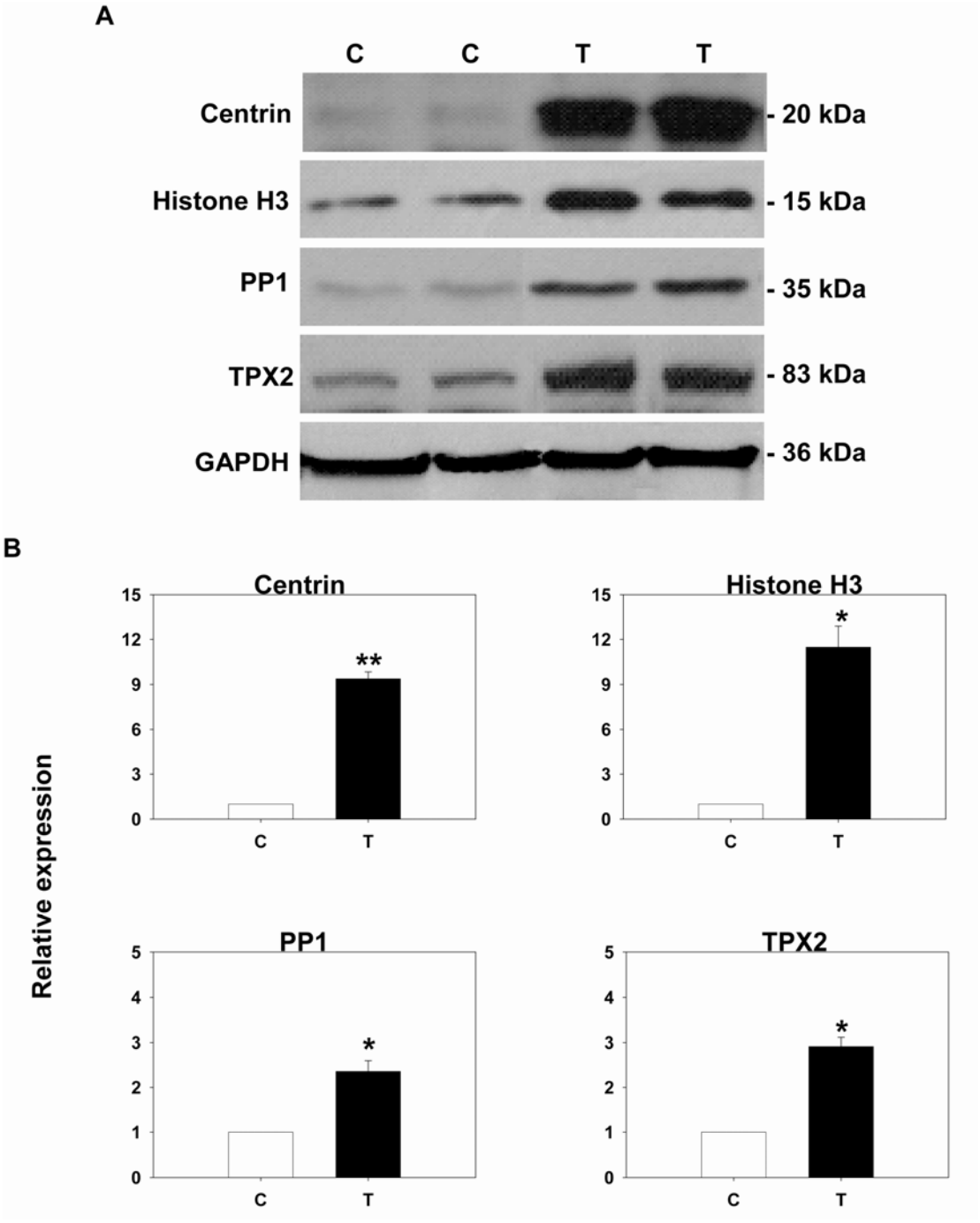

A
C
C
T
T
Centrin
- 20 kDa
Histone H3
- 15 kDa
PP1
- 35 kDa
TPX2
- 83 kDa
GAPDH
- 36 kDa
B
Relative expression
Centrin
Histone H3
PP1
TPX2
**
*
*
*
C
T

Figure 17. Estrogen modulation of the protein expression of centrin and histone H3 in Syrian hamster E_2-induced tumors of the kidney

A. The protein expression of centrin and histone H3 was determined in 6-month age-matched kidney samples from control cholesterol-treated animals (C) and in tumors from animals continuously treated with E_2 (T), after a 10-day E_2 withdrawal (T-Eout) and after a 10-day concomitant Tx (T-tam) treatment period. B. A significant 80% decline in centrin expression was observed after 10-day concomitant treatment with Tx. No significant changes in histone H3 protein expression were observed after estrogen modulation. GAPDH was used as a loading control. Data represent the mean ± SE, n=3/group. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, * $p < 0.05$ vs C, ♠ $p < 0.05$ vs T.

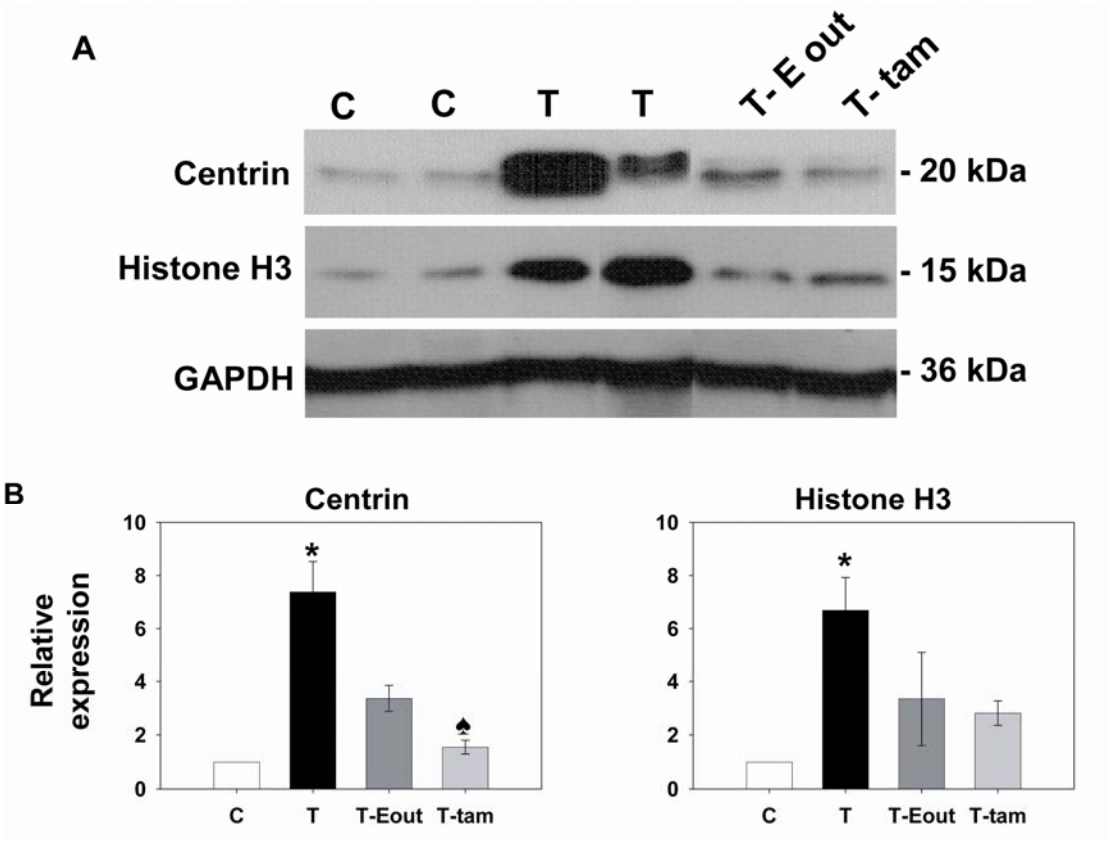

A
C
C
T
T
T- E out
T- tam
Centrin
- 20 kDa
Histone H3
- 15 kDa
GAPDH
- 36 kDa
B
Centrin
Histone H3
Relative expression
10
8
6
4
2
0
*
♠
C
T
T-Eout
T-tam

Figure 18. Protein expression of centrin, histone H3, PP1 and TPX2 in ACI rat mammary glands and in E_2-induced mammary tumors

A. The expression of centrin, histone H3, PP1 and TPX2 was significantly increased, 2.9, 15.3, 1.8 and 2.1-fold, respectively, in E_2-induced tumors (T) when compared to control cholesterol-treated (C) samples (B). β-actin was used as a loading control. Data represent the mean ± SE, n=3. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, * $p < 0.05$ vs C.

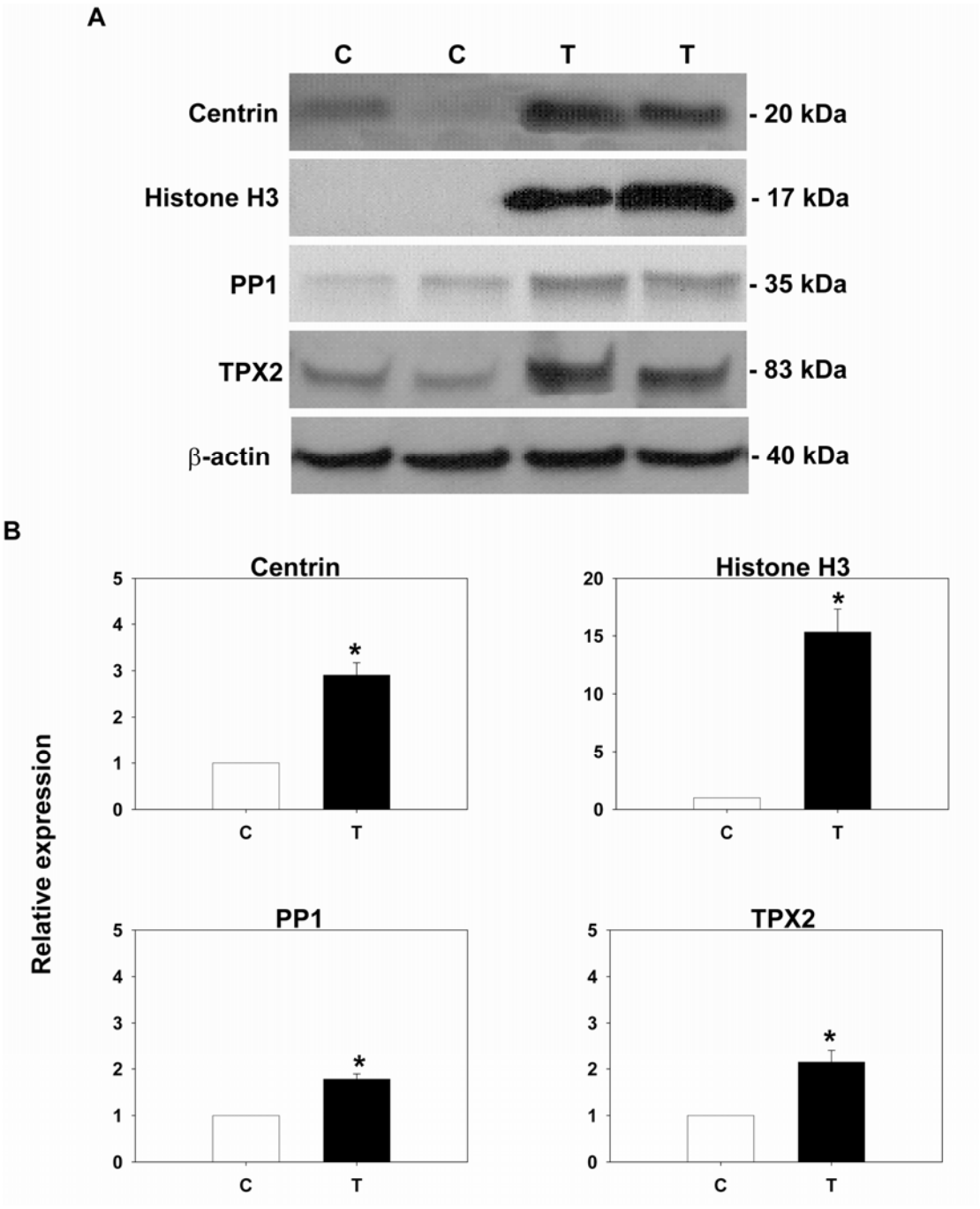

A
C
C
T
T
Centrin
- 20 kDa
Histone H3
- 17 kDa
PP1
- 35 kDa
TPX2
- 83 kDa
β-actin
- 40 kDa
B
Relative expression
Centrin
Histone H3
PP1
TPX2
C
T
*

Figure 19. Estrogen modulation of the protein expression of centrin and histone H3 in ACI rat mammary glands and in E_2-induced mammary tumors

A. The expression of centrin and histone H3 was determined after 6-month cholesterol-treatment (C) and in E_2-induced tumors from animals continuously treated with E_2 (T), after an 8-day E_2-withdrawal period (T-Eout) and after an 8-day concomitant Tx treatment (T-tam). β-actin was used as a loading control. A significant decline in centrin expression was observed after either an 8-day E_2-withdrawal period or after 8-days of concomitant Tx treatment, 73% and 61%, respectively (B). No significant changes in histone H3 protein expression were observed after estrogen modulation. B-actin was used as a loading control. Data represent the mean ± SE, n=3/group. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, ** $p < 0.001$ vs C, * $p < 0.05$ vs C, ♠ $p < 0.05$ vs T.

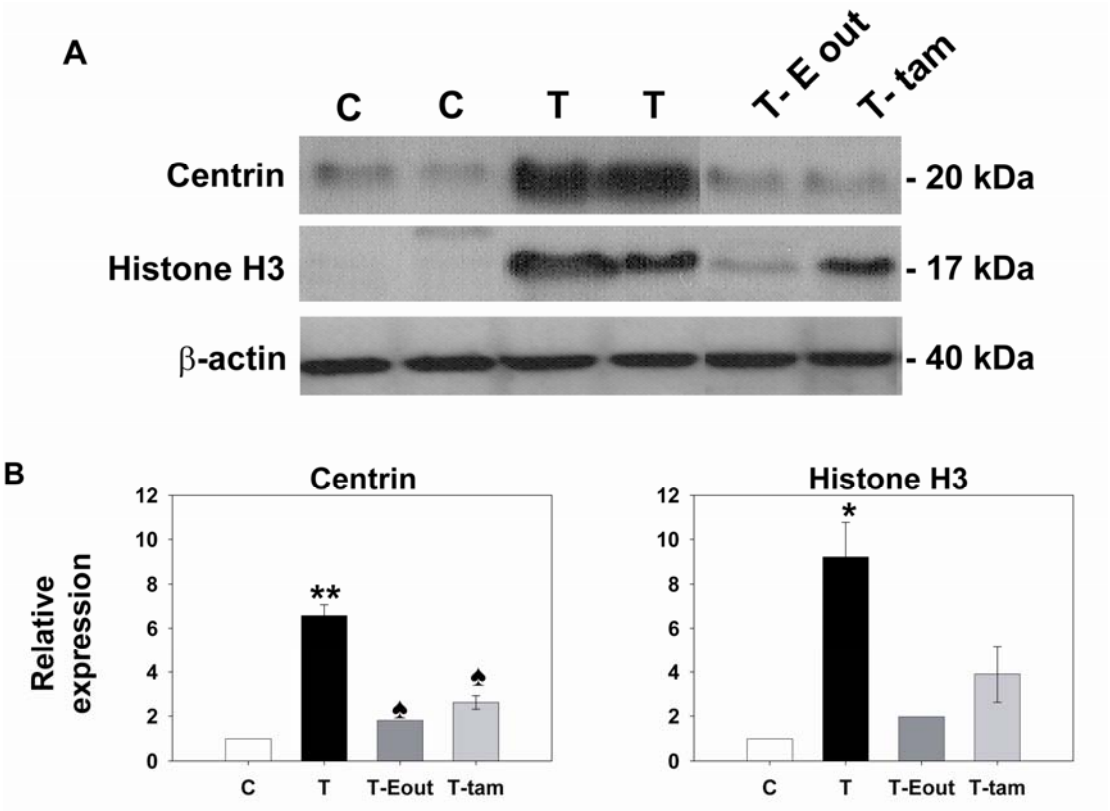

A
C
C
T
T
T- E out
T- tam
Centrin
- 20 kDa
Histone H3
- 17 kDa
β-actin
- 40 kDa
B
Centrin
Relative expression
12
10
8
6
4
2
0
**
♠
♠
C
T
T-Eout
T-tam
Histone H3
12
10
8
6
4
2
0
*
C
T
T-Eout
T-tam

Figure 20. Effect of estrogen treatment in the protein expression of MDM2 and p53 in the Syrian hamster kidney and in E_2-induced tumors of the kidney

A. The expression of MDM2 was low in samples from cholesterol-treated controls (C), however, MDM2 and p53 expression was significantly increased, 8.8- and 2.9-fold, respectively, in E_2-induced tumors (T) when compared to C samples (B). GAPDH was used as a loading control. Data represent the mean ± SE, n=6. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, ** $p < 0.001$ vs C.

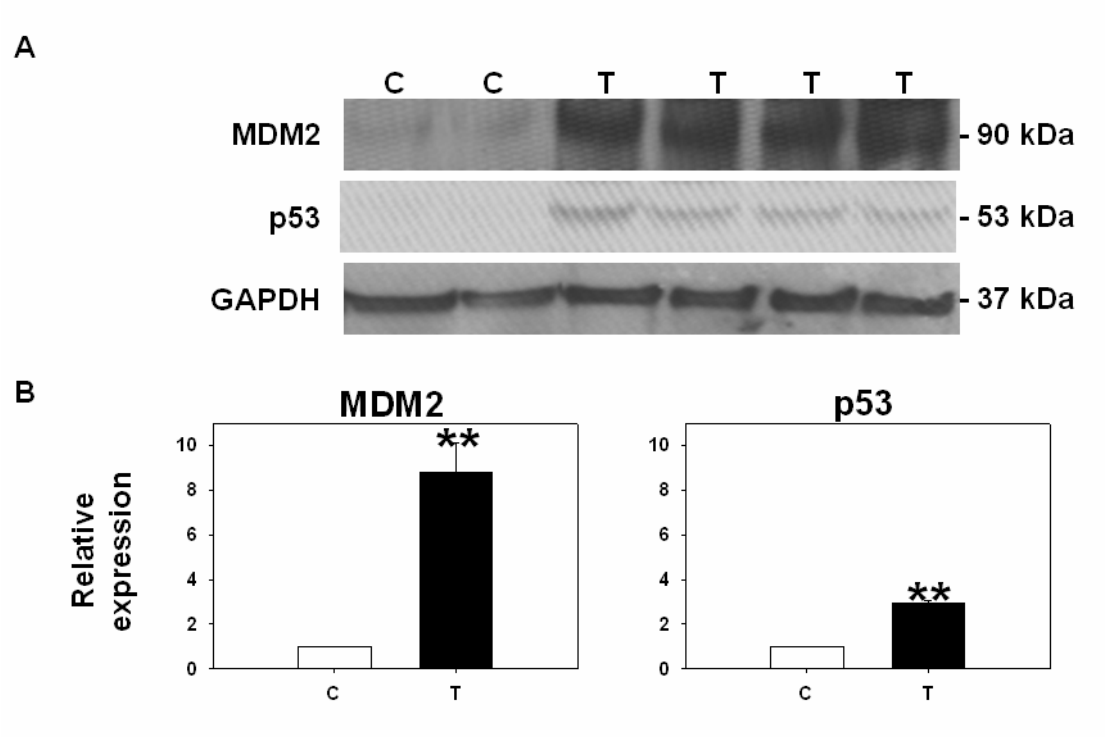

A
C
C
T
T
T
T
MDM2
- 90 kDa
p53
- 53 kDa
GAPDH
- 37 kDa
B
MDM2
p53
Relative expression
10
8
6
4
2
0
**
**
C
T

Figure 21. Effect of E_2 in the mRNA expression of MDM2 and $p53^{wt}$, measured by real-time PCR, in the Syrian hamster kidney

In E_2-induced tumors of the kidney (T), a significant 3.5-fold increase in mRNA MDM2 expression was detected, but no significant changes were observed in $p53^{wt}$ expression when compared to cholesterol-treated control samples (C). In addition, no significant changes in the expression of MDM2 or $p53^{wt}$ were observed in either 3- (E3) or 5-month (E5) E_2-treated kidney samples. Data represent the mean ± SE, n=6. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, * $p < 0.05$ vs C, E3 and E5.

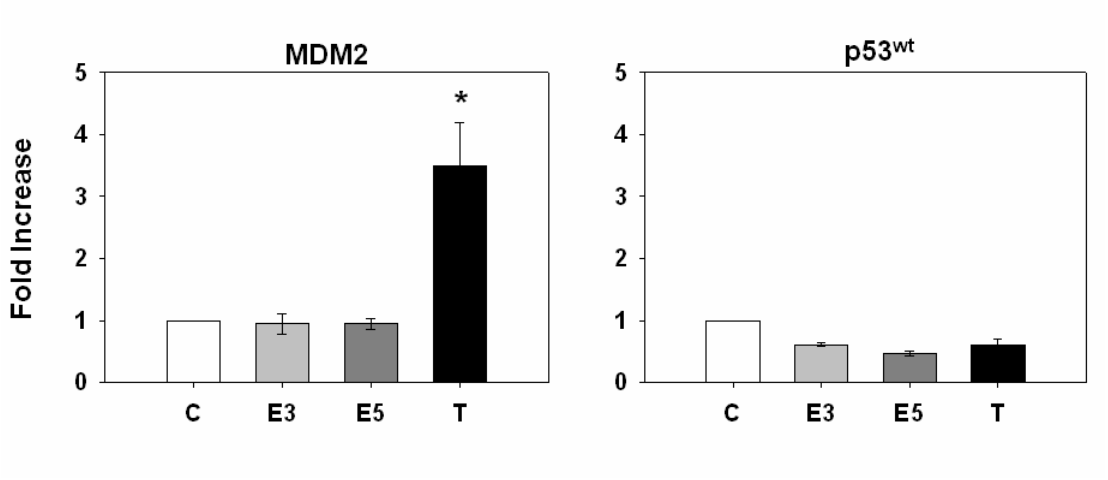

MDM2
p53wt
Fold Increase
5
4
3
2
1
0
*
C
E3
E5
T

Figure 22. Estrogen modulation of the protein expression of MDM2 and p53 in Syrian hamster E_2-induced tumors of the kidney

A. A significant decline, 48 and 47%, in MDM2 protein expression was observed in E_2-induced tumors of the kidney after a 10-day withdrawal period of E_2 (T-Eout) and after concomitant treatment with Tx (T-tam), respectively (B). No significant changes in p53 protein expression were observed after estrogen modulation. GAPDH was used as a loading control. Data represent the mean ± SE, n=3/group. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, ** $p < 0.001$ vs C, ♠ $p < 0.05$ vs T.

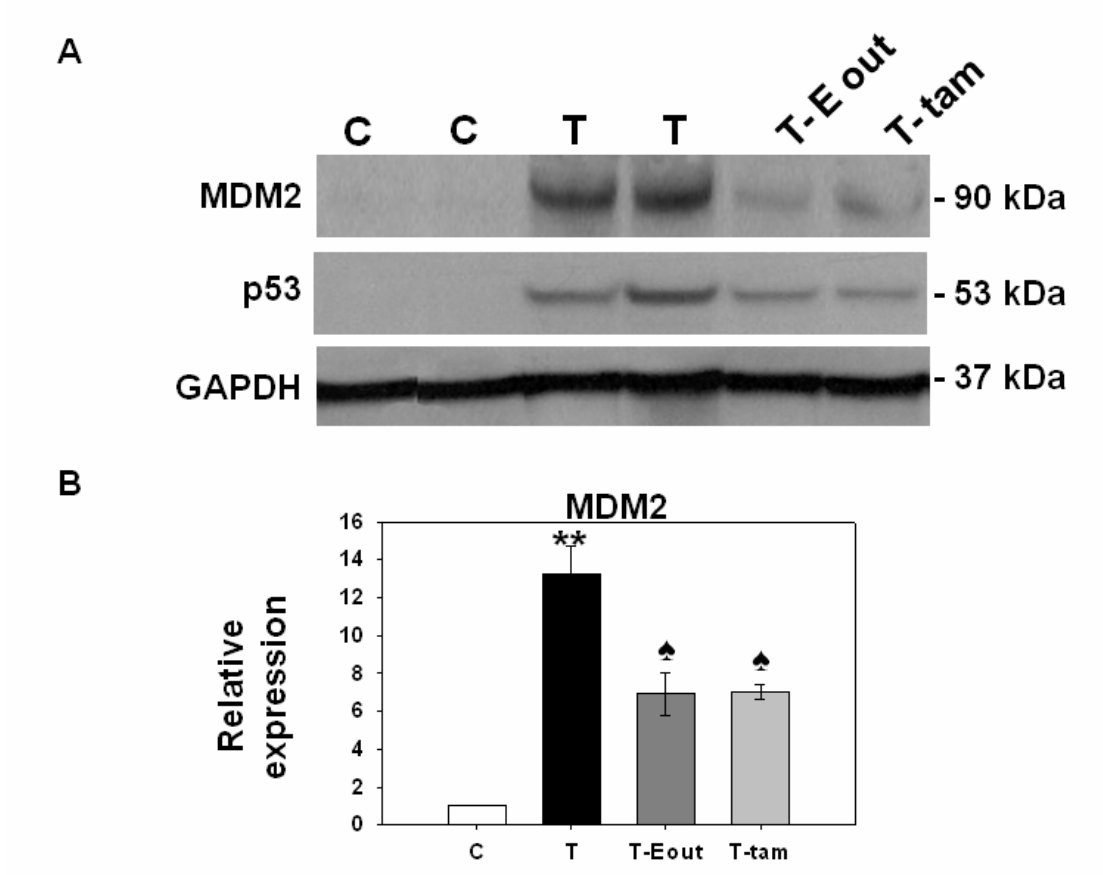

A
C
C
T
T
T-E out
T-tam
MDM2
- 90 kDa
p53
- 53 kDa
GAPDH
- 37 kDa
B
MDM2
Relative expression
16
14
12
10
8
6
4
2
0
**
♠
♠
C
T
T-Eout
T-tam

Figure 23. Binding of $p53^{wt}$ and MDM2 in Syrian hamster kidneys and in E_2-induced tumors of the kidney

$p53^{wt}$ was immunoprecipitated from Syrian hamster kidneys treated with cholesterol (HKC) and E_2-induced kidney tumors (HKT) and subjected to Western blot analysis for the presence of MDM2.

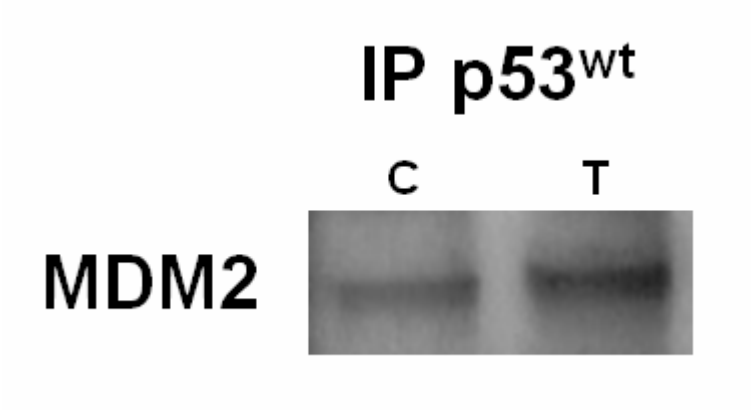

IP p53wt
C
T
MDM2

Figure 24. Effect of the MDM2 inhibitor RITA on the protein expression of MDM2 and p53 in Syrian hamster kidneys and in E_2-induced tumors of the kidney

A. A significant increase in MDM2 and p53 expression, 13 and 64%, respectively (B), was observed after a 15-day concomitant treatment period with 0.75 mg (T+0.75mg RITA) or 1.0 mg RITA (T+1.0mg RITA). GAPDH was used as a loading control. Data represent the mean ± SE, n=4. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, *$p < 0.05$ vs C, ♦$p < 0.05$ vs T.

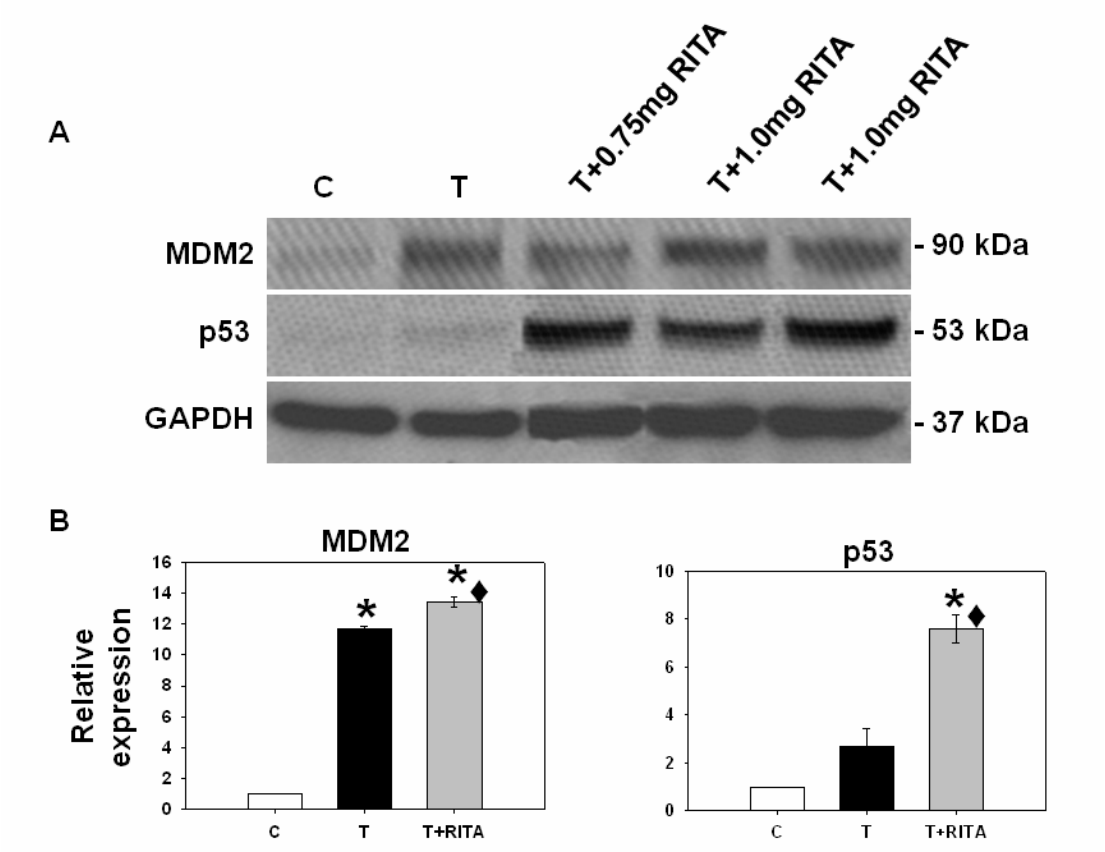

A
C
T
T+0.75mg RITA
T+1.0mg RITA
T+1.0mg RITA
MDM2
p53
GAPDH
- 90 kDa
- 53 kDa
- 37 kDa
B
MDM2
Relative expression
16
14
12
10
8
6
4
2
0
C
T
T+RITA
p53
10
8
6
4
2
0
C
T
T+RITA

Figure 25. Effect of the MDM2 inhibitor RITA on the protein expression of Bax and p21 in Syrian hamster kidneys and in E_2-induced tumors of the kidney

A. A significant increase in the expression of Bax, 60% (B), was observed after a 15-day concomitant treatment of 0.75 mg (T+0.75mg RITA) or 1.0 mg RITA (T+1.0mg RITA). No significant increase in p21 expression was observed after concomitant RITA treatment. GAPDH was used as a loading control. Data represent the mean ± SE, n=4. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, *$p < 0.05$ vs C, ♦$p < 0.05$ vs T.

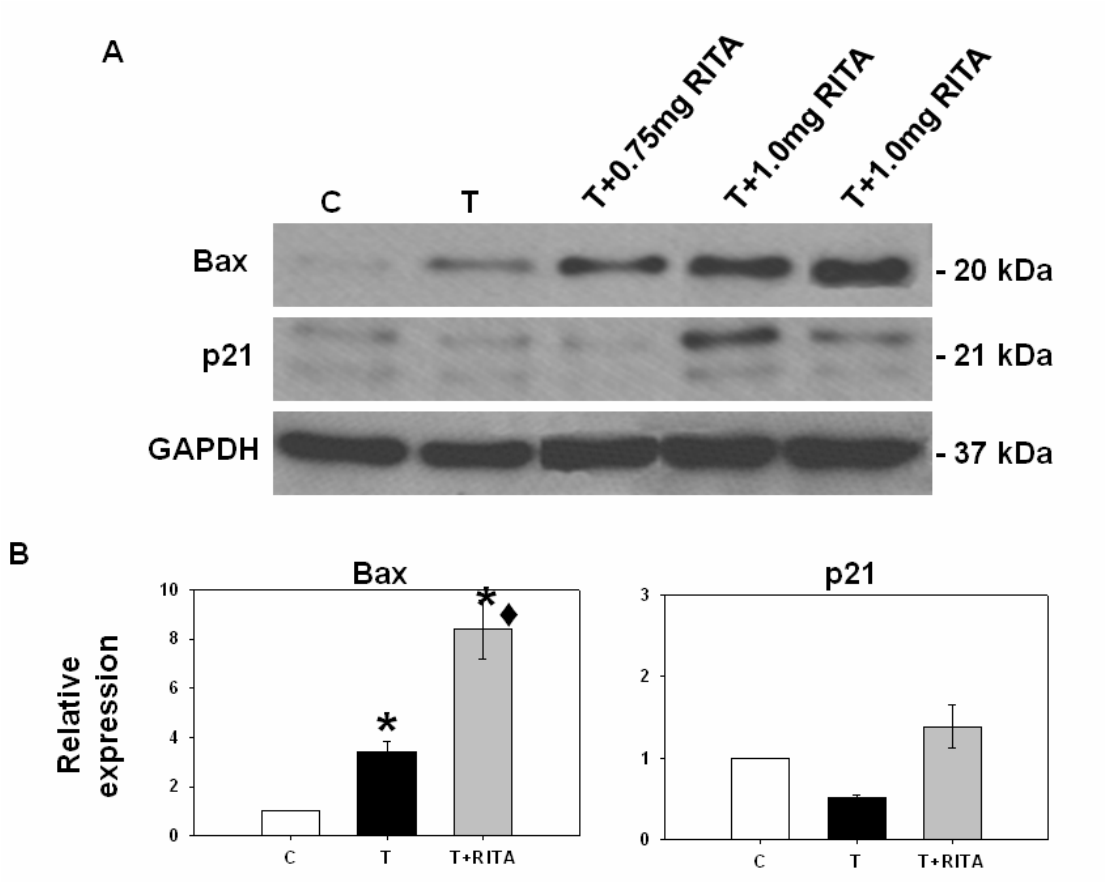

A
C
T
T+0.75mg RITA
T+1.0mg RITA
T+1.0mg RITA
Bax
- 20 kDa
p21
- 21 kDa
GAPDH
- 37 kDa
B
Bax
p21
Relative expression
10
8
6
4
2
0
3
2
1
0
C
T
T+RITA
C
T
T+RITA

Figure 26. Protein expression of MDM2 and p53 in ACI rat mammary glands and in E_2-induced mammary tumors

MDM2 expression was markedly increased, 7.7-fold (B), in E_2-induced tumors (T), when compared to control cholesterol-treated mammary gland samples (C), while p53 expression was very low and only detected in T samples. β-actin was used as a loading control. Data represent the mean ± SE, n=6. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, * $p < 0.05$ vs C.

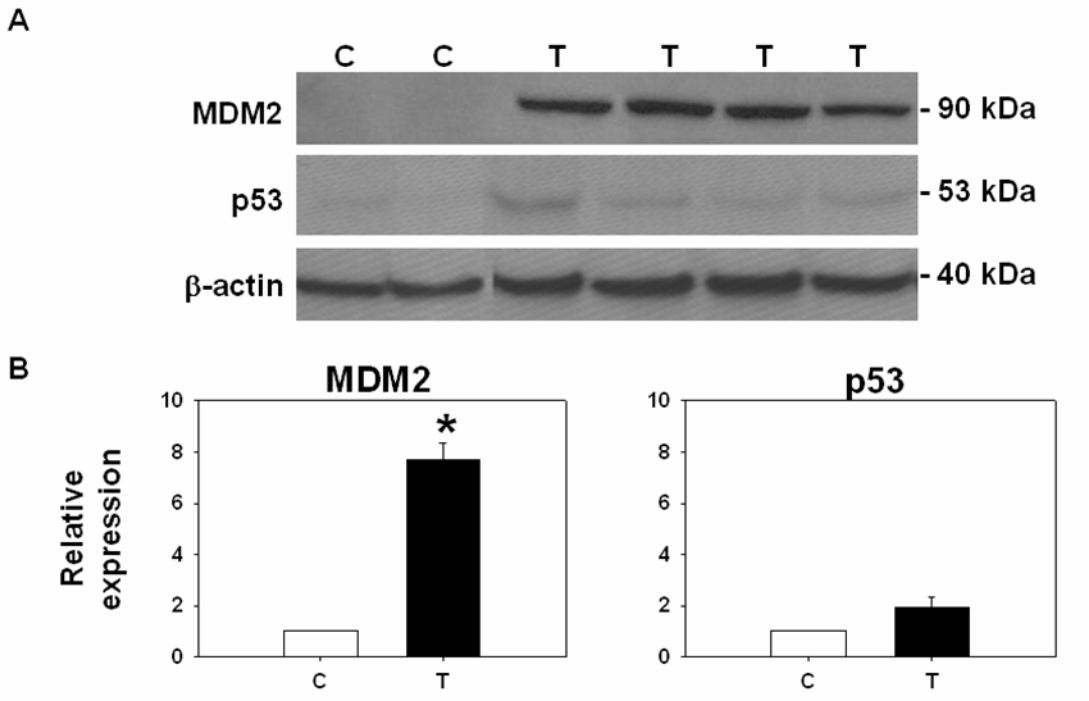

A
C
C
T
T
T
T
MDM2
- 90 kDa
p53
- 53 kDa
β-actin
- 40 kDa
B
MDM2
p53
Relative expression
10
8
6
4
2
0
*
C
T

Figure 27. mRNA expression of MDM2 and $p53^{wt}$, measured by real-time PCR, in ACI rat mammary glands and E_2-induced mammary tumors

A significant 4.1-fold increase in MDM2 mRNA expression was detected in E_2-induced tumor samples (T) compared with cholesterol-treated control samples (C), while no significant changes were detected in $p53^{wt}$. In addition, no significant changes in the expression of MDM2 or $p53^{wt}$ were observed in either 3- (E3) or 5-month (E5) E_2-treated kidney samples. Data represent the mean ± SE, n=6. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, ** $p < 0.001$ vs C, E3 and E5.

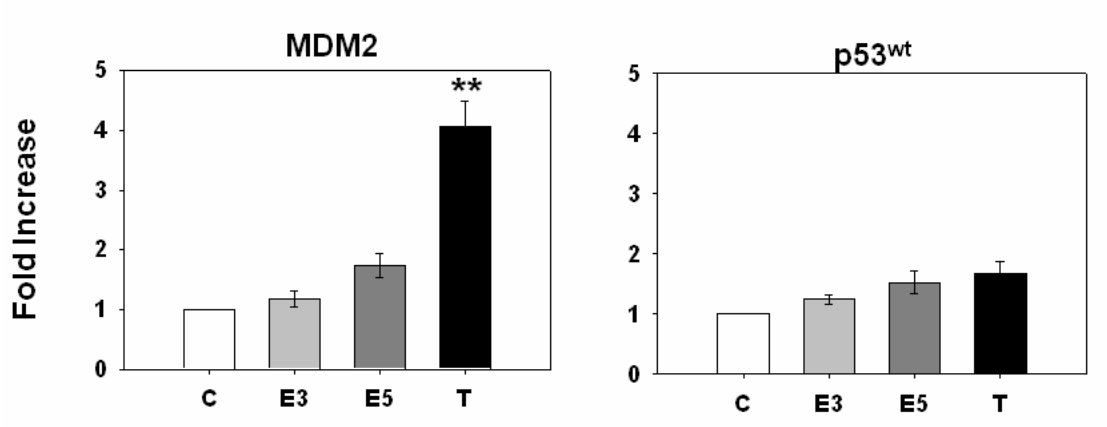

MDM2
p53wt
Fold Increase
0
1
2
3
4
5
**
C
E3
E5
T

Figure 28. Estrogen modulation of MDM2 and p53 protein expression in ACI rat mammary glands and in E_2-induced mammary tumors

A. A significant decline, 66 and 46%, respectively (B), in MDM2 expression was observed in E_2-induced mammary tumors after an 8-day withdrawal period of E_2 (T-Eout) and after concomitant treatment with Tx (T-tam). No significant changes in p53 protein expression were observed after estrogen modulation. β-actin was used as a loading control. Data represent the mean ± SE, n=3/group. Statistical significance was determined by one way ANOVA with a Tukey post hoc test, * $p < 0.05$ vs C, ♠ $p < 0.05$ vs T.

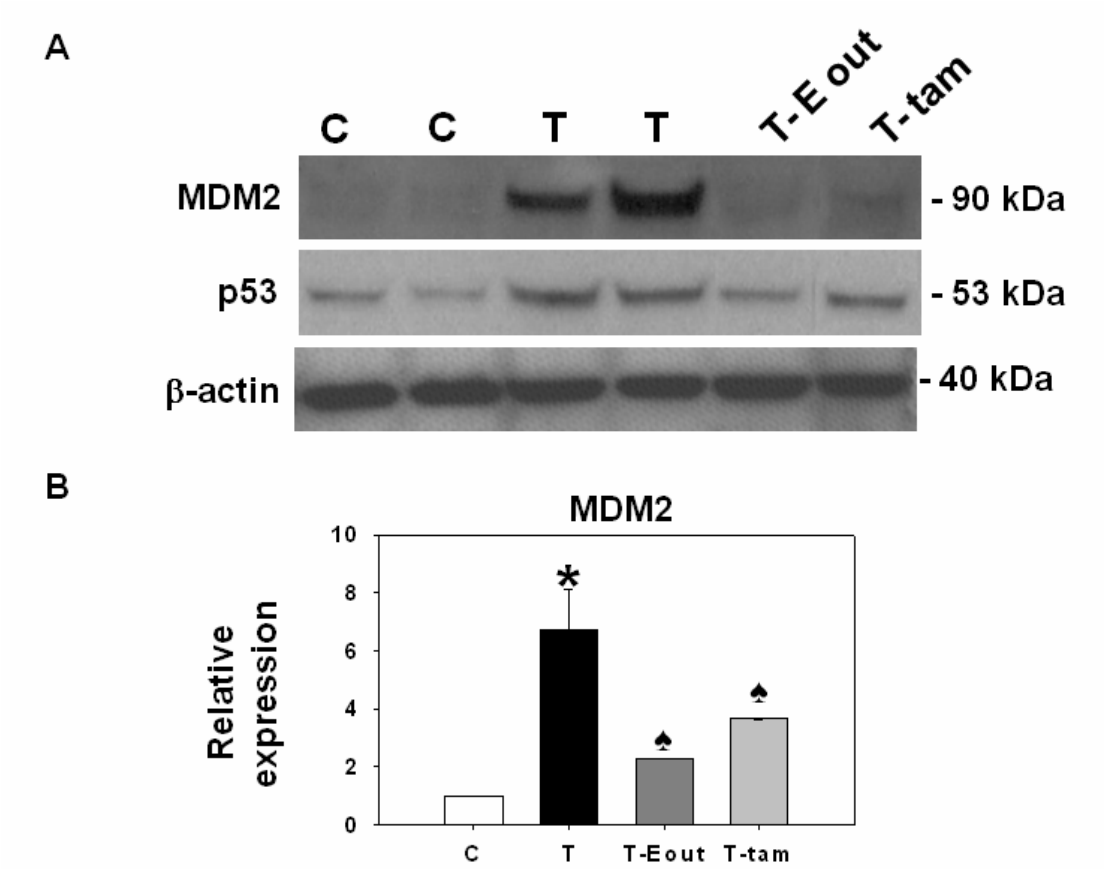
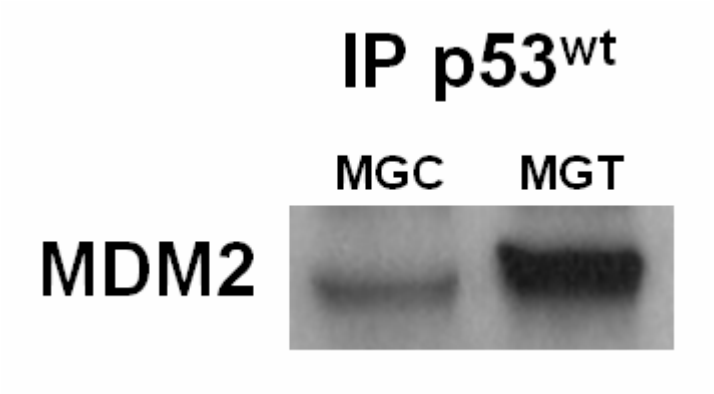

A
C
C
T
T
T-E out
T-tam
MDM2
- 90 kDa
p53
- 53 kDa
β-actin
- 40 kDa
B
MDM2
Relative expression
10
8
6
4
2
0
*
♠
♠
C
T
T-Eout
T-tam

Figure 29. $p53^{wt}$ and MDM2 binding in ACI rat mammary glands and in E_2-induced mammary tumors

$p53^{wt}$ was immunoprecipitated from ACI rat mammary glands treated with cholesterol (MGC) and from E_2-induced mammary gland tumors (MGT) and subjected to Western blot analysis for the presence of MDM2.


IP p53wt
MGC
MGT
MDM2

Chapter 5: Discussion

5.1 General Discussion

CIN and aneuploidy are defining features of human sporadic BC. Almost a century ago, Boveri proposed that aneuploidy in cancer cells may arise through errors in chromosome segregation due to centrosome defects (Boveri, 1914). CA has been correlated with CIN and aneuploidy as defining features of human BC (Makris et al., 1997; Lingle et al., 1998; Arnerlov et al., 2001) as well as in E_2-induced tumors of the ACI rat mammary gland (Li et al., 2002a; Li et al., 2004) and the Syrian hamster tumors of the kidney (Li and Li, 2003; Hontz et al., 2007). Additionally, these defining features of estrogen-induced oncogenesis have been causally linked to the over-expression of the mitotic kinase, AurA in human BC (Katayama et al., 2003) and in E_2-induced tumors (Li et al., 2004; Hontz et al., 2007).

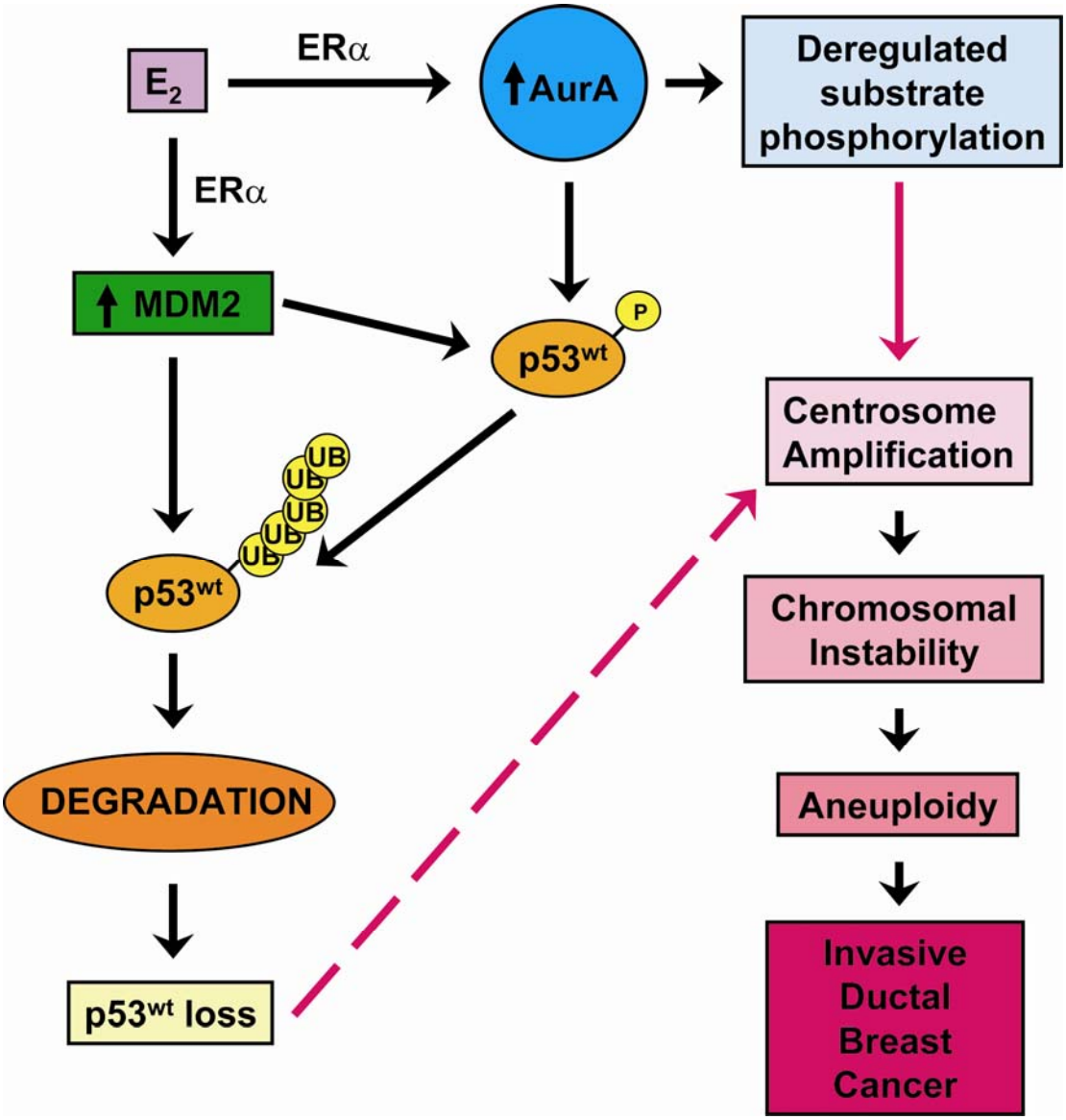
The G1/S and G2/M checkpoints act through the tumor suppressor p53 to ensure the orderly progression of the cell cycle. In some malignant tumors a $p53^{wt}$ mutation has been correlated with CA (Weber et al., 1998; Carroll et al., 1999; Ouyang et al., 2001; Jeng et al., 2004; Zhu et al., 2005). In BC tumors that retain $p53^{wt}$, MDM2 over-expression is often observed in synchrony with CA (Carroll et al., 1999). MDM2 over-expression results in the degradation of $p53^{wt}$ and the loss of function which is enhanced by its phosphorylation by AurA (Katayama et al., 2004).

For the first time, in the current study, the data demonstrate a link between the estrogen-induced over-expression of the oncoproteins AurA and

MDM2 and the molecular cascade of CA, CIN and aneuploidy observed in the E_2-induced Syrian hamster tumors of the kidney. Based on these results and similar previous reports in both human BC and the ACI rat, a correlative sequence of events is proposed for E_2-induced oncogenesis beginning with E_2 interacting with its receptor, ERα, leading to the subsequent over-expression of AurA and MDM2, CA, CIN, aneuploidy and eventual neoplastic transformation (Figure 30). These early molecular and cellular changes during E_2-induced oncogenesis may provide new novel targets for the prevention and therapeutic intervention of human sporadic BC.

Figure 30. Schematic representation of the proposed pathway of events leading to neoplastic transformation during E_2-induced oncogenesis

E_2, acting through ERα, results in the sustained over-expression of AurA and MDM2. Over-expressed AurA leads to CA through the deregulated phosphorylation of its centrosomal protein substrates. Over-expressed MDM2 leads to CA through the loss of function of $p53^{wt}$. Loss of function of $p53^{wt}$ can be enhanced by AurA phosphorylation of $p53^{wt}$. CA leads to the development of CIN, aneuploidy and eventual BC.


E2
ERα
AurA
Deregulated substrate phosphorylation
ERα
MDM2
p53wt
P
Centrosome Amplification
UB
UB
UB
UB
UB
p53wt
Chromosomal Instability
DEGRADATION
Aneuploidy
p53wt loss
Invasive Ductal Breast Cancer

5.2 Overall Conclusions

AurA and B expression

The Auroras are a family of mitotic kinases essential for the proper execution of various mitotic events including: centrosome duplication, maturation and separation, spindle assembly and stability, chromosome duplication and segregation and cytokinesis (Carmena and Earnshaw, 2003; Katayama et al., 2003).

The over-expression of AurA and B in the early tumor foci of the Syrian hamster kidney is in agreement with previously published reports showing AurA over-expression in E_2-induced tumors of the ACI rat mammary gland (Li et al., 2004) and in human BC (~94%) (Tanaka et al., 1999). The over-expressed AurA in the E_2-induced tumors shows the presence of a doublet which may represent the native and phosphorylated forms of AurA.

IHC analysis of these E_2-induced tumors showed that their expression was confined to cells present in tumor foci. Positively stained cells were observed as early as 3.5 months of E_2 treatment suggesting that the AurA and B over-expression is an early event, that can only be observed by IHC, as these small early foci are probably diluted during the processing of whole tissue lysates for Western blot anaylsis.

CA, monitored by the over-expression of centrin and γ-tubulin, was observed in concert with AurA and B over-expression in E_2-induced tumors of the Syrian hamster kidney, as well as in similarly induced tumors of the ACI

rat mammary gland (Li et al., 2004). In the hamster kidney model, the over-expressed centrin showed a clear shift in the centrin band between cholesterol-treated control and E_2-treated animals and E_2-induced tumor samples. A doublet was also present in γ-tubulin expression E_2-induced tumors of the kidney similar to differential phosphorylation states reported in E_2-induced ACI rat mammary gland tumors (Li et al., 2004) and non-mammalian species (Oakley and Oakley, 1989; Lajoie-Mazenc et al., 1996). AurA expression was associated with an amplified tumor centrosome fraction isolated from a combined sample of E_2-induced tumors of the kidney. Additionally, AurA co-localized to amplified centrosomes in E_2-induced tumor foci as identified by immunofluorescence. The over-expression of the Aur kinases, specifically AurA, is consistent with the postulation of its involvement in eliciting CA (Zhou et al., 1998).

The discovery that AurA and B are persistently over-expressed in early tumorous foci of the kidney and AurA in mammary dysplasias and DCIS in female ACI rats (Li et al., 2004), both induced by E_2, suggests that these kinases may be under direct or indirect estrogen control. This is now supported by our finding that AurA protein expression showed a marked decline in tumors upon E_2 withdrawal or after the co-administration of Tx in the presence of E_2 as compared with corresponding tumors maintained on E_2 alone. The relatively brief period of E_2 withdrawal or E_2 plus Tx co-administration did not result in a decrease of CA. This result was not

unexpected as centrosomes are complex structures, containing the relatively stable proteins centrin and γ-tubulin, which likely require many cell cycles to be depleted from the centrosome structure.

In the hamster kidney model, AurA mRNA was over-expressed 6.0-fold in E_2-induced tumors of the kidney compared with age-matched cholesterol-treated control kidneys. These data are consistent with elevated AurA mRNA levels reported in E_2-induced tumors of the ACI rat (Li et al., 2004) and human BC (~62%) (Miyoshi et al., 2001). In addition, over-expressed AurA led to a concomitant increase in AurA kinase activity in E_2-induced tumors of the kidney compared with age-matched cholesterol-treated control kidneys. The over-expression of active AurA kinase has the ability to transform NIH3T3 cells and induce tumor formation when implanted in nude mice, thus supporting the role of AurA as an oncoprotein (Bischoff et al., 1998; Zhou et al., 1998).

Persistant over-expression of AurA and B has been associated with CA, CIN and aneuploidy (Carmena and Earnshaw, 2003; Katayama et al., 2003; Li et al., 2004; Fu et al., 2007), key molecular changes observed during oncogenesis. Previous work in our lab has established CIN and aneuploidy as early events in solely E_2-induced tumors of the hamster kidney (Li et al., 1999; Li et al., 2001; Papa et al., 2003), and in the E_2-induced ACI rat mammary tumor model. These traits have been detected in 55-78% of human DCISs and 85-92% of human IDBCs (Makris et al., 1997; Arnerlov et al., 2001; Li et

al., 2002a). Similarly, CA, CIN and aneuploidy have been reported in 84% and 91% of E_2-induced rat mammary DCISs and primary mammary tumors (Li et al., 2002a), respectively. While the precise relationship between sustained AurA and B over-expression and CA leading to CIN and aneuploidy has yet to be defined, there is growing correlative evidence that these characteristics occur early and therefore, may be important molecular alterations representing a common early pathway in E_2-driven neoplastic transformation, rather than a consequence of tumor progression.

Aur kinase substrates

CA, detected in nearly all cancers, has long been implicated as a cause of CIN and aneuploidy leading to neoplastic transformation (Boveri, 1914; Lingle et al., 1998; Pihan et al., 2001). However, the mechanism by which CA occurs is not well understood. The two prevailing views are that CA arises from either cell division failure or a disruption of the centriole duplication cycle (Nigg, 2002; Duensing et al., 2007). In combination, AurA and B phosphorylate more than 25 currently known centrosomal and mitotic protein substrates (Li and Li, 2006). The phosphorylation of any individual or combination of these substrates might affect the deregulation of the centrosome cycle leading to CA and downstream molecular changes leading to tumor formation. The data show that four of these Aur substrates, centrin,

histone H3, PP1 and TPX2, are over-expressed in the Syrian hamster tumor foci of the kidney and the ACI rat mammary gland.

Centrin is a calcium binding protein that is required for centriole duplication and is one of the first proteins to localize at sites of newly forming centrioles (Salisbury, 2007). Centrin and AurA expression overlap throughout the cell cycle and *in vitro* kinase assays demonstrated the ability of AurA to phosphorylate centrin (Lukasiewicz, 2007). In addition, over-expression of AurA in HeLa cells led to increased levels of phospho-centrin suggesting that this phosphorylation has a stabilizing effect on the expression of centrin (Lukasiewicz, 2007). Therefore, we can speculate that the over-expression of centrin we are observing may be due to phosphorylation by AurA. This stabilizing phosphorylation may be contributing to the CA observed in the Syrian hamster tumors of the kidney and ACI rat mammary gland by interfering with proper centriole/centrosome duplication.

Chromatin condensation is essential for cell division in eukaryotes. Phosphorylation of histone H3 is considered to be a crucial event required for chromatin condensation and cell cycle progression (Hans and Dimitrov, 2001). Studies indicate that both AurA and B have the ability to effectively phosphorylate histone H3 at Ser-10 *in vitro* and *in vivo* (Crosio et al., 2002). The over-expression of the Aur kinases may be leading to the constituitive phosphorylation of histone H3 and accelerated progression through the cell cycle thus increasing proliferation, as reported for hepatocellular carcinoma

(Sistayanarain et al., 2006). The link between increased histone H3 phosphorylation and CA remains elusive. The stages of centrosome duplication are tightly linked with the cell cycle. Therefore, one effect of cell cycle deregulation, via the over-expression of histone H3, could be the accumulation of centrosome defects in the cell and eventual CA.

The current results showed that both centrin and histone H3 were over-expressed at the protein level in E_2-induced Syrian hamster tumors of the kidney and the ACI rat mammary gland, however, we were unable to determine the differential expression of phospho-centrin and -histone H3 during E_2-induced oncogenesis due to the use of unstable and unpredictable phospho-antibodies. Additionally, our attempts to localize phospho-centrin and -histone H3 in isolated amplified tumor centrosomes were unsuccessful, presumably due to the lack of reliable phospho-antibodies. Nevertheless, we were able to show a decline in centrin protein expression in tumors upon E_2 withdrawal or the co-administration of Tx in the presence of E_2 as compared with corresponding tumors maintained on E_2 alone, suggesting that centrin is under direct or indirect estrogen control. In contrast, we observed no significant changes in histone H3 protein expression upon E_2 withdrawal or the co-administration of Tx in the presence of E_2 as compared with corresponding tumors maintained on E_2 alone.

Further, we examined the expression of PP1 and TPX2, proteins that interact with AurA as both regulators and substrates. Their roles in the

regulation of AurA have been well defined with TPX2 as an activator (Eyers and Maller, 2004) and PP1 as an inhibitor of AurA kinase activity (Satinover et al., 2004). The roles of PP1 and TPX2 as substrates of AurA are not as clear. Upon phosphorylation by AurA, the phosphatase activity of PP1 is reduced (Katayama et al., 2001) and TPX2 is believed to play a crucial role in the localization of AurA to the spindle microtubules (Kufer et al., 2002). TPX2 is required for spindle pole formation, and it has been reported that altering TPX2, either by depletion or over-expression, leads to a failure in spindle assembly (Gruss et al., 2002).

The results show that both PP1 and TPX2 show an increase in protein expression in E_2-induced tumors of the Syrian hamster kidney and the ACI rat mammary gland. The importance of these results, while interesting, remains unclear in the absence of phospho-antibody data. In addition, TPX2 primarily localizes to the cells in the tumor foci. The localization of TPX2, shortly after 4.0-months of E_2 treatment, suggests its involvement during early stages of tumor development.

These results, while preliminary, suggest that these four Aur substrates may prove to be important players in eliciting the alterations observed during early stages of E_2-induced oncogenesis. However, the roles of these substrates will only be clearly defined when improved and stable phospho-antibodies are developed. Thus, the significance of the over-expression of

these Aur substrates during E_2-induced oncogenesis, and their role in CA and eventual neoplastic transformation remains elusive.

MDM2-p53

The G1/S and G2/M cell cycle checkpoints, that normally ensure the orderly progression of cell cycle events, can be inactivated during oncogenesis (Hartwell and Weinert, 1989). In normal cells, DNA damage triggers checkpoint activation through the tumor suppressor p53 and up-regulation of its downstream targets. Mutations in p53 are common in many human tumors (~50%) (Caron de Fromentel and Soussi, 1992; Greenblatt et al., 1994) leading to a loss of its tumor suppressor properties. However, p53 mutations in BC are less common (~20%) (Pharoah et al., 1999; Gasco et al., 2002; Lacroix et al., 2006). In tumors that retain $p53^{wt}$, like BC, p53 inactivation occurs mainly through the deregulation and over-expression of MDM2 (Oliner et al., 1992; Finlay, 1993). Our results show a marked increase in MDM2 protein and mRNA expression in E_2-induced Syrian hamster tumors of the kidney and in ACI rat mammary gland tumors, providing additional evidence that the inactivation of $p53^{wt}$ through MDM2 over-expression may be a common pathway in estrogen-driven oncogenesis.

MDM2 is regulated by p53 through a p53 binding site present in the MDM2 gene (Wu et al., 1993). In addition to p53 regulation, the expression of ERα has been shown to induce MDM2 transcription (Phelps et al., 2003). In

$ERα^+$ BC tumors, MDM2 gene amplification is uncommon, although MDM2 mRNA and protein levels are often increased, 73 and 71%, respectively (Marchetti et al., 1995; Bueso-Ramos et al., 1996; Turbin et al., 2006). Additionally, in MCF-7 cells, mitogen stimulation, such as E_2, induced elevated expression of MDM2 (D'Assoro et al., 2008). These results suggest that MDM2 expression is under estrogen direct or indirect control as supported by our finding that upon E_2 withdrawal or the co-administration of Tx in the presence of E_2, MDM2 protein expression was markedly decreased in these tumors as compared with tumors maintained on E_2 alone.

Binding studies of MDM2 oncoprotein and p53 provided evidence that the bound p53 is wild type and not mutated in E_2-induced Syrian hamster tumors of the kidney and in the ACI rat mammary gland. The binding of over-expressed MDM2 oncoprotein to $p53^{wt}$ facilitates the degradation of $p53^{wt}$ via ubiquitin ligase and the proteasome, contributing to the CA, CIN and aneuploidy seen in E_2-induced Syrian hamster tumors of the kidney and the ACI rat breast. Human breast DCISs exhibit a high frequency of CA (Lingle et al., 1998; Carroll et al., 1999; Lingle et al., 2002). In tumors that retain $p53^{wt}$, high levels of MDM2 were also observed (Carroll et al., 1999). Similarly, over-expression of MDM2 in Swiss 3T3 cells (Carroll et al., 1999) and E_2 stimulation of MDM2 expression in MCF-7 cells ($p53^{wt}$) (D'Assoro et al., 2008) resulted in CA and CIN. These results suggest that MDM2 over-expression, resulting in a loss of $p53^{wt}$, commonly results in CA.

In addition, the phosphorylation of $p53^{wt}$ by the oncoprotein AurA at serine 315 makes it more susceptible to ubiquitination by MDM2 and proteolysis (Katayama et al., 2004). AurA also phosphorylates $p53^{wt}$ at serine 215 which abrogates its function. This phosphorylation inhibits $p53^{wt}$ DNA binding and transcriptional activation leading to an AurA override causing cell cycle progression, survival and transformation (Liu et al., 2004). Previously, we have shown over-expression of AurA and its link to CA, CIN and aneuploidy in the E_2-induced Syrian hamster tumors of the kidney (Hontz et al., 2007) and the ACI rat mammary gland (Li et al., 2004). Interestingly, both the over-expression of AurA and loss of $p53^{wt}$ function via MDM2 over-expression lead to a similar molecular cascade of CA, CIN and aneuploidy.

Disrupting the MDM2-$p53^{wt}$ interaction during oncogenesis is of vital importance for reactivating $p53^{wt}$ and restoring its tumor suppressor function. The small molecule inhibitor RITA prevents the MDM2-p53 interaction *in vitro* and *in vivo* and displays anti-tumor activity without significant adverse effects (Issaeva et al., 2004). RITA functions by binding to p53, inhibiting the MDM2-p53 binding leading to p53 inactivation. Treatment of tumor-bearing animals with RITA led to a significant increase in $p53^{wt}$ and a corresponding increase in p53 downstream targets, MDM2 and Bax. In contrast, no significant changes were observed in p21 protein expression after RITA treatment. Previous reports show that treatment with RITA correlates with increased expression of MDM2 in a $p53^{wt}$ dependent manner (Issaeva et al., 2004).

Additionally, p53 restoration in murine leukemia cells lacking p53 led to a correlated increase in Bax mRNA and protein levels (Miyashita et al., 1994a; Miyashita et al., 1994b; Selvakumaran et al., 1994). While Bax leads to apoptosis and a cytotoxic cell response, p21 leads to cell cycle arrest and a cytostatic cell response. Previous studies showed a decreased binding of p53 to p300 which can activate p53 by acetylation (Issaeva et al., 2004). Additionally, previous studies in HCT116 cells show that disruption of the p53-p300 interaction leads to a failure of p21 activation which then favors apoptosis over cell cycle arrest (Iyer et al., 2004). Our results suggest that RITA is leading to an apoptotic response in the damaged tumor cells via p53 stimulation of Bax. Therefore, previous RITA studies, which showed an induction of apoptosis mediated by $p53^{wt}$ using TUNEL assays (Issaeva et al., 2004), are in support of our current findings.

Our findings suggest that RITA can directly inhibit the MDM2 mediated inactivation of $p53^{wt}$ observed during estrogen-induced oncogenesis. If used as an early intervention, before significant oncoprotein and chromosomal changes, RITA may maintain $p53^{wt}$ function, thus potentially inhibiting CA, CIN and transformation.

5.3 Future Directions

Future directions should include further study of the regulation of AurA and MDM2 by estrogens, the study of Aur kinase substrates as better

antibodies, both non- and phosphorylated, become commercially available, the study of additional mitotic kinases involved in oncogenesis, like PLK1, and their possible roles in E_2-driven oncogenesis and further refinements in the targeting of specific entities, AurA and MDM2, with small molecule inhibitors.

The ultimate goal of these studies was to identify early molecular and cellular changes during E_2-induced oncogenesis that could provide new novel targets for the prevention and therapeutic intervention of human sporadic BC. The over-expression of the two oncoproteins AurA and MDM2 seemed likely initial starting points. Preliminary studies from our lab using the Aur kinase small molecule inhibitor MK-0457 (originally known as VX-680) in the ACI rat showed that while tumor incidence was unaffected, tumor multiplicity and size were significantly reduced (~60%) (Li S.A., 2008). In addition, preliminary studies in the Syrian hamster show that RITA has the ability to restore endogenous $p53^{wt}$ function. Both results, while preliminary, represent initial steps in the chemoprevention of E_2-driven oncogenesis targeting specific entities. The over-expression of AurA and MDM2 appear to be early events during oncogenesis. Therefore, early intervention could hold the promise to effective tumor prevention by blocking the cascade of CA, CIN and aneuploidy.

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