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THE ROLE OF AN ONCOPROTEIN CIP2A IN BREAST CANCER

by

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To my family

Anni Laine

The role of an oncoprotein CIP2A in breast cancer

The Department of Pathology, University of Turku, Turku Centre for Biotechnology, University of Turku and Åbo Akademi University and Turku Doctoral Programme of Biomedical Sciences (TuBS), University of Turku, Turku, Finland

ABSTRACT

Cancerous inhibitor of PP2A (CIP2A) is an oncoprotein expressed in several human cancer types. Previously, CIP2A has been shown to promote proliferation of cancer cells. Mechanistically, CIP2A is known to inhibit activity of a tumor suppressor protein phosphatase 2A (PP2A) towards an oncoprotein MYC, further stabilizing MYC in human cancer. However, the molecular mechanisms how CIP2A expression is induced during cellular transformation are not well known. Also, expression, functional role and clinical relevance of CIP2A in breast cancer had not been studied before.

The results of this PhD thesis work demonstrate that CIP2A is highly expressed in human breast cancer, and that high expression of CIP2A in tumors is a poor prognostic factor in a subset of breast cancer patients. CIP2A expression correlates with inactivating mutations of tumor suppressor p53 in human cancer. Notably, we demonstrate that p53 inactivation up-regulates CIP2A expression via increased expression of an oncogenic transcription factor E2F1. Moreover, CIP2A promotes expression of E2F1, and this novel positive feedback loop between E2F1 and CIP2A is demonstrated to regulate sensitivity to both p53-dependent and -independent senescence induction in breast cancer cells. Importantly, in a CIP2A deficient breast cancer mouse model, abrogation of CIP2A attenuates mammary tumor formation and progression with features of E2F1 inhibition and induction of senescence. Furthermore, we demonstrate that CIP2A expression defines the cellular response to a senescence-inducing chemotherapy in breast cancer. Taken together, these results demonstrate that CIP2A is an essential promoter of breast cancer tumor growth by inhibiting senescence. Finally, this study implicates inhibition of CIP2A as a promising therapy target for breast cancer.

KEYWORDS: Cancerous inhibitor of PP2A, E2F1, Protein phosphatase 2A, p53, senescence, breast cancer, cancer

Anni Laine
CIP2A-onkoproteiinin rooli rintasyövässä

Patologia, Turun yliopisto, Turun biotekniikan keskus, Turun yliopisto ja Åbo Akademi, Turun biolääketieteellinen tutkijakoulu (TuBS), Turun yliopisto

TIIVISTELMÄ

CIP2A-onkoproteiinin ilmentyminen on havaittu useissa ihmisen eri syöpätyypeissä. CIP2A:n on osoitettu lisäävän syöpäsolujen kasvua ja jakaantumista. CIP2A toimii onkoproteiinina estämällä proteiini fosfataasi 2A:n (PP2A:n) kasvunestäjä aktiivisuutta MYC-onkoproteiinia kohtaan, johtaen MYC:in stabilisoitumiseen syöpäsoluissa. Sen sijaan mekanismit, jotka aiheuttavat CIP2A:n määrän lisääntymistä normaalien solujen muuttuessa syöpäsoluiksi ja jotka lisäävät CIP2A:n ilmentymistä syövässä, eivät ole vielä tunnettuja. CIP2A:n ilmentyminen, toiminnallinen rooli ja kliininen merkitys rintasyövässä ovat myös vielä selvittämättä.

Tässä väitöskirjatyössä osoitetaan CIP2A-proteiinin korkea ilmentyminen rintasyövässä sekä todetaan kasvaimen CIP2A:n ilmentymisen olevan ennustetta huonontava tekijä tietyssä joukossa rintasyöpäpotilaita. Lisäksi CIP2A:n ilmentyminen korreloi mutatoituneen p53 kasvunestäjäproteiinin ilmentymisen kanssa syövässä. Tässä työssä tunnistetaan myös mekanismi, jossa p53:n aktiivisuuden estyminen lisää E2F1-transkriptiotekijän välityksellä CIP2A:n määrää soluissa. Lisäksi tunnistetaan uusi mekanismi, jolla E2F1 ja CIP2A tukevat toistensa ilmentymistä syöpäsoluissa. Tämän mekanismin näytetään säätelevän sekä p53-riippuvaista että p53-riippumatonta peruuttamatonta solujen kasvun pysähtymistä, senesenssiä. Tärkeä löydös väitöskirjatyössäni on, että rintasyöpää mallintavassa hiirikannassa CIP2A:n poisto vähentää rintarauhaskasvainten muodostumista ja kasvua samalla estäen E2F1-proteiinin määrää ja senesenssille tyypillisten piirteiden lisääntymistä kasvaimissa. Lisäksi E2F1:n ja CIP2A:n välisen mekanismin osoitetaan säätelevän rintasyöpäpotilaiden vastetta senesenssiä aiheuttavalle hoitomuodolle. Kaiken kaikkiaan tämän työn tulokset osoittavat, sekä CIP2A-onkoproteiinin kliinisen merkityksen ihmisen rintasyövässä, että CIP2A-välitteisen rintasyövän kasvua lisäävän vaikutuksen joka välittyy syöpäsolujen senesenssiherkkyyden lisääntymisen kautta. Nämä tulokset osoittavat lisäksi CIP2A:n eston mahdolliseksi uudeksi lähestymistavaksi kehitettäessä uusia hoitoja rintasyöpäpotilaille.

AVAINSANAT: CIP2A, E2F1, PP2A, p53, senesenssi, rintasyöpä, syöpä

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ABBREVIATIONS

AML	Acute myeloid leukemia
ANP32a	Acidic (leucine-rich) nuclear phosphoprotein 32 family
ARF-BP1/Mule	ARF-binding protein/Mcl1-ubiquitin ligase E3
ARPP19a	cAMP-regulated phosphoprotein, 19kDa
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
BAX	Bcl-2 associated protein
Bcl-2	B-cell CLL/lymphoma 2
B-MYB	Myb-related protein B
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
BUB1B	Budding uninhibited by benzimidazoles 1 homolog beta
CCND1	Cyclin D1
CDC2	Cell division cycle 2
Cdk	Cyclin dependent kinase
ChIP	Chromatin immunoprecipitation
CDKN2A	Cyclin-dependent kinase inhibitor 2A
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CIP2A	Cancerous inhibitor of PP2A
CML	Chronic myeloid leukemia
DcR2	Decoy receptor 2
DEC1	Deleted in esophageal cancer 1
DHFR	Dihydrofolate reductase
DMBA	Demethylbenzathracene
DNA	Deoxyribonucleic acid
E2F1	E2 treanscription factor 1
EMT	Epithelial-mesenchymal transition
ENSA	Endosulfine alpha
ER	Estrogen receptor
ERK	Extracellular signal regulated kinase
Fas	Tumor necrosis factor receptor superfamily, member 6
FOXM1	Forkhead box M1
GATA3	GATA binding protein 3
GEFs	Guanine nucleotide exchange factors
GSK3	Glycogen synthase kinase 3
HDAC1	Histone deacetylase 1
HER2	Human epidermal growth factor receptor 2
HCC	Hepatocellular carcinoma
HEC	Highly expressed in cancer
HMECs	Human mammary epithelial cells
HNSCC	Head and neck squamous cell carcinoma

Hsp72	Heat shock protein 72
hTERT	Human telomerase reverse transcriptase
K	Lycine
Killer/DR5	Tumor necrosis factor receptor superfamily, member 10b
LCMT1	Leucine carboxyl methyltransferase 1
LT	Large T antigen
M	Mitosis
MAPK	Mitogen activated protein kinase
MAP2K4	Mitogen-activated protein kinase kinase 4
MAP3K1	Mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase
MDM2	Mouse double minute 2
MEF	Mouse embryonic fibroblasts
MEK	Mitogen activated/ Extracellular signal regulated kinase kinase
MK2	Mitogen activated protein kinase –activated protein kinase 2
MLL3	Myeloid/lymphoid or mixed-lineage leukemia 3
MMTV	Mouse mammary tumor virus
MOMP	Mitochondrial outer-membrane permeabilization
mRNA	Messenger RNA
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
NGS	Next-generation sequencing
NPCs	Neural progenitor cells
NSCLC	Non-small cell lung cancer
OIS	Oncogene-induced senescence
PI3KCA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PMAIP1	PMA induced protein 1
PME-1	Protein phosphatase methylesterase 1
PP2A	Protein phosphatase 2A
PR	Progesterone receptor
PRIMA-1	P53-dependent reactivation of massive apoptosis
PTEN	Phosphatase and tensin homolog
PTPA	Phosphotyrosyl phosphatase activator
PUMA	P53 up-regulated mediator of apoptosis
PyMT	Polyomavirus middle antigen
PyST	Polyomavirus small antigen
RNA	Ribonucleic acid
RB	Retinoblastoma
RITA	Reactivation of p53 and induction of *tumor* cell apoptosis
RUNX1	Runt-related transcription factor 1
S	Serine
SA-β-gal	Senescence associated-β-galactoside
SAHF	Senescence-associated heterochromatic foci
SASP	Senescence-associated secretory phenotype
SCC	Squamous cell carcinoma
SCF	Stem cell factor

siRNA	Small interfering RNA
Skp2	S-phase kinase-associated protein 2
SNP	Single nucleotide polymorphisms
SPC	Spermatogonial progenitor cell
ST	Small T antigen
SV40 ER	Simian virus 40 early region
T	Threonine
TCC	Transitional cell carcinoma
TCF	Transcription factor 14
TFIIH	Transcription factor II H
TNBC	Triple negative breast cancer
TPA	Tetradecanoylphorbol 13-acetate
UCC	Urothelial cell carcinoma
WNT	Wingless-type MMTV integration site family
ZEB1	Zinc finger E-box binding homeobox 1

LIST OF ORIGINAL PUBLICATIONS

I Come C, <u>Laine A</u>, Chanrion M, Edgren H, Mattila E, Liu X, Jonkers J, Ivaska J, Isola J, Darbon JM, Kallioniemi O, Thezenas S, Westermarck J. (2009).
CIP2A is associated with human breast cancer aggressivity.
Clinical Cancer Research 15: 5092-5100

II <u>Laine A</u>, Sihto H, Come C, Rodenfeldt M, Zwolinska A, Niemelä M, Khanna A, Chan EK, Kähäri V-M, Kellokumpu-Lehtinen P-L, Sansom O, Evan GI, Junttila MR, Ryan KM, Marine JC, Joensuu H, Westermarck J. (2013).
Senescence sensitivity of breast cancer cells is defined by positive feedback loop between CIP2A and E2F1.
Cancer Discovery 3:182-97

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1. INTRODUCTION

Cancer is the third most common cause of death worldwide and the second most common cause of death in developed countries (Jemal et al., 2011). Globally, according to estimations, almost 13 million new cancer cases and almost 8 million cancer related deaths occur every year (Jemal et al., 2011). Among women, the most common cancer type is breast cancer (Jemal et al., 2011). Breast cancer is a heterogeneous disease. Different breast cancer subtypes vary, for instance, in prognosis and in response to therapies. Prognosis of certain breast cancer subtypes have improved during the past decades, due to improved therapies. However, new therapeutic approaches are still needed for breast cancer.

As a disease, different cancer types may vary substantially in clinical picture and in prognosis. Even so, they still share molecular and phenotypical similarities. One of the barriers that cells have to bypass during carcinogenesis is irreversible growth arrest, senescence, in order to transform into continuous proliferating cancer cells (Hanahan and Weinberg, 2011). At later stages, senescence has been identified to suppress tumor progression by inhibiting the conversion of tumors into a more malignant form (Sanchez-Tillo et al., 2012; Weinberg, 2008).

Importantly, minimal requirements that enable transformation of several types of normal cells to cancer cells have been identified. These include activation of oncoproteins H-Ras and human telomerase catalytic subunit (hTERT), together with inactivation of tumor suppressor proteins p53, retinoblastoma (RB) and protein phosphatase 2A (PP2A)(Hahn et al., 1999a; Hahn et al., 2002). Mechanisms of inactivation of the tumor suppressor PP2A in human cancer has long been elusive. Cancerous inhibitor of PP2A (CIP2A) has been shown to inhibit PP2A in human cancer cells and elevated expression of CIP2A has been demonstrated in several types of human cancer (Table 1)(Junttila et al., 2007). CIP2A has been shown to promote cancer cell growth by stabilizing MYC (Junttila et al., 2007; Khanna et al., 2009). However, the mechanisms how CIP2A expression is induced during normal cell transformation into cancer cell have been obscure. Importantly, the *in vivo* significance of CIP2A in cancer formation has not been studied thus far.

This thesis study investigates the expression of CIP2A and the functional role of CIP2A in human breast cancer. Mechanisms mediating CIP2A induction during cell transformation are also investigated. Importantly, a mechanism describing how high expression of CIP2A and another oncoprotein, E2 transcription factor 1 (E2F1), are maintained in breast cancer is identified. Furthermore, we study the role of this mechanism in senescence induction and breast cancer development and progression. Finally, the clinical relevance of this mechanism in breast cancer therapy response is demonstrated.

2. REVIEW OF THE LITERATURE

2.1. Cancer

2.1.1. Biology of cancer

As a disease entity cancer is a group of various different types of diseases that share common features. The formation of human cancer is a multistep process where cells gain biological features that enables cells to grow and form tumors without limitations. In 2000, Hanahan and Weinberg proposed six hallmarks of cancer (Hanahan and Weinberg, 2000). First of all, cancer cells have unlimited potential to proliferate without extracellular mitogenic stimulus, enabling replicative immortality and capability to evade growth suppressing signaling (Hanahan and Weinberg, 2000). Cancer cells can also escape programmed cell death and induce angiogenesis to supply nutrients and oxygen to cancer cells in tumors (Hanahan and Weinberg, 2000). The final capability of the cancer cells is to invade surrounding tissues and further to form distant metastasis (Hanahan and Weinberg, 2000). Still over a decade later these hallmarks of cancer are widely accepted. Recently, these characteristics were updated with addition of two new hallmarks, ‘energy metabolism reprogramming’ and ‘evasion of immune destruction’ (Hanahan and Weinberg, 2011; Metzker, 2009).

2.1.1.1. Genetic alterations in cancer

The underlying mechanism for the above mentioned biological changes in cell function towards a more cancer-like phenotype, is genome instability (Hanahan and Weinberg, 2011). In normal cells, when mutations happen during cell growth or division, cellular maintenance systems detect these and prevent spontaneous mutations by driving damaged cells to programmed cell death, apoptosis, or irreversible cell cycle arrest, senescence (Houtgraaf et al., 2006). Apoptosis and also senescence, is a phenomenon that cells need to overcome in order to fully transform into cancer cells (Hanahan and Weinberg, 2011).

Nowadays, the genetic alterations in cancers has been widely studied due to well-developed next-generation sequencing (NGS) techniques to screen the cancer cell genome and compare it with normal cell genome (Metzker, 2009). In cancer, many proto-oncogenes are activated into oncogenes and tumor suppressors inactivated. Oncogenes are capable of promoting tumor growth, while tumor suppressors inhibit cell growth and proliferation. Alterations in gene expressions are cancer type specific. However, there are also certain driver genes, whose mutation is essential in the initiation of carcinogenesis of several cancer types. It is relevant to dissociate these from passenger genes,

which mutations do not bring growth advantages to cells. However, the functional component within cells are proteins and alterations in protein functions are not always due to mutation in protein coding gene. Proteins can be regulated also during translation from ribonucleic acid (RNA) to protein and by post-translational modifications, including phosphorylation and methylation, resulting in altered signaling pathway activity in cancer cells (Hanahan and Weinberg, 2011).

Studies using primary rodent cells implicated that alteration of only two pathways, such as Ras and MYC, can transform rodent cells into tumorigenic cells (Land et al., 1983). However, later studies revealed that more changes are needed to transform human cells. Over a decade ago, Hahn and colleagues characterized minimal alterations needed to transform human cells into cancer cells (Hahn et al., 1999a; Hahn et al., 2002). They found that activation of oncogenic active H-Ras and human telomerase catalytic subunit (hTERT) in combination with expression of simian virus 40 early region (SV40 ER) large antigen (LT) and small antigen (ST) is enough to transform different types of human cells into tumorigenic cancer cells (Figure 1)(Hahn et al., 1999a; Hahn et al., 2002). SV40 LT inactivates tumor suppressors retinoblastoma (RB) and p53 and ST inactivates protein phosphatase 2A (PP2A)(Ali and DeCaprio, 2001; Pallas et al., 1990; Yang et al., 1991). However, there are some cell type specificities in these requirements, such as in requirements of activation of Ras downstream effectors in different human cell types. For example, in addition to inactivation of p53, RB and PP2A and activation of TERT, human fibroblasts need activation of Raf and Ral-guanine nucleotide exchange factors (GEFs) and immortalized human mammary epithelial cells need activation of Raf, PI3K and Ral-GEFs in order to transform (Rangarajan et al., 2004).

Figure 1. Minimal genetic alterations required for human cellular transformation. Activated oncogenes are presented in red boxes and inactivated tumor suppressors in green boxes.

2.1.2. Cancer types

Cancer is a wide disease entity referring to multiple different kinds of cancer types and diseases. First of all, cancers can be grouped by the tissue where the primary tumor arises, such as breast or prostate. Secondly, cancer can be grouped by the cell type where they are originate. The most common type of cancers, are carcinomas arising from epithelial cells. Sarcomas are rare cancers forming from mesenchymal cells, including muscle and bone tissue. Cancers derived of hematopoietic cells are divided into leukemias and lymphomas. Neuroblastoma, glioma, glioblastomas and medulloblastomas are neuronal cancers arising from different neuronal cell types.

2.1.2.1. Breast cancer

As mentioned, breast cancer is the most common type of cancer affecting women worldwide (Jemal et al., 2011). Even though incidence for breast cancer has increased in many developed countries in past decades, mortality has decreased mainly due to earlier detection of tumors through mammography and due to improved therapies (Jemal et al., 2011). Also breast cancer is a heterogeneous disease, which can be further divided into subtypes via their histopatological and gene expression profiles (Perou et al., 2000; Polyak, 2007; Sorlie et al., 2001). The mammary gland forms from 15-20 lobus from each milk duct that leads to a nipple. Furthermore, each lobus consists of smaller milk secreting glands including lobules. 95% of the breast cancer cases are carcinomas, which can be further divided into ductal and lobular carcinomas (Yoder et al., 2007). Ductal carcinoma arise from epithelial in milk glandular ducts, whereas lobular carcinoma form from lobular epithelial (Yoder et al., 2007). Ductal and lobular carcinoma *in situ* are benign pre-stage forms of ductal and lobular carcinomas, respectively, but there is a high risk that *in situ* forms can progress into a malignant state (Yoder et al., 2007). Most breast cancer cases are ductal. However, lobular carcinomas have been associated with worse survival rates (Yoder et al., 2007). In addition to ductal and lobular carcinomas, also other invasive breast cancer types exist, such as tubular, medullary and inflammatory breast carcinomas (Malhotra et al., 2010).

Breast cancer subtypes

Breast cancers are divided into subgroups according to expression of hormonal proteins human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER) and progesterone receptor (PR). Most breast cancers are ER-positive and can be treated with selective estrogen receptor modulator, usually tamoxifen, and HER2-positive cancers can be treated with monoclonal antibody against HER2 protein, such as trastuzumab (Lin et al., 2010b).

Analyzing gene expression profiles of breast cancer cases has revealed that breast cancers can be divided into five subtypes: basal-like, luminal A and B, HER2+/ER- and normal breast-like (Hu et al., 2006; Perou et al., 2000; Sorlie et al., 2001). In molecular classification of breast cancer expression of a gene set shown to have the most variation between different tumors studied, were analyzed and compared with the gene expression pattern of normal breast components, mainly to basal and luminal epithelial cells (Perou et al., 2000; Sorlie et al., 2001). Basal-like and luminal tumor subtypes were shown to have similar gene expression patterns than basal and luminal epithelial cells (Perou et al., 2000). The normal breast-like tumor sub-group also has a more similar gene expression pattern than normal breast tissue (Perou et al., 2000). Later, the luminal subgroup was further divided into two groups, as it was noted that their gene expression pattern clusters into two groups: A and B (Sorlie et al., 2001). Luminal A and B types are estrogen-dependent, whereas HER2+/ER-

and basal like cancers are ER-independent (Lin et al., 2010b; Perou et al., 2000). Most of the basal like breast cancers are triple negative for ER, HER and PR (Lin et al., 2010b). Patients with different molecular subgroup breast tumors have been demonstrated by several studies to have differences in incidence, survival and treatment response (Prat and Perou, 2011). Luminal A tumor types are the most common type of breast cancers (Carey et al., 2006; Millikan et al., 2008). Patients carrying luminal A tumor type have the best prognosis and basal-like subgroup have the worst (Carey et al., 2006; Cheang et al., 2009; Hu et al., 2006; Sorlie et al., 2001). Another molecular subtype, a Caludin-low group, was later discovered (Prat et al., 2010). Most of the Claudin-low tumors are triple-negative, invasive ductal carcinomas with poor prognosis (Prat et al., 2010). Recently, novel breast cancer subtypes have been identified by using next-generation sequencing techniques (Curtis et al., 2012; Koboldt, 2012; Shah et al., 2012). In the near future, this information can be used to develop new targeted therapies for certain subtypes or to improve patient stratification for existing therapies (Ellis and Perou, 2013).

Genetic alterations in breast cancer

Even though alterations in oncogenes and tumor suppressors have been indicated in breast cancer, the etiologic events of breast cancer are still unclear. However, family history of breast cancer is a strong risk factor indicating inherited mutations of cancer related genes (Polyak, 2007; Walerych et al., 2012). Germ line mutations of tumor suppressor genes, *BRCA1* and *BRCA2,* are known to predispose women for breast and ovarian cancer (Miki et al., 1994; Powell and Kachnic, 2003; Wooster et al., 1995). Mutations in BRCA1 or BRCA2 occur in about 1% of all breast cancer cases (Shuen and Foulkes, 2011). BRCA1 and BRCA2 proteins are essential factors in repairing DNA damage in cells (Chen et al., 1999; Cortez et al., 1999; Nagaraju and Scully, 2007). Women with inherited mutations in *BRCA1* gene have been shown to develop predominately basal-like breast cancers (Carey et al., 2010). However, most basal-like breast cancer patients do not have germ-line mutations in *BRCA1* (Carey et al., 2010). In addition to *BRCA1* and *BRCA2*, mutations in several other genes have been associated with hereditary breast cancer, such as *CHEK2*, *PTEN*, *MAP3K1* and *CASP8* (Kenemans et al., 2004; Polyak, 2007)

Tumor suppressor gene, *p53*, is mutated both in spontaneous and in hereditary breast cancer (Forbes et al., 2011; Petitjean et al., 2007); (Olivier et al., 2010; Walerych et al., 2012). Previously, p53 mutations were identified in about 25% of breast cancer cases (Olivier et al., 2010; Petitjean et al., 2007). However, recent NGS studies have revealed that p53 is mutated in 12-80% of breast cancer cases depending on subtype (Koboldt, 2012). This indicates that p53 mutation is a driver of breast cancer. The presence of p53 mutation associates with more aggressive breast cancers, especially with HER2+/ER- and with basal-like breast cancer types (Langerod et al., 2007; Olivier et al., 2006;

Perou et al., 2000; Sorlie et al., 2001). In several studies, mutated p53 was shown to predict poor prognosis of breast cancer patients (Joensuu et al., 2006; Koboldt, 2012; Langerod et al., 2007; Lundin et al., 2001; Olivier et al., 2006; Ozcelik et al., 2007). Especially missense mutations in deoxyribonucleic acid (DNA) binding domain of p53 seems to be associated with poor survival of breast cancer patients (Olivier et al., 2006; Ozcelik et al., 2007).

HER2 is an oncogene that is overexpressed in about 20% of all breast cancer cases (Burstein, 2005; Osborne et al., 2004). A recent NGS study presented altered expression of HER2 in 15% of the invasive breast tumors (Koboldt, 2012). In most of the cases, *HER2* is overexpressed due to amplification of the gene, rarely by mutation (Burstein, 2005; Koboldt, 2012; Roy and Perez, 2009). HER2 overexpression leads to marked activation of proliferation and cell survival promoting signaling pathways, such as the Ras-Raf-MEK-ERK and the PI3K-Akt pathways (Chang, 2007). Moreover, HER2 overexpression has been identified to predict poor survival of breast cancer patients (Berchuck et al., 1990; Slamon et al., 1987; Yarden, 2001).

Overall, recent NGS studies have shown that in sporadic breast tumors several genes are mutated, but mainly at low frequency (Ellis et al., 2012; Ellis and Perou, 2013; Shah et al., 2012; Stephens et al., 2012). However, there are some genes that are mutated at higher frequency. In luminal type breast tumors, mutations are found most commonly in Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PI3KCA), Mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase (MAP3K1), Mitogen-activated protein kinase kinase 4 (MAP2K4), GATA binding protein 3 (GATA3), Myeloid/lymphoid or mixed-lineage leukemia 3 (MLL3) and Runt-related transcription factor 1 (RUNX1) (Banerji et al., 2012; Ellis et al., 2012; Koboldt, 2012; Stephens et al., 2012). Out of these genes, PI3KCA, MAP3K1 and MAP2K4 function in stress-induced PI3K-Akt pathway leading to cell death (Ellis and Perou, 2013). Additionally, other genes influencing this pathway are also mutated in luminal breast cancer at lower frequencies, indicating that alteration of the PI3K-Akt pathway is a driving event in luminal breast cancer (Ellis and Perou, 2013). In basal-like tumors, *p53, BRCA1, PI3KCA* and *phosphatase and tensin homolog* (*PTEN*) are mutated in relatively high frequency (Koboldt, 2012). Whereas, in HER2+/ER- tumors, the most frequently mutated genes are *PI3KCA, GATA3* and *p53* (Koboldt, 2012).

Nowadays, there is an emerging amount of information regarding gene and protein expression profiles in breast cancer and their correlation with response to different cancer therapies. This data serves a basis to find more tailored and hopefully more efficient therapy options for patients with different types of breast cancers.

2.2. Cellular senescence and cancer

2.2.1. Replicative and premature senescence

Terminal cell growth arrest, cellular senescence, was first identified in primary cells after multiple replications *in vitro* (Hayflick, 1976; Hayflick and Moorhead, 1961). This replicative senescence, Hayflick's limit, is associated with shortened telomeres and aging of cells. In senescence, cells stop proliferating despite available nutrition and mitogenic signalling and they arrest cell cycle progression (Campisi and d'Adda di Fagagna, 2007; Collado and Serrano, 2010; Kuilman et al., 2010).

In addition to replicative senescence, another type of senescence can occur in response to various stress stimuli, such as DNA damage, oxidative stress and acute expression of oncogenes or inactivation of tumor suppressors (Figure 2)(Ben-Porath and Weinberg, 2005; Kuilman et al., 2010). Originally, oncogene-induced senescence (OIS) was identified in mutant oncogenic H-Ras transfected human and mouse fibroblasts (Serrano et al., 1997). Later OIS and tumor suppressor inactivation-induced senescence have been reported in context of several different oncogenes and tumor suppressors in many tissue types both *in vitro* and *in vivo* (Collado and Serrano, 2010).

2.2.2. Pathways regulating senescence and biomarkers for senescence

During induction of senescence, major growth restricting pathways are activated. Classical pathway induced in senescence is p53-regulated signaling (Ben-Porath and Weinberg, 2005; Polager and Ginsberg, 2009). p53 activates its downstream effector p21 which further binds cyclin dependent kinases (Cdk) and inhibits activity of the cyclin E/Cdk2 complex resulting in activation of RB and inhibition of transcription factor E2F1 (Figure 2)(Ben-Porath and Weinberg, 2005; Polager and Ginsberg, 2009). ARF protein, induced in response to different stress stimuli such as acute expression of oncogenic Ras, inhibits negative regulator of p53, human homolog of double minute 2 (MDM2) (Ben-Porath and Weinberg, 2005; Ferbeyre et al., 2002; Polager and Ginsberg, 2009). However, p53 can also promote senescence without inducing downstream pathways via p21, as p21 deficient mouse embryonic fibroblasts (MEFs) undergo senescence similarly as wild type MEFs (Pantoja and Serrano, 1999). Another important regulator of senescence is $p16^{INK4a}$ which can inhibit the activity of cyclin D/Cdk4 or cyclin D/Cdk6 complex, resulting in activation of retinoblastoma (RB) protein and inhibition of E2F1 (Figure 2)(Ben-Porath and Weinberg, 2005; Kuilman et al., 2010). Similarly as ARF, $p16^{INK4a}$ is activated in different kind of stress situations (Lowe and Sherr, 2003). Notably, $p16^{INK4a}$-induced senescence seems to be more relevant in human cell senescence as compared to mice (Ben-Porath and Weinberg, 2005). Interestingly, oncogene MYC has been reported to induce OIS by activating both ARF-p53 and p16-RB pathways (Figure 2)(Campaner et al., 2010a).

Furthermore, Cdk2 can inhibit MYC-induced senescence by targeting both ARF-p53 and p16-RB pathways (Campaner et al., 2010a; Campaner et al., 2010b).

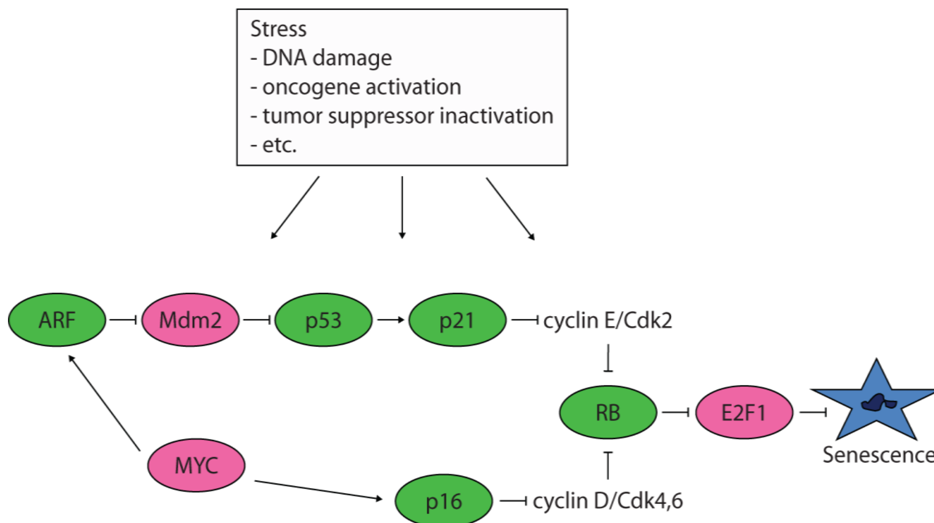

Figure 2. Pathways regulating premature senescence induction. Tumor suppressors are indicated with green and oncoproteins with red color.

In cell culture conditions, senescent cells often show clear morphological changes. Even though morphological changes are somewhat cell type specific, senescent cells generally become flattened, enlarged and multinucleated (Kuilman et al., 2010). In addition to morphological changes, there are several biomarkers that can be used to detect senescent cells *in vitro* and *in vivo*. As above mentioned pathways are activated in senescence, their gene and protein expression, especially p21 and p16, are used as markers for senescence. General DNA damage related proteins can also be used as a marker for stress-induced senescence. A classical biomarker for senescence is increased activity of senescence associated-β-galactoside (SA-β-gal) enzyme (Dimri et al., 1995). β-galactoside (β-gal) is a lysosomal enzyme that is detectable in normal cells at pH 4,0 and in senescent cells it is detected at pH 6,0 (Lee et al., 2006). In senescent cells, β-gal activity is likely increased due to increased lysosomal content and increased lysosomal β-gal expression (Lee et al., 2006). Notably, SA-β-gal expression is not needed for senescence as human fibroblast and cancer cells deficient for β-gal can be driven to senescence (Lee et al., 2006).

Another commonly used marker for senescence is senescence-associated heterochromatic foci (SAHF)(Narita et al., 2003). Senescent cells have been reported to accumulate heterochromatin structure *in vitro* and that SAHF was associated with repression of pro-proliferative E2F target genes (Narita et al.,

2003). However, SAHF seems to be highly cell type and context specific and promoted via p16-induced senescent signaling (Kosar et al., 2011).

Especially as a result of DNA damage induced senescence, cells have alterations in their secretome, termed senescence-associated secretory phenotype (SASP), which secrete several inflammatory cytokines and chemokines (Coppe et al., 2008; Rodier et al., 2009). Additionally, microarray analysis of senescent cells has revealed novel markers for senescence, such as proteins deleted in esophageal cancer 1 (DEC1) and decoy receptor 2 (DcR2)(Collado et al., 2005).

2.2.3. Senescence and cancer

Cellular senescence is a phenomenon that cells have to bypass in order to transform into cancer cells (Figure 3). In order for human cells to overcome senescence and immortalize, tumor suppressors p53 and RB have to be inactivated and oncogene hTERT activated, which is consistent with alterations that are needed for transformation of normal human cells into cancer cells (Figure 3)(Shay and Wright, 2005). However, in order for full transformation of human cells inactivation of PP2A is also required (Figure 3)(Hahn et al., 2002).

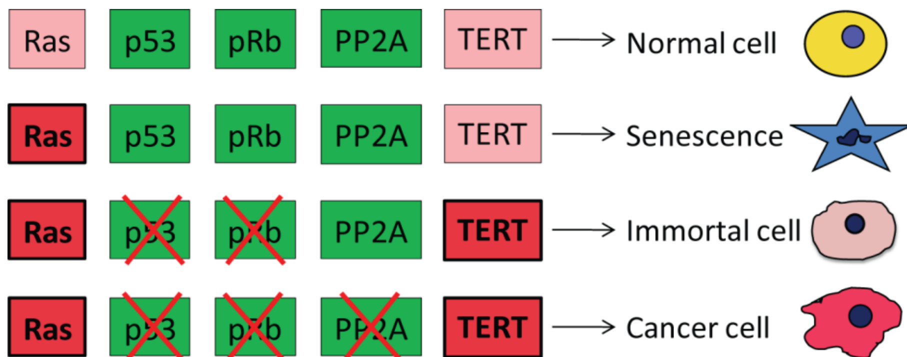

Figure 3. Premature senescence as a barrier in cellular transformation. Alteration in a single oncogene or tumor suppressor leads to senescence in human cells. Activated oncogenes presented in dark red boxes and inactivated tumor suppressors in green boxes with crosses.

Senescence has been indicated to oppose tumor formation in several human and mouse *in vivo* studies. In human naevi, oncogenic mutation in BRAF induces senescence and arrests malignant melanoma progression, which can be overcome via activation of PI3K pathway (Michaloglou et al., 2005; Vredeveld et al., 2012). Inactivation of tumor suppressor, Pten, inactivation was shown to trigger p53-dependent senescence in a prostate specific mouse model (Chen et al., 2005b). Similarly, in a mutant oncogenic *KRAS*-driven mouse model, multiple senescent markers are expressed in premalignant lung and pancreas tumors, whereas malignant lung and pancreas adenocarcinomas

have overcame senescence and proliferation is increased (Collado et al., 2005). In line, demethylbenzathracene (DMBA) and tetradecanoylphorbol 13-acetate (TPA) treatment-induced mouse skin papillomas, harbor oncogenic mutation in *H-RAS* and express senescent markers (Collado et al., 2005). Also, senescence has been detected in early stage human prostate cancer lesions, indicating that senescence is a restrictive phenomenon in human prostate carcinogenesis (Chen et al., 2005b). Additionally, in a lymphoma mouse model, Suv39h1 was shown to restrict lymphomagenesis by inducing senescence (Braig et al., 2005). Furthermore, in a HER2 expressing MMTVneu breast cancer mouse model, loss of heat shock protein 72 (Hsp72) was shown to suppress tumorigenesis by inducing senescence (Guy et al., 1992; Meng et al., 2011).

Invasion of epithelial cells to surrounding tissues is possible if cells transit from epithelial-like to mesenchymal-like cells, a phenomenon called the epithelial-mesenchymal transition (EMT)(Sanchez-Tillo et al., 2012; Thiery, 2002). Several proteins activate EMT in cancer cells, of which some have also been shown to regulate senescence. For example, Zinc finger E-box binding homeobox 1 (ZEB1), Twist1 and Twist2 (Sanchez-Tillo et al., 2012). ZEB1$^{-/-}$ MEFs have been shown to undergo senescence and additionally ZEB1 has been shown to be inhibited by p16-RB pathway activity (Liu et al., 2007; Liu et al., 2008b). Ansieau and colleagues have shown that inhibition of Twist2 in the MMTVneu breast cancer mouse model induced senescence (Ansieau et al., 2008). In addition, inhibition of both Twist1 and Twist2 induced senescence in human breast cancer cells (Ansieau et al., 2008). Furthermore, Twist1 and Twist2 can bypass Ras-induced senescence in primary human mammary epithelial cells (HMECs) and further promote EMT in HMECs (Ansieau et al., 2008). Taken together, senescence has been shown to restrict tumorigenesis in various different tumor types both in cancer mouse models and in human cancer.

2.2.3.1. Senescence as a cancer therapy target

Senescence has been shown to be induced in response to cancer therapies and consequently senescence induction has been implicated as a novel approach to develop new cancer therapies. Many chemotherapies and irradiation have been reported to induce senescence in both wild type p53 harboring and p53 deficient human cancer cell lines (Chang et al., 1999; Ewald et al., 2010; Gewirtz et al., 2008; te Poele et al., 2002). Some reports have also shown that therapy-induced senescence occurs in human tumors after chemotherapy, such as in breast cancer after neoadjuvant chemotherapy, including cyclophosphamide, doxorubicin and 5-fluorouracil (Ewald et al., 2010; te Poele et al., 2002). Notably, senescence was detected only in tumor cells not in normal breast tissue following treatment (te Poele et al., 2002). Importantly, these tumors in which senescence was induced, have better responses to chemotherapy as senescence in diagnostic colorectal cancer

patients predicted better outcome for combination therapy of 5-fluorouracil and leucovorin (Haugstetter et al., 2010). In contrast, in a lymphoma mouse model, doxorubicin treatment was reported to induce senescence and release of pro-survival factor into the microenvironment of lymphoid thymus leading to chemo-resistant sites in thymus (Gilbert and Hemann, 2010). Similarly in a breast cancer mouse model, doxorubicin-induced p53-mediated senescence protected tumor cells from chemotherapy (Jackson et al., 2012).

In addition to existing chemotherapies, there are several novel prospects on how to induce senescence in tumors. In contrast to oncogene induced senescence in normal cells, oncogene inactivation in cancer cells, such as MYC, may lead to senescence (Collado and Serrano, 2010). MYC inactivation *in vivo* induces p53- and p16-dependent senescence in lymphoma, osteosarcoma and hepatocellular carcinoma (Wu et al., 2007). Also, MYC inactivation has also been reported to suppress Ras-driven lung adenocarcinoma by inducing senescence (Soucek et al., 2008). Especially in MYC overexpressing tumors, Cdk2 is known to inhibit both p16- and p53-dependent senescence (Campaner et al., 2010a; Nardella et al., 2011).

Probably the most studied way to induce senescence in tumors is wild type p53 reactivation. p53 restoration represses lymphoma, sarcoma and liver tumors in mouse models, due to senescence induction in sarcoma and in liver, and due to induction of apoptosis in lymphoma (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). Several small molecule compounds which reactivate p53 in human cancer cells have been identified. Of which, some are currently being tested clinically (Frezza and Martins, 2012; Lane et al., 2010; Wade et al., 2013). Especially, Nutlin-3, which induces potent senescence by disrupting Mdm2-p53 interactions in cancer cells harboring wild type p53 (Brummelkamp et al., 2006; Van Maerken et al., 2006; Vassilev et al., 2004). In several different *in vivo* models Nutlin-3 has shown efficient tumor suppressive activity and it is under clinical studies (Kunkele et al., 2012; Sarek et al., 2007; Tovar et al., 2006; Vassilev et al., 2004). However, higher p53 mutation range in cancers detected with NGS compared to previously used methods has decreased number of cancer cases that could benefit from wild type p53 targeting therapies (Koboldt, 2012; Shah et al., 2012).

Paradoxically, senescence can also be induced in cancer cells by inactivating a tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (Pten)(Alimonti et al., 2010; Trotman et al., 2003). Pten inhibitor VO-OHpic inhibits Pten activity and activates PI3K-Akt pathway leading to p53-dependent senescence in human prostate cancer cells and tumor regression in a human xenograft model of prostate cancer (Alimonti et al., 2010). As many prostate tumors harbor one allele loss of Pten, tumor cells should be more sensitive for Pten inhibitor compared to normal cells making Pten inactivation a potential therapy approach (Nardella et al., 2011; Trotman et al., 2003).

Senescence can also be induced in tumors independently of p53. Lin and colleagues showed that inactivation of an oncoprotein S-phase kinase-associated protein 2 (Skp2) restricted total Pten-loss induced prostate tumorigenesis in mice by inducing p21- and p27-mediated senescence (Lin et al., 2010a). Also, inactivation of the Skp2-Stem cell factor (SCF) complex, with inhibitor MLN4924, was reported to suppress xenograft growth of p53-deficient human prostate cancer cells (Lin et al., 2010a). In melanoma, a transcription factor forkhead box M1 (FOXM1) was identified as a critical target of Cdk4 and Cdk6, which suppresses senescence (Anders et al., 2011). In the same study, inhibitor of Cdk4/6, was shown to down-regulate FOXM1 and induce senescence specifically in melanoma cells, harboring either wild type or mutant p53, but not in normal melanocytes (Anders et al., 2011). In fact, Cdk inhibitors are under investigation as potential anticancer therapies (Nardella et al., 2011).

Another potential senescence inducing therapy approach is to target telomerase complex, whose activation is necessary for cellular transformation (Hahn et al., 1999a; Hahn et al., 1999b; Mocellin et al., 2013). Small molecule inhibitors, immune therapy and gene therapy options are under studies to inhibit telomerase activity in cancer and some of them are in clinical trials (Harley, 2008; Mocellin et al., 2013).

2.2.3.2. Senescence, tumor dormancy and clearance of senescent cells

Tumor dormancy is a state during tumor development where tumor mass is not increased either by a balance between proliferating and dying cells or via reversible cell cycle arrest, quiescence, of tumor cells (Paez et al., 2012). However, tumor dormancy can also occur in response to cancer therapy enabling cancer cells to escape the treatment (Hensel et al., 2013; Paez et al., 2012). During recent years, it has been noted that dormant cells can also be reversible senescent in addition to being quiescent. Although senescence was originally defined as a irreversible growth arrest, it has been suggested that in under certain, thus far unclear, circumstances senescent cell arrest could be reversed to proliferative state (Campisi, 2013; Gordon and Nelson, 2012). This could be either due to inefficient induction of senescence program or due to inactivation of tumor suppressors and strong oncogenic signaling in senescent cells (Ewald et al., 2010; Gordon and Nelson, 2012; Kuilman et al., 2010). Also, it has been indicated that tumor cells could be driven to reversible senescence, in response to cancer therapies, leaving a possibility that cells can re-enter proliferation (Gewirtz et al., 2008; Wang et al., 2011b).

Interestingly, there is some evidence that tumor regression upon senescence induction is due to the immune response. Xue and colleagues showed that in mouse liver cancer, reactivation of p53 regressed tumor growth by inducing senescence and chemokine expression which triggered an innate immune response to target senescent cells resulting in tumor clearance (Xue et al.,

2007). Later, an intact immune system, and particularly $CD4^+$ T-cells, was demonstrated to be essential for clearance of senescent cells and for tumor suppression in mouse lymphoma, leukemia and in hepatocellular carcinoma models (Kang et al., 2011; Rakhra et al., 2010).

2.3. Signaling oncoproteins and tumor suppressors in cancer

2.3.1. Tumor suppressors in cancer: p53

2.3.1.1. p53 in different cellular functions

Regulation of p53

p53 is a transcription factor that was found more than 30 years ago by several research groups (DeLeo et al., 1979; Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero et al., 1979; Smith et al., 1979). p53 protein consists of five domains. Two N-terminal transactivation domains, needed to activate transcription of the p53 target genes (Kruse and Gu, 2009; Mirzayans et al., 2012). The central DNA binding domain and the C-terminal regulatory domain facilitates the binding of p53 to DNA (Kruse and Gu, 2009; Mirzayans et al., 2012). The C-terminal oligomerization domain is needed for tetramerization of the protein (Kruse and Gu, 2009; Mirzayans et al., 2012). In normal cells, p53 is expressed at low levels and upon stress signals, either from outside or inside the cell, p53 is activated inhibiting cell growth and proliferation (Hayon and Haupt, 2002; Mirzayans et al., 2012; Zilfou and Lowe, 2009).

A classical mechanism of how p53 is stabilized upon DNA damage is mediated by kinases, Ataxia telangiectasia mutated (ATM), Ataxia telangiectasia and Rad3-related (ATR), Checkpoint kinase 1 (Chk1) and Checkpoint kinase 2 (Chk2)(Kruse and Gu 2009). ATM and ATR can phosphorylate serine 15 and 37 residues of p53 (Banin et al., 1998; Tibbetts et al., 1999). ATM and ATR can also phosphorylate and activate Chk2 and Chk1, respectively (Chaturvedi et al., 1999; Liu et al., 2000). Chk1 and Chk2 can further phosphorylate and stabilize p53 on multiple serine and threonine residues (Mirzayans et al., 2012; Shieh et al., 2000). In addition to these mechanisms, many other kinases phosphorylate p53 in order to stabilize it and induce p53-mediated transcription after DNA damage (Kruse and Gu, 2009; Velez-Cruz and Johnson, 2012).

In addition to phosphorylation, p53 is regulated by many other post-translational modifications, such as acetylation, methylation and sumoylation, and importantly by ubiquitin-mediated proteasomal degradation (Kruse and Gu, 2009). In the absence of stress expression, p53 is tightly controlled in normal cells mainly by ubiquitin ligase human homolog of Mdm2 (Hayon and Haupt, 2002; Kruse and Gu, 2009; Michael and Oren, 2003; Mirzayans et al., 2012;

Momand et al., 2000). p53 can in turn directly bind to the *Mdm2* promoter and induce its transcription (Barak et al., 1993; Wu et al., 1993). Mdm2 protein is an E3 ubiquitin ligase which binds the N-terminal part of p53 and further ubiquitinates p53 (Honda et al., 1997; Kubbutat et al., 1997; Oliner et al., 1993). Thus, promoting degradation of p53 via the proteasome degradation pathway (Honda et al., 1997; Kubbutat et al., 1997; Oliner et al., 1993). Taken together, these results show that p53 negatively auto-regulates itself by promoting *Mdm2* expression. Additionally, Mdm2-related protein, MdmX, contributes to Mdm2-p53 regulation by hetero-dimerizing with Mdm2 and binding to p53 thus inhibiting transcriptional activity of p53 (Gembarska et al., 2012; Gu et al., 2002; Melo and Eischen, 2012; Sharp et al., 1999).

p53 inhibits growth signaling

As a result to stress stimuli, activated p53 can regulate multiple different cellular functions, such as apoptosis, senescence, DNA repair and autophagy, by affecting transcription of various genes. The role of p53 in senescence regulation was discussed in the previous section and below, two other p53-regulated cellular functions, cell cycle checkpoint activity and apoptosis, are described.

p53 has an essential role in regulating the cell cycle progression through the checkpoints in G1/S and G2/M of the cell cycle. p53 accumulation induces G1 arrest mainly by transactivation of *p21* (Brugarolas et al., 1995; Deng et al., 1995). p21 can inhibit cell cycle progression from G1 by several mechanisms. p21 inhibits cyclin-Cdk complexes whose activity is needed to release E2F proteins from the RB/E2F complex and further activate E2Fs to promote entering into the S phase of the cell cycle (Harper et al., 1993; Xiong et al., 1993). In contrast, p21 has also been shown to inactivate RB by promoting its degradation (Broude et al., 2007). In addition, p21 inhibits an important proliferation promoting protein, Proliferating cell nuclear antigen (PCNA)(Rousseau et al., 1999). Importance of p21 induction in G1 cell cycle arrest is pointed out in studies demonstrating that $p21^{-/-}$ mouse embryonic fibroblasts cannot undergo G1 arrest in response to DNA damage (Brugarolas et al., 1995; Deng et al., 1995). Furthermore, p21 can ensure that damaged cells do not enter mitosis by inhibiting cyclin B1, whose activity is needed for G2/M transition (Gillis et al., 2009). In addition to regulation through p21, p53 can also prevent cyclin B1-Cdc2 complex activity upon DNA damage by activating 14-3-3σ, thus preventing the cyclin B1-Cdc2 complex from entering to the nucleus (Chan et al., 1999).

p53 can promote apoptosis by inducing transcription of pro-apoptotic genes, such as *Tumor necrosis factor receptor superfamily, member 6* (*Fas*), *Tumor necrosis factor receptor superfamily, member 10b (Killer/DR5*), *Bcl-2 associated protein* (*BAX)*, *PMA induced protein 1* (*PMAIP1*, also named as *NOXA*) and *p53 up-regulated mediator of apoptosis* (*PUMA*)(Riley et al., 2008).

In addition to transactivation of pro-apoptotic genes, p53 can also accumulate in the cytoplasm and activate mitochondrial outer-membrane permeabilization (MOMP) (Dumont et al., 2003; Mihara et al., 2003). This further activates the mitochondrial apoptosis cascade, leading to caspase activation (Danial and Korsmeyer, 2004). The induction of MOMP is mediated by the B-cell CLL/lymphoma *2 (*Bcl-2) family of proteins, such as Bax and PUMA, whose transcript expression is also induced by p53 (Chipuk and Green, 2009).

2.3.1.2. p53 in cancer

A decade after p53 was discovered, wild type p53 was found to prevent oncogene-induced transformation of cells and both alleles of *p53* were found to be mutated in murine tumors (Eliyahu et al., 1989; Finlay et al., 1989). Important results to support this idea were notions that patients with Li-Fraumeni syndrome have a hereditable mutant allele of *p53* and that they all develop tumors; and that $p53^{-/-}$ mice develop tumors at their early age (Donehower et al., 1992; Malkin et al., 1990). Given the fact that p53 regulates multiple different cellular functions, inhibition of p53 is expected to be essential for cellular transformation. Nowadays, it is known that overall about 50% of all human cancers harbor mutated *p53* and tumors having wild type *p53* have gained other mechanisms to inhibit p53 (Junttila and Evan, 2009; Olivier et al., 2010; Robles and Harris, 2010).

Mutations of p53

Somatic mutations of *p53* are found in almost all kind of human cancer types, but there is an enormous tissue specificity in the mutation rate (Petitjean et al., 2007). The most *p53* mutation prone tumor sites are lung and ovary harboring *p53* mutation in 90% and 94% of tumors at these sites respectively (Bell, 2011; Hammerman, 2012). In addition, somatic *p53* mutations are more common in aggressive tumors, such as in Her2+ and in triple negative breast cancers, compared to breast cancer subgroups with more favorable outcomes (Koboldt, 2012; Langerod et al., 2007; Shah et al., 2012). Most of the *p53* mutants are missense mutants (73%), which is a point mutation of a single nucleotide leading to a coding of different amino acid, which may result in altered function of the protein (Petitjean et al., 2007). Other kinds of *p53* mutations are also found in tumors, such as nonsense, frameshifts, rarely silent, splice site, intronic mutations and large deletions (Petitjean et al., 2007). Importantly, mutations are most often found in the codons between 125-300, which almost totally represents the DNA binding domain of p53 (Petitjean et al., 2007). Despite the fact that several different codons in p53 are mutated, there are three hot spot codons, 175, 248, 273, that are commonly mutated in several different cancer types (Olivier et al., 2010). Importantly, there are several *p53* polymorphisms, a mutation that occurs in more than 1% of the people, of which few of them have been shown to be cancer-related (Whibley et al., 2009). As

an example, polymorphism in the *p53* codon 36 has been shown to promote degradation of *p53* messenger RNA (mRNA) and to abrogate p53's ability to induce apoptosis (Candeias et al., 2008). Although it has been thought that *p53* mutations are predominantly somatic, excluding rare Li-Fraumeni patients, it has been suggested that 2-3% or even 17%, of *p53* mutations could be germline mutations (Gonzalez et al., 2009; Lalloo et al., 2006).

Originally, loss of tumor suppressor gene activity was thought to require mutations in both of the gene alleles (Santarosa and Ashworth, 2004). However, there are emerging amount of evidence that even a loss of function mutation of one wild type allele (haploinsufficiency) of *p53* results in reduced p53 protein production and function (Rivlin et al., 2011; Santarosa and Ashworth, 2004). In addition, mutated p53 can have dominant negative function in cells. Mutated p53 can form a mixed tetramers with wild type p53 resulting in DNA binding deficiency and inhibition of transcription regulation (Oren and Rotter, 2010; Rivlin et al., 2011). However, heterozygous *p53* gene allele state is usually followed by a loss of heterozygosity (LOH), a loss of a wild type allele, resulting in a total inhibition of wild type p53 function (Rivlin et al., 2011; Santarosa and Ashworth, 2004).

In addition to inactivating p53 mutations, several studies have reported that mutated p53 has gain of function properties in cancer cells (Freed-Pastor and Prives, 2012; Oren and Rotter, 2010; Rotter, 1983; Walerych et al., 2012). Thus far mutated p53 has been shown to have a role in increasing cancer cell genomic instability, suppressing apoptosis and promoting cancer cell migration and invasion (Freed-Pastor and Prives, 2012; Oren and Rotter, 2010; Walerych et al., 2012).

Proteins inhibiting p53 in cancer

In addition to mutation of *p53*, it is also inactivated by other inhibitory mechanisms in human tumors, like altered functions of Mdm2, MdmX and ARF. Mdm2 has been found overexpressed in various human cancers including melanoma, colorectal cancer and in breast cancer (Wade et al., 2013). In many cancer types, *Mdm2* is amplified (Gannon and Jones, 2012; Wade et al., 2013). Single nucleotide polymorphisms (SNP), SNP309G, in the Mdm2 promoter, have also been found in human tumors, enhancing Mdm2 expression to inhibit p53 activity and further increasing the risk of sarcomas and breast cancer (Bond et al., 2004). MdmX is also overexpressed in various human cancer types (Marine, 2011; Wade et al., 2013). For example, 19% of breast carcinomas were shown to overexpress MdmX and importantly *MdmX* amplification was shown to correlate with wild type p53 status and with normal copy number of the *Mdm2* gene (Danovi et al., 2004). Tumorigenic role of Mdm2 and MdmX have been verified in transgenic mouse models overexpressing either *Mdm2* or *MdmX* and both models develop spontaneous tumors similar to those seen in p53$^{-/-}$ mice (Jones et al., 1998; Xiong et al.,

2010). p53 is mutated at low frequency in melanoma, whereas MdmX is up-regulated in large proportions (Gembarska et al., 2012). Furthermore, MdmX has been shown to promote melanoma carcinogenesis *in vivo* by inhibiting induction of p53-driven apoptosis (Gembarska et al., 2012).

p14 alternative reading frame (ARF) protein, is an alternative reading frame product of *CDKN2A* gene and a tumor suppressor (Quelle et al., 1995). ARF is not an isoform of p16, another protein encoded by *CDKN2A*, and they have different functions. ARF positively regulates p53 activity by interacting with Mdm2 and by inhibiting Mdm2-mediated ubiquitination and proteasomal degradation of p53 (Honda and Yasuda, 1999; Stott et al., 1998). A ubiquitin ligase ARF-binding protein/Mcl1-ubiquitin ligase E3 (ARF-BP1/Mule) has also been identified to directly bind and ubiquitinate p53 (Chen et al., 2005a). Moreover, inactivation of ARF-BP1 has been shown to be essential for ARF-mediated stabilization of p53 (Chen et al., 2005a). In human cancer, homozygous deletion of *ARF* encoding locus, *INK4a/ARF*, is common (Ozenne et al., 2010; Sharpless, 2005). ARF is silenced also by other mechanisms in cancer, such as its promoter methylation (Ozenne et al., 2010). Similarly as $p53^{-/-}$ mice, $ARF^{-/-}$ mice develop spontaneous tumors at early age (Kamijo et al., 1999). Although $ARF^{-/-}$ mice develop sarcomas as also $p53^{-/-}$ mice do, 28% of $ARF^{-/-}$ mice develop carcinomas and tumors of the nervous system that are infrequently observed in $p53^{-/-}$ mice (Kamijo et al., 1999).

2.3.1.3. p53 reactivation as a cancer therapy

Several mouse models have indicated the efficacy of p53 reactivation in tumor suppression (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). Importantly, p53 reactivation has been shown to suppress malignant tumor growth but not low grade tumors (Feldser et al., 2010; Junttila et al., 2010). Multiple p53 targeting therapy approaches have been developed during past years. The major approach for p53 reactivation, is via inhibition of the interaction between MDM2 or MDMX and p53 (Wade et al., 2013). Small molecules such as Nutlin-3, MI-219 and reactivation of p53 and induction of *tumor* cell apoptosis (RITA), are able to release p53 from inhibition in cancer cells expressing wild type p53 (Issaeva et al., 2004; Shangary et al., 2008; Vassilev et al., 2004). However, a large number of cancers express mutated p53, which has inspired to identify strategies to reactivate mutated p53 in cancer cells, such as p53-dependent reactivation of massive apoptosis (PRIMA-1) and very recently stictic acid (Bykov et al., 2002; Lane et al., 2010; Wassman et al., 2013). PRIMA-1 is able to restore DNA binding capacity of p53 leading to transcriptional transactivation of p53 and further apoptosis in p53 mutant cancer cells (Bykov et al., 2002). Stictic acid can bind to the newly identified binding pocket of p53 more potently than PRIMA-1 resulting in induction of p53 targets *p21* and *PUMA* expression in p53 mutant osteosarcoma cells (Wassman et al., 2013). In addition, two thiosemicarbazone compounds have recently been reported to significantly

restore wild type p53 function and induce apoptosis in the $p53^{R172H}$ mutant mouse model and inhibits xenograft growth of cancer cells harboring mutant p53 (Yu et al., 2012).

Interestingly, several studies have implicated p53's role in tumor suppression by inducing senescence rather than apoptosis or growth arrest *in vivo* (Brady et al., 2011; Chen et al., 2005b; Cosme-Blanco et al., 2007; Dankort et al., 2007). Nutlin-3 has been reported to reduce tumor growth by inducing p53-dependent senescence in several cancer types, such as in melanoma, neuroblastoma and carcinomas (Verhaegen et al., 2012) (Arya et al., 2010; Lehmann et al., 2007; Van Maerken et al., 2006). However, in some cancer types, Nutlin-3 has also been shown to induce apoptosis (Manfe et al., 2012; Villalonga-Planells et al., 2011; Wang et al., 2012; Ye et al., 2012). Due to high p53 mutation frequency in human cancers, activation of p53 downstream effectors could be a more effective approach for senescence induction in tumors. For instance, induction of p21-mediated senescence has been shown to efficiently reduce tumor growth *in vivo* without inducing p53 (Lin et al., 2010a; Ruan et al., 2012). Taken together, there is an urgent need to identify proteins that could be targeted in order to induce p53-independent senescence in tumors.

2.3.2. Tumor suppressors in cancer: PP2A

2.3.2.1. Stucture and regulation of PP2A

Highly conserved protein phosphatase 2A (PP2A) is responsible for a large fraction of the serine/threonine phosphatase activity in cells, largely by its numerous cellular targets (Eichhorn et al., 2009; Janssens et al., 2008; Kalev and Sablina, 2011). PP2A heterotrimeric holoenzyme, consists of a catalytic subunit C, a structural subunit A and a regulatory subunit B (Janssens et al., 2008; Sents et al., 2012). The A and C subunits both have two isoforms, α and β, and both are encoded by two different genes, PPP2R1A and PPP2R1B for the A and PPP2CA and PPP2CB for the C subunit (Figure 4)(Eichhorn et al., 2009; Janssens et al., 2008; Sents et al., 2012). Notably, PP2A Cα knock-out mice are not viable, indicating the importance of PP2A in various cellular functions (Gotz et al., 1998). Subunits A and C forms PP2A's core enzyme ($PP2A_D$) and PP2A exists also in a dimeric complex in cells (Eichhorn et al., 2009; Janssens et al., 2008; Sents et al., 2012). However, the majority of PP2A is in heterotrimeric complex consisting dimeric $PP2A_D$ and a regulatory B subunit (Sents et al., 2012). Theoretically, 96 different PP2A holoenzyme complexes can exist in human cells, due to the great amount of different regulatory B subunits (Figure 4)(Janssens et al., 2008; Sents et al., 2012). Different B subunits have tissue specific expressions, as well as different subcellular localizations (Eichhorn et al., 2009; Janssens and Goris, 2001; Janssens et al., 2008; Kalev and Sablina, 2011). In addition, substrate specificity and PP2A phosphatase activity is determined by holoenzyme

composition and especially by regulatory B subunits (Eichhorn et al., 2009; Kalev and Sablina, 2011; Sents et al., 2012).

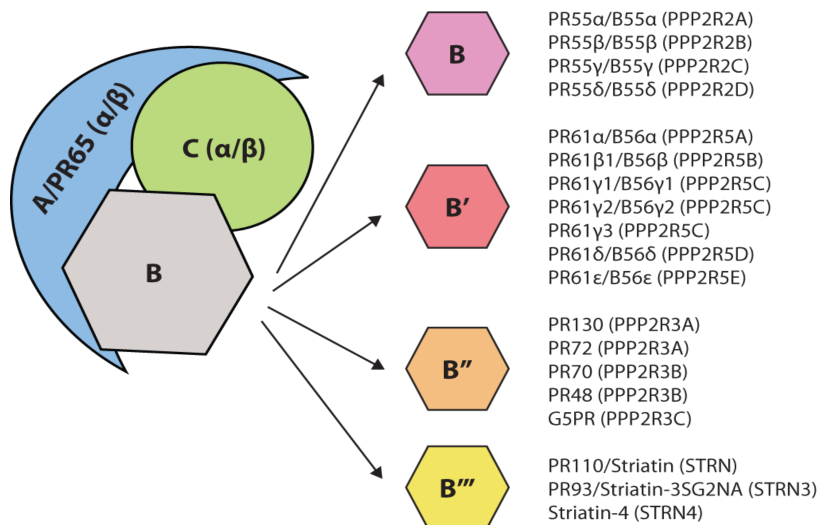

Figure 4. Structure of PP2A. Nomenclature of B subunits is presented.

In addition to different B subunit composition, PP2A activity is regulated by post-translational modifications and by interacting proteins. Catalytic subunit PP2A (PP2A C) has been shown to be phosphorylated on threonine 304 and tyrosine 307 residues and these phosphorylations seems to inactivate PP2A activity (Chen et al., 1992; Schmitz et al., 2010). These phosphorylations seem to also play a role in selecting a B subunit to bind the PP2A dimer. Phosphorylation of tyrosine 307 of C subunit seems to inhibit formation of holoenzyme with PR61 subunit family, whereas threonine 304 phosphorylation can inhibit binding of PR55 subunit family to the dimer enzyme (Chen et al., 1992; Longin et al., 2007). Moreover, PP2A has been reported to activate its phosphatase activity by auto-dephosphorylating tyrosine and threonine residues of catalytic subunit, meaning that PP2A seems to also have tyrosine phosphatase activity (Chen et al., 1992; Guo and Damuni, 1993). Some studies have also reported phosphorylations towards PR61 B subunits which result in alteration of PR61-PP2A holoenzyme assembly and PP2A activity (Letourneux et al., 2006; Margolis et al., 2006). Recently, in neuronal cells, phosphorylation of B55β serine residues 20, 21 and 22 was shown to regulate localization of B55β between the cytosol and mitochondria further regulating neuronal cell death (Merrill et al., 2012).

Another crucial post-translational modification of PP2A is reversible methylation. PP2A catalytic subunit is methylated on C-terminal leucine 309 residue by leucine carboxyl methyltransferase 1 (LCMT1) and reversed by a methylesterase protein phosphatase methylesterase 1 (PME-1) (Figure 5)(De Baere et al., 1999; Ogris et al., 1999; Westermarck and Hahn, 2008). The

methylation of PP2A C leucine 309 residue plays a role in regulating holoenzyme assembly as methylation has been shown to be required for PR55 subunit recruitment on the PP2A dimer (Ikehara et al., 2007). This methylation also increases the activity of PP2A and in contrast, demethylation of PP2A C by PME-1 and binding of PME-1 to catalytic active site of PP2A results in inhibition of PP2A phosphatase activity (Hombauer et al., 2007; Lee and Stock, 1993; Xing et al., 2008). When the crystal structure of PME-1 alone and in complex with PP2A core dimer was studied, it was noted that PME-1 actually has a dual role in regulating PP2A activity (Xing et al., 2008). PME-1 binding to the active site of PP2A can lead either to the activation of PME-1 and demethylation of PP2A C or removal of manganese ions from PP2A that are necessary for phosphatase activity (Xing et al., 2008). However, PME-1-mediated inhibition of phosphatase activity of PP2A can be reversed by phosphotyrosyl phosphatase activator (PTPA)(Figure 5)(Cayla et al., 1994; Longin et al., 2004). Furthermore, inhibition of PTPA can transform immortalized human cells (Sablina et al., 2010). Contradictory results have been shown in human cancer cells where silencing of PTPA was reported to induce apoptosis (Fellner et al., 2003).

2.3.2.2. Tumor suppressor activity of PP2A

The tumor suppressor role of PP2A was discovered in experiments using okadaic acid, a marine-sponge toxin shown to selectively inhibit phosphatase activity of PP2A, to induce progression of mitosis and to promote tumor growth in mice (Bialojan and Takai, 1988; Gliksman et al., 1992; Suganuma et al., 1990). Although the inhibition of PP2A is not essential for transformation of mouse cells, PP2A also functions as a tumor suppressor in mice as a knock-in mice carrying mutant PP2A Aα significantly promotes tumor formation in lungs (Ruediger et al., 2011). Importantly, inhibition of PP2A has been demonstrated as a prerequisite for the cellular transformation of human cells into cancer cells (Chen et al., 2007; Hahn et al., 2002; Sablina et al., 2007; Sablina et al., 2010). In line with these data, PP2A is inhibited by several viral oncoproteins, adenovirus early region 4 open reading frame 4 (E4orf4), polyomavirus small antigen (PyST) and middle antigen (PyMT) and simian virus 40 small antigen (SV40 ST)(Pallas et al., 1990; Shtrichman et al., 1999). Out of these viral proteins, SV40 ST is especially interesting as it was shown to bind to PP2A by displacing B subunits from the PP2A dimer (Figure 5)(Chen et al., 2007; Cho et al., 2007). In addition, SV40 ST, and specifically the PP2A binding domain, can also transform human fibroblasts together with oncogenic active Ras, SV40 large antigen (LT), which inhibits both RB and p53, and hTERT (Hahn et al., 2002). For these reasons, ST is widely used in structural and functional PP2A studies.

As PP2A is a major phosphatase of serine phosphorylated proteins in cells, it is not unexpected that PP2A activity is involved in almost all critical cellular functions. Recently, it was shown that in cellular transformation, the importance

of inactivation of PP2A was linked to its ability to induce certain oncogenic signaling pathways (Sablina and Hahn, 2008; Sablina et al., 2010). In fact, inhibition of B56α, B56γ, PR72/PR130 and PTPA regulatory units were able to transform human cells similarly as SV40 ST (Sablina et al., 2010). Furthermore, perturbation of PP2A complexes containing B56α, B56γ and PR72/PR130 led to activation of MYC, wingless-type MMTV integration site family (WNT) and PI3K/Akt pathways (Sablina et al., 2010). In addition to these pathways, PP2A tumor suppressor activity has been linked to many other cellular functions, such as regulation of Ras-Raf-MEK-ERK pathway and apoptosis (Sablina and Hahn, 2008; Westermarck and Hahn, 2008).

PP2A tumor suppressor role in regulating proliferation

One of the well characterized tumor suppressive roles of PP2A is to regulate the Ras-Raf-MEK-ERK pathway. This pathway is a classical mitogen activated protein kinase (MAPK) pathway which generally mediates extracellular growth stimulus from the cell surface to the nucleus where it promotes the transcription of proliferation promoting genes (Roberts and Der, 2007). Overexpression of SV40 ST can activate MAPK and further induce its target gene AP-1 (Alberts et al., 1993; Frost et al., 1994). In cancer, the Ras-Raf-MEK-ERK signaling cascade is usually constitutively active (Roberts and Der, 2007). However, PP2A can both inhibit and promote this pathway depending on the cell type and context (Eichhorn et al., 2009). PP2A can dephosphorylate and inactivate kinases MEK and ERK (Sontag et al., 1993; Westermarck et al., 1998). Especially PP2A complexes including either B55α, B55δ, B56β and B56γ has been reported to inactivate ERK1 and ERK2 (Letourneux et al., 2006; Van Kanegan et al., 2005). PP2A can also inactivate an adapter protein Shc, which mediates the proliferation promotion signal from cell surface receptors to Ras (Ugi et al., 2002). Interestingly, by dephosphorylation PP2A complex including B55α or B55δ can re-activate Raf-1 and promote MEK-ERK signaling (Abraham et al., 2000; Adams et al., 2005; Dougherty et al., 2005). Additionally, B55γ containing PP2A can suppress c-SRC activity, shown to promote the Raf-MEK-ERK pathway by inducing Raf-1 activity without altering Ras protein expression (Chao et al., 1997; Stokoe and McCormick, 1997).

In addition to the MAPK cascade, PP2A can inhibit proliferation by suppressing oncoprotein MYC (Yeh et al., 2004). Inhibition of the PP2A B subunits which are essential for human cell transformation, B56α, PR72/PR130, and PTPA, as well as SV40 ST can induce MYC expression (Sablina et al., 2010). Previous studies demonstrated that B56α-containing PP2A enzyme can dephosphorylate serine 62 residue of MYC leading to decreased stability of MYC (Arnold and Sears, 2006; Yeh et al., 2004). Phosphorylation of serine 62 and threonine 58 residues are essential regulators of MYC stability (Lutterbach and Hahn 1994; Sears et al. 1999). ERK kinase has been reported to phosphorylate serine 62 of MYC upon Ras activation and in turn phosphorylation of threonine 58 is mediated by Glycogen synthase kinase 3

(GSK3)(Sears et al., 2000; Welcker et al., 2004). Accordingly, inhibition of B56α induces expression of both total and serine 62-phosphorylated MYC and can induce activity of MYC towards its target genes (Arnold and Sears, 2006). Recently, B55α and B56β containing PP2A complexes were identified to mediate dephosphorylation of serine 62 MYC in HeLa cells (Niemelä et al., 2012). Additionally, PP2A can inhibit phosphorylation of serine 62 MYC by inhibiting MEK-ERK and the Akt pathway, known to promote phosphorylation of serine 62 MYC (Henriksson et al., 1993; Pulverer et al., 1994; Puustinen et al., 2009; Westermarck et al., 1998).

Another level of MYC regulation by PP2A is mediated by WNT signaling (Eichhorn et al., 2009). Unperturbed WNT signaling is an important regulator of embryogenesis. In adult cells perturbed WNT signaling can also lead to increased proliferation and tumorigenesis (Barker and Clevers, 2000; Eichhorn et al., 2009). Especially, B56 subunits containing PP2A holoenzymes, which have been shown to both positively and negatively regulate WNT signaling (Eichhorn et al., 2009). Oncoprotein β-catenin, a central mediator of WNT signaling, is widely expressed in human cancer (Jamieson et al., 2012). Importantly, activation of β-catenin is followed by transformation of human cells in cooperation with inhibition of B56γ (Sablina et al., 2010).

PP2A regulates cell fate

In addition to MYC and WNT signaling, inhibition of total PP2A activity by depletion of either Cα, B56γ or PTPA was found to increase the phosphorylation of Akt and to transform immortalized human cells (Sablina et al., 2010). Other studies have also indicated that PP2A inhibits the cell survival by suppressing PI3K-Akt pathway (Yuan et al., 2002; Zhao et al., 2003). B55α containing PP2A has been shown to dephosphorylate threonine 308 residue of Akt leading to decreased Akt activity and proliferation (Kuo et al., 2008). Importance of PP2A-mediated Akt regulation has been implicated also in transformation experiments with mammary epithelial cells, where PP2A inhibition can be replaced by activation of the PI3K-Akt pathway (Zhao et al., 2003)

In addition to B subunit specific functions, Aβ containing PP2A complex can dephosphorylate small GTPase RalA, leading to inhibition of RalA activity (Sablina et al., 2007). RalA regulates multiple signaling pathways and cellular functions, such as apoptosis and cell migration (Sablina et al., 2007). RalA activity was also shown to be required for cellular transformation associated with Aβ PP2A inhibition (Sablina et al., 2007).

PP2A can also influence cell fate by regulating apoptosis machinery (Janssens and Rebollo, 2012; Van Hoof and Goris, 2003). PP2A regulates apoptosis on many levels and this seems to also be caused by different holoenzyme compositions (Janssens and Rebollo, 2012; Van Hoof and Goris, 2003).

PP2A in senescence

Considering the role of PP2A as a tumor suppressor, PP2A could be expected to positively regulate senescence. In fact, in cervical cancer HeLa cells, PP2A C subunit has been shown to mediate histone deacetylase 1 (HDAC1) overexpression-induced proliferation inhibition and senescence (Chuang and Hung, 2011). In this study, HDAC1 overexpression repressed HeLa cell xenograft tumor growth and HDAC1 was reported to induce transcription of PP2A C, accelerate PP2A phosphatase activity towards RB, resulting in decreased proliferation and induction of senescence (Chuang and Hung, 2011). In melanoma B56α protein expression was down-regulated as compared to normal human melanocytes and overexpression of B56α in melanoma cells induced senescent phenotypes (Mannava et al., 2012). Mannava and colleagues also showed that B56α depletion can rescue oncogenic BRaf- and NRas-induced senescence in human melanocytes (Mannava et al., 2012). Inhibition of B56α seems to have an important role in transformation of melanocytes into malignant melanoma as B56α was shown to be significantly more frequently expressed in benign nevi, than in malignant melanoma tissue (Mannava et al., 2012).

In contrast to above mentioned results, there are some evidence that PP2A inhibits senescence induction. First, okadaic acid has been reported to induce senescent phenotype in human prostate cancer cells measured with senescence associated β-galactoside staining and with flattened cell morphology (Park et al., 2007). Inhibition of PP2A C was also reported to be essential in oxidative stress induced senescence in lungs (Volonte and Galbiati, 2009). Upon oxidative stress Caveolin-1 was shown to translocate PP2A C from ATM and inhibit PP2A mediated ATM dephosphorylation, leading to activation of ATM-p53-p21 pathway and senescence (Volonte and Galbiati, 2009).

Recently, EMT inducers, TWIST1, ZEB1 and ZEB2, were shown to transform human mammary epithelial cells in cooperation with oncogenic HRas and also inhibit PP2A phosphatase activity (Morel et al., 2012). Previously, TWIST proteins and ZEB proteins were shown to prevent oncogene induced senescence (Ansieau et al., 2008; Kwok et al., 2007; Ohashi et al., 2010). Therefore, it is likely that inhibition of PP2A activity is involved in malignant conversion of tumors by EMT and EMT-induced senescence inhibition.

Mutations of PP2A in cancer

Several different mutations in PP2A subunits have been found in many kinds of human cancers, but mainly at low frequency, indicating that other inhibitory mechanisms are more essential. Mutations in Aα have been reported mainly in ovarian and endometrial cancer (McConechy et al., 2011; Zhao et al., 2013). Mutations in Aβ have been found in lung tumors, colon tumors and in colorectal

cancer (Takagi et al., 2000; Wang et al., 1998). Mutations in B subunits have been found mainly in *PP2R2A* encoding B55α (Cheng et al., 2011; Curtis et al., 2012; Mosca et al., 2013).

2.3.2.3. PP2A inhibiting proteins in cancer

In addition to viral proteins inhibiting PP2A, endogenous proteins have also been identified in cells that are capable to inhibit activity of PP2A including ANP32A, SET, PME-1, CIP2A, endosulfine alpha ENSA and cAMP-regulated phosphoprotein, 19kDa (ARPP19) (Figure 5). Most of these proteins have been found overexpressed in human cancers and inhibition of these proteins provides a potential anti-cancer approach. CIP2A, as a PP2A inhibitor and its other functions within cells will be discussed in chapter 2.3.2.1.

SET and ANP32A

PP2A inhibitors SET and ANP32A were originally extracted from bovine (Li et al., 1995). However, it has been shown that under certain circumstances these proteins can also inhibit protein phosphatase 1 (PP1)(Katayose et al., 2000). Notably, it was shown that SET is a truncated version from previously known protein SET which also inhibits PP2A (Li et al., 1996). Overexpression of *SET* has been reported in various human cancers, including testicular cancer, head and neck squamous cell carcinoma, lung cancer and childhood form of kidney cancer, Wilm’s tumor (Carlson et al., 1998; Ginos et al., 2004; Irie et al., 2012; Korkola et al., 2006; Sun et al., 2006; Westermarck and Hahn, 2008). SET is also overexpressed in different kinds of leukemia (Andersson et al., 2007; Fornerod et al., 1995; Westermarck and Hahn, 2008). Importantly, in acute myeloid leukemia and in chronic lymphocytic leukemia SET overexpression predicts poor survival in patients (Christensen et al., 2011; Cristobal et al., 2012). In non-lymphocytic leukemia, SET was found to be fused with Nup214 protein (Kalev and Sablina, 2011; Li et al., 1996; von Lindern et al., 1992). In addition, in leukemogenesis of chronic myelogenous leukemia (CML), PP2A inhibition has been shown to result from activation of oncogenic BCR/ABL which induce expression of SET (Neviani et al., 2005). Taken together, these results suggest that inhibition of PP2A by SET plays a relevant role in induction and progression of leukemia and some solid cancers.

In lung tumors, SET inhibiting sphingosine analogue drug, FTY720, promoted PP2A reactivation and PP2A activity towards pro-necrotic serine/threonine kinase, RIPK1 (Saddoughi et al., 2013). Notably, FTY720 induced lung cancer cell death by inducing necrosis, but not apoptosis and further suppressed tumor growth of lung cancer cells in a xenograft model (Saddoughi et al., 2013). Additionally, in HeLa cells, SET can induce MEK-ERK signaling cascade and inhibit Fas-mediated apoptosis (Harmala-Brasken et al., 2003). In contrast, another study demonstrated that SET might inhibit MEK-ERK

mediated cell proliferation (Fukukawa et al., 2005). Additionally, SET overexpression was shown to increase phosphorylation of c-jun serine 63 and threonine 73 residues and activity of transcription factor, AP-1, a dimer of c-jun and c-fos (Al-Murrani et al., 1999). Interestingly, Switzer and colleagues suggested that SET is a relevant cancer therapy target based on their findings that a novel SET inhibitor, COG112, an apolipoprotein-E mimetic peptide, inhibits multiple oncogenic functions of SET in human glioblastoma and in breast cancer cell lines (Christensen et al., 2011; Switzer et al., 2011). In this study, COG112 was reported to promote PP2A activity towards dephosphorylation of Akt activity and c-Myc, to increase metastasis suppressor nm23-H1 activity and to inhibit migration and invasion of cancer cells by releasing Rac1 from SET (Switzer et al., 2011). In summary, SET has multiple oncogenic targets, mainly PP2A-mediated and appears to have cancer specific functions.

ANP32A has a contradictory role in regulating cell functions. Phosphorylation of ANP32A has been found to disassociate it from PP2A leading to elevated PP2A activity and suppression of ERK signaling pathway (Yu et al., 2004). In contrast, ANP32A also has tumor suppressor activity as it can inhibit oncogene-induced cell transformation (Bai et al., 2001; Chen et al., 1996). In line with this notion, ANP32A expression is decreased in prostate and pancreatic cancers (Brody et al., 2007; Schramedei et al., 2011).

PME-1

As mentioned previously, PME-1 demethylates PP2A catalytic subunit and inactivates phosphatase activity of PP2A (Lee et al., 1996; Lee and Stock, 1993; Xing et al., 2008). Taken into account the inhibitory role of PME-1, it is expected that PME-1 would be expressed in various human cancers. In glioma, PME-1 is overexpressed and correlates positively with increased malignant grade of glioma (Puustinen et al., 2009). Mechanistically, PME-1 was shown to demethylate PP2A C in glioblastoma cells and inhibition of PME-1 was found to inhibit phosphorylation of ERK via PP2A activity (Puustinen et al., 2009). In line with these findings, expression of demethylated form of PP2A C subunit and PP2A activity was decreased in PME-1$^{-/-}$ mouse brain, liver and heart, when compared to wild type mice (Ortega-Gutierrez et al., 2008). In addition, Longin and colleagues reported that PME-1 overexpression in human cancer cells increases demethylation of PP2A C subunit (Longin et al., 2008). Moreover, taking into account facts that PME-1-/- mice died the day after birth due to severe breathing and suckling malfunctions and that PME-1-mediates PP2A C demethylation both in normal mouse brain tissues and in human glioblastoma cells, PME-1 seems to have a specific role in regulating PP2A demethylation and activity of PP2A during formation of brain tumors (Ortega-Gutierrez et al., 2008; Puustinen et al., 2009).

Recently, reduced PP2A C methylation either by PME-1 overexpression or by LCMT-1 inhibition, was reported to promote full transformation of immortalized human cells that have been weakly transformed by down-regulation of PP2A B56γsubunit (Jackson and Pallas, 2012). Mechanistically, transformation was reported to result from increased Akt and p79/p85 S6 kinase (S6K) pathway activity, previously known PP2A-targeted survival promoting pathways (Jackson and Pallas, 2012). Identification of two specific inhibitors of PME-1 demethylation activity, ABL127 and AMZ30, have been reported and most likely these will be used to study the role of PME-1 in human cancer in a more detail (Bachovchin et al., 2011; Zuhl et al., 2012).

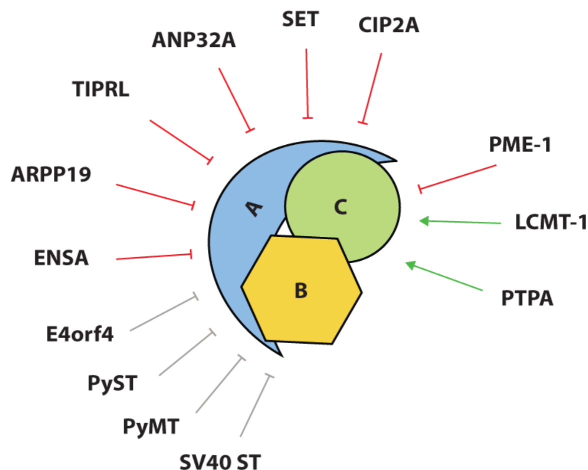

Figure 5. Proteins regulating function of PP2A. Red (bar-headed lines) indicates endogenous and grey bar-headed lines indicate viral PP2A inhibiting proteins. Green (arrow-headed lines) indicate positive regulators of PP2A.

ENSA, ARPP19 and TIPRL

Endosulfine alpha (ENSA) and cAMP-regulated phosphoprotein, 19kDa (ARPP19) have been shown to promote mitosis by inhibiting PP2A (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). Both of these proteins are phosphorylated by Greatwall kinase which enables them to bind PP2A and inhibit PP2A's mitotic substrates (Gharbi-Ayachi et al., 2010; Lorca and Castro, 2013; Mochida et al., 2010). Despite their growth inhibiting role in mitosis the role of ENSA and ARPP19 in cancer cells remains obscure.

TOR signaling pathway regulator-like (TIPRL) was initially found to interact with PP2A-like phosphatases in yeast (Gingras et al., 2005). Later it was verified to also interact with PP2A in mammalian cells (McConnell et al., 2007; Smetana and Zanchin, 2007). TIPRL has been reported to have an oncogenic role in human hepatocellular carcinoma (HCC) and TIPRL is overexpressed in HCC (Song et al., 2012). Mechanistically, TIPRL has been shown to inhibit PP2A-mediated apoptosis in HCC cells in cooperation with TRAIL (Song et al., 2012). Importantly, inhibition of TIPRL and TRAIL expression decreased tumor growth by inducing apoptosis in HCC xenograft tumors (Song et al., 2012).

2.3.2.4. PP2A as a cancer therapy target

As PP2A is a tumor suppressor inhibited in human cancer, one approach to target PP2A in cancer is to reactivate it. One way to do this is to inhibit PP2A inhibiting proteins, such as SET or PME-1. In addition, PP2A activators have also been developed. Forskolin has been shown to indirectly increase PP2A activity by increasing protein kinase A (PKA)(Ding and Staudinger, 2005; Feschenko et al., 2002; Kalev and Sablina, 2011; Seamon et al., 1981). However, forskolin derivatives can also activate PP2A independently of PKA (Neviani et al., 2005). FTY720 has been investigated as an anti-cancer therapeutic approach in leukemia and it has been proposed to activate PP2A (Liu et al., 2008a; Roberts et al., 2010). In B cell lymphocytic leukemia xenograft tumors, FTY720 treatment prolonged survival of tumor bearing mice compared to control mice (Liu et al., 2008a). Recently, FTY720 was shown to induce programmed necrosis in lung tumors by inhibiting PP2A-SET interactions (Saddoughi et al., 2013). Notably, FTY720 is an immunosuppressant and it has been investigated for treatment of several diseases in addition to cancer (Pitman et al., 2012; Pyne et al., 2011). FTY720 seems to have several targets in cells among SET and PP2A and the exact molecular mechanism of action is lacking (Pitman et al., 2012; Pyne et al., 2011).

In addition to activation of PP2A, inhibition of PP2A has been suggested as a strategy to inhibit cancer growth. The idea behind this is to drive cells into cell death by accelerating the cell cycle and overriding cell cycle checkpoints (Buck et al., 2003; Kalev and Sablina, 2011). Interestingly, some PP2A inhibitors have been shown to induce cell death in cancer cells, such as fostriecin, norcantharidin and LB1.2 (Bonness et al., 2006; Cheng et al., 1998; Hart et al., 2004; Lewy et al., 2002; Roberge et al., 1994). The most promising inhibitor is LB1.2, a synthetic derivative of norcantharidin (Lu et al., 2009). LB1.2 inhibits PP2A leading to increased phosphorylation and activation of Akt-1, Plk-1 and inhibition of p53 by increasing phosphorylation of Mdm-2 (Lu et al., 2009). Combined treatment with LB1.2 and a chemotherapy drug commonly used for the treatment of mice bearing glioblastoma multiforme and neuroblastoma xenografts, temozolomide, resulted in a dramatic inhibition of tumor growth (Lu et al., 2009). Similar results were obtained by treating mice bearing glioblastoma xenografts with a combination of LB1.2 and the drug, doxorubicin (Lu et al., 2009). However, all of these PP2A inhibitors, fostriecin, norcantharidin and LB1.2, are not PP2A specific as they can also inhibit other phosphatases (Kalev and Sablina, 2011). Therefore, specific PP2A targeting compounds are needed.

2.3.3. Oncoproteins in cancer: Cancerous inhibitor of PP2A

CIP2A was originally identified as a PP2A interacting protein by purifying proteins which bind PP2A A subunit protein in human cancer cells (Junttila et al., 2007). Ever since, CIP2A has been found to be overexpressed in various human cancers, including colon cancer, head and neck squamous cell carcinoma, ovarian cancer, prostate cancer, gastric cancer and in non-small cell lung cancer (Table 1)(Bockelman et al., 2011; Dong et al., 2011; Junttila et al., 2007; Khanna et al., 2009; Vaarala et al., 2010). In most of the studied cancer types, CIP2A has clinical relevance: it correlates with progression of cancer, patient's survival or in some cancer types it is even an independent prognostic factor (Table 1). These clinical results support the notion that CIP2A has an essential role in the development of cancer.

Table 1. CIP2A expression and clinical relevance in human cancer.

Cancer	Overexpression frequency (n)	Clinical correlations	References
AML	77% (70) 10% (30)	In remission CIP2A expressed in 6% of the patients (n=32)	(Wang et al., 2011a) (Cristóbal et al., 2011)
Bladder (TCC) Bladder (UCC)	42% (43) 73% (117)	Correlates with high tumor grade Correlates with poor survival and with high tumor grade	(Huang et al., 2012b) (Xue et al., 2013)
Breast Breast (TNBC)	45% (1028) 64% (57)	Correlates with high tumor grade n.d.	(Niemelä et al., 2012) (Tseng et al., 2012)
Cervical	73% (15) mRNA 53% (72) protein	n.d.	(Liu et al., 2011)
CML	(31)	Prognostic role (high expression at the time of diagnosis predicts for poor survival)	(Lucas et al., 2011)
Colon	32% (167)	Prognostic role and correlates with poor survival	(Teng et al., 2012)
Colorectal	88% (752)	Correlates with high tumor grade	(Böckelman et al., 2012)
Esophageal SCC	90% (40)	n.d.	(Qu et al., 2012)
Gastric	87% (37) 65% (223)	n.d. Prognostic role	(Li et al., 2008) (Khanna et al., 2009)
HCC	63% (136)	Correlates with poor survival	(He et al., 2012)
HNSCC	79% (14)	n.d.	(Junttila et al., 2007)
Lung	83% (29) mRNA 72% (90) protein 67% (58) mRNA 66% (39) protein	Prognostic role Only tumor stage studied, no association	(Dong et al., 2011) (Ma et al., 2011)
Oral SCC	100% (8)	n.d.	(Katz et al., 2010)
Ovarian	83% (524) 66% (152)	Correlates with poor survival and with high tumor grade Correlates with high tumor grade	(Bockelman et al., 2011) (Fang et al., 2012)
Prostate	73% (59)	Correlates with high tumor grade	(Vaarala et al., 2010)
RCC	35% (107)	Correlates with poor survival	(Ren et al., 2011)
Tongue SCC	45% (71)	Correlates with poor survival and with high tumor grade	(Böckelman et al., 2011)

AML=acute myeloid leukemia, TCC=transitional cell carcinoma, TNBC=triple negative breast cancer, CML=chronic myeloid leukemia, UCC=urothelial cell carcinoma, TNBC=triple negative breast cancer, SCC=squamous cell carcinoma, HCC=hepatocellular carcinoma, HNSCC=head and neck hepatocellular carcinoma, n=number of patients, n.d.=not determined.

2.3.3.1. Function of CIP2A in normal tissues

In normal human tissues, CIP2A is mainly expressed at low levels, except in testis (Huang et al., 2012a; Junttila et al., 2007; Liu et al., 2011; Ventelä et al., 2012; Xu et al., 2012). In line with these data, CIP2A hypomorphic mutant ($CIP2A^{HOZ}$) mice are viable and without any clear anatomical phenotypes (Ventelä et al., 2012). However, in $CIP2A^{HOZ}$ male mouse, as a result of decreased expression of CIP2A, sperm count was reduced and also the epididymis was smaller compared to wild type mice (Ventelä et al., 2012). In addition, in spermatogonial progenitor cell (SPC) population of testis, CIP2A was found to co-express with proliferation marker KI-67 and with self-renewal protein PLZF (Ventelä et al., 2012). Importantly, in $CIP2A^{HOZ}$ mice, PLZF-positive SPCs were reduced (Ventelä et al., 2012). Expression of different self-renewal genes in seminiferous tubuli cells, were also decreased (Ventelä et al., 2012). These results indicate that CIP2A promotes sperm production by promoting proliferation and self-renewal of SPCs.

In addition to testis, the function of CIP2A in normal cells and tissues is largely unknown. However, CIP2A is expressed in neurogenic areas in mouse embryos and in adult mouse brain (Kerosuo et al., 2010). Functionally, CIP2A promotes self-renewal and proliferation of neural progenitor cells (NPCs) isolated from the lateral ventricle wall of mouse embryos and during differentiation of NPCs, CIP2A expression is decreased (Kerosuo et al., 2010). Furthermore, CIP2A was shown to promote MYC expression in NPCs and it appears to regulate the self-renewal and proliferation of NPCs together with MYC (Kerosuo et al., 2010). Results from SPCs and NPCs suggest that CIP2A regulates stem and progenitor cell self renewal.

2.3.3.2. Regulation of CIP2A in cancer

The mechanisms up-regulating CIP2A in cellular transformation and in cancer are still largely unknown. Some mutations in CIP2A have been found in human cancers, but the biological relevance of these is unknown (http://www.cbioportal.org/public-portal/cross_cancer.do). In addition, according to the Tumorscape database analysis of 3131 tumors, CIP2A is not significantly amplified in cancer (http://www.broadinstitute.org/tumorscape). Notably, two studies have reported SNPs in CIP2A. In the first study, in two cancer cell lines, one SNP was identified per cell line (Khanna et al., 2011). However, these SNPs alone cannot explain general CIP2A up-regulation in cancer (Khanna et al., 2011). In the second study, two cancer-specific SNPs were identified in human HCC patients (Li et al., 2012). However, these SNPs were not associated with increased risk for HCC (Li et al., 2012). Interestingly, hepatitis virus B and C infections and one of these two SNPs, were identified to increase the risk for HCC (Li et al., 2012). Given these facts, it is expected that instead of mutations and amplification, CIP2A is up-regulated in cancer by other proteins regulating transcription and post-transcriptional modifications of

CIP2A. To support this idea, some mechanisms that promote CIP2A expression in cellular transformation and in cancer cells have been found.

Analysis of the *CIP2A* promoter revealed existence of CpG rich regions (Khanna et al., 2011). As methylation of CpG regions has been suggested to associate with transcriptional silencing of the gene, methylation of the CpG regions on the *CIP2A* promoter was suggested as a mechanism to down-regulate CIP2A in normal cells and tissues (Khanna et al., 2011). Analysis of transcriptional regulation of *CIP2A* identified several putative transcription factor binding sites on the *CIP2A* promoter (Khanna et al., 2011). Transcription factor, ETS1, mediates positive regulation of CIP2A via the MEK-ERK pathway (Khanna et al., 2011; Pallai et al., 2012). Ras-MEK1/2 signaling is known to activate ETS1 in response to growth factor signaling (Sharrocks, 2001). Later, mainly in urogenital cancer cell lines, member of the ETS family ETS1 and ELK1, were shown to promote *CIP2A* transcription together (Pallai et al., 2012). In addition to ETS1, transcription factor MYC has also been reported to promote *CIP2A* expression in cancer cells (Khanna et al., 2009; Mannava et al., 2012).

Oncogenic pro-proliferative functions of CIP2A

During cellular transformation, *CIP2A* expression is increased (Mathiasen et al., 2012). However, CIP2A expression alone cannot transform mouse embryonal fibroblasts (MEFs), but CIP2A can promote both oncogenic Ras-induced transformation of MEFs and transformation of human embryonal kidney (HEK) fibroblasts that were manipulated by inactivating p53 and RB and expressing human telomerase (hTERT) and oncogenic Ras (Junttila et al., 2007). Previously, it was known that the activity of both JNK and ERK are necessary for Ras-induced transformation (Junttila et al., 2008; Nielsen et al., 2007). Transformation assays performed with $JNK2^{-/-}$ MEFs expressing oncogenic Ras, revealed that ectopic expression of either ATF2, CIP2A or MYC are capable to replace JNK2 activity and to transform $JNK2^{-/-}$ MEFs (Mathiasen et al., 2012). Moreover, this study identified that in response to oncogenic Ras expression, JNK2 positively regulates transcription of *CIP2A* via ATF2 (Mathiasen et al., 2012).

In addition to transformation assays, multiple differing assays have also demonstrated the oncogenic role of CIP2A in human cancer. Inhibition of CIP2A *in vitro,* leads to reduced proliferation of various different human cancer cells in several assays, including anchorage independent growth, colony formation and thymidine incorporation assay (Junttila et al., 2007; Khanna et al., 2009; Niemelä et al., 2012). Later, the proliferation-promoting role of CIP2A *in vitro,* was confirmed in many different cancer cell lines (Dong et al., 2011; Fang et al., 2012; Li et al., 2008; Liu et al., 2011; Ma et al., 2011; Teng et al., 2012; Xue et al., 2013). Importantly, CIP2A depletion with small interfering RNA (siRNA), delays growth of various tumor xenografts, including cervical

cancer, head and neck squamous cell carcinoma (HNSCC), urothelial cell carcinoma (UCC) and non-small cell lung carcinoma (NSCLC) tumor xenografts (Junttila et al., 2007; Ma et al., 2011; Xue et al., 2013).

Interestingly, one study has reported that in addition to the inhibition of clonogenic potential of various cancer cell lines after depletion of CIP2A via siRNA, the depletion of CIP2A also induced senescence associated β-gal expression in gastric cancer AGS cells (Li et al., 2008). These results indicate that CIP2A may also support proliferation capabilities by suppressing senescence in cancer cells.

Mechanistically, CIP2A was originally identified to stabilize oncoprotein MYC via PP2A inhibition in cancer cells (Junttila et al., 2007). As mentioned previously, PP2A promotes degradation of MYC by dephosphorylating serine 62 residue of MYC (Arnold and Sears, 2006; Yeh et al., 2004). Whereas, CIP2A inhibits PP2A activity towards serine 62 phosphorylated MYC, leading to MYC stabilization in cancer cells (Junttila et al., 2007). Several other studies demonstrating CIP2A mediated MYC regulation in human cancer cells and in normal cells verify these findings (Fang et al., 2012; Kerosuo et al., 2010; Khanna et al., 2009; Liu et al., 2011; Lucas et al., 2011; Niemelä et al., 2012; Ren et al., 2011). In gastric cancer cell lines, MYC was also identified to promote CIP2A mRNA and protein expression, forming a positive feedback loop between CIP2A and MYC (Khanna et al., 2009). The CIP2A regulation of proliferation and MYC, was verified when signature genes and pathways regulated by CIP2A transcriptome was analyzed (Niemelä et al., 2012). In this study, CIP2A depletion caused suppression of HeLa cell colony growth, which was identified as a MYC-dependent function of CIP2A (Niemelä et al., 2012). Importantly, depletion of either B56α or B56β were able to reverse CIP2A depletion caused alterations in MYC expression, in almost all effected MYC target gene expressions and in colony growth (Niemelä et al., 2012).

Oncogenic activity of CIP2A towards other cellular functions

By studying CIP2A-regulated transcriptome, MYC-independent functions were found for CIP2A. Importantly, CIP2A promotes migration and invasion capacities of cancer cells independently of MYC, but in a PP2A-dependent manner (Niemelä et al., 2012; Ren et al., 2011; Xue et al., 2013). Other studies have shown similar effects on migration and invasion following CIP2A depletion in renal cell carcinoma (RCC) and UCC cells (Ren et al., 2011; Xue et al., 2013). In addition, CIP2A was noted to promote JNK2 expression in cancer cells independently of MYC (Niemelä et al., 2012).

Even though CIP2A promotes proliferation, it is not regulated during cell cycle (Junttila et al., 2007; Khanna et al., 2009). However, some studies have shown that CIP2As oncogenic role might be at least partly be explained by suppression of apoptosis induction. In hepatocellular carcinoma (HCC), head

and neck squamous cell carcinoma (HNSCC), ovarian cancer, breast cancer and in lung cancer cells, CIP2A was shown to suppress apoptosis by promoting phosphorylation of Akt (Chen et al., 2010; Chen et al., 2011; Fang et al., 2012; Lin et al., 2012; Ma et al., 2011; Tseng et al., 2012). Regulation of Akt activity towards apoptosis by CIP2A, has also been associated with bortezomib resistance in HCC and in HNSCC cells (Chen et al., 2010; Lin et al., 2012; Tseng et al., 2012). Bortezomib currently used clinically for the treatment of multiple myeloma and mantle cell lymphoma (Du and Chen, 2013). Inhibition of Akt was previously shown to sensitize HCC cells to bortezomib (Chen et al., 2008). Interestingly, in the bortezomib-resistant PLC5 HCC cell line, inhibition of CIP2A by siRNA re-sensitized the cells to bortezomib by inducing PP2A activity and apoptosis (Chen et al., 2010). These findings were further verified in bortexomib-sensitive Huh-7 HCC tumor xenografts, in which bortezomib down-regulated CIP2A expression, increased PP2A activity and reduced tumor growth (Chen et al., 2010).

CIP2A has also been reported to inhibit UNC5H2/B-induced apoptosis, which is known to limit cancer progression (Guenebeaud et al., 2010). UNC5H2/B promotes apoptosis by recruiting PR65β-containing PP2A to serine/threonine kinase DAPk (Guenebeaud et al., 2010). For apoptosis induction, PP2A is needed to activate and dephosphorylate DAPk (Guenebeaud et al., 2010). Finally, CIP2A was shown to promote cell survival by inhibiting activity of PP2A towards DAPk and further suppress UNC5H2/B-mediated apoptosis (Guenebeaud et al., 2010).

2.3.3.3. CIP2A in breast cancer

At the time of initiating my PhD thesis projects there was no data about CIP2A in breast cancer. However since then, several studies regarding CIP2A in breast cancer have been published. By comparing the CIP2A-regulated microarray signature with previously published breast cancer signatures, CIP2A signature was noted to mostly cluster with basal-like and with HER2+ breast cancer subtypes (Niemelä et al., 2012). CIP2A protein expression was also found to be significantly higher in basal-like (61%) and in HER2+ (60%) breast cancers as compared to 45% of CIP2A positive tumors in all breast tumors studied (Niemelä et al., 2012). In another study expression of CIP2A was analyzed from 57 triple negative breast cancer patient tumors and CIP2A was shown to be expressed in 64% of tumors (Tseng et al., 2012). Additionally, CIP2A regulated gene expression signature clustered most clearly with basal-like and with HER2+ breast cancer subtypes in two different human breast cancer cohorts studied (Niemelä et al., 2012). Furthermore, in a basal-like breast cancer cell line, MDA-MB-231, CIP2A was identified to promote MYC-mediated gene expression, indicating that CIP2A promotes MYC stability in basal-like breast cancers (Niemelä et al., 2012). Also, *MYC* amplification was found to associate with CIP2A expression in human breast cancer, suggesting

that CIP2A-mediated MYC stabilization and *MYC* amplification are not mutually exclusive in breast cancer (Niemelä et al., 2012).

Mechanistically, it was recently suggested that CIP2A promotes resistance to bortezomib-induced apoptosis via promotion of Akt phosphorylation in triple negative breast cancer cell lines (Tseng et al., 2012). These results are supported by similar findings in HCC and in HNSCC cells (Chen et al., 2010; Lin et al., 2012). Additionally, CIP2A expression was shown to be inhibited by doxorubicin and to mediate doxorubicin resistance, which was also shown to be associated with the phosphorylation of Akt in human breast cancer cells (Choi et al., 2011).

Thus, CIP2A is overexpressed in most of the human cancers studied thus far. Several studies have also implicated the pro-proliferative role of CIP2A in cancer. Taken together, these findings suggest that the inhibition of CIP2A is a viable cancer therapeutic approach. Further studies are however needed to develop this strategy.

2.3.4. Oncoproteins in cancer: E2F1

E2 transcription factor (E2F) protein family consists of multiple transcription factors that are essential regulators of cell cycle and cell fate (Polager and Ginsberg 2009). E2F proteins are divided into subfamilies according to which RB family protein they bind, their structure and transcription activity (DeGregori and Johnson 2006, Polager and Ginsberg 2009). Nowadays, there are eight known E2F family genes, *E2F1-8*, and each of them produce one protein except *E2F3, which* produces two proteins (DeGregori and Johnson 2006). E2F1, E2F2 and E2F3 are termed activator E2Fs, since they positively regulate cell cycle progression (DeGregori and Johnson 2006). In contrast, E2F4, E2F5, E2F6, E2F7 and E2F8 mainly function as repressors of cell cycle progression (DeGregori and Johnson 2006). However, it should be noted that most of the conclusions, based on which E2F proteins are divided into activator or repressor function groups, are generated based on *in vitro* experimental settings and lack extensive *in vivo* verification (Chen et al. 2009). Division of E2F proteins based on their interaction with RB proteins is more straightforward. E2F1-5 proteins share the C-terminal transactivation domain needed for RB protein family binding (Chen et al. 2009). E2F6-8 lack this domain and function independently of Rb (Chen et al. 2009). Moreover, E2F1-E2F6 share dimerization domain. Hetero-dimerization with DP family proteins enables their DNA binding and transcriptional activity (DeGregori and Johnson 2006). E2F7 and E2F8 lack this domain and can bind DNA independently of DP proteins (DeGregori and Johnson 2006).

E2F1 was the first identified E2F family protein. Originally, E2F1 was noted to work as a transcription factor by binding and activating adenovirus gene E2 (Kovesdi et al., 1986; Reichel et al., 1987). Importantly, multiple research

groups have identified E2F1 as an interacting partner of RB (Bagchi et al., 1991; Bandara et al., 1991; Chellappan et al., 1991; Chittenden et al., 1991). Soon thereafter, the importance of E2F1 in the regulation of cellular proliferation and apoptosis was noted (Johnson et al., 1993; Wu and Levine, 1994). Also, Johnson and colleagues, published that E2F1 expression can induce S-phase entry in immortal quiescent cells (Johnson et al., 1993). Later, several other studies have confirmed that E2F1 promotes cellular proliferation (Johnson et al., 1994; Neuman et al., 1994; Chen et al. 2009).

2.3.4.1 E2F1 in different cellular functions

E2F1 promotes cellular proliferation

E2F1 has multiple target genes which are involved in the regulation of various cellular functions, such as apoptosis, cell cycle control, proliferation, development and differentiation (Polager and Ginsberg, 2009; Wong et al., 2011).

RB binding is the key mechanism regulating E2F1 activity in the control of cell cycle and proliferation (Figure 6). In normal cells, as a response to growth factor stimulation, cyclin dependent-kinase complexes are activated (Malumbres and Barbacid 2009). Cyclin-dependent kinase 4 (Cdk4)-cyclin D and Cyclin-dependent kinase 6 (Cdk6)-cyclin D are expressed through Gap 1 (G1) phase and Cdk2-cyclin E in late G1 phase (Malumbres and Barbacid 2009). Both Cdk4/Cdk6-cyclin D and Cdk2-cyclin E complexes activate E2F1 by releasing it from Rb/E2F1 complex via Rb hyper-phosphorylation (Figure 6)(Mittnacht 1998). This leads to dimerization of E2F1 with DP1 and DNA binding of E2F1/DP1 complex (Polager and Ginsberg, 2008). Active E2F1 induces transcription of its target genes and progression of cell cycle from phase G1 to synthesis (S) phase (Figure 6)(DeGregori et al., 1995). In resting cells, Rb is hypo-phosphorylated and binds E2F1 in order to keep it inactivated. Released E2F1 binds to its target genes via a DNA-binding motif that is conserved between all E2Fs (Cao et al., 2011; Chen et al., 2009; Singh et al., 2010).

E2F1 has multiple target genes associated with cell growth and proliferation (Polager and Ginsberg, 2009). Recently, RB/E2F complex were reported to also have tissue-specific target genes which can explain why E2Fs regulate several cellular functions (Kudron et al., 2013). In particular, E2F1 activates genes associated with progression of cell cycle phases G1 and S, such as *Cyclin E*, *Myb-related protein B* (*B-MYB)*, *cyclin A*, *Cell division cycle 2* (*CDC2*) and *Dihydrofolate reductase* (*DHFR*) (Dalton, 1992; DeGregori et al., 1995; Dimri et al., 1994; Fry et al., 1997). E2F1 was also later shown to activate the transcription of genes required for phase gap 2 (G2) and mitosis progression, cell cycle checkpoint responses and DNA repair, such as *CDC2*, *Highly expressed in cancer* (*HEC*), *Budding uninhibited by benzimidazoles 1 homolog*

beta (*BUB1B*) and *Breast cancer 1, early onset* (*BRCA1*) (Polager et al., 2002; Ren et al., 2002; Zhu et al., 2004). It has also been shown that cells can ensure expression of pro-proliferative genes via positive feedback through replication initiation proteins to E2F1 (Herr et al., 2012).

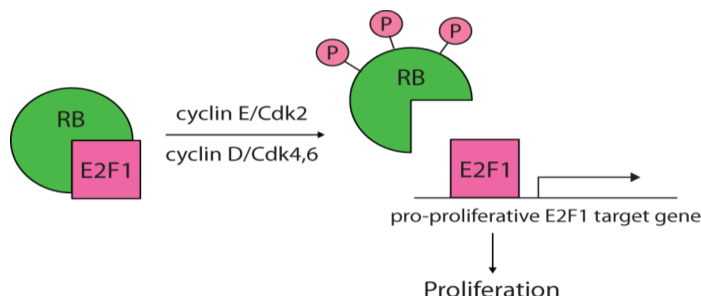

Figure 6. RB/E2F1 complex regulates proliferation.

E2F1 can also auto-regulate itself by binding to its own promoter and activating transcription of *E2F1* (Johnson et al., 1994b; Wade et al., 2010). In normal murine cells and in human cancer cells, in response to growth stimulation, E2F1 is released from Rb and can positively auto-regulate its own activity to promote transition from G1 to DNA synthesis phase (Johnson et al., 1994b; Wade et al., 2010).

E2F1 regulates senescent growth arrest

In line with cell cycle regulation, E2F1 also regulates the permanent exit of cells from the cell cycle, cellular senescence (Polager and Ginsberg, 2009). Nevertheless, the role of E2F1 in senescence regulation is not straightforward. Inhibition of E2F1 in senescent cells has been reported by several studies (Dar et al., 2011; Moiseeva et al., 2011; Vernier et al., 2011). In addition, RB was recently noted to selectively repress E2F1 target genes expression, like *Proliferating cell nuclear antigen* (*PCNA*) and *Cyclin A2* (*CCNA2)*, independently of E2F1 during senescence by associating with the TAAC element present in a subset of E2F1's target genes (Chen et al., 2012). This further strengthens the inhibition of E2F1 proliferation-promoting targets, in senescent cells. Inhibition of E2F1 can also induce the senescence phenotype in tumor and immortalized cells (Park et al., 2006; Polager and Ginsberg, 2009; Verhaegen et al., 2012). Importantly, ectopic expression of E2F1, can also rescue the senescence phenotype in cancer cells (Dar et al., 2011; Verhaegen et al., 2012; Vernier et al., 2011). On the other hand, E2F1 overexpression was shown to induce cellular senescence in normal human fibroblasts (Dimri et al., 2000). This can be explained in the following way: in normal cycling cells, ectopic E2F1 cannot further promote S phase entry and as a consequence, ectopic E2F1 causes oncogene induced senescence (OIS), like oncogenic active H-Ras (Dimri et al., 2000; Polager and Ginsberg, 2009).

E2F1 promotes apoptosis

E2F1 has a contradictory role in regulating both cellular proliferation and apoptosis (DeGregori et al., 1997; Hallstrom and Nevins, 2003; Lazzerini Denchi and Helin, 2005). In addition to E2F1 overexpression experiments performed in cell culture, mutant E2F1 mouse models have shown that E2F1 can induce apoptosis and that this has a physiological significance (Field et al., 1996; Meng et al., 1999; Pruschy et al., 1999; Yamasaki et al., 1996). In 1996, two research group reported that E2F1-deficient mice are viable and reproduce normally (Field et al., 1996; Yamasaki et al., 1996). However, they found that E2F1-deficient mice experienced testicular atrophy, exocrine gland dysplasia and defects in T lymphocyte development, which was caused by defects in thymocyte apoptosis. Importantly, E2F1-deficient mice were noted to develop tumors, although with a long latency (Yamasaki et al., 1996). These results suggested that E2F1 can act both as an inducer of proliferation and apoptosis.

Interestingly, E2F1 can induce both p53-dependent and p53-independent apoptosis. E2F1 promotes p53-dependent apoptosis by activating transcription of genes, such as positive p53 regulator, *Cyclin-dependent kinase inhibitor 2A* (*CDKN2A*) encoding ARF protein, *Ataxia telangiectasia mutated* (*ATM*) and *Checkpoint kinase 2* (*CHK2*), which can phosphorylate and activate p53 (Bates et al., 1998; Berkovich and Ginsberg, 2003; Powers et al., 2004; Rogoff et al., 2004). Furthermore, E2F1 can induce expression of pro-apoptotic co-factors of p53, thus supporting p53 induced activation of apoptosis-related genes (Polager and Ginsberg, 2009). On the other hand, E2F1 can induce p53-independent apoptosis by activating many pro-apoptotic genes, including caspases, *P53 up-regulated modulator of apoptosis* (*PUMA*), *PMA-induced protein 1* (*PMAIP1*, also named as *NOXA*) and *P73* (Stanelle and Putzer, 2006).

Taken together, E2F1-mediated regulation can decide whether cells continue to proliferate, enter apoptosis or even senescence. However, in normal cells E2F1 is tightly regulated and it seems to be that E2F1 is regulating the balance between these different outcomes.

2.3.4.2. Regulation of E2F1 activity

Post-translational regulation of E2F1

In addition to previously described RB-dependent regulation, E2F1 can be regulated by several post-translational modifications (Figure 7). However, most of the post-translational studies have been done by inducing DNA damage in cells in order to stabilize E2F1. The role of post-translational modifications of E2F1 in regulating normal cell cycle progression, are less understood. In response to DNA damage, ATM and Chk2 phosphorylate E2F1 residues serine 31 and serine 364, respectively (Lin et al., 2001; Stevens et al., 2003). In both

of these studies, DNA damage induction stabilized E2F1 and promoted apoptosis (Lin et al., 2001; Stevens et al., 2003). Recently, it was reported that in response to DNA damage, serine 364 phosphorylated E2F1 forms a complex with RB and a serine 31 phosphorylated form of E2F1, is not bound to RB (Carnevale et al., 2012). Interestingly, both forms induced pro-apoptotic p73, and were needed for a potent apoptosis induction following DNA damage (Carnevale et al., 2012). Additionally, in breast cancer cells treated with epirubicin, p38 mitogen activated protein kinase (MAPK) was revealed to induce E2F1 and it was mediated by a downstream kinase of p38 Mitogen activated protein kinase –activated protein kinase 2 (MK2)(de Olano et al., 2012). Furthermore, MK2 directly phosphorylates E2F1 at serine 364 and this phosphorylation is partly responsible for epirubicin-induced E2F1 expression (de Olano et al., 2012). In addition, *in vitro* assays have shown that p38 can phosphorylate E2F1 serine 403 and threonine 433 and mutation in one or another of these residues, impaired the ability of E2F1 to be exported from nucleus for degradation (Ivanova et al., 2009). Phosphorylation of serine 403 of E2F1 is also associated with the DNA damage response induced by long term doxorubicin treatment in cancer cells and increased E2F1 activity towards apoptosis-related genes (Real et al., 2010). Whether this phosphorylation is relevant *in vivo,* remains obscure. Furthermore, general transcription factor II H (TFIIH) protein complex, has been reported to mediate phosphorylation of E2F1 on serine 403 and threonine 433 during S phase (Vandel and Kouzarides, 1999). TFIIH-mediated phosphorylation of E2F1 was suggested to support E2F1 turnover by ubiquitin-dependent proteasomal degradation (Vandel and Kouzarides, 1999).

Despite the fact that many of the studies related to E2F1 regulation are associated with E2F1's ability to induce apoptosis, there are studies indicating that E2F1 associated with cell cycle progression and is also subject to phosphorylation. Soon after E2F1 was discovered, Cdk4-cyclin D complex were found to directly phosphorylate serine 332 and 337 residues of E2F1, which prevents interaction of RB with E2F1 (Fagan et al., 1994; Mundle and Saberwal, 2003). Furthermore, during S phase, E2F1's DNA binding affinity is inhibited by binding of Cdk2-cyclin A to N-terminal domain of E2F1 between residues 67-108. This enables Cdk2-cyclin A to form a stable complex with E2F1 and further phosphorylate the serine 375 residue of E2F1, resulting in release of the E2F1-DP1 heterodimer from DNA binding (Guida and Zhu, 1999; Krek et al., 1994; Mundle and Saberwal, 2003; Xu et al., 1994). Recently, Cyclin-dependent kinase 8 (Cdk8), an oncogene overexpressed in colorectal cancer, was reported to phosphorylate E2F1 serine 375 and this phosphorylation was required for interaction of E2F1 to Cdk8 (Zhao et al., 2012). The phosphorylation by Cdk8 repressed the transcriptional potential of E2F1 to inhibit β-catenin/Transcription factor 14 (TCF) –dependent genes, essential regulators of colorectal cancer, and to activate other E2F1 target genes (Zhao et al., 2012). In addition to phosphorylation, E2F1 is regulated by acetylation and methylation as shown in figure 7 (Cho et al., 2012; Ianari et al., 2004; Kontaki and Talianidis, 2010; Martinez-Balbas et al., 2000; Marzio et al.,

2000; Pediconi et al., 2003; Xie et al., 2011). Taken together, the activity of E2F1 is tightly regulated by several post-translational mechanisms. However, the mechanisms regulating E2F1 functions associated with proliferation need further clarifications.

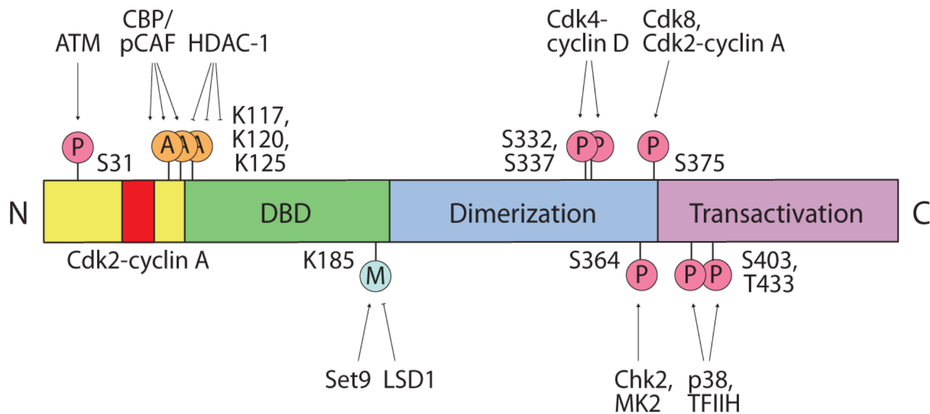

Figure 7. Domain structure and post-translational modifications of E2F1.
437 amino acids long E2F1 consists of DNA binding domain (DBD), dimerization domain needed for heterodimerization with DP1 and transactivation domain needed for RB binding. Cdk2-cyclin A binds to residues 68-107 (red). Post-translational modifications, phosphorylations (P), acetylations (A) and methylations (M) of E2F1 and factors that mediate the modifications are shown within corresponding residues. N and C indicate N-terminal and C-terminal parts respectively. S=serine, T=threonine, K=lysine.

Proteasomal degradation of E2F1

Like most of the cell cycle regulators, E2F1 is also inhibited by proteasomal degradation. E2F1 is targeted for ubiquitination through the C-terminal transactivation domain mediating RB binding (Campanero and Flemington, 1997). As a consequence, RB binding masks this sequence and protects E2F1 from ubiquitination and degradation (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996). In late S-G2 phase E2F1 degradation is promoted by binding of S-phase kinase-associated protein 2 (Skp2) ubiquitin ligase to the N-terminus of E2F1 (Marti et al., 1999). Importantly, Skp2 regulates E2F1 stability together with Cdk2-cyclin A, as they are all present in the same protein complex (Wong et al., 2011). However, it is still obscure how Skp2 binding on E2F1 is regulated. Martelli and colleagues, proposed that E2F1 is ubiquitinated in response to ARF protein binding especially in cells defective for p53 function (Martelli et al., 2001). It was later confirmed that ARF promotes ubiquitination and degradation of E2F1, but only in the presence of functional p53 (Rizos et al., 2007). Also, Mdm2 has been shown to prolong E2F1 half-life by inhibiting ubiquitination of E2F1 by replacing Skp2 from E2F1 (Zhang et al., 2005). Interestingly, binding of Mdm2 to E2F1 and down-regulation of E2F1 upon Mdm2 inhibition was not caused either by ARF or pRB (Zhang et al., 2005).

2.3.4.3. E2F1 in cancer

In the early days of E2F1 studies, it was noted that viral oncoproteins, type 5 adenovirus early region 1A (E1A), human papilloma virus (HPV) E7 and simian virus 40 large T (SV40 LT), can inhibit RB protein leading to promotion of E2F1 activity and simultaneously stimulation of cell proliferation (Chellappan et al., 1992; DeCaprio et al., 1988; Dyson et al., 1989; Whyte et al., 1988). These results indicated that E2F1 might have oncogenic capacity in carcinogenesis. However, this hypothesis was challenged especially by results from E2F1$^{-/-}$ mice which suggests that E2F1 can also function as a tumor suppressor (Yamasaki et al., 1996).

Expression and regulation of E2F1 in cancer

The oncogenic role of E2F1 is supported by several studies which found E2F1 overexpressed in multiple different cancer types, such as in hepatocellular carcinoma, cervical cancer, glioblastoma, ovarian cancer and breast cancer (Alonso et al., 2005; De Meyer et al., 2009; Han et al., 2003; Midorikawa et al., 2004; Reimer et al., 2006; Wilting et al., 2008; Zondervan et al., 2000). E2F1 is overexpressed both due to the positive regulatory mechanisms and via amplification (Chen et al., 2009). Given the fact that E2F1 also has tumor suppressive roles in regulating cellular functions by promoting apoptosis and that loss of E2F1 in mice leads to tumorigenesis, E2F1 could be expected to also be found silenced in human cancer. However, decreased expression of E2F1 in cancer has rarely been found and loss-of-function mutations or silenced E2F1 have not been reported (Bramis et al., 2004; Kwong et al., 2003; Lee et al., 2008; Rabbani et al., 1999). In contrast, the upstream effectors of E2F1, such as *RB* and *p53,* are commonly mutated in cancer (Knudsen and Knudsen, 2006; Petitjean et al., 2007). In fact, several mutations in the same signaling pathway are rarely found in cancer and this partially explains why mutations of E2F1 have not been established (Chen et al., 2009). In addition to p53 and RB proteins, other factors also in RB/E2F pathway, can be deregulated. Cyclin kinase inhibitor p16^{INK4a} holds RB in a dephosphorylated state and E2F1 in an inactive form, by suppressing cyclin D/Cdk4 (Nevins, 2001). In cancer, such as melanoma and pancreatic cancers, p16^{INK4a} has been found mutated and lost and this results in RB phosphorylation and E2F1 activation (Nevins, 2001; Sherr and McCormick, 2002). Amplification and translocation of the gene, *cyclin D1* (*CCND1),* has also been reported in multiple cancer types (Hallstrom and Nevins, 2009; Nevins, 2001). In addition, amplification of the *CDK4* gene, has been recognized in sarcomas and gliomas (Hallstrom and Nevins, 2009; Nevins, 2001).

E2F1 activity in cancer can also be promoted by RB-independent mechanisms. MYC oncoprotein can induce E2F1 directly by enhancing E2F1 transcription and indirectly by inducing Cdk4 expression (Hermeking et al., 2000). It has also been reported that proper MYC function promoting S phase and

apoptosis, requires E2F1, E2F2 and E2F3 activities and that MYC-induced apoptosis is E2F1-dependent (Leone et al., 2001). Interestingly, E2F1 can also promote transcription of MYC (Polager and Ginsberg, 2009). Taken together, these data suggest that E2F1 and MYC share functional properties and that these two oncogenes are connected via regulation of each other's transcription function.

It has been a paradox, especially in cancer, that E2F1 can promote both cell proliferation and apoptosis. In cancer, the PI3K-Akt pathway can mediate oncogenic activity of E2F1 by inhibiting E2F1-mediated apoptosis (Hallstrom and Nevins, 2003). In response to growth factor stimulus, the PI3K-Akt pathway suppresses E2F1-induced pro-apoptotic proteins (Hallstrom and Nevins, 2003, 2009). In addition, the PI3K-Akt pathway can inhibit E2F1 activity towards apoptosis by phosphorylating TopBP1 (Topoisomerase II-binding protein 1) which then binds and represses E2F1 (Liu et al., 2003; Liu et al., 2006). TopBP1 specifically inhibits E2F1 pro-apoptotic function (Liu et al., 2004b). In line with the results from cultured cells, TopBP1 is overexpressed in 58% of breast cancer patients and associates with high tumor grade and poor patient survival (Liu et al., 2009). Interestingly, PI3K-Akt can also inhibit MYC-induced apoptosis, further supporting cooperation of E2F1 and MYC in cancer regulation (Hallstrom and Nevins, 2009; Kauffmann-Zeh et al., 1997). In a recent study, it was revealed that a subset of E2F1-induced pro-apoptotic target genes, are inhibited via the activation of the PI3K-Akt pathway (Hallstrom et al., 2008). Moreover, low expression of this subset of E2F1 target genes, are associated with poor prognosis in breast and ovarian cancer patients (Hallstrom et al., 2008). These results indicate that the balance between E2F1-induced proliferation and apoptosis has clinical relevance for patient survival (Hallstrom et al., 2008). Furthermore, E2F1 seems to regulate its own ability to induce apoptosis, as E2F1 positively regulates Akt (Chaussepied and Ginsberg, 2004).

In addition to the pro-proliferative role of E2F1 in cancer, it can prevent senescent growth arrest in tumor cells (Dar et al., 2011; Park et al., 2006; Verhaegen et al., 2012). Recently, it was shown that Mdm2 depletion-mediated reduction in melanoma tumor xenograft growth, was caused by senescence induction and importantly, the tumors had less E2F1 expression compared to control tumor xenografts (Verhaegen et al., 2012). *In vitro*, ectopic expression of E2F1 could rescue p53-driven senescence in melanoma cells (Verhaegen et al., 2012). Verhaegen et al., suggested that E2F1 down-regulation is an active driver of the senescent phenotype in melanoma cells (Verhaegen et al., 2012).

E2F1 has both oncogenic and tumor suppressive role in cancer

The first indications of the oncogenic role of E2F1 came from *in vitro* transformation assays performed with cultured cells. E2F1 overexpression

alone promoted cell proliferation in quiescent fibroblasts and in combination with oncogenic Ras, transformed fibroblasts (Johnson et al., 1994a; Lukas et al., 1996). E2F1 overexpression alone fails to prevent terminal differentiation of keratinocytes and villus enterocytes, but it prevented terminal differentiation of megakaryocytes *in vivo* (Chandrasekaran et al., 1996; Guy et al., 1996; Pierce et al., 1998a). This suggests that the ability of E2F1 to promote proliferation is tissue-specific. In a transgenic mouse model expressing E2F1 under a keratin 5 (K5) promoter (K5-E2F1), E2F1 overexpression in mouse squamous epithelial tissue caused epidermal hyperplasia (Pierce et al., 1998a; Pierce et al., 1998b). However, in this K5-E2F1 transgenic mouse model, by 42 weeks of age, E2F1 overexpression cannot induce skin carcinomas alone, but rather with either partial or total loss of p53 or with oncogenic active H-Ras mice developed skin tumors (Pierce et al., 1998a; Pierce et al., 1998b). Moreover, E2F1 overexpression alone in 47 to 95 weeks of age, K5-E2F1 transgenic mice was found to have both oncogenic and tumor suppressive roles (Pierce et al., 1999). Pierce et al. have found that 50% of old K5-E2F1 mice developed spontaneous tumors in various tissues expressing K5, mainly in skin (Pierce et al., 1999). In contrast, K5-E2F1 mice were resistant to carcinogen-induced skin tumorigenesis due to increased apoptosis in epidermis (Pierce et al., 1999). As mentioned previously, spontaneous tumors arise also in E2F1$^{-/-}$ mice (Yamasaki et al., 1996). 56% of these mice formed tumors, mainly reproductive tract sarcomas, lung adenocarcinomas and lymphomas, between 8 and 18 months of age (Yamasaki et al., 1996). Taken together, these studies show that deregulation of E2F1 alone can have either oncogenic or long latency tumor suppressive role *in vivo* (Pierce et al., 1999; Yamasaki et al., 1996).

2.3.4.4. E2F1 in breast cancer

As in many other cancer types, E2F1 is overexpressed in breast cancer (Baldini et al., 2006; Han et al., 2003; Zhang et al., 2000). High E2F1 protein and mRNA expression in tumors has been reported to associate with high tumor grade and poor survival of breast cancer patients (Baldini et al., 2006; Han et al., 2003; Verlinden et al., 2007; Vuaroqueaux et al., 2007; Zhang et al., 2000). In contrast, in one study, low E2F1 expression in mammary tumors has been associated with poor patient’s survival (Worku et al., 2008). However, this finding might be explained by two ways. Firstly, given the fact that in mouse models, E2F1 has been reported to have tumor suppressive properties with long latency, it can be speculated that in certain types of breast tumors E2F1 may play a tumor suppressive role (Pierce et al., 1999; Yamasaki et al., 1996). Secondly, *E2F1* mRNA expression was normalized against a different mRNA transcript (Cytokeratin 19) in the study by Worku et al., as compared to the transcript (18s ribosomal RNA) used in the two other studies analyzing *E2F1* mRNA expression in breast cancer (Verlinden et al., 2007; Vuaroqueaux et al., 2007; Worku et al., 2008).

Interestingly, Zhang et al. studied E2F1-positive cells in normal human breast tissue, in carcinoma *in situ* and in invasive carcinoma and reported that E2F1 expression gradually increases during breast neoplasia (Zhang et al., 2000). Furthermore, a study performed with wild type and E2F1$^{-/-}$ mice support these results. According to a microarray data, activator E2Fs, promote mouse mammary gland proliferation during puberty when epithelial branches are penetrating mammary fat pad and upon pregnancy when additional mammary ductal branching occurs (Andrechek et al., 2008). Interestingly, during involution of mammary gland after lactation, which happens by apoptosis and by remodeling, E2F1 induced pathways are not activated (Andrechek et al., 2008). The phenotypes in puberty predicted by microarray results were verified in E2F1$^{-/-}$ mice (Andrechek et al., 2008). In summary, these results wouls suggest that E2F1 promotes proliferation and not apoptosis in normal mammary and this supports the notion that E2F1 acts mainly as an oncogene in mammary carcinogenesis rather than as a tumor suppressor.

2.3.4.5. E2F1 as a therapeutic target in cancer

As E2F1 has been shown to induce apoptosis it has been suggested that up-regulation of E2F1 might be an effective approach to inhibit tumor growth (Putzer, 2007; Wu and Yu, 2009). In line with this, DNA damage-inducing and apoptosis-promoting compounds, such as epirubicin, have been shown to increase E2F1 expression (de Olano et al., 2012). Also, E2F1 overexpression has been shown to sensitize cancer cells for apoptosis-inducing chemotherapies (Hao et al., 2006; Meng et al., 1999; Putzer, 2007). However, as mentioned above, E2F1 is commonly up-regulated in many human cancer types (Alonso et al., 2005; Midorikawa et al., 2004; Reimer et al., 2006; Wilting et al., 2008; Zondervan et al., 2000). Based on E2F1 up-regulation in cancer and its role in promoting proliferation, induction of E2F1 in cancer might cause support of proliferation programs rather than apoptosis. Additionally, inhibition of E2F1 induces senescence in tumor cells; in melanoma cells inhibition of E2F1 was shown to be even essential for senescence induction (Park et al., 2006; Verhaegen et al., 2012). Taken together, these results suggest that inhibition of E2F1 provides safer and more efficient approach to target cancer than E2F1 up-regulation.

3. AIMS OF THE STUDY

The CIP2A oncoprotein has been shown to be overexpressed in various different human cancer types. Notably, normal human tissues express CIP2A at low or undetectable level. However, the *in vivo* role of CIP2A in carcinogenesis has not been studied thus far. Moreover, the molecular mechanisms of CIP2A induction during cellular transformation are still obscure. Also, the clinical significance of CIP2A overexpression in human breast cancer is not clear.

The specific aims of this thesis study:

I. To study the clinical relevance of CIP2A in human breast cancer

II. To characterize the role of CIP2A as a possible therapy target in breast cancer

III. To identify the mechanisms of CIP2A up-regulation during cellular transformation and in cancer

IV. To study the role of CIP2A in breast cancer *in vivo*

4. MATERIALS AND METHODS

The materials and methods used in this study are listed below and detailed descriptions are found in the original publications. The materials used in the figure 8 (unpublished data) are described in this chapter.

Experimental procedures

Methods	**Used in**
Anchorage-independent soft agar growth	I
Adenoviral infection	II, Fig.8
cDNA synthesis	I, II
Cell proliferation assay	I
Chromatin immunoprecipitation	II
IHC staining	II
Ingenuity Transcription Factor analysis	II
Luciferase assay	II, Fig.8
MEF isolation	II
Mouse mammary tumor cell isolation	II
Mouse xenografts	I
RNA extraction and purification	I, II
RT-qPCR	I, II
SA-β-gal staining	II
siRNA transfection	I, II, Fig.8
Wound healing assay	I

Materials

Cell lines	**Cell type**	**Used in**
HCT116	Human colorectal carcinoma	II
HCT116 $p21^{-/-}$	Human colorectal carcinoma	II
HCT116 $p53^{-/-}$	Human colorectal carcinoma	II
HeLa	Human cervical adenocarcinoma	II
MCF-7	Human mammary adenocarcinoma	I, II, Fig.8
MDA-MB-231	Human mammary adenocarcinoma	I, II
MEF WT	Mouse embryonic fibroblasts isolated from wild type mouse embryos	II
MEF $CIP2A^{HOZ}$	Mouse embryonic fibroblasts isolated from $CIP2A^{HOZ}$ mouse embryos	II

neu/WT	Isolated cells from parental MMTVneu mouse mammary tumors	II
neu/HOZ	Isolated cells from MMTVneu x CIP2A^{HOZ} mouse mammary tumors	II
T47D	Human mammary ductal carcinoma	I
SAOS-2	Human osteosarcoma	II

Chemicals	**Used in**
Doxorubicin	II
Doxocycline	II
Nutlin-3	II
RITA	II
Tamoxifen	II
Vinorelbine	II

Antibodies		**Used in**
p-Akt	Rabbit polyclonal	I
CIP2A	Rabbit polyclonal	I, II, Fig.8
CIP2A	Mouse monoclonal	II, Fig.8
DcR2	Rabbit polyclonal	II
E2F1	Mouse monoclonal	II
p-E2F1 S364	Rabbit polyclonal	II
Ki-67	Rat monoclonal	I, II
MYC	Mouse monoclonal	I, Fig.8
p-MEK	Rabbit polyclonal	I
p21	Rabbit polyclonal	II
p53	Mouse monoclonal	II
p53	Rabbit polyclonal	II
PP2A B55α	Mouse monoclonal	II
RB	Rabbit polyclonal	II
p-RB S807/811	Rabbit polyclonal	II
β-actin	Mouse monoclonal	I, II

Materials and methods used in Figure 8

Cell culture and Nutlin-3 treatment

MCF-7 breast cancer cell line was obtained from ATCC. Cells were cultured in RPMI-1640 medium supplemented with β-estradiol, insulin and 10% of inactivated fetal bovine serum. Cells were exposed to indicated concentrations of Nutlin-3 (Cayman Chemicals).

SiRNA transfections

MCF-7 cells were transfected at 30% confluency, with 250 pmol of siRNAs by using Oligofectamine transfection reagent according to manufacturer's instructions (Invitrogen). Cells were transfected either with non-targeting (scrambled) or with MYC siRNA or with combination of MYC and CIP2A siRNAs. Sequences of siRNAs are listed in table 2.

Table 2. SiRNA sequences used in Figure 8.

siRNA	Sequence (from 5' to 3' end)
scrambled	GUAACAAUGAGAGCACGGCTT
MYC	UCCCGGAGUUGGAAAACAATT
CIP2A	CUGUGGUUGUGUUUGCACUTT

Adenoviral transduction

MCF-7 cells at 40% confluency, were transduced either with control (AdCTL) or with CIP2A expressing adenovirus in medium containing 1% of inactivated fetal bovine serum. 24 hours after transduction, medium was changed to normal MCF-7 culture medium containing 10% of inactivated fetal bovine serum.

Luciferase reporter assay

MCF-7 cells were first transduced on 96-well plate either with control (AdCTL) or with CIP2A expressing adenovirus (AdCIP2A) 24 hours before transfection of MYC responsive luciferase reporter and ubiquitin promoter-driven *Renilla* luciferase reporter (Ayer et al., 1996; Schorpp et al., 1996). MYC luciferase reporter construct is driven by four copies of MYC responsive elements (Ayer et al., 1996). Firefly and renilla luciferase activities were measured with Dual-Glo Luciferase Assay System (Promega) and renilla luciferase values were used for data normalization.

SA-β-gal staining

In order to detect senescent cells, cells were fixed and stained for senescence associated-β-galactoside (SA-β-gal) at pH 6.0 according to manufacturer's protocol (Sigma). Morphologically flattened and SA-β-gal positive cells were counted as senescent cells under the microscope.

Antibodies

Antibodies used in Western blots against CIP2A, MYC, p53, p21 and β-actin proteins are the same than listed above.

5. RESULTS

5.1. CIP2A expression, correlation with clinicopathological markers and CIP2A as a prognostic factor in human breast cancer (I, II)

In order to study CIP2A expression in human breast cancer, *CIP2A* mRNA expression in 159 previously characterized breast cancer tumors was compared to 5 normal breast tissue samples (Chanrion et al., 2007). CIP2A protein expression was also analyzed in a three different tissue arrays containing a) 33 human breast cancer tumor samples, b) from FinProg study: 1228 breast cancer tumor samples (Lundin et al., 2001) and c) from FinHer study: 1010 advanced breast cancer patient tumor samples (Joensuu et al., 2006). FinHer cohort included only patients with advanced breast cancer, the most of them (89%) had axillary node-positive breast cancer and the rest had high-risk node negative cancer (Joensuu et al., 2006). At mRNA level *CIP2A* is significantly overexpressed compared to normal breast cancer tissues (I Figure 1). CIP2A protein expression is also induced in human breast cancer as compared to normal breast tissue (I Figure 2A). CIP2A protein was found to be expressed at the same level in the unselected tissue arrays of 33 breast cancer samples (I Figure 2B) and in the FinProg breast cancer samples, 39% and 46%, respectively (II Figure 1A and Supplementary figure 1A, B). Strikingly, in the advanced breast cancer patient samples (FinHer study) CIP2A was expressed in 79% of tumors (II Figure 7A).

In previously established microarray data set of 251 of human breast tumors (Miller et al., 2005), CIP2A expression significantly correlated with lower histologic grade of differentiation, lymph node positivity, expression of proliferation markers Ki-67 and PCNA, p53 mutation and progesterone receptor negativity (I Table 3). On the other hand, CIP2A does not correlate with patients age or with estrogen receptor status of the tumors (I Table 3). In FinProg and in FinHer breast cancer patient cohorts CIP2A expression correlates positively with several different clinicopathological poor prognostic markers (II Figure 1B, 7A, Supplementary figure 1C, D). In FinProg and in FinHer breast cancer samples CIP2A correlated also with Ki-67 proliferation marker, lower histologic grade of differentiation and with p53 immunopositivity indicative for mutated p53 and in addition with larger tumor size (II Figure 1B, 7A, Supplemental figure 1C, D). Additionally, *CIP2A* mRNA level was significantly higher in histologically classified aggressive breast cancers, invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC) and invasive ductal carcinoma with intraductal comedo carcinoma (IDC+ICC) compared to good prognosis mucinous carcinomas (MUC)(I Figure 1B). Notably, CIP2A expression in mucinous carcinoma and in normal breast tissue samples were at comparable levels (I Figure 1B).

As CIP2A expression associates with several aggressive breast cancer markers, it was hypothesized that high CIP2A expression in tumors might be a

prognostic factor for poor survival in breast cancer patients. However, when 159 breast cancer patients were divided into two groups according to their tumors CIP2A expression, either CIP2A expression over the median (high CIP2A) or lower than median (low CIP2A), overall survival between these groups was nearly statistically different (I Figure 1E). Interestingly, patients with high CIP2A expressing tumors had worse survival 5 years after surgery of mammary tumors (I Supplemental figure 1). Similarly, in FinHer study cohort CIP2A had nearly significant prognostic role in overall survival (II Supplemental figure 5A). Importantly, in a subgroup of FinHer patients, which breast tumors are Her2-negative, CIP2A expression associates with survival of the patients (II Figure 7B, Supplemental figure 5B). Notably, majority (68%) of the FinHer patients had HER2-negative breast tumors (II Figure 7A,B). However, in a multivariate analysis, CIP2A associated with poor outcome of HER2-negative breast tumor patients, but it is not an independent prognostic factor (p=0.058; for $CIP2A^{++}$ vs. $CIP2A^{-}$, hazard ratio (HR) = 4.26; 95% confidence interval (CI), 1.29-14.08; p=0.017; for CIP2A++ vs. CIP2A+, HR=1.54; 95% CI, 0.75-3.15; p=0.241). Moreover, neither tumor size, axillary nodal status, histologic grade or p53 expression were associated with survival of HER2-negative patients. In line with these data, CIP2A does not have prognostic role in survival of patients with Her2-positive tumors (II Supplemental figure 5C).

In conclusion, we found that CIP2A is expressed in human breast cancer and its expression is further increased during progression of breast cancer. Even though CIP2A associates with several clinicopathological markers of aggressive disease, CIP2A cannot be used as a common prognostic factor in breast cancer. However, in certain subgroups of breast cancer, CIP2A expression can still have a prognostic value.

5.2. CIP2A promotes proliferation and inhibits senescence in breast cancer (I, II)

5.2.1. CIP2A promotes proliferation in breast cancer (I)

In order to study whether CIP2A promotes breast cancer cell growth, CIP2A expression was silenced with siRNA and effects on proliferation, migration and tumorigenic growth were studied. CIP2A depletion with siRNA in MDA-MB-231 and in T47D breast cancer cells resulted in decreased proliferation in methylthiazolyldiphenyl-tetrazolium bromide (MTT) proliferation assay (I Figure 3C). In addition, inhibition of CIP2A in MDA-MB-231 and in T47D cells also decreases MYC expression (I Figure 3A, B), which has been previously shown to be associated with CIP2As action to promote proliferation in cancer (Junttila et al., 2007; Khanna et al., 2009). Notably, CIP2A inhibition did not have effect on phosphorylated Akt or phosphorylated MEK, indicating that CIP2A selectively inhibits phosphatase activity of PP2A towards MYC (I Figure 3A). Interestingly, CIP2A inhibition did not perturb the migration capacities of MDA-MB-231 cells in a scratch wound migration assay (I Figure 3D). Instead, CIP2A

clearly promotes tumorigenic growth of breast cancer cells, as CIP2A depletion in MDA-MB-231 cells by single siRNA transfection inhibited anchorage independent growth *in vitro* and orthotopic tumor xenograft growth *in vivo* (I Figure 4A, B). Single transfection of CIP2A siRNA, resulted in efficient inhibition of CIP2A protein expression for 7 days, which partly explains the significant difference in tumor volume and weight, 31 days after injecting control and CIP2A siRNA transfected MDA-MB-231 cells in mice (I Figure 4C, D).

5.2.2. Inhibition of CIP2A induces senescence in breast cancer cells (II)

In order to further study the ability of CIP2A to promote breast cancer growth, CIP2A siRNA transfected breast cancer cells were stained with senescence associated β-galactosidase (SA-β-gal) to detect senescent cells. Inhibition of CIP2A in MCF-7 and in MDA-MB-231 breast cancer cells, induces flattened cell morphology and SA-β-gal positivity (II Figure 3A, C). In addition, senescence marker decoy receptor 2 (DcR2) is also induced upon loss of CIP2A in MCF-7 cells (II Figure 3B). Despite that $CIP2A^{HOZ}$ mice do not have any obvious phenotypes, mouse embryonic fibroblasts (MEFs) isolated from $CIP2A^{HOZ}$ mice are growth arrested, show senescence-associated flattened morphology and positive SA-β-gal staining after a few passages in cell culture conditions (II Figure 5A, B, C, Supplemental figure 4A-H). In addition, when wild type and $CIP2A^{HOZ}$ mice skin were treated with DMBA carcinogen, which causes oncogenic mutation in H-Ras, leading to oncogene-induced senescence, $CIP2A^{HOZ}$ mice skin expressed statistically more SA-β-gal staining than wild type mice skin (Supplemental figure 4I, J).

In order to study whether CIP2A is an essential regulator of senescence, senescence was induced with different previously known senescence-inducers, small molecule Nutlin-3, doxorubicin and p21 expression, in human breast cancer cells transduced with adenovirus expressing CIP2A. Importantly, in MCF-7 human breast cancer cells transduction of CIP2A adenovirus prevented both Nutlin-3- and doxorubicin-induced p53-dependent senescence (II Figure 3E, F, I, J). In MDA-MB-231 cells, CIP2A expression also prevented p21-induced senescence (II Figure 4J, K). Although CIP2A expression prevented Nutlin-3-induced alterations in senescence-associated genes, CIP2A did not prevented induction of p21 expression (II Figure 3D, G). In $CIP2A^{HOZ}$ MEFs Nutlin-3 treatment could not potentiate senescence induced by loss of CIP2A, further indicating that inhibition of CIP2A mediates p53-induced senescence (II Figure 5D). Importantly, even though CIP2A is down-regulated after RITA treatment, constitutive CIP2A expression cannot rescue RITA-induced cell death in MCF-7 cells (II Supplemental figure 2F, G). This result further indicate that CIP2A promotes proliferation of breast cancer cells by inhibiting senescence and not by inhibiting cell death.

5.3. p53-induced pathways inhibit CIP2A expression (I, II, Figure 8)

5.3.1. p53 negatively regulates CIP2A expression (I, II)

In most of the normal tissues, including mammary glands of both human and mice, CIP2A is not expressed or expressed at low levels (I Figure 1A, 2A, II Figure 6A)(Junttila et al., 2007). However, in spontaneous mouse mammary tumors of a breast cancer mouse model presenting inducible deletion of Brca1 and p53 under keratin 14 promoter (Jonkers et al., 2001), *CIP2A* expression was increased (I Figure 2C). Importantly, CIP2A and Ki-67 protein expression co-localized in these mammary tumors (I Figure 2C).

As p53 mutation positively correlated with CIP2A expression in clinical breast cancer cohorts, and as inactivation of p53 function is a necessary for cell transformation, we hypothesized that p53 could negatively regulate *CIP2A* expression during cellular transformation (I Figure 1D, II Figure 1A, B, 7A). To study the mechanisms responsible for *CIP2A* up-regulation upon cellular transformation, MEFs, which also have low CIP2A expression, and human cancer cells were used as tools (II Figure 1C). Following *p53* inactivation by siRNA in MEFs, CIP2A protein was clearly up-regulated (II Figure 1C). Similar mechanism was also seen in MCF-7 breast cancer cells, which have inactivated wild type p53. After p53 re-activation, by Nutlin-3 or RITA, CIP2A was suppressed both at mRNA and at protein level in MCF-7 cells (II Figure 1D, E Supplemental figure 2A, B). Notably, Nutlin-3 had no effect on CIP2A expression in MDA-MB-231 breast cancer cells, harboring mutated and inactivated *p53* (II Figure 1F, G). Nevertheless, transduction of wild type p53 expressing adenovirus into MDA-MB-231 cells, resulted in CIP2A down-regulation (II Figure 1F, G). Interestingly, by analyzing activities of transcription factor pathways from previously published CIP2A transcriptome in human cervical cancer HeLa cells, CIP2A inhibition was found to resemble with p53 activation state (II Figure 1K). The analysis was performed by using Ingenuity Transcription Factor Analysis software. Importantly, in the Eμ-myc lymphoma mouse model carrying tamoxifen-inducible p53 allele (Martins et al., 2006), CIP2A expression was down-regulated following tamoxifen-treatment (II Figure 1I, J).

To confirm that regulation of CIP2A is truly p53-dependent, isogenic wild type and *p53*$^{-/-}$ colon carcinoma HCT116 cells were treated with doxorubicin, which activates p53. Following doxorubicin treatment, *p21* expression was only increased in wild type cells and *CIP2A* was suppressed in wild type cells but not in *p53*$^{-/-}$ cells (II Figure 1H). In order to study whether p53 regulates *CIP2A* transcription, previously published luciferase reporter construct containing the -1802 base pair *CIP2A* promoter fragment (Khanna et al., 2011), was transfected into MCF-7 cells before Nutlin-3 or RITA treatment. The *EGF receptor* (*EGFR*) promoter was used as a control promoter (Johnson et al., 2000). Nutlin-3 and RITA treatment inhibited transcriptional activity of the *CIP2A* promoter but not that of the *EGFR* (II Figure 2A, Supplemental figure

2C). Two predicted p53 binding sites were found on the *CIP2A* promoter by bioinformatic tools and the next step was to study by chromatin immunoprecipitation (ChIP), whether p53 binds to these predicted sites on the *CIP2A* promoter (II Figure 2B, Supplemental figure 2D). Although in HCT116 cells after doxorubicin treatment, p53 efficiently bound to its target gene promoters, *Mdm2* and *p21*, p53 did not bind to predicted sites on *CIP2A* promoter (II Figure 2C). As a result, we hypothesized that p53 regulates transcriptional activity of *CIP2A* through its downstream targets.

5.3.2. The transcription factor E2F1 downstream of p53 positively regulates CIP2A (II)

Nutlin-3 has been previously shown to induce a p53-dependent pathway including up-regulation of a direct target of p53, p21, leading to dephosphorylation of RB and inhibition of E2F1 (II Figure 2D)(Huang et al., 2009). By using isogenic wild type and $p21^{-/-}$ HCT116 cells, we studied whether CIP2A is regulated by p21. Following doxorubicin treatment in wild type HCT116 cells, *p21* expression was induced and *CIP2A* was decreased (II Figure 2E). However, in doxorubicin treated $p21^{-/-}$ HCT116 cells, CIP2A expression was unchanged (II Figure 2E). Moreover, similarly as in Nutlin-3-treated MCF-7 cells, both E2F1 and CIP2A expression was down-regulated by p21 expression in MDA-MB-231 cells (II Figure 2D, F). In MCF-7 cells, inhibition of E2F1 by siRNA inhibited CIP2A expression (II Figure 2G). In addition, in doxycycline-inducible E2F1 expressing Saos-2 osteosarcoma cells, *CIP2A* mRNA expression was also increased after doxycycline treatment (II Figure 2H). Together these results lead to a hypothesis that, downstream of inactivated p53, the transcription factor E2F1 positively regulates transcriptional activity of *CIP2A*.

By using a Genomatix software, a putative E2F1 binding site was found on the *CIP2A* promoter and possible binding of E2F1 to this site was studied by ChIP. Saos-2 cells transfected with E2F1 expressing vector, clearly enriched E2F1 on the predicted E2F1 binding site on the *CIP2A* promoter as compared to an empty vector transfected cells (II Figure 2I). This result was confirmed in ChIP-sequencing from HA-tagged E2F1 transfected MCF-7 cells (II Supplemental figure 2E). These results show that upon tumor suppression CIP2A expression is inhibited by p53-induced pathway via p21 induction and E2F1 suppression (II Figure 2J). *Vice versa*, during cellular transformation and tumor progression, due to p53 inactivation, E2F1 and CIP2A are overexpressed (II Figure 2J).

5.4 CIP2A-E2F1 feedback loop prevents cellular senescence (II)

5.4.1. CIP2A positively regulates E2F1 in breast cancer cells (II)

In order to study how breast cancer cells constitutively expressing CIP2A, avoid senescence induction, expression of downstream effectors in Nutlin-3-induced p53 pathway was studied. As expected, in CIP2A expressing and Nutlin-3-treated MCF-7 cells p53 and p21 were induced (II Figure 4A). In addition, phosphorylation of serine residues 807 and 811 of RB was also decreased (II Figure 4A), which has been shown before (Huang et al., 2009). However, in CIP2A expressing cells E2F1 was not down-regulated 8 hours after Nutlin-3 treatment, compared to control cells were both E2F1 and CIP2A were efficiently inhibited (II Figure 4A). However, at a later time point E2F1 expression was inhibited following Nutlin-3 treatment (II Figure 4E). CIP2A-mediated positive regulation of E2F1, was mainly post-transcriptional as at the same 8 hour time point *E2F1* mRNA expression was down-regulated in CIP2A expressing Nutlin-3-treated MCF-7 cells (II Figure 4B). Surprisingly, Nutlin-3 treatment also inhibited *E2F1* at the mRNA level, and we speculated that this is most likely due to a negative auto-regulation of E2F1. It has been shown that as a response to dephosphorylation of RB, E2F1 can inhibit its own promoter activity (Johnson et al., 1994b).

Stable CIP2A expression did not have any effect on RB phosphorylation in Nutlin-3-treated cells and a simultaneous depletion of RB and CIP2A by siRNAs, resulted in similar senescent phenotype as upon the depletion of CIP2A alone in MCF-7 cells. We concluded that RB inhibition cannot reverse senescence induced by CIP2A depletion (II Figure 4A, Supplemental figure 3B). As a conclusion, E2F1-CIP2A feedback loop was identified to suppress senescence in breast cancer cells.

5.4.2. Inhibition of PP2A regulatory subunit, B55α, increases phosphorylation of E2F1 serine 364 (II)

Interestingly, CIP2A overexpression was identified to up-regulate phosphorylation of serine 364 residue of E2F1 (II Figure 4C), which has been previously shown to stabilize E2F1, as this form is more resistant to proteosomal degradation as compared to dephosphorylated form (Kontaki and Talianidis, 2010; Stevens et al., 2003). CIP2A expression also inhibited Nutlin-3-induced down-regulation of serine 364 phosphorylated form of E2F1 for 24 hours, much longer than total E2F1 expression (II Figure 4D, E). Taken together, our results suggest that CIP2A promotes especially stable phosphorylated form of E2F1 in breast cancer cells.

CIP2A inhibition-induced changes in gene expression and in cancer cell growth, has shown to be rescued by inhibition of PP2A regulatory subunits, B55α and B56β (Niemelä et al., 2012). By depleting these B subunits via

siRNAs in MCF-7 cells, we tested whether inhibition of either B55α or B56β also are responsible for CIP2A-mediated senescence regulation (II Supplemental figure 3A, B). Inhibition of B55α, but not B56β, increased phosphorylation of E2F1 serine 364 (II Figure 4F). Similarly, as in CIP2A expressing cells, in B55α-depleted cells E2F1 down-regulation was inhibited following Nutlin-3 treatment (II Figure 4G). These results indicate that CIP2A positively regulates E2F1 protein expression by inhibiting dephosphorylation of E2F1 serine 364 via B55α-containing PP2A complex.

5.4.3. E2F1 down-regulation is needed for senescence induction (II)

In order to study whether E2F1 inhibition induces senescence in MCF-7 cells, as it does in many other cancer cell lines, E2F1 was silenced by short hairpin RNA (shRNA). As expected, E2F1 silencing resulted in senescence induction in MCF-7 cells (II Figure 4H). Importantly, Nutlin-3 treatment and E2F1 inhibition were relatively as efficient in senescence induction, but Nutlin-3 could not further increase E2F1 inhibition-induced senescence (II Figure 4H, I).

Similarly to human breast cancer cells, in MEFs Nutlin-3 decreased E2F1 expression and continuous CIP2A expression rescued E2F1 inhibition by Nutlin-3 (II Figure 5E). These results demonstrate that E2F1 inhibition is essential for senescence induction downstream of p53 activation.

5.4.4. p53-induced down-regulation of MYC via CIP2A is not linked to senescence induction (Figure 8)

Interestingly, Nutlin-3 was found to also inhibit MYC expression in MCF-7 cells (Figure 8A). In the bioinformatic transcription factor pathway analysis, in addition to increased p53 activity, MYC activity was decreased in CIP2A-depleted HeLa cells (II Figure 1K). As previously, CIP2A has been shown to promote MYC stability and as inhibition of MYC in cancer cells has been shown to induce senescence (Junttila et al., 2007; Wu et al., 2007), we studied whether CIP2A-mediated MYC regulation affects to senescence induction. Importantly, CIP2A expression rescued MYC inhibition by Nutlin-3 in MCF-7 cells, similarly as E2F1 expression (Figure 8B). Additionally, stable CIP2A expression inhibited p53-induced down-regulation of the transcriptional activity of MYC (Figure 8C), as measured by the activity of a promoter construct that is driven by four copies of MYC responsive elements (Ayer et al., 1996). However, MYC depletion by siRNA in MCF-7 cells did not result in senescent phenotype, but rather induced cell death (Figure 8D,E). Moreover, combined inhibition of MYC and CIP2A in MCF-7 cells, provoked both cell death and senescent phenotype. These results indicate that CIP2A depletion can induce senescence in MYC negative cells.

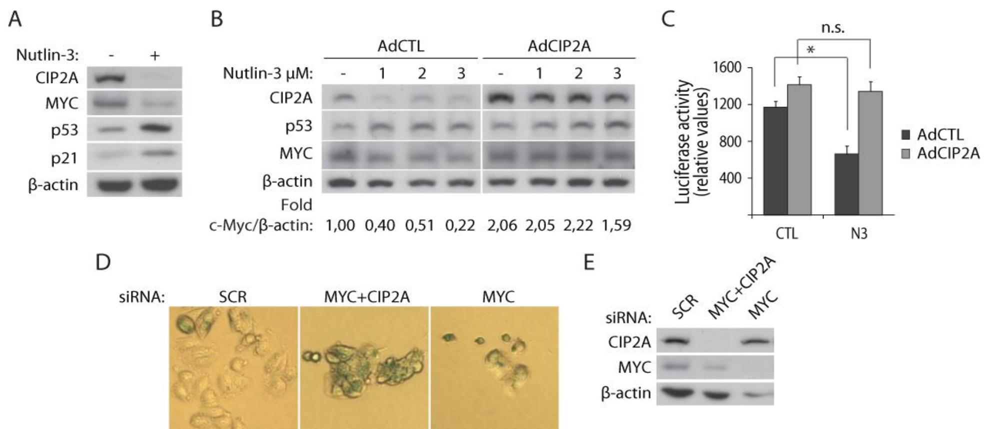

Figure 8. MYC inhibition by p53 does not regulate CIP2A inhibition –induced senescence.
A) Western blot analysis of CIP2A, MYC, p53 and p21 protein expression in MCF-7 cells treated with Nutlin-3 (5 µM) for 2 days. B) Western blot analysis of CIP2A, p53 and MYC protein expression in MCF-7 transduced either with control adeno (AdCTL) or with CIP2A expressing adenovirus (AdCIP2A)(MOI=40) and treated with Nutlin-3 for 24 hours. Quantitation of MYC expression normalized to β-actin is shown below the blot. C) AdCTL and AdCIP2A transduced (MOI=40) MCF-7 cells were transfected with E-box luciferase reporter and treated with Nutlin-3 (N3, 2µM) for 24 hours. Transfection efficiency was monitored by co-expressing cells with Ubi-Renilla luciferase construct. Shown is mean + SEM of two independent experiments. *p=0,0337, n.s.=0,8299 by Student's t-test. D) SA-β-gal staining of MCF-7 cells 5 days after transfection either with scrambled (SCR), MYC or both MYC and CIP2A (MYC+CIP2A) siRNAs. E) Western blot analysis of CIP2A and MYC expression 4 days after transfection either with SCR, MYC or both MYC and CIP2A siRNAs.

5.4.5. CIP2A promotes breast cancer growth by suppressing senescence (II)

To show that CIP2A also is an essential mediator of carcinogenesis and senescence in breast cancer *in vivo*, CIP2A^{HOZ} mice were crossed with a breast cancer mouse model MMTVneu. MMTVneu mice express proto-oncogene *HER2* specifically in mammary glands (Guy et al., 1992). Spontaneous tumors in MMTVneu mice have high *CIP2A* expression (II Figure 6A). As compared to parental MMTVneu mice (neu/WT), MMTVneu x CIP2A^{HOZ} (neu/HOZ) mice had significantly less mammary gland tumors. In addition, in neu/HOZ mice, as compared to neu/WT mice, tumor progression was delayed, when counting days when tumor appeared until mice had to be sacrificed due to maximum allowed size of tumor was reached (II Figure 6A, D, E). Notably, proliferation was reduced in neu/HOZ mouse mammary glands as studied by Ki-67 immunohistochemically in macroscopically tumor-free mammary glands (II Figure 6B, C). Importantly, neu/HOZ mouse mammary tumors showed senescence-associated genes expression changes, and induced expression of senescence marker DcR2 (II Figure 6F, G). Moreover, isolated neu/HOZ mammary tumor cells entered rapidly spontaneous senescence in cell culture as measured with SA-β-gal staining (II Figure 6H). Notably, senescent phenotype in tumors was associated with decreased E2F1

protein expression and also decreased expression of E2F1 target genes (II Figure 6I, J, Supplemental figure 4K). In summary, E2F1-CIP2A feedback loop promotes proliferation of breast cancer by suppressing senescence *in vivo*.

5.5 The role of CIP2A in breast cancer therapy response (II)

Finally, we studied whether CIP2A expression in breast cancer tumors can define their response to chemotherapies. In order to study this, FinHer cohort of advanced breast cancer tumor samples were used. Patients involved in FinHer study were divided according their HER2 expression status to HER2-negative and HER2-positive groups (Joensuu et al., 2006). HER2-positive tumor bearing patients were mainly treated with trastuzumab-therapy, a monoclonal antibody targeting HER2 (Joensuu et al., 2006). In contrast, patients with HER2-negative breast cancer tumors lack efficient therapy options. In FinHer study, patients with HER2-negative tumors were randomly given either vinorelbine or docetaxel chemotherapy followed by three cycles of fluorouracil, epirubicin and cyclophosphamide (FEC) in both groups (II Figure 7C). Importantly, in vinorelbine-treated group patients with CIP2A expressing tumors had significantly worse survival than patients with CIP2A-negative tumors (II Figure 7D). However, CIP2A did not have a prognostic role in docetaxel treated patients (II Supplemental figure 5D).

Interestingly, vinorelbine induced senescent phenotype in MCF-7 breast cancer cells and in addition, it down-regulated E2F1 and CIP2A mRNA and protein expression without inducing *p53* or *p21* (II Figure 7E, F, G, Supplemental figure 5E, F). Importantly, CIP2A-deficient MCF-7 cells were more sensitive for vinorelbine-induced E2F1 down-regulation (II Figure 7H). Stable CIP2A expression also rescued vinorelbine-induced E2F1 inhibition in MCF-7 cells, similarly as shown with Nutlin-3 (II Figure 7I). Taken together, these results show that low CIP2A expression in breast tumors sensitizes for senescence-inducing vinorelbine treatment. These results also indicate that E2F1-CIP2A feedback loop mediates the resistance for senescence-inducing chemotherapies in breast cancer (II Figure 7J).

6. DISCUSSION

6.1. Mechanisms promoting CIP2A-mediated cancer growth

6.1.1. Mechanisms up-regulating CIP2A in cancer

Inhibition of PP2A is one of the prerequisites for transformation of normal cells into cancer cells (Hahn et al., 1999a; Hahn et al., 2002). Recently, in the context of this work, transformed human mammary epithelial cells (HMECs) were demonstrated to exhibit less PP2A activity compared to immortalized HMECs (Morel et al., 2012). Thereby, it is important to understand the underlying mechanisms that inactivate PP2A. One possible mechanism is overexpression of endogenous inhibitors of PP2A, such as CIP2A. CIP2A is expressed at low or even undetectable levels in most of the normal human tissues, but in multiple different cancer types studied thus far CIP2A is overexpressed (Table 1). Several mechanisms for CIP2A up-regulation have been proposed. Firstly, a transcription factor ETS1 promotes *CIP2A* expression in human cancer via the EGFR-MEK pathway (Figure 9)(Khanna et al., 2011). Secondly, in Ras transformed mouse fibroblasts, JNK2 was shown to promote CIP2A expression (Figure 9)(Mathiasen et al., 2012). In addition, CIP2A and MYC have been shown to feed each other's expression, as CIP2A promotes MYC stability by increasing MYC serine 62 phosphorylation and, in human gastric cancer and in melanoma, MYC has been shown to induce CIP2A expression (Figure 9)(Junttila et al., 2007; Khanna et al., 2009; Mannava et al., 2012). Interestingly, bioinformatic analysis revealed that, in HeLa cells, the transcriptional response to CIP2A depletion mimicked inhibition of MYC activity, and in addition, induction of p53 activity (II). Additionally, p53 mutation was shown to positively correlate with CIP2A expression in human breast cancer tumor samples in three different patient cohorts, leading to a hypothesis that p53 inactivation in transforming cells might up-regulate CIP2A (I,II). p53-mediated negative regulation of CIP2A, was confirmed in MEFs and in several experiments in cancer cells and also in a lymphoma mouse model (II). However, it was noted that CIP2A is not a direct target of p53 and that the p53-RB-E2F1 pathway mediates the regulation. Based on these findings, it can be concluded that during transformation the impaired p53-RB-E2F1 pathway leads to up-regulation of CIP2A (II).

Out of four thus far identified CIP2A regulators, MYC, ETS1, JNK2 and E2F1, JNK2-mediated regulation of CIP2A is the only mechanism that has been confirmed to have an essential role in transformation of mouse fibroblasts (Mathiasen et al., 2012). ETS1- and MYC-mediated CIP2A regulation have been shown in cancer cells, leading to a possibility that these mechanisms are more essential in promoting oncogenic properties of CIP2A during cancer progression (Khanna et al., 2009; Khanna et al., 2011; Mannava et al., 2012). However, as ETS1, MYC and p53 mechanisms are identified in different

cancer types, there might also be tissue specificity in CIP2A regulation (Khanna et al., 2009; Khanna et al., 2011; Mannava et al., 2012).

Our finding that the p53-RB-E2F1 pathway regulates expression of the PP2A inhibitor, CIP2A, in cancer suggests that function of two major tumor suppressors, p53 and PP2A, are connected (II). Interestingly, several publications have shown that PP2A regulates p53 (Li et al., 2002; Ruediger et al., 2011; Seeling et al., 1999; Seshacharyulu et al., 2013; Shouse et al., 2008; Shouse et al., 2010). These results indicate that there is a feedback regulation between p53 and PP2A. The p53-PP2A regulation will be discussed later.

6.1.2. Post-translational regulation of E2F1 by PP2A

E2F1 is regulated by multiple post-translational modifications (Figure 7). Notably, most of the studies demonstrate that post-translational modifications of E2F1 increase its stability, leading to induction of apoptosis machinery upon DNA damage response (Polager and Ginsberg, 2009). However, post-translational modifications of E2F1 involved in regulation of proliferation, are not that well understood. Here, in breast cancer cells CIP2A was identified to promote E2F1 phosphorylation into serine 364 (II). Phosphorylation of serine 364 site has been previously shown to promote more stable form of E2F1 (de Olano et al., 2012; Stevens et al., 2003). Our results, showing that the E2F1-CIP2A feedback loop promotes proliferation of breast cancer by inhibiting senescence, suggest that serine 364 phosphorylation of E2F1 is a novel post-translational modification regulating E2F1 in proliferation.

Feedback loop between E2F1 and CIP2A was identified to regulate senescence induction both in human and in mouse cells (II). Although, phoshorylation of serine 364 residue of E2F1 is conserved in primates, but not in rodents (Carnevale et al., 2012), E2F1-CIP2A feedback loop is still present in mouse cells (II). However, there are multiple post-translational modification sites in E2F1, making it possible that CIP2A promotes phosphorylation of E2F1 on several sites, in addition to serine 364, both in human and in mouse cells. In addition, other phosphorylation sites are also known to increase the stability of E2F1, such as phosphorylation of serine 332 and 337, which prevents interaction between RB and E2F1 (Fagan et al., 1994; Mundle and Saberwal, 2003). Studies are needed to clarify, which post-translational modifications of E2F1 are regulated by CIP2A in cancer.

CIP2A promoted serine 364 phosphorylation of E2F1 by B55α containing PP2A complex (II). Shortly, inhibition of B55α-PP2A complex mimics CIP2A overexpression by shifting E2F1 protein more to serine 364 phosphorylated form (II). Previously, inhibition of B55α by siRNA was shown to efficiently rescue CIP2A depletion-induced alterations in cancer cell growth and in gene expression profiles (Niemelä et al., 2012). These results indicate that inhibition of B55α-PP2A complex is a crucial mediator of CIP2A's oncogenic properties

in cancer. Importantly, clinical data has implicated a tumor suppressive role for B55α. In prostate cancer 67% of tumors had deleted *B55α* (Cheng et al., 2011). A recent publication also demonstrated decreased expression of *B55α* in non-small cell lung cancer (Kalev et al., 2012). Importantly, deletion of *B55α* in human luminal B type breast cancer samples, was reported as a potential driver mutation (Curtis et al., 2012). Additionally, an oncogenic microRNA-31 has been shown to repress B55α in mouse and in human lung cancer cells (Liu et al., 2010). However, when requirements of different B subunit inhibition in transformation of immortalized human embryonic kidney cells was studied, inhibition of B55α was not necessary (Sablina et al., 2010). These results might be explained by tissue specific requirements, as different cell types have been shown to have distinctive requirements for alterations in oncogene and tumor suppressor activities, in order to transform into cancer cells (Rangarajan et al., 2004). Interestingly, recent publications have demonstrated an opposite role for B55α in cancer. Inhibition of B55α in HeLa cells was demonstrated to result in increased activity of ATM and Chk2, leading to G1/S cell cycle arrest (Kalev et al., 2012). Another study reported that upon glutamine deprivation-induced stress, B55α is elevated, leading to activation of p53 and increased cancer cell survival (Reid et al., 2013). These results indicate that although clinical evidence strongly support that B55α is a tumor suppressor, the role of B55α as a tumor suppressor may be highly context dependent.

PP2A is a serine/threonine phosphatase that regulates almost all functions in cell (Eichhorn et al., 2009). Thereby, it is plausible that serine 364 residue of E2F1 is also a direct dephosphorylation target of PP2A. However, some upstream effectors of E2F1 are known to be regulated by PP2A. PP2A dephosphorylates and inhibits activity of Chk2 and its upstream activator ATM (Dozier et al., 2004; Liang et al., 2006; Petersen et al., 2006). On the other hand, serine 364 residue of E2F1 was shown to be phosphorylated by Chk2, as a response to DNA damage (Stevens et al., 2003). Although most of the studies are performed under DNA damage conditions, inhibition of PP2A by okadaic acid was shown to increase phosphorylation of Chk2 without DNA damage induction (Carlessi et al., 2010). Notably, as mentioned above, inhibition of B55α-containing PP2A complex has been reported to increase phosphorylation of ATM, leading to elevated activity of Chk2 (Kalev et al., 2012). Based on this information, CIP2A overexpression-triggered decrease in B55α-PP2A-mediated dephosphorylation towards serine 364 E2F1 could happen either directly or indirectly through previously known dephosphorylation targets of PP2A, ATM or Chk2.

Additionally, PP2A has been shown to indirectly regulate E2F1 through c/EBPα and TRIP-Br1 (Yin et al., 2009; Zang et al., 2009). Inhibition of PP2A was shown to increase interaction of E2F1 and c/EBPα in prostate cancer cells (Yin et al., 2009). Overexpression of c/EBPα induces E2F1, decreases PP2A activity and accelerates prostate cancer cell growth (Yin et al., 2009). In another study, in normal kidney and in renal carcinoma cells, B55α-containing PP2A complex was shown to dephosphorylate and regulate protein expression

of TRIP-Br1 proto-oncoprotein (Zang et al., 2009). Furthermore, TRIP-Br1 was shown to promote transcription of E2F1 (Zang et al., 2009). Based on these studies, a slight increase in *E2F1* mRNA after Nutlin-3 treatment in stable CIP2A expressing cells (II) might be explained, in addition to auto-regulation of *E2F1*, by altered expression of CIP2A/PP2A-regulated proteins, c/EBPα and TRIP-Br1.

6.2. CIP2A regulates multiple oncogenic pathways during breast carcinogenesis

Previously and also in this thesis, CIP2A has been shown to sustain proliferation of cancer cells by inhibiting proteolytic degradation of MYC protein (I)(Junttila et al., 2007; Khanna et al., 2009; Lucas et al., 2011; Niemelä et al., 2012). Interestingly, bioinformatic analysis of CIP2A transcriptome showed that the gene expression profile of CIP2A-depleted cells, resembled that of cells in which MYC is inactivated, in addition to cells upon p53 activation (II). Moreover, even though p53 reactivation inhibited MYC via CIP2A, CIP2A inhibition-induced senescence was MYC-independent (Figure 8). MYC inhibition has been shown to induce apoptosis and decrease proliferation in cancer (Cappellen et al., 2007; Huang et al., 2006; Koh et al., 2011; Wu et al., 1996). Thereby, we propose that CIP2A promotes breast carcinogenesis via at least two different pathways, MYC and E2F1 (Figure 9).

In addition to E2F1, there is evidence that CIP2A can also inhibit apoptotic pathways in cancer. CIP2A has been shown to support the activity of anti-apoptotic Akt and suppress UNC5H2-induced apoptosis (Chen et al., 2010; Guenebeaud et al., 2010). Likely, CIP2A-mediated regulation of senescence, proliferation and apoptosis might be cancer type-specific. However, since CIP2A is widely expressed in human cancer, CIP2A most likely regulates multiple pathways in order to promote tumor growth (Figure 9).

Even though CIP2A has been shown to work as an oncogene in several different *in vitro* assays and in xenograft experiments with human cancer cells (Junttila et al., 2007; Khanna et al., 2009; Niemelä et al., 2012), the results of this thesis provides the first genetic evidence that CIP2A is an oncogene in a spontaneous cancer mouse model (I,II). These results are interesting, as it is known that inhibition of PP2A is not needed for rodent cell transformation (Rangarajan et al., 2004). Specifically, in our study, loss of CIP2A in the MMTVneu model did not prolong the tumor initiation of the first tumor in mammary glands (data not shown). Instead, CIP2A-deficient MMTVneu mice had prolonged tumor progression (II). These results indicate that in rodents, PP2A has tumor suppressive role: even though CIP2A is not necessary for transformation, it promotes tumor progression. However, in a recent publication, mice expressing mutated and inactive PP2A Aα subunit, had increased incidence of carcinogen-induced lung cancer (Ruediger et al., 2011). This result indicates a role for PP2A also in tumor initiation in mice.

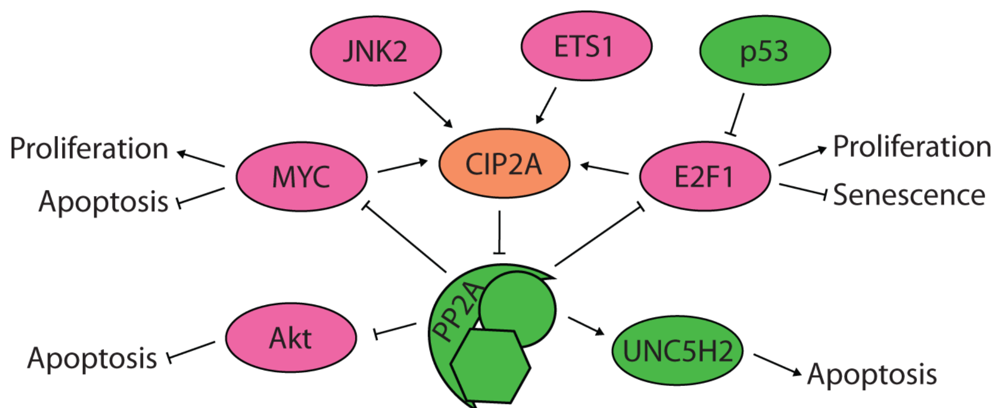

Figure 9. CIP2A is regulated by and regulates multiple cancer relevant pathways. Oncogenic proteins are indicated in pink and in orange. Tumor suppressive proteins are indicated in green.

As discussed previously, a link between two tumor suppressors, p53 and PP2A, was identified in this study. In rodent cells, p53-PP2A connection could explain why inactivation of p53, but not PP2A, is needed for rodent cell transformation. As mentioned, CIP2A regulates multiple oncogenic pathways (Figure 9). Thereby, p53 inhibition-induced inactivation of CIP2A-selective PP2A activities, might induce the essential PP2A regulated pathways needed for rodent cell transformation. However, for human cell transformation, in addition to perturbation of only two pathways as in rodents, alterations of five pathways are needed, including inactivation of PP2A (Hahn et al., 1999a; Hahn et al., 2002; Rangarajan et al., 2004). Overall, human cells have more complicated signaling pathways as compared to rodent cells. In transformation studies, SV40 small T antigen (ST) is a widely used tool to inhibit PP2A (Sablina and Hahn, 2008). Interestingly, CIP2A expression, as well as ST, has been shown to transform immortalized human embryonal kidney cells (Hahn et al., 2002; Junttila et al., 2007). However, ST transformed cells can form more colonies in soft agar than CIP2A transformed cells. This indicates that ST also inhibits, in addition to CIP2A-selective PP2A functions, the remaining tumor suppressive PP2A complexes, resulting in activation of more oncogenic pathways (Hahn et al., 2002; Junttila et al., 2007).

6.3. E2F1-CIP2A feedback loop inhibits senescence

Senescence has been shown to be a barrier that normal cells have to bypass in order to transform into cancer cells (Collado and Serrano, 2010; Kuilman et al., 2010). In addition, senescence can delay tumor progression (Collado and Serrano, 2010; Kuilman et al., 2010). Several mechanisms, regulating senescence induction, have been identified (Collado and Serrano, 2010). However, one well-defined regulator of senescence is p53 (Polager and Ginsberg, 2009). In addition to senescence, p53 regulates several other

cellular functions (Polager and Ginsberg, 2009). Interestingly, several publications have indicated that tumor suppressive effect of p53, is actually mediated by its ability to induce senescence rather than to induce apoptosis or DNA damage response (Brady et al., 2011; Chen et al., 2005b; Liu et al., 2004a; Xue et al., 2007).

In my thesis study, a novel mechanism regulating senescence was identified. Inhibition of E2F1-CIP2A feedback loop, downstream of p53, was demonstrated as an essential requirement for senescence induction in human breast cancer cells (II). Importantly, this feedback loop was identified to delay tumor progression in MMTVneu breast cancer mouse model (II). Thereby, inhibition of E2F1-CIP2A feedback mechanism is a novel tumor suppressive function for p53. However, we showed that this feedback loop can also inhibit senescence independently of p53 activation (II). In addition, several other research groups have demonstrated p53-independent mechanisms for senescence induction (Aliouat-Denis et al., 2005; Ha et al., 2007; Jacobs and de Lange, 2004; Lin et al., 2010a; Zou et al., 2002). Notably, a large fraction of human cancers harbor mutated p53 and thereby, senescence induction without involvement of p53, is a prospective strategy for tumor suppression in p53 mutant cancers. In addition, E2F1-CIP2A feedback loop-regulated senescence provides a plausible explanation for our results that inhibition of CIP2A by siRNA reduced xenograft growth of human breast cancer cells harboring mutated p53 (I).

The role of PP2A in senescence has not been widely studied thus far. Two publications have shown before that PP2A can mediate senescence induction, another study was performed with HeLa cells and another one with melanoma (Chuang and Hung, 2011; Mannava et al., 2012). Interestingly, in melanoma study, CIP2A expression was increased in melanoma cells as compared to normal melanocytes (Mannava et al., 2012). Notably, in same melanoma cell lines inhibition of E2F1, and increased PP2A B subunit expression, was demonstrated to induce senescence (Mannava et al., 2012; Verhaegen et al., 2012). In support to our results, ectopic expression of E2F1 in melanoma cells, similarly to ectopic expression of CIP2A in breast cancer cells, was reported to inhibit Nutlin-3-induced senescence (Verhaegen et al., 2012). These findings indicate that PP2A could also mediate CIP2A-E2F1 feedback regulation in other cancer types than in breast cancer. However, in melanoma, PP2A B56γ was shown to regulate senescence (Mannava et al., 2012), as compared to B55α in breast cancer in our study (II). Further studies are needed to investigate whether, in addition to B55α and B56γ, other B subunits also are involved in senescence regulation. In contrast, PP2A inhibition has been shown to mediate senescence in some models (Park et al., 2007; Volonte and Galbiati, 2009). Thereby, the tumor suppressive role of PP2A in senescence regulation needs further clarification.

Interestingly, *TWIST1* was one of the senescence inhibiting genes that was down-regulated in *CIP2A*-depleted MMTVneu mammary tumors (II). Studies

have shown that some proteins, such as ZEB and TWIST, regulating epithelial-mesenchymal transition (EMT), can also regulate cellular senescence (Sanchez-Tillo et al., 2012). Recently, EMT inducers, TWIST1 and ZEB1/2, were reported to promote transformation of immortalized human mammary epithelial cells (HMECs)(Morel et al., 2012). Importantly, TWIST1 and ZEB1/2-induced transformation resulted in reduction of PP2A activity (Morel et al., 2012). Taken together, these results suggest an interesting possibility that senescence is a limiting factor in tumor metastasis. In addition, the results of Morel and colleagues show that PP2A is inhibited during EMT, further indicating that by this mechanism PP2A may inhibit metastasis (Morel et al., 2012). This was supported also by finding that CIP2A expression correlates with lymph node positivity in human breast cancer (I). Whether CIP2A is a promoter of conversion of primary cancer cells to metastatic cells by supporting EMT, is an interesting research question that should be studied in the future.

6.4. CIP2A as a prognostic factor for survival and in a therapy response in breast cancer

In three cohorts of breast tumor samples studied, CIP2A expression correlated with many factors that are associated with poor prognosis of breast cancer, such as with big tumor size, high histological grade, lymph node positivity and with expression of proliferation markers, Ki-67 and PCNA (I,II). Similarly, in many other cancer types, CIP2A associates positively with high tumor grade and with poor prognostic markers (Table 1). However, in all of these breast cancer cohorts, high CIP2A expression does not predict poor prognosis in all patients, even in the FinHer study where CIP2A is expressed in 79% of all tumors of patients with advanced breast cancer (II). However, when patients in the FinHer study were divided according their tumors' HER2 expression status (Joensuu et al., 2006), it was noted that CIP2A expression predicts for unfavorable survival for patients bearing HER2-negative breast tumor (II). Moreover, in a multivariate analysis of patients with HER2-negative tumors, CIP2A expression was nearly an independent prognostic factor. Interestingly, in this analysis neither tumor size, axillary nodal status, histologic grade or p53 expression correlate significantly with survival of HER2-negative tumor patients. Only loss of estrogen receptor (ER) expression was an independent prognostic marker in a group of patients with HER2-negative tumors. It is possible that HER2 is so strong oncogene in breast cancer that it hides the effect of CIP2A in patients with HER2-positive breast tumors. Based on this, there might be two different pathways in breast cancer development and in one of them HER2 is a driver oncogene, whereas in HER2-negative tumors the major oncogene is CIP2A.

Notably, most of the tumors, expressing low levels of E2F1 also are HER2-negative (Vuaroqueaux et al., 2007). Importantly, patients with HER2-negative breast cancer have significantly better outcome, if they have low expression of E2F1 in tumors than high E2F1 expression, similarly as with CIP2A positivity

(Vuaroqueaux et al., 2007). The results of Vuaroqueaux and colleagues and the results of this thesis study indicate that activation of E2F1-CIP2A feedback loop in cancer promotes specifically the progression of HER2-negative breast cancer and thus high E2F1 and CIP2A predict for a poor outcome.

The results of this thesis study show that E2F1-CIP2A feedback loop determines the sensitivity for senescence-inducing therapies in HER2-negative breast cancer (II). Previously, in one publication, CIP2A inhibition has been indicated to sensitize hepatocellular carcinoma cells to bortezomib treatment (Chen et al., 2010). Notably, another publication have showed that overexpression of CIP2A in breast cancer cells can overcome doxorubicin-induced inhibition of proliferation (Choi et al., 2011). Similarly, in our studies stable expression of CIP2A prevented doxorubicin-induced senescence in human breast cancer cells (II). These results indicate that low CIP2A expression in tumors might be a general mechanism to make tumor cells more sensitive for chemotherapies. This further demonstrates that CIP2A expression could be used in breast cancer patient stratification, when selecting senescence-inducing chemotherapies.

In this study, B55α containing PP2A complex was shown to mediate the feedback signaling from CIP2A to E2F1 thereby, making CIP2A-negative and B55α expressing breast cancer cells more sensitive for senescence-inducing therapies (II). However, recently the suppression of *B55α* was demonstrated to inhibit homologous recombination DNA repair by modulating phosphorylation of ATM (Kalev et al., 2012). Inhibition of *B55α* also sensitized human cancer cells for PARP inhibitor, providing an alternative treatment method for CIP2A-positive cancers (Kalev et al., 2012). Thereby, further studies are needed to clarify the role of B55α and, more generally, PP2A in regulating response to different chemotherapies.

6.5. Feasibility and consequences of targeting E2F1-CIP2A feedback loop for pro-senescence cancer therapy

Traditionally, cancers have been treated with cytotoxic drugs, which are expected to result in dramatic cell death in tumors, but also they can cause severe side effects. One option to reduce proliferation of cancer cells without massive cell death, is to induce senescence in tumors (Ewald et al., 2010; Roninson, 2003). In fact, several chemotherapies nowadays used in clinics exert at least partly their therapeutic efficacy by inducing senescence (Ewald et al., 2010). However, new approaches to induce specifically senescence response in cancer are needed. Some approaches have been suggested, such as inhibition of Pten by an inhibitor, VO-OHpic, and inhibition of Skp2 by an inhibitor of the SCF-Skp2 complex, MLN4924 (Alimonti et al., 2010; Lin et al., 2010a). Both of these inhibitors were shown to induce senescence in *in vivo* prostate cancer models and MLN4924 is currently under clinical trials (Alimonti et al., 2010; Lin et al., 2010a; Nawrocki et al., 2012). E2F1-CIP2A feedback

loop serves as a novel target for senescence-inducing chemotherapies. The clear benefit of targeting E2F1-CIP2A mechanism is a possibility to induce senescence independently of p53, as a large fraction of cancers express mutated p53. Additionally, CIP2A inhibition was demonstrated to induce senescence without functional RB pathway (II), further increasing the group of patients that could benefit from inhibition of E2F1-CIP2A feedback loop. Importantly, another study verified that E2F1 inhibition induced senescence without p16-RB signaling (Verhaegen et al., 2012). Even though E2F1-CIP2A feedback loop was identified only in breast cancer (II), a finding that E2F1 inhibition is crucial for senescence induction in melanoma, as mentioned before (Verhaegen et al., 2012), indicates that E2F1-CIP2A feedback loop regulates senescence in different human cancer types. These facts, and the overexpression of E2F1 (Alonso et al., 2005; De Meyer et al., 2009; Han et al., 2003; Midorikawa et al., 2004; Reimer et al., 2006; Wilting et al., 2008; Zondervan et al., 2000) and CIP2A in various cancer types (Table 1), makes E2F1-CIP2A feedback loop a very attractive target in multiple cancer types.

In most of the normal human and mouse tissues, including mammary glands, CIP2A is expressed at low levels as a contrast to cancer, where CIP2A is highly expressed in most of the cases (Table 1)(Junttila et al., 2007; Ventelä et al., 2012). An only exception is testis which express CIP2A and where loss of CIP2A reduces sperm count in mice (Ventelä et al., 2012). Additionally, CIP2A-depleted ($CIP2A^{HOZ}$) mice do not show any obvious anatomical or physiological phenotypes, making it highly unlikely that CIP2A inhibition would have severe side effects in cancer treatments (Ventelä et al., 2012).

E2F1 is known to have both tumor suppressive and oncogenic capabilities (Pierce et al., 1999; Yamasaki et al., 1996). E2F1 has been shown to promote apoptosis and therefore up-regulation of E2F1 has been proposed as a therapy approach (Hallstrom and Nevins, 2009). However, to avoid possible E2F1-mediated proliferation promotion effects in cancer cells, a safer option is to inhibit E2F1 which should also result in down-regulation of CIP2A and senescence induction (Park et al., 2006; Verhaegen et al., 2012). $E2F1^{-/-}$ mice are viable even though they have some abnormalities, including testicular atrophy, exocrine gland dysplasia, defects in T lymphocyte development and even tumors at older age (Field et al., 1996; Yamasaki et al., 1996). Thereby, targeting E2F1-CIP2A feedback loop and therefore only E2F1 targets associated with senescence induction, side effects which could be possibly associated with E2F1 inhibition could be avoided.

Although targeting of E2F1-CIP2A feedback loop appears an attractive approach to inhibit tumor growth, tools to target selectively this loop are lacking. Inhibitors targeting E2Fs have been generated, however, they are not specific for certain E2F proteins (Bandara et al., 1997; Fabbrizio et al., 1999; Ma et al., 2008; Montigiani et al., 2003). Notably, inhibition of CIP2A by siRNA has been shown to result in significant reduction of human cancer cell growth *in vivo* (I)(Junttila et al., 2007; Xue et al., 2013). As discussed above, in

addition to E2F1, CIP2A has multiple oncogenic targets, MYC, Akt and UNC5H2 (Figure 9)(Chen et al., 2010; Guenebeaud et al., 2010; Junttila et al., 2007; Khanna et al., 2009). For this reason, it can be speculated that inhibition of CIP2A, for example by RNA interference therapy, could be advantageous as a cancer therapy by inhibiting two pro-proliferative feedback loops, E2F1-CIP2A and MYC-CIP2A, and by inducing apoptotic machinery via inhibition of anti-apoptotic Akt and inducing pro-apoptotic UNC5H2 (Chen et al., 2010; Guenebeaud et al., 2010; Khanna et al., 2009; Mannava et al., 2012). Notably, regulation of all these CIP2A targets are mediated by PP2A. Thereby, there is a demand for the identification of PP2A binding site in CIP2A and further development of an inhibitor against CIP2A-PP2A interaction.

7. SUMMARY

CIP2A is an oncoprotein that is overexpressed in many human cancer types. However, the *in vivo* importance of CIP2A has not been shown. This study demonstrated for the first time that CIP2A is an oncogene *in vivo* in a cancer mouse model. Moreover, this study identified a novel mechanism for CIP2A induction by p53 inactivation in cellular transformation (Figure 10). Importantly, in this study a previously unknown feedback loop maintaining CIP2A and E2F1 expression in cancer has been identified, and this mechanism was demonstrated to promote breast cancer progression by inhibiting cellular senescence (Figure 10). Notably, E2F1-CIP2A loop affects the senescence-inducing cancer therapy response, and thereby our results could be useful in stratifying patients for breast cancer therapies (Figure 10). Finally, this thesis demonstrates that CIP2A is a potential senescence-inducing cancer therapy target.

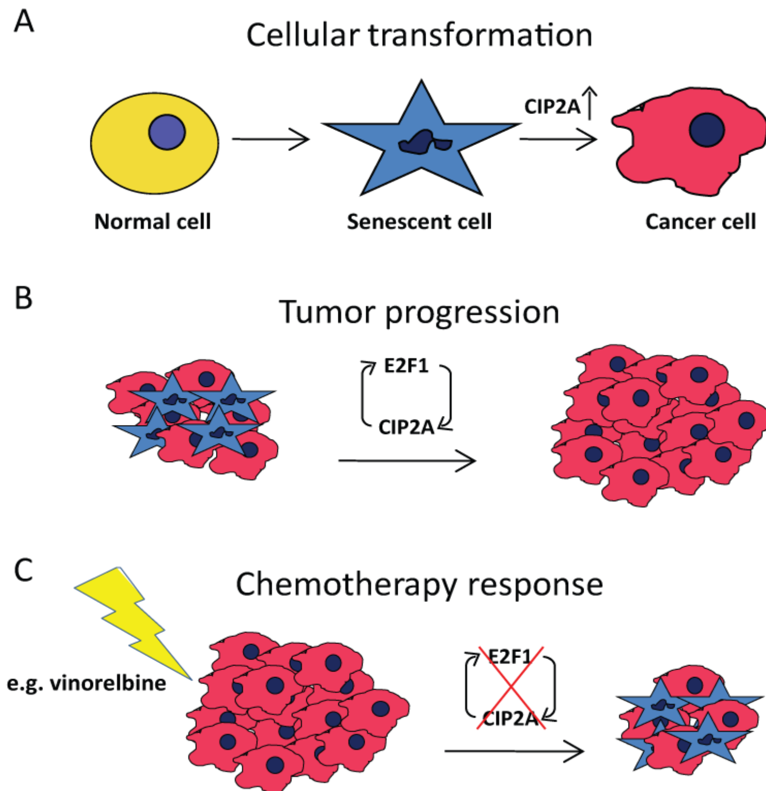

Figure 10. The role of senescence in cellular transformation, tumor progression and in chemotherapy response.
A) Senescence is a phenomenon that cells have to overcome in order to transform into cancer cells. B) Senescence is also known to inhibit tumor progression. C) Different chemotherapies can induce senescence in tumors, such as vinorelbine. CIP2A expression and status of the E2F1-CIP2A feedback loop in different situations are presented.

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ORIGINAL PUBLICATIONS I-II

Human Cancer Biology

CIP2A Is Associated with Human Breast Cancer Aggressivity

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I

Abstract

Purpose: To investigate the clinical relevance of the recently characterized human oncoprotein cancerous inhibitor of protein phosphatase 2A (CIP2A) in human breast cancer.

Experimental Design: CIP2A expression (mRNA and protein) was measured in three different sets of human mammary tumors and compared with clinicopathologic variables. The functional role of CIP2A in breast cancer cells was evaluated by small interfering RNA–mediated depletion of the protein followed by an analysis of cell proliferation, migration, anchorage-independent growth, and xenograft growth.

Results: CIP2A mRNA is overexpressed (n = 159) and correlates with higher Scarff-Bloom-Richardson grades (n = 251) in samples from two independent human breast cancer patients. CIP2A protein was found to be overexpressed in 39% of 33 human breast cancer samples. Furthermore, CIP2A mRNA expression positively correlated with lymph node positivity of the patients and with the expression of proliferation markers and p53 mutations in the tumor samples. Moreover, CIP2A protein expression was induced in breast cancer mouse models presenting mammary gland–specific depletion of p53 and either BRCA1 or BRCA2. Functionally, CIP2A depletion was shown to inhibit the expression of its target protein c-Myc. Loss of CIP2A also inhibited anchorage-independent growth in breast cancer cells. Finally, CIP2A was shown to support MDA-MB-231 xenograft growth in nude mice.

Conclusions: Our data show that CIP2A is associated with clinical aggressivity in human breast cancer and promotes the malignant growth of breast cancer cells. Thus, these results validate the role of CIP2A as a clinically relevant human oncoprotein and warrant further investigation of CIP2A as a therapeutic target in breast cancer treatment. (Clin Cancer Res 2009;15(16):5092–100)

Breast cancer is the most common malignancy that affects women, with >1 million cases occurring worldwide annually. Further, breast cancer is the most important cause of cancer-related deaths in women. However, the understanding of the molecular mechanisms that maintain the malignant growth of breast cancer cells remains incomplete (1).

The oncogenic transformation of human cells requires the perturbation of a distinct set of oncogenes and tumor suppressors (2). It was recently shown that the tumor suppressor activity of protein phosphatase 2A (PP2A) prevents the transformation of human breast epithelial cells (3). The role of PP2A as a relevant breast cancer tumor suppressor was further strengthened by a recent study showing that somatic mutations occurred in one of the subunits of the functional PP2A trimer (PP2A Aβ) in 13% of human breast cancers and that PP2A trimers containing this mutation fail to suppress the oncogenic activity of RalA (4, 5). In

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Translational Relevance

Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a recently identified human oncoprotein that inhibits c-Myc protein degradation in cancer cells. CIP2A has been found to be overexpressed in different human cancers, but its clinical relevance has not yet been established. In addition, the role of CIP2A in human breast cancer has not yet been studied. In this study, we show that CIP2A expression strongly correlates with aggressive characteristics of human breast cancer tumors (high Scarff-Bloom-Richardson grade, lymph node positivity, and expression of proliferation markers). Importantly, these results show for the first time that CIP2A expression is linked with clinical markers of aggressivity in human cancer. We also show that CIP2A depletion decreases the proliferation of human breast cancer cell lines and inhibits the growth of xenograft MDA-MB-231 cells *in vivo*. Altogether, these results warrant further investigation of CIP2A as a therapeutic target in the treatment of breast cancer.

addition to RalA inactivation, regulation of the proteolytic stability of the oncogenic transcription factor c-Myc is an important mechanism by which PP2A exerts its tumor suppressor activity (6, 7). PP2A-mediated dephosphorylation of serine 62 on c-Myc results in the ubiquitination and proteolytic degradation of c-Myc and thereby the inhibition of malignant cell growth and cellular transformation (6, 7). Increased protein stability of c-Myc has been detected in malignant cells isolated from hematologic cancers (8). On the other hand, the overexpression of c-Myc protein has been reported in 40% to 45% of human breast cancers, whereas the amplification of the *c-Myc* gene is observed only in 20% to 25% of human breast cancers (9, 10). These data suggest that, in addition to gene amplifications, the stabilization of c-Myc protein might contribute to its oncogenic activity in breast cancer.

We have recently characterized a human oncoprotein designed cancerous inhibitor of PP2A (CIP2A; ref. 11). CIP2A promotes c-Myc protein stability in human cancer cells by its capacity to inhibit PP2A activity directed toward serine 62 on c-Myc (11). Moreover, CIP2A promotes the proliferation and *in vivo* tumor growth of HeLa cells and cells derived from human head and neck squamous cell carcinomas (11). In addition, CIP2A was found overexpressed in tissue samples derived from human head and neck squamous cell carcinoma, human colon cancer, and human gastric cancer (11, 12). However, the clinical role of CIP2A and its association with disease progression is yet to be clearly defined. In addition, the role of CIP2A in human breast cancer has not been studied thus far.

Here we show that CIP2A expression correlates with the invasive and aggressive characteristics of human breast tumors [high Scarff-Bloom-Richardson (SBR) grade, lymph node–positive tumors, and high proliferation]. We also show that CIP2A depletion inhibits c-Myc expression as well as the proliferation and tumorigenic growth of human breast cancer cells.

Materials and Methods

Tumor samples and clinical material. Tumor samples used for the mRNA expression analysis as well as protocols for mRNA extraction and real-time PCR have been described previously (13, 14). To summarize, a total of 159 primary breast carcinomas and 5 normal breast tissues were analyzed in this study. These carcinomas were obtained from patients who had undergone initial surgery between 1989 and 2001 at the Cancer Research Center of Val d'Aurelle in Montpellier, the Bergonié Institute in Bordeaux, or the Department of Obstetrics and Gynecology of Turin. Informed consent was obtained from the patients before surgery. The patients' age at diagnosis varied from 27 to 92 years (mean,

Table 1. Summary of characteristics of the breast cancer patients (mRNA expression study; Fig. 1)

Tumors of the quantitative PCR study (159 patients)	No. patients
Histologic type	
IDC	82
ILC	27
IDC + intraductal comedo carcinoma	20
IDC + micropapillary	9
IDC + ILC	8
Mucinous	3
Papillary	3
Tubular	3
Ductal carcinoma *in situ*	1
Other	3
SBR grade	
1	27
2	88
3	43
Not defined	1
Stage	
I	34
IIa	63
IIb	45
IIIa	2
Unknown	15

Tumors of the microarray study (251 patients)	No. patients
Estrogen receptor status	
Positive	213
Negative	34
Unknown	4
Progesterone receptor status	
Positive	190
Negative	61
Elston SBR modified grade	
1	67
2	128
3	54
Unknown	2
Lymph node status	
Positive	84
Negative	158
Unknown	9
Patient age (y)	
<50	51
50-65	83
>65	117
p53 status	
Wild-type	193
Mutant	58

Table 2. Primers used for quantitative real-time PCR

Primer	Sequence (5′-3′)
36B4-fwd	GTCACTGTGCCAGCCCAGAA
36B4-rev	TCAATGGTGCCCCTGGAGAT
β-actin-fwd	CCAACCGCGAGAAGATGA
β-actin-rev	CCAGAGGCGTACAGGGATAG
CIP2A-fwd	GAACAGATAAGAAAAGAGTTGAGCATT
CIP2A-rev	CGACCTTCTAATTGTGCCTTTT
HPRT1-fwd	ACGTCTTGCTCGAGATGTGAT
HPRT1-rev	TGTAATTCCAGCAGGTCAGCAA

63 years; median, 65 years). For the 159 patients, the median follow-up time was 65.9 months. The tumors were sampled from patients at stage I (21%), stage IIa (40%), stage IIb (28%), and stage IIIa (1%), whereas 9% of the tumors were at an unknown stage (Table 1). Two tumors presenting approximately three times higher CIP2A expression than the next highest expressing tumor in the rest of the group (23.1 and 19.1 compared with 6.9) were excluded from the study because they were not within a normal distribution of the values. Fresh tissues were formalin-fixed and paraffin-embedded immediately after surgical removal. Frozen sections were stained with H&E to select samples consisting of at least 50% tumor cells and to establish the histologic type and the histologic grade (Table 1).

RNA extraction and purification. Frozen breast samples were homogenized using the Fast-Prep System from Q-Biogene. Briefly, ~40 mg frozen tissues were broken up in lysing buffer on a lysing matrix for 40 s. Total RNA was extracted and cleaned up from the lysate using the Qiagen RNeasy Mini Kit. The RNA purity and integrity were controlled by using a Bioanalyzer 2100 (Agilent). Only RNAs with a score of 8 to 10 were included in this study.

cDNA synthesis. After DNase treatment, 1 μg total RNA was incubated with 250 ng random hexamer for 10 min at 70°C. Total RNA was reverse transcribed in a final volume of 20 μL containing 1× first-strand buffer, 0.1 mol/L DTT, 10 mmol/L deoxynucleotide triphosphate, and 200 units SuperScript reverse transcriptase. The samples were incubated at 25°C for 10 min, and then at 42°C for 1 h. The reverse transcriptase was finally inactivated by heating at 70°C for 15 min.

PCR amplification. CIP2A primers have been designed using the Universal ProbeLibrary for Humans from Roche Applied Science (forward primer 5′-GAACAGATAAGAAAAGAGTTGAGCATT-3′ and reverse primer 5′-CGACCTTCTAATTGTGCCTTTT-3′). The quantification was based on the standard curve method. The data were normalized using the expression median of three reference genes (36B4, HPRT1, and β-actin; primer sequences are indicated in Table 2). Oligonucleotides were obtained from Proligo. For quantitative real-time PCR, 2 μL of diluted reverse transcription reaction samples (1/15) were added to 13 μL of a PCR mixture made up of 7.5 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems), 0.075 μL of each primer at a concentration of 100 μmol/L, and RNase-free water. The thermal cycling conditions comprised an initial step at 50°C for 2 min and a denaturation step at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All PCRs were carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The specificity of each primer couple was shown by a dissociation curve analysis. To generate a calibration curve, a serially diluted cDNA mixture was used as a standard and quantified for each primer set. The standard concentration was plotted against the cycle number at which the fluorescence signal increased above the background (threshold) value (Ct value). The amplification efficiency [E (%) = $(10^{(1/-s)} - 1) * 100$ (s = slope)] of each standard curve was determined and appeared to be >95% and <105% over a wide dynamic range.

Histologic staining. CIP2A protein was detected using a rabbit anti-CIP2A antibody (ref. 15; diluted 1:1,000) and an anti-mouse peroxidase polymer. 3,3′-Diaminobenzidine was used as the chromogen (ImmunoVision), and hematoxylin was used as a counterstain. Human breast cancer tissue microarrays (n = 33) were used for immunohistochemical stainings. The staining methods have been described previously (16). These samples were independent from other samples used in this study.

Microarray reanalysis. Data from Affymetrix U133B arrays hybridized with mRNA from 251 breast tumors (17) were reprocessed using the R language (R Development core team) and the MAS5 algorithm implemented in the Bioconductor package *affy*. A boxplot of CIP2A expression (probe set 231855_at) was drawn using R by dividing the samples into groups based on respective clinical parameters. Ki-67 and proliferating cell nuclear antigen (PCNA) status was estimated from the expression data itself. The Affymetrix probe sets and cutoffs for dividing tumors into "high"/"low"–expressing groups were 212020_s_at >500 units for Ki-67 and 201202_s at >1,500 units for PCNA. Other clinical data were derived from the published information (17). The statistical significance of differences between groups was assessed using a Mann-Whitney test implemented in R.

Small interfering RNA transfections. Double-stranded small interfering RNA (siRNA) oligonucleotides (50 nmol per 35 mm plate; CIP2A: 5′-CUGUGGUUGUGUUUGCACUTT-3′; scrambled: 5′-UAACAAUGAGAGCACGGCTT-3′) were transfected with Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Antibodies. The following antibodies were used: rabbit polyclonal anti-CIP2A (11); mouse monoclonal anti-human Ki-67, clone Mib-1 (DAKO); rat monoclonal anti-mouse Ki-67, clone TEC-3 (DAKO); mouse monoclonal anti-c-Myc, clone 9E10 (BD Pharmingen); mouse monoclonal anti-β-actin, clone AC-74 (Sigma); rabbit polyclonal anti-phospho-Akt (Santa Cruz Biotechnology); and rabbit polyclonal anti-phospho-MEK (Cell Signaling technology).

Cell proliferation assay. MDA-MB-231 or T47D cells were transfected on 96-well plates with CIP2A or scrambled siRNA for 3 days, and the number of living cells was subsequently analyzed by using a CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega) following the manufacturer's instructions.

Anchorage-independent soft-agar growth. MDA-MB-231 cells (10×10^3) were seeded in a 6-well plate 48 h after siRNA transfection. Soft-agar assays were done in medium containing 10% fetal bovine serum as described previously (11). The number and size of colonies were analyzed using ImageJ 1.38x software from microscopy images (magnification, ×35).

In vivo *tumor formation.* MDA-MB-231 cells were transfected with CIP2A or scrambled siRNAs for 72 h, and down-regulation of CIP2A protein expression was confirmed from parallel samples before injection. siRNA-transfected cells (2×10^6) mixed with Matrigel (BD Bioscience) were injected into the mammary fat pad of an immunocompromised mouse. Twenty-four injections were done for each condition (siCIP2A or siSCR). The size of the palpable tumors was evaluated every third day by the use of a precision instrument and the tumor weight was analyzed at the end of the experiment.

Migration assay. MDA-MB-231 cells (10×10^4) were cultivated on a monolayer and treated for 3 days with the indicated siRNA in a 24-well plate. Then, a wound (2 mm width) was scratched into confluent cultures of siCIP2A- or siSCR-treated MDA-MB-231 cells. Randomly marked wound regions with an identical width were studied, and wound closure (the percentage of closed scratch area) was measured using ImageJ 1.38x software from microscopy images (magnification, ×35).

Results

CIP2A expression correlates with human breast cancer aggressivity. To study CIP2A expression in human breast cancer, 159 previously characterized human mammary tumors and 5 normal breast samples (refs. 13, 14; Table 1) were evaluated for CIP2A mRNA expression by real-time PCR analysis. We

found that CIP2A is significantly overexpressed in human mammary tumors when compared with normal tissue (P = 0.027; Fig. 1A). Regarding histologic subtypes of breast cancer, statistically significant overexpression of CIP2A, compared with normal breast samples, was found in invasive ductal carcinoma (IDC; P = 0.027), invasive lobular carcinoma (ILC; P = 0.012), and IDC with intraductal comedo carcinoma (P = 0.032; Fig. 1B). Importantly, mucinous carcinomas, which are mammary tumors with a good prognosis, displayed CIP2A mRNA expression at a level that is comparable with expression levels in normal breast samples and significantly lower than in the invasive IDC, ILC, and IDC with intraductal comedo carcinoma tumors (P < 0.044; Fig. 1B).

The above results suggest that CIP2A expression may be linked with human breast cancer aggressivity. To support this, analysis of the data set shown in Fig. 1A and B revealed that CIP2A expression correlates with a higher tumor grade of ILC; SBR grade 2 and 3 samples presented significantly higher CIP2A expression levels (P = 0.011) than grade 1 lobular carcinomas (Fig. 1C). In IDC, CIP2A expression levels correlated with higher SBR grades. However, this correlation was not quite statistically significant (P = 0.066; data not shown). On the other hand, no association existed between CIP2A expression and the SBR grade of the tumors derived from IDC with intraductal comedo carcinoma (P = 0.663; data not shown).

To further substantiate the observed correlation of CIP2A expression with the increase in breast cancer aggressivity, the expression levels of CIP2A were correlated with several markers of tumor progression in a published microarray data set of 251 human breast tumors (17). Again, CIP2A mRNA expression was found to correlate with a higher SBR grade of the tumor (grade 3 versus 1 and grade 3 versus 2; P < 0.001; Fig. 1D; Table 3). Using

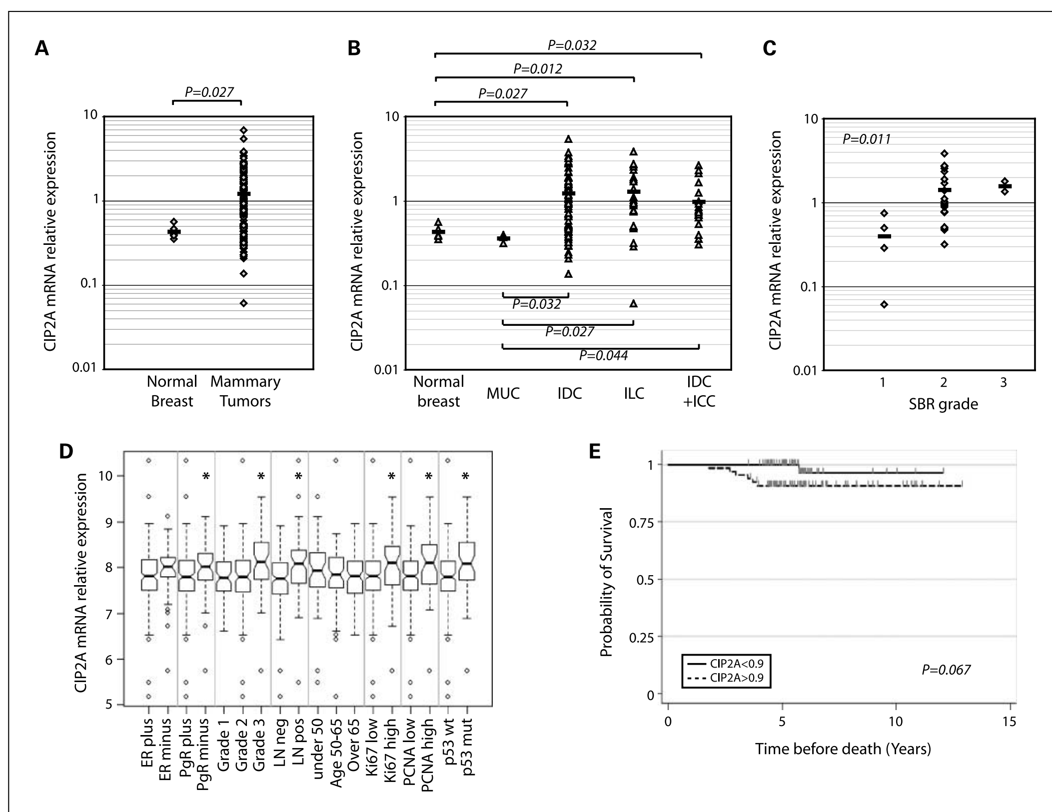

Fig. 1. CIP2A expression correlates with breast cancer aggressivity. *A,* real-time PCR analysis for CIP2A mRNA from 159 human mammary tumors and 5 normal breast samples. Two-sample Wilcoxon (Mann-Whitney) rank-sum test was used for statistical analysis. *Small bold line,* average. *B,* CIP2A mRNA expression in the indicated breast cancer tumor types. *MUC,* mucinous carcinoma. Two-sample Wilcoxon (Mann-Whitney) rank-sum test was used for statistical analysis. *Small bold line,* average. *C,* correlation of CIP2A mRNA expression and SBR grade of ILC from the tumors presented in *A* and *B*. Mann-Whitney rank-sum test was used for statistical analysis. *Small bold line,* average. *D,* correlation of CIP2A expression with the indicated clinical parameters on the microarray data from 251 human mammary tumors (17). The statistical significance of differences between groups was assessed using a Mann-Whitney test implemented in R. *, P < 0.05 (exact values are indicated in Table 3). *E,* Kaplan-Maier curves of the overall survival of the patients from the quantitative-PCR analysis (*A* and *B*). Tumors are separated in two groups based on their CIP2A expression levels compared with the median value (0.9) for the total tumor material. *Solid line,* tumors with a lower CIP2A expression compared with the median (<0.9; 77 tumors, 48.4% of the tumors); *dashed line,* tumors with a higher CIP2A expression than the median (>0.9; 82 tumors, 51.6% of the tumors); *vertical ticks,* censored patients.

Table 3. Correlation between CIP2A expression and the clinicopathologic variables of the tumors from the Miller et al. microarray data (*P* values of a regular two-sided Wilcoxon rank-sum test, Mann-Whitney test was used)

	P
Estrogen receptor + vs -	0.130
Progesterone receptor + vs -	0.024
Grade 1 vs 3	<0.001
Grade 2 vs 3	<0.001
Lymph node - vs +	<0.001
Age (y) <50 vs >65	0.129
Low vs high Ki-67	0.009
Low vs high PCNA	0.018
p53 wild-type vs mutant	<0.001

the same microarray data set, we also investigated the association of CIP2A with other clinicopathologic variables and found that CIP2A mRNA expression associates with progesterone receptor negativity ($P = 0.024$), lymph node positivity ($P < 0.001$), p53 mutation ($P < 0.001$), and expression of the proliferative markers Ki-67 ($P = 0.009$) and PCNA ($P = 0.018$; Fig. 1D; Table 3). However, there was no significant association between CIP2A expression and patient age or estrogen receptor status (Fig. 1D; Table 3).

The correlation between CIP2A mRNA expression and overall survival of the patients was studied in the samples shown in Fig. 1A and B. Patients were separated into two groups based on the median value of their CIP2A expression (0.9): the "low-expressing" group contained patients with CIP2A expression levels lower than the median of 0.9 and the "high-expressing" group contained patients with higher CIP2A expression than the median. During the first 5 years, no deaths occurred in the group of patients with low CIP2A expression, whereas six patients died due to breast cancer in the population with high CIP2A expression during the same period (Fig. 1E). Although the difference in overall survival of patients with high or low CIP2A-expressing tumors was not quite statistically significant ($P = 0.067$), patients with high CIP2A expression showed worse survival during the first 5 years post-surgery ($P = 0.016$; Supplementary Fig. S1).

These results show that CIP2A is overexpressed in invasive human mammary carcinomas. Importantly, these findings show for the first time that CIP2A expression correlates with the tumor grade and lymph node positivity in human cancer patients. Moreover, the association of CIP2A expression with markers of increased cellular malignancy (Ki-67 and PCNA expression) further supports the role of CIP2A in promoting the aggressive behavior of breast cancer.

CIP2A protein expression in breast cancer. The above results show the overexpression of CIP2A mRNA in human mammary tumors. To confirm these findings, we also wanted to study CIP2A protein status in breast cancer. CIP2A is a cytoplasmic protein (11, 12, 15). As shown in Fig. 2A, normal mammary tissue did not express detectable levels of cytoplasmic CIP2A, whereas high cytoplasmic expression of CIP2A protein was observed in human breast carcinomas. In a tissue array of 33 human mammary tumors, clear cytoplasmic CIP2A positivity was observed in 39% of the tumors (Fig. 2B). To further evaluate the observation of CIP2A expression in breast cancer tissue, CIP2A and Ki-67 protein expression was analyzed in a well-defined mouse model of breast cancer presenting a mammary gland–specific deletion of p53 and either BRCA1 or BRCA2 (*K14cre;Brca1*$^{F/F}$*;p53*$^{F/F}$ or *K14cre;Brca2*$^{F/F}$*;p53*$^{F/F}$; refs. 18, 19). All analyzed tumors (10 of 10) displayed strong CIP2A staining in epithelial carcinoma cells, whereas no CIP2A positivity was observed in the normal mammary gland tissue (Fig. 2C). In concordance with the correlation of CIP2A and Ki-67 mRNA expression in human breast cancer material (Fig. 1D), CIP2A-positive cancer cells displayed clear nuclear expression of Ki-67 in both breast cancer mouse models (Fig. 2C). These results, taken together with the observed correlation of p53 mutation and CIP2A mRNA expression in human tumor material (Fig. 1D), further suggest that CIP2A may be involved in mammary gland tumorigenesis induced by the inhibition of p53 tumor suppressor activity.

CIP2A promotes c-Myc protein expression and cell proliferation in breast cancer cells. Our group and others have recently shown that CIP2A promotes c-Myc protein stability in HeLa, head and neck squamous cell carcinoma, and gastric cancer cells (11, 12, 15). Here, we show that the depletion of CIP2A in MDA-MB-231 and T47D breast cancer cell lines results in the inhibition of c-Myc steady-state protein levels (Fig. 3A and B). The specificity of the siRNA used for CIP2A depletion has been reported previously (11). Importantly, CIP2A depletion did not inhibit the phosphorylation of Akt or MEK (Fig. 3A). We have shown previously that CIP2A depletion in HeLa cells inhibits bromodeoxyuridine incorporation but does not induce programmed cell death (11). Here, CIP2A depletion inhibited the number of living MDA-MB-231 and T47D breast cancer cells cultured under high-serum conditions as measured by MTT assay (Fig. 3C). As CIP2A depletion has been shown to inhibit bromodeoxyuridine incorporation but not induce apoptosis (ref. 11; data not shown), we conclude that the observed decrease in the number of living cells in response to CIP2A depletion is most likely due to the inhibition of proliferation. A positive correlation between CIP2A expression and lymph node positivity in human breast cancer (Fig. 1D) suggests that CIP2A may be involved in regulating the migration properties of breast cancer cells. However, we did not observe any significant effect of CIP2A depletion on MDA-MB-231 cell migration in a standard scratch wound assay (Fig. 3D).

CIP2A depletion inhibits tumor growth of MDA-MB-231 cells. The ability of cells to grow and form colonies on semisolid agar (anchorage-independent growth) is a hallmark of malignantly transformed cells. To study the role of CIP2A on the malignant growth of breast cancer cells, MDA-MB-231 cells were transfected with scrambled or CIP2A siRNA, and their capacity to form colonies on semisolid agar was evaluated. For this purpose, we first studied the efficiency of CIP2A depletion by a single transfection of siRNA. A very potent reduction of CIP2A protein expression was still found after 10 days (Supplementary Fig. S2). We found that CIP2A depletion resulted in a statistically significant decrease of the anchorage-independent growth of MDA-MB-231 cells 14 days post-transfection (Fig. 4A). To study whether CIP2A is required for breast cancer tumor growth, MDA-MB-231 cells transfected with either CIP2A or scrambled siRNA were injected into the mammary fat pad of athymic mice and tumor growth was monitored over 31 days. Beginning at day 18 after injection, CIP2A depletion

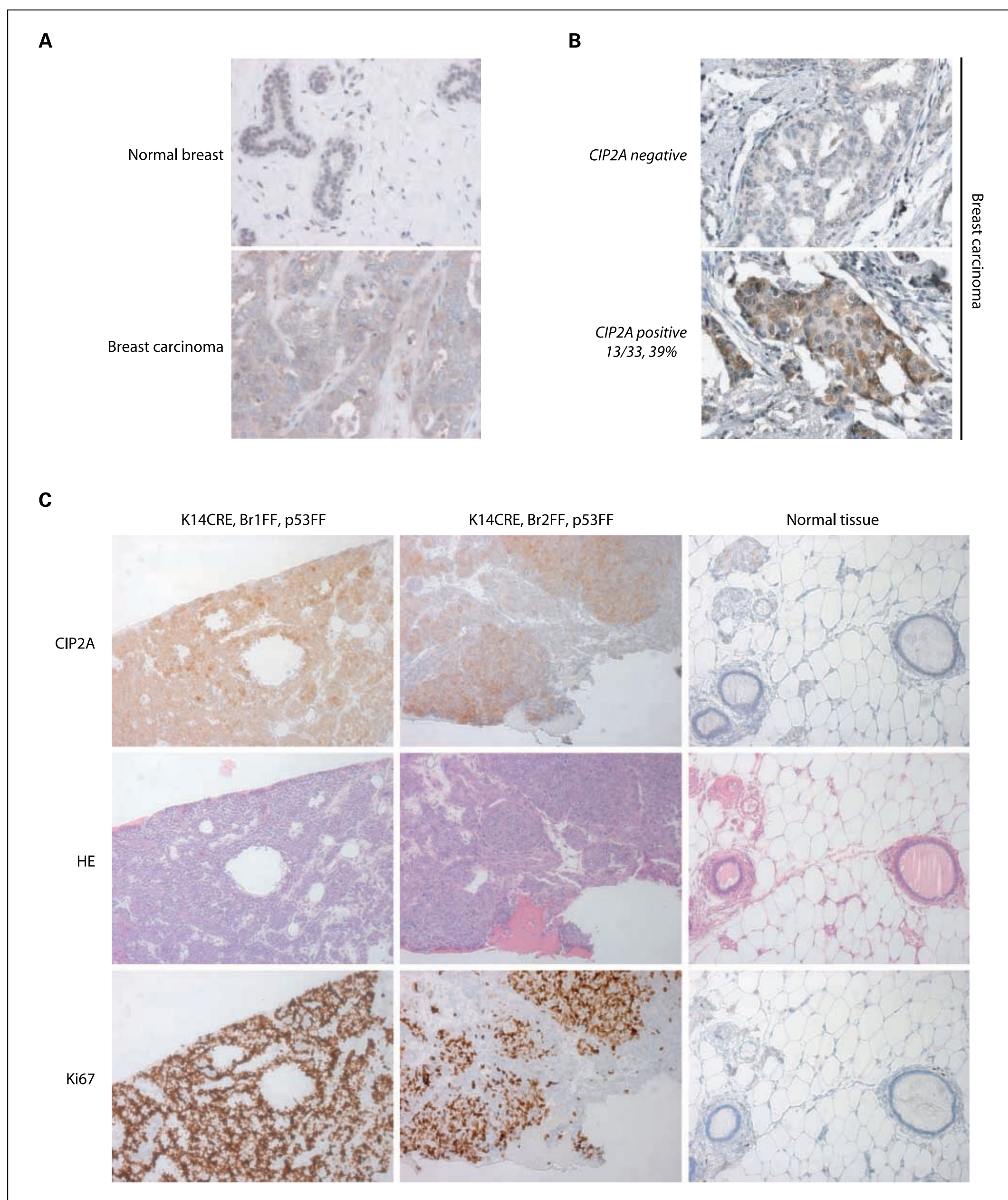

Fig. 2. CIP2A protein expression in human breast cancer. *A,* CIP2A immunoreactivity in normal breast and breast cancer tissue was evaluated by immunohistochemistry. As reported previously in other tissue types (11), CIP2A antibody displayed nonspecific nuclear staining of normal tissue, whereas clearly distinguishable cytoplasmic staining was observed for breast cancer tissue. *B,* CIP2A immunostaining of human breast cancer tissue array. Cytoplasmic CIP2A expression was found in 39% of the tumors analyzed. *C,* CIP2A and Ki-67 immunostaining of mouse mammary tumors. CIP2A and Ki-67 expression was studied by immunohistochemistry on mammary tumor sections from two different breast cancer mouse models, *K14cre*;*Brca1*$^{F/F}$;*p53*$^{F/F}$ or *K14cre*;*Brca2*$^{F/F}$;*p53*$^{F/F}$. Representative samples of 10 tumors per group are shown, and all display strong CIP2A and Ki-67 staining in the tumor cells. Magnification, ×20.

resulted in a significant reduction in tumor volume (Fig. 4B) as determined by the ANOVA for repeated-measures test, which indicates the difference in global evolution of xenograft growth over time. In addition, CIP2A depletion resulted in a significant decrease in the tumor volume and weight as measured at the end of the experiment at day 31 (Fig. 4C and D). Together, these results show that CIP2A promotes malignant growth of human breast cancer cells.

Discussion

CIP2A was recently identified as a human oncoprotein based on its capacity to transform human immortalized cells, its overexpression in human head and neck squamous cell carcinomas and colon cancer, and its capacity to promote tumor growth (11). However, the clinical role of CIP2A and its association with disease progression has not yet been established.

We show here that CIP2A is overexpressed in the most common types of human mammary carcinomas compared with normal breast. Importantly, CIP2A overexpression was observed at both mRNA and protein levels by using two independent sets of human breast cancer material (Figs. 1A and 2B). Moreover, CIP2A expression correlates with the aggressive characteristics of the tumors (high SBR grade, lymph node–positive tumors, and high Ki-67 and PCNA levels; Fig. 1C and D). At this point, the difference in the overall survival of patients with high or low CIP2A-expressing tumors does not advocate the use of CIP2A as a prognostic marker ($P = 0.067$). However, our observation that high CIP2A expression did predict a worse prognosis for the patients at a 5-year follow-up (Fig. 1E; Supplementary Fig. S1) indicates that the predictive role of CIP2A in association with other genes overexpressed in high-grade human breast cancers should be further evaluated in the future by studying more patient samples. It is also possible that the lack of a significant survival effect is due to the relatively long overall survival of patients in the studied cohort, which is likely due to the fact that most of the studied tumors were from patients with early stages of disease (Table 1).

On the molecular level, we show that CIP2A depletion results in the reduction of c-Myc protein levels in two distinct human breast cancer cell lines. Interestingly, the combination of our results together with the previously published studies reveals that *c-Myc* amplification and CIP2A overexpression correlate with the shared features of human mammary tumors. Indeed, both *c-myc* amplification and CIP2A expression correlate with a higher tumor grade and with lymph node positivity (refs. 20, 21; Fig. 1C and D). Similarities in clinical roles of *c-myc* amplification and CIP2A expression, together with the role of CIP2A in promoting c-Myc protein expression (Fig. 3A and B), clearly strengthen the functional link between these two human oncoproteins. The role of CIP2A in promoting c-Myc expression in breast cancer cells is an important observation in the light of previous evidence that overexpression of c-Myc protein has been observed in the majority of human breast cancer patient

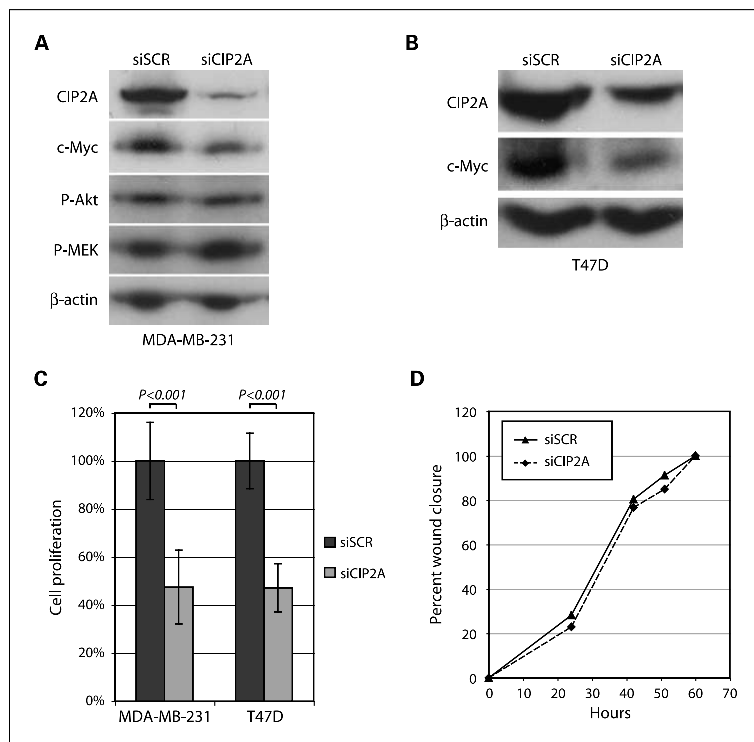

Fig. 3. CIP2A promotes c-Myc expression and cell proliferation in breast cancer cells. Western blot analysis of MDA-MB-231 (*A*) or T47D (*B*) cells treated with CIP2A or scrambled siRNA for 72 h. The expression and phosphorylation status of the indicated proteins was studied by previously validated antibodies. Representative results of three to five independent experiments with similar results. *C,* cell proliferation assay of MDA-MB-231 or T47D cells treated with CIP2A or scrambled siRNA for 3 d. Mean ± SD of three independent experiments with six replicates. Kruskal-Wallis equality of populations test was used for statistical analysis. *D,* scratch wound assays of MDA-MB-231 cells. Cells were treated for 3 d with the indicated siRNA. Subsequently, a 2 mm scratch wound was introduced into confluent cultures and the wound closure (percentage of closed scratch area) was measured in photographs at the time points indicated.

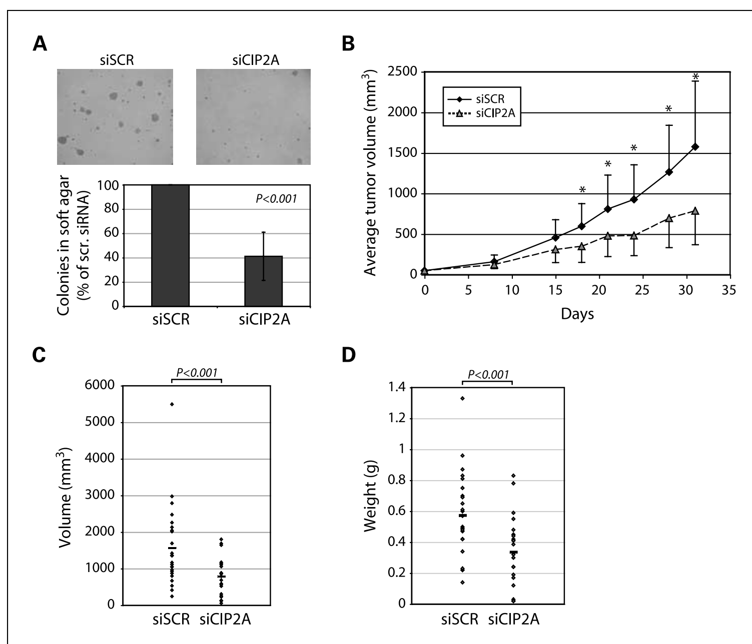

Fig. 4. CIP2A promotes tumor growth of MDA-MB-231 cells. *A,* anchorage-independent growth on soft agar of MDA-MB-231 cells transfected with CIP2A or scrambled siRNA. *Top,* representative phase-contrast microscopy images of the indicated cultures; *bottom,* number of colonies measured 15 d after replating by ImageJ software. Average ± SD of four experiments. Kruskal-Wallis test was used for statistical analysis. *B,* xenograft growth of MDA-MB-231 cells treated with CIP2A or scrambled siRNA. Cells were treated with the siRNA for 3 d. Two million cells were subsequently injected with Matrigel into the mammary fat pad of Hsd: athymic nude mice. Mean ± SE of tumor volumes at the indicated time points. Multiple-sample ANOVA for repeated-measures test was used (*, $P < 0.0001$). *C,* final volume of tumors from the experiment shown in *B* at day 31. Kruskal-Wallis test was used for statistical analysis. *Small bold line,* average. *D,* weight of tumors from the experiment shown in *B* at day 31. Kruskal-Wallis test was used for statistical analysis. *Small bold line,* average.

samples, whereas *c-Myc* gene amplification occurs only in a far smaller fraction of cancers (9, 10). Taken together, these results suggest that, along with other mechanisms that stabilize c-Myc protein (6, 22), CIP2A expression may be important for sustaining the malignant behavior of breast cancers. Importantly, our data show that although CIP2A depletion inhibited c-Myc protein expression, it did not markedly alter the activity of either extracellular signal-regulated kinase or Akt/protein kinase B pathway, supporting previously reported data that CIP2A depletion does not stimulate general PP2A activity (11). However, based on the data presented here, we cannot exclude the possibility that, in addition to its role in regulating c-Myc protein expression, CIP2A could promote breast cancer aggressivity by regulating other, yet to be defined signaling pathways.

The current treatment for breast cancer includes traditional regimens such as surgery and radiotherapy, whereas the only molecularly targeted drugs for breast cancer treatment today are the HER2-targeting antibody trastuzumab, the antiestrogen drugs such as tamoxifen, and the humanized anti–vascular endothelial growth factor antibody bevacizumab in limited cases. In this regard, there is an urgent need to identify novel potential target proteins for breast cancer therapy. The results shown in this study support the potential role for CIP2A as a target protein for future cancer therapies. Importantly, CIP2A expression is very low in most human tissues (11). In this study, we further showed that CIP2A protein is not detectable in human and mouse mammary gland tissues (Fig. 2A and C). This suggests that CIP2A targeting may not have severe side effects that could limit the efficacy of such a therapy.

In conclusion, the results of this study show that CIP2A is associated with human breast cancer aggressivity. Importantly, these results provide the first indications for the clinical relevance of this recently characterized human oncoprotein in human breast cancer. Based on the functional characteristics and cancer-specific expression of CIP2A, it is evident that the role of CIP2A in promoting the aggressivity of breast cancer and other types of human malignancies deserves further attention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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II

RESEARCH ARTICLE

Senescence Sensitivity of Breast Cancer Cells Is Defined by Positive Feedback Loop between CIP2A and E2F1

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Tumor Suppression

p53 activity

p21

E2F1

CIP2A

Tumor Progression

p53 activity

p21

E2F1

CIP2A

ABSTRACT Senescence induction contributes to cancer therapy responses and is crucial for p53-mediated tumor suppression. However, whether p53 inactivation actively suppresses senescence induction has been unclear. Here, we show that E2F1 overexpression, due to p53 or p21 inactivation, promotes expression of human oncoprotein CIP2A, which in turn, by inhibiting PP2A activity, increases stabilizing serine 364 phosphorylation of E2F1. Several lines of evidence show that increased activity of E2F1-CIP2A feedback renders breast cancer cells resistant to senescence induction. Importantly, mammary tumorigenesis is impaired in a CIP2A-deficient mouse model, and CIP2A-deficient tumors display markers of senescence induction. Moreover, high CIP2A expression predicts for poor prognosis in a subgroup of patients with breast cancer treated with senescence-inducing chemotherapy. Together, these results implicate the E2F1-CIP2A feedback loop as a key determinant of breast cancer cell sensitivity to senescence induction. This feedback loop also constitutes a promising prosenescence target for therapy of cancers with an inactivated p53–p21 pathway.

SIGNIFICANCE: It has been recently realized that most currently used chemotherapies exert their therapeutic effect at least partly by induction of terminal cell arrest, senescence. However, the mechanisms by which cell-intrinsic senescence sensitivity is determined are poorly understood. Results of this study identify the E2F1-CIP2A positive feedback loop as a key determinant of breast cancer cell sensitivity to senescence and growth arrest induction. Our data also indicate that this newly characterized interplay between 2 frequently overexpressed oncoproteins constitutes a promising prosenescence target for therapy of cancers with inactivated p53 and p21. Finally, these results may also facilitate novel stratification strategies for selection of patients to receive senescence-inducing cancer therapies. *Cancer Discov; 3(2); 182–97. ©2013 AACR.*

INTRODUCTION

Cellular senescence functions as a barrier that normal cells have to overcome to transform into cancer cells (1). Accordingly, analysis of several types of premalignant tumors, most notably benign skin nevi, has revealed the existence of senescent pretumorigenic cells (1, 2). The functional relevance of spontaneous senescence induction in preventing tumor initiation and progression has been shown by several recent mouse studies (3–5).

Notably, although traditionally considered as apoptosis-inducing agents, most of the currently used chemotherapies exert their therapeutic effect at least partly by senescence induction (6, 7). Similarly, evidence is accumulating that despite the essential role of the tumor suppressor p53 in mediating apoptosis induction by genotoxic stimuli and chemotherapies, its *in vivo* tumor suppressor activity is not dependent on apoptosis, but rather on senescence induction (8–11). However, p53 function is inactivated in the majority of human cancers, and p53 inactivation correlates with poor patient survival in several cancer types including breast cancer (12). Traditionally, resistance of p53-mutant cells to chemotherapy has been linked to defective checkpoint function of p53 (13). However, we cannot exclude the possibility that, in addition to defective checkpoint activity, p53 inhibition actively promotes a mechanism or mechanisms that confer cancer cells' general resistance to chemotherapy-induced senescence. In addition to mutations, p53 is known to be inactivated in cancer cells by enhanced proteolytic degradation driven by the ubiquitin ligases MDM2 and MDMX (14). Although therapeutic strategies to activate senescence via inhibition of MDM2/MDMX-p53 interactions have been under intense research lately (14), because of p53 mutations, they are unlikely to be efficient in a large fraction of human

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tumors. Therefore, the need to identify novel mechanisms that promote senescence resistance and tumor progression downstream of inactivated p53 is urgent. Identification of such mechanisms would not only provide novel insights into senescence regulation but could also facilitate development of novel prosenescence therapeutic strategies for cancers harboring inactivated p53 (6, 7).

E2F1 is an oncogenic transcription factor that is overexpressed in various human cancer types (15). Recent studies have indicated that E2F1's classic function in transcriptional activation of S-phase–associated genes only partially explains its oncogenic activity (15, 16). Its transcriptional activity is negatively regulated by p53 through p21-mediated regulation of retinoblastoma (Rb) protein phosphorylation (15, 16), but expression and activity of E2F1 are also regulated directly by phosphorylation, independently of Rb (16, 17). The p53 reactivation by small-molecule activator Nutlin-3 inhibits protein expression of E2F1 and induces senescence-like growth arrest (18). Accordingly, knockdown of E2F1 expression also induces cellular senescence in p53-deficient cancer cells and blocks tumor growth (19–21). However, the mechanisms by which E2F1 prevents senescence induction in p53-deficient cells are currently unclear.

The human oncoprotein cancerous inhibitor of PP2A (CIP2A) is overexpressed in 65% to 90% of tissues from patients in almost all human cancer types studied thus far, and its expression correlates with cancer progression in a large variety of human malignancies (Supplementary Table S1; refs. 22–25). Even though CIP2A protein expression correlates with proliferation in human cancers (22–25), expression of CIP2A is not regulated by cell-cycle activity (24). Overexpressed CIP2A transforms immortalized cells of either human or mouse origin (23, 26), whereas its depletion by RNA interference (RNAi) inhibits anchorage-independent growth of several types of tumor cells (22–26). CIP2A's tumor-promoting role has been shown by several xenograft studies (22, 23, 25, 26), but the genetic evidence that it contributes to tumor progression is yet lacking. CIP2A's oncogenic function has been mostly linked to its capacity to prevent proteolytic degradation of MYC by promoting its serine 62 phosphorylation (23, 24, 27, 28). As CIP2A overexpression is one of the most frequent alterations in human cancers (Supplementary Table S1), identification of novel mechanisms that regulate CIP2A, and oncogenic targets that could explain its significant correlation with human cancer progression, would be of general interest.

Here, we show that *CIP2A* is a direct transcriptional target of E2F1 and that CIP2A overexpression increases expression of E2F1, phosphorylated at serine 364. The positive feedback loop between these 2 human oncoproteins is stimulated by p53 inactivation, and is critical for inhibition of senescence induction in human breast cancer cells. Moreover, our results strongly indicate that the E2F1-CIP2A positive feedback loop plays a role in the resistance toward senescence-inducing chemotherapy in patients with breast cancer. Furthermore, we provide the first genetic evidence for CIP2A's role in promoting breast cancer progression. Our data also indicate that this newly identified oncogenic mechanism is a potential prosenescence target for treatment of cancers with inactivated p53.

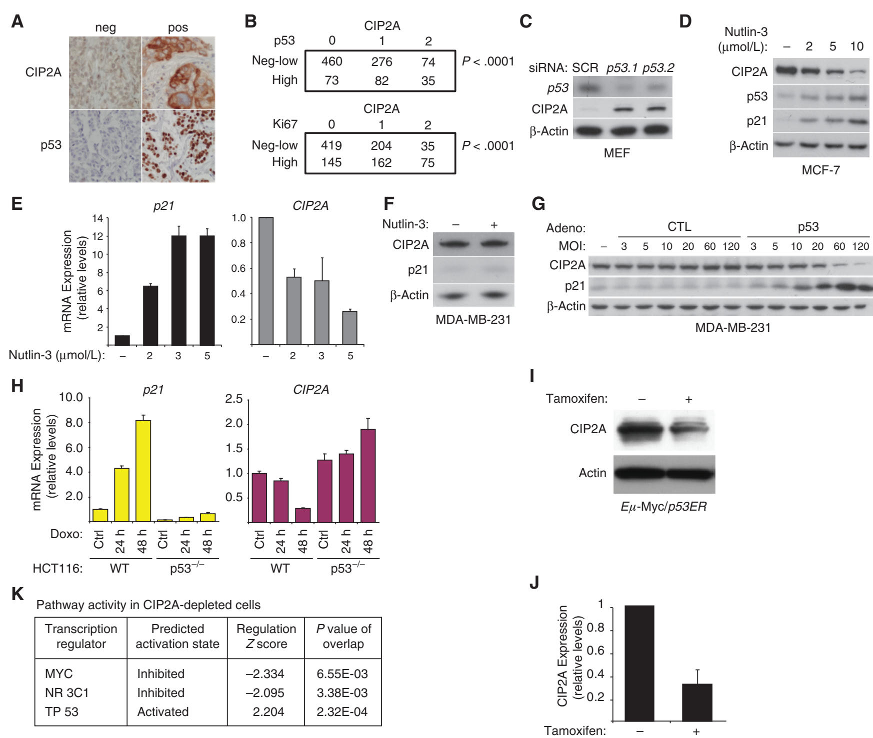
RESULTS

CIP2A Expression Is Associated with p53 Expression and Adverse Prognostic Factors in Human Breast Cancer

High *CIP2A* mRNA expression positively correlates with the presence of *p53* mutation in human breast cancer samples (22). To confirm that p53 inactivation in breast cancer cells correlates with CIP2A protein expression, a series of unselected human breast cancers were stained for CIP2A and p53 protein expression, by using a p53 antibody that we have recently shown to be indicative of p53 mutation (29). Of the 1228 cancers investigated, 46% were positive for CIP2A (Supplementary Fig. S1A and S1B), and CIP2A expression significantly correlated with high p53 immunopositivity (Fig. 1A and B). However, despite statistical correlation between high p53 immunopositivity and increased CIP2A protein expression (Fig. 1B), this analysis identified tumors in which CIP2A was highly expressed even in the absence of p53 immunopositivity. It is possible that in these cases CIP2A overexpression is due to high expression of MYC or ETS1 transcription factors, both shown recently to stimulate CIP2A expression in human cancer cells (24, 30). Moreover, CIP2A expression correlated significantly with several markers of aggressive disease, such as a high Ki-67 proliferation index, a large tumor size, and a low histologic grade of differentiation (Fig. 1B and Supplementary Fig. S1C and S1D).

Wild-type p53 Downregulates CIP2A Expression

To study whether wild-type (WT) p53 negatively regulates CIP2A expression, p53 expression was inhibited by siRNA in cultured mouse embryonic fibroblasts (MEF), and CIP2A expression was subsequently studied by Western blotting. As shown in Fig. 1C, inhibition of p53 expression in MEFs by 2 different siRNA sequences resulted in robust induction of CIP2A protein expression. Moreover, reactivation of WT p53 in MCF-7 human breast cancer cells with small-molecule inhibitors of the Mdm2–p53 interaction, Nutlin-3 (31) or RITA (32), inhibited CIP2A expression at both the mRNA and protein levels (Fig. 1D and E and Supplementary Fig. S2A and S2B). To confirm that CIP2A downregulation by Nutlin-3 is dependent on WT p53 function, we treated MDA-MB-231 human breast cancer cells, harboring inactive mutant p53, with Nutlin-3. Nutlin-3 treatment had no effect on either p21 or CIP2A protein expression in MDA-MB-231 cells (Fig. 1F). However, when WT p53 was introduced to these cells, CIP2A protein expression was inhibited in a concentration-dependent manner (Fig. 1G). To further confirm that CIP2A expression is regulated by a p53-dependent mechanism, we treated isogenic WT and *p53*$^{-/-}$ HCT116 human colorectal cancer cells with the p53-activating chemotherapy doxorubicin. In contrast to WT cells, *p53*$^{-/-}$ HCT116 cells were resistant to doxorubicin-induced inhibition of *CIP2A* mRNA expression (Fig. 1H). In addition to *in vitro* models, we analyzed CIP2A expression in lymphoma tissue derived from a transgenic *Eμ-Myc* mouse model carrying tamoxifen-inducible p53 (33). As shown in Fig. 1I and J, *in vivo* restoration of p53 function resulted in inhibition of CIP2A expression in lymphoma tissue, thus confirming that p53 also negatively regulates oncoprotein CIP2A expression *in vivo*. Interestingly,



Transcription regulator	Predicted activation state	Regulation Z score	P value of overlap
MYC	Inhibited	–2.334	6.55E-03
NR 3C1	Inhibited	–2.095	3.38E-03
TP 53	Activated	2.204	2.32E-04

Figure 1. WT p53 negatively regulates CIP2A expression. **A,** representative immunohistochemical stainings of CIP2A and p53 expression in human breast cancer tumors. **B,** CIP2A expression positively correlates with p53 expression and with proliferation marker Ki-67 in human breast tumors ($n = 1228$). *P* value calculated by χ^2 test. **C,** Western blot analysis of CIP2A expression in MEFs 48 hours after transfection with scrambled (SCR) or 2 different p53 siRNAs (p53.1 and p53.2). **D,** Western blot analysis of CIP2A, p53, and p21 expression in MCF-7 cells treated with 2, 5, or 10 μmol/L of Nutlin-3 for 36 hours. **E,** *p21* and *CIP2A* mRNA expression in MCF-7 cells treated with 2, 3, and 5 μmol/L of Nutlin-3 for 24 hours. Shown is mean ± SEM of 2 independent experiments. **F,** CIP2A protein expression in MDA-MB-231 human breast cancer cells harboring DNA binding–deficient p53 treated with 5 μmol/L of Nutlin-3 for 24 hours. **G,** Western blot analysis of CIP2A expression in MDA-MB-231 cells 48 hours after transduction either with control (CTL) or with WT p53-expressing (p53) adenovirus using different multiplicity of infections (MOI). **H,** *p21* and *CIP2A* mRNA expression from WT and *p53*$^{-/-}$ HCT116 cells treated with 0.2 μg/μL of doxorubicin (Doxo) for 0 (Ctrl), 24, or 48 hours. Shown is mean ± SD of 2 experiments analyzed by qBasePLUS 1.0 analysis software. **I,** Representative Western blot analysis of CIP2A expression from tamoxifen-inducible *Eμ-MYC:p53ER* lymphomas treated systemically either with vehicle (–) or with tamoxifen (+). **J,** Quantitation of CIP2A protein levels from **I**. CIP2A protein expression normalized to β-actin. Shown is mean ± SD of 3 vehicle- and 4 tamoxifen-treated lymphoma lysates. **K,** Ingenuity Transcription Factor analysis of CIP2A-regulated gene expression changes in HeLa cells. **C, D,** and **G,** representative result of 2 independent experiments with similar results.

in addition to the experimental data above, bioinformatic analysis of a recently published CIP2A-regulated gene signature (34) with Ingenuity Transcription Factor Analysis software, which reads transcription factor activities, showed that the transcriptional response to CIP2A knockdown mimicked most significantly the situation in which p53 is activated (Fig. 1K and Table S2). These results together identify CIP2A as a novel *in vivo* target of WT p53 activity and indicate that p53-mediated CIP2A downregulation functionally contributes to the p53 response.

E2F1 Upregulates CIP2A Expression Downstream of Inactivated p53

To study whether p53 regulates CIP2A expression at the transcriptional level, MCF-7 cells transfected with a *CIP2A* promoter luciferase construct containing the 1802 bp upstream promoter fragment (30) were treated with Nutlin-3 or RITA. The p53 reactivation by either of these compounds inhibited the activity of the *CIP2A* promoter but not the activity of the *EGF receptor* (EGFR) promoter (35) that was used as a control (Fig. 2A and

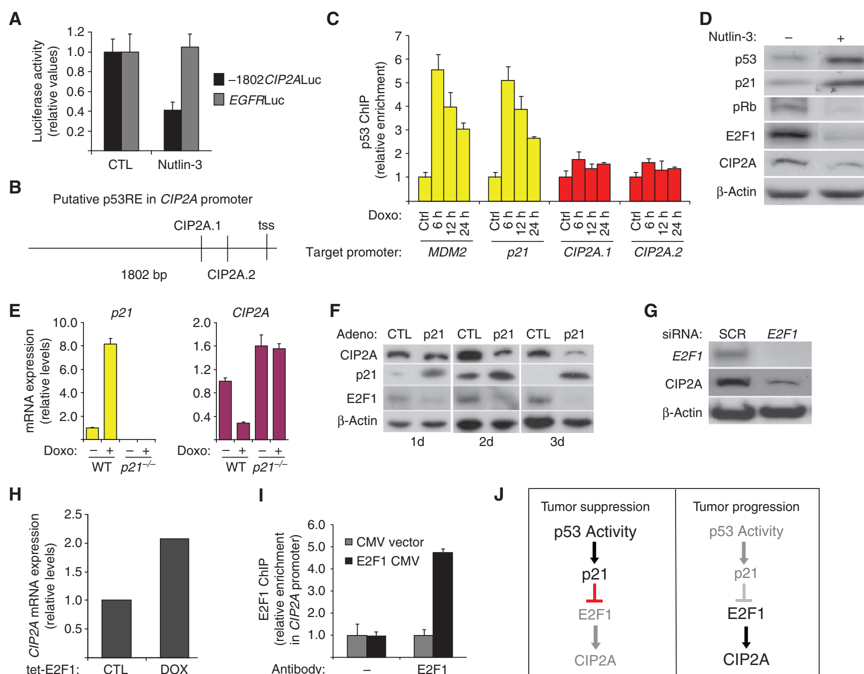


Figure 2. E2F1 upregulates CIP2A expression downstream of inactivated p53. **A,** MCF-7 cells transfected either with *CIP2A* promoter (–1802*CIP2A*luc) or with *EGF receptor* promoter (*EGFR*luc) luciferase reporter plasmid were treated with Nutlin-3 (2 μmol/L) for 24 hours and luciferase activity was measured. Shown is mean ± SD of 2 independent experiments. **B,** putative p53-responsive elements in *CIP2A* –1802 promoter according to Genomatix and ConTra softwares (tss, transcription start site). **C,** ChIP was conducted with p53 antibody from HCT116 cells treated with 0.2 μg/mL of doxorubicin for 0 (ctrl), 6, 12, or 24 hours. ChIP DNA was analyzed by real-time PCR with 2 different sets of primers against putative p53 binding sites in *CIP2A* promoter and as a positive control against the p53 binding site in *p21* and *Mdm2* promoters. Results were analyzed by qBasePLUS 1.0 analysis software and shown is mean ± SD from a representative of 2 independent experiments. **D,** Western blot detecting p53, p21, phosphorylated (serine 807/serine 811) Rb (ppRb), E2F1, and CIP2A expression from MCF-7 cells treated with 3 μmol/L of Nutlin-3 for 8 hours. Irrelevant data have been removed from the original graph. **E,** *p21* and *CIP2A* mRNA expression analyzed by RT-PCR from isogenic WT and *p21*–/– HCT-116 cells treated with 0.2 μg/mL of doxorubicin for 48 hours. Shown is mean ± SD of 2 experiments analyzed by qBasePLUS 1.0 analysis software. **F,** Western blot analysis of CIP2A, p21, E2F1, and β-actin expression from MDA-MB-231 cells transduced with either control (CTL) or p21-expressing adenovectors (p21) with MOI 80 for 1, 2, or 3 days. Irrelevant data have been removed from the original graph. **G,** Western blot analysis of *CIP2A* and *E2F1* expression in MCF-7 cells 24 hours after transfection either with scrambled (SCR) or with E2F1 siRNA. Irrelevant data have been removed from the original graph. **H,** *CIP2A* mRNA expression in doxycycline (DOX)-inducible wild-type E2F1 expressing Saos-2 cells treated for 24 hours with DOX. **I,** E2F1 ChIP was conducted in Saos-2 cells transfected either with empty CMV vector or with CMV vector expressing E2F1 (E2F1 CMV). Shown is mean ± SD of replicates from a representative of 2 experiments with similar results. **J,** Schematic model of CIP2A regulation by p53 activity. Inactive molecules and functions are shown in gray. **D, F,** and **G,** representative result of 2 independent experiments with similar results.

Supplementary Fig. S2C). Bioinformatic analysis of the −1802 fragment of the *CIP2A* promoter revealed 2 putative p53 binding sites (Fig. 2B and Supplementary Fig. S2D). However, when a chromatin immunoprecipitation assay for p53 was conducted in doxorubicin-treated HCT-116 cells, we could not detect any enrichment for these 2 putative binding sites, although p53 clearly accumulated on *Mdm2* or *p21* promoters (Fig. 2C). In support of these results, p53 was found not to bind to the *CIP2A* promoter in chromatin immunoprecipitation sequencing (ChIP-Seq) analysis conducted with control or Nutlin-3–treated MCF-7 cells (data not shown; S. Aerts; personal communication). These results indicate that although p53 activity inhibits *CIP2A* gene transcription, *CIP2A* is not a direct target gene of p53.

The p53 downstream target, p21, regulates gene expression by inhibiting cyclin-dependent kinases (CDK), which in turn leads to dephosphorylation of Rb protein and consequent inhibition of an oncogenic transcription factor E2F1 (15, 16). We confirmed that Nutlin-3–induced CIP2A downregulation is associated with the activation of the above-described p21 cascade, leading also to the previously observed inhibition of

E2F1 protein expression (ref. 18; Fig. 2D). To study whether p21 induction is required for p53-mediated CIP2A downregulation, we used isogenic HCT-116 WT and *p21*$^{-/-}$ cells. In the unperturbed *p21*$^{-/-}$ cells, CIP2A expression was increased, as compared with that in WT cells (Fig. 2E). Interestingly, similar to *p53*$^{-/-}$ HCT-116 cells, *p21*$^{-/-}$ HCT-116 cells also were resistant to doxorubicin-induced CIP2A inhibition (Fig. 2E). Moreover, p21 expression by adenoviral transduction inhibited E2F1 and CIP2A expression in MDA-MB-231 cells harboring mutated p53 (Fig. 2F). Importantly, p21-elicited E2F1 inhibition was detected already at a 24-hour time point (1 day) and preceded downregulation of CIP2A protein expression (Fig. 2F). These results suggest that increased E2F1 expression may stimulate CIP2A expression in cells with inactive p53 and p21. In support of this hypothesis, CIP2A expression was inhibited in cells transfected with *E2F1*–targeting siRNA (Fig. 2G). Of note, CIP2A downregulation by E2F1 RNA interference (RNAi) is unlikely to be caused by general inhibition of cell-cycle activity, as CIP2A expression neither is sensitive to aphidicolin-elicited cell-cycle arrest nor is associated with serum-induced cell-cycle progression (24). Furthermore, conditional tetracycline-induced overexpression of E2F1 resulted in *CIP2A* upregulation at the mRNA level (Fig. 2H). To verify that *CIP2A* is a direct E2F1 target, we conducted E2F1 ChIP in cells transfected with an E2F1 expression construct. The E2F1 binding site at −378 to −361 in the −1802 fragment of *CIP2A* promoter was predicted by using Genomatix software. As shown in Fig. 2I, E2F1 antibody immunoprecipitation clearly enriched this putative *CIP2A* promoter E2F1 binding site from E2F1-overexpressing cells as compared with cells transfected with control vector or nonantibody controls. E2F1 binding to *CIP2A* promoter was further verified by ChIP-Seq analysis from MCF-7 cells by using the ENCODE database (Supplementary Fig. S2E).

Taken together, these results strongly imply downregulation of CIP2A oncoprotein expression as a novel target mechanism for p53 tumor suppressor activity (Fig. 2J). Moreover, these results show that E2F1 stimulates CIP2A expression in cells with inactive p53 and p21 (Fig. 2J).

Inhibition of CIP2A Expression Is a Prerequisite for p53-Mediated Senescence Induction

In line with the indicated role for CIP2A as a p53 effector protein (Fig. 1K), CIP2A depletion by RNAi in MCF-7 cells mimicked p53-activated senescence, as characterized by increased senescence-associated β-galactoside (SA-β-gal) activity and flattened cell morphology in most of the cells (Fig. 3A). Induction of senescence was verified in CIP2A siRNA-transfected MCF-7 cells by increased expression of the p53-induced senescence marker decoy receptor 2 (DcR2; ref. 11; Fig. 3B). Importantly, CIP2A depletion also induced the appearance of the senescence phenotype in p53-mutant MDA-MB-231 cells (Fig. 3C), in which depletion of CIP2A causes long-term inhibition of xenograft tumor growth (22). Previously, we have shown that inhibition of CIP2A does not induce programmed cell death in HeLa cells (23). As hypothesized, stable expression of CIP2A did not reverse the obvious cell death phenotype in MCF-7 cells treated with RITA, a known inducer of p53-dependent cell death (Supplementary Fig. S2F and S2G; ref. 32). These results indicate that CIP2A downregulation is linked to p53-induced senescence.

To study whether CIP2A inhibition is truly required for p53-mediated senescence induction, Nutlin-3–induced CIP2A inhibition was prevented by infection of MCF-7 cells with CIP2A-expressing adenovirus. Of note, even though CIP2A overexpression did not prevent Nutlin-3–induced p21 induction (Fig. 3D), it prevented senescence induction in MCF-7 cells. This finding was indicated by a significant decrease in the number of cells displaying SA-β-gal activity and flattened cell morphology (Fig. 3E and F), as well as inhibition of induction of several Nutlin-3–regulated genes that previously have been shown to be functionally involved in p53-induced senescence (refs. 36–38; Fig. 3G).

Overexpression of CIP2A was recently shown to induce resistance to cell proliferation inhibition in doxorubicin-treated MCF-7 cells (39). In line with doxorubicin-elicited inhibition of *CIP2A* mRNA expression in a p53- and p21-dependent manner (Figs. 1I and 2E), protein expression of both E2F1 and CIP2A was also inhibited by doxorubicin treatment (Fig. 3H). Importantly, as for Nutlin-3, stable expression of CIP2A rescued MCF-7 cells from doxorubicin-induced senescence (Fig. 3I and J).

Positive Feedback Loop between CIP2A and E2F1 Functions as a Barrier for Senescence Induction

To investigate the underlying mechanism by which p53 reactivation-induced inhibition of CIP2A induces senescence, we studied the effect of CIP2A expression on Nutlin-3–induced p53–p21–Rb–E2F1 pathway function. As shown above (Fig. 3D), stable expression of CIP2A did not affect Nutlin-3–induced p21 activation (Fig. 4A). This finding suggests that the mechanism through which CIP2A inhibits senescence may function downstream of p21. Moreover, p21-mediated CDK inhibition seemed to be intact in CIP2A-overexpressing cells, as Rb dephosphorylation in Nutlin-3–treated cells was not affected (Fig. 4A). However, stable expression of CIP2A did effectively prevent Nutlin-3–induced E2F1 protein downregulation (Fig. 4A). Importantly, CIP2A seems to regulate E2F1 at the posttranscriptional level, as *E2F1* mRNA was downregulated by Nutlin-3 in CIP2A adenovirus–transduced cells at the same 8-hour time point (Fig. 4B), at which E2F1 protein was inhibited only in control virus–transduced cells (Fig. 4A). E2F1 is known to negatively autoregulate its promoter activity followed by hypophosphorylation of Rb (40), and this most likely explains the downregulation of *E2F1* at the mRNA level by Nutlin-3. In support of posttranslational effects of CIP2A on E2F1, CIP2A overexpression clearly increased expression of the serine 364 phosphorylated form of E2F1 (Fig. 4C), previously shown to be relatively resistant to proteolytic degradation (17, 41). The stable nature of serine 364 phosphorylated E2F1 is further shown by high levels of phosphoserine 364 E2F1 in Nutlin-3–treated and CIP2A-overexpressing cells at the 24-hour time point (Fig. 4D). At this time point, expression of nonphosphorylated E2F1 was already inhibited, along with inhibition of *E2F1* mRNA expression (Fig. 4E and data not shown). Slightly reduced expression of total E2F1 in CIP2A-overexpressed cells (Fig. 4A and C) suggests that CIP2A overexpression drives E2F1 protein to a serine 364 phosphorylated form that may not be as readily detected by the total E2F1 antibody.

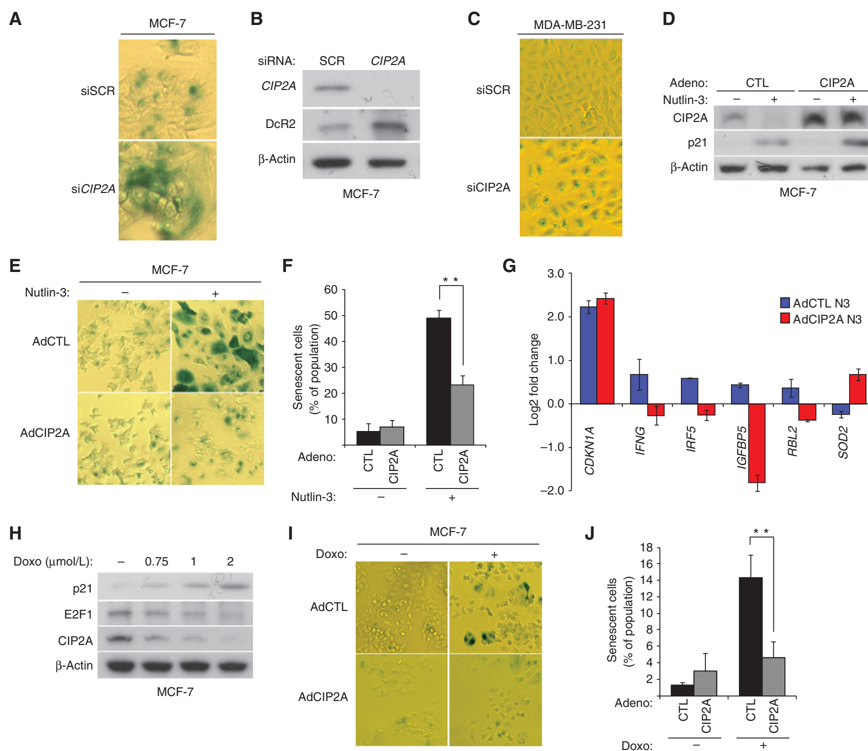


Figure 3. Inhibition of CIP2A expression is a prerequisite for p53-mediated senescence induction. **A,** SA-β-gal staining of MCF-7 cells 5 days after transfection either with scrambled (siSCR) or with *CIP2A* siRNA (si*CIP2A*). **B,** Western blot analysis of senescence marker DcR2 expression in MCF-7 cells 5 days after transfection either with scrambled (SCR) or with *CIP2A* siRNA. **C,** SA-β-gal staining of MDA-MB-231 cells 5 days after transfection either with siSCR or with siCIP2A. **D,** Western blot analysis of CIP2A and p21 expression in either control (AdCTL) or CIP2A (AdCIP2A)–transduced (MOI = 40) MCF-7 cells treated with Nutlin-3 (3 μmol/L) for 3 days. Irrelevant data have been removed from the original graph. **E,** SA-β-gal staining of AdCTL- or AdCIP2A-transduced (MOI = 40) MCF-7 cells treated with Nutlin-3 (3 μmol/L) for 3 days. **F,** percentage of SA-β-gal–positive and morphologically flattened cells of AdCTL- or AdCIP2A- transduced (MOI = 40) MCF-7 cells treated with Nutlin-3 (3 μmol/L) for 3 days. Shown is mean ± SD from 2 experiments. **, $P = 0,0022$ by Student *t* test. **G,** RT-PCR analysis of cellular senescence–associated p53-regulated genes *IFNG, IRF5, IGFBP5, RBL2,* and *SOD2* from AdCTL- or AdCIP2A-transduced (MOI = 40) MCF-7 cells treated with Nutlin-3 for 3 days. Shown is log2 fold change ± SEM of 2 replicates from a representative experiment of **E**. **H,** Western blot analysis of CIP2A, p21 and E2F1 expression in MCF-7 cells treated with doxorubicin (Doxo) with indicated concentrations. **I,** SA-β-gal staining of AdCTL- or AdCIP2A-transduced (MOI = 40) MCF-7 cells treated with Doxo (2 μmol/L) for 3 days. **J,** percentage of SA-β-gal–positive and morphologically flattened cells of AdCTL- or AdCIP2A-transduced (MOI = 40) MCF-7 cells treated with Doxo (2 μmol/L) for 3 days. Shown is mean ± SD of 3 replicates from a representative experiment in **I**. **, $P = 0,0082$ by Student *t* test. **A–E, H,** and **I,** representative result of at least 2 independent experiments with similar results.

CIP2A inhibits phosphatase activity of serine/threonine phosphatase PP2A (23, 42). Furthermore, inhibition of 2 regulatory B subunits of PP2A, B55α and B56β, rescues CIP2A depletion–induced effects on colony growth and gene expression (34). As a result, we hypothesized that PP2A holoenzymes consisting of either B55α or B56β subunits could be responsible for dephosphorylation of the serine 364 residue of E2F1 in cancer cells. In fact, inhibition of B55α, but not B56β, resulted in increased phosphorylation of serine 364 in E2F1 (Fig. 4F and Supplementary Fig. S3A). In addition, as with CIP2A overexpression, depletion of B55α rescued E2F1 protein downregulation induced by Nutlin-3 (Fig. 4G). Moreover, this effect was not observed with depletion of B56β (Fig. 4G). Taken together, these results suggest that the positive feedback mechanism from CIP2A to E2F1 is mediated by inhibition of the PP2A complex containing the B55α subunit.

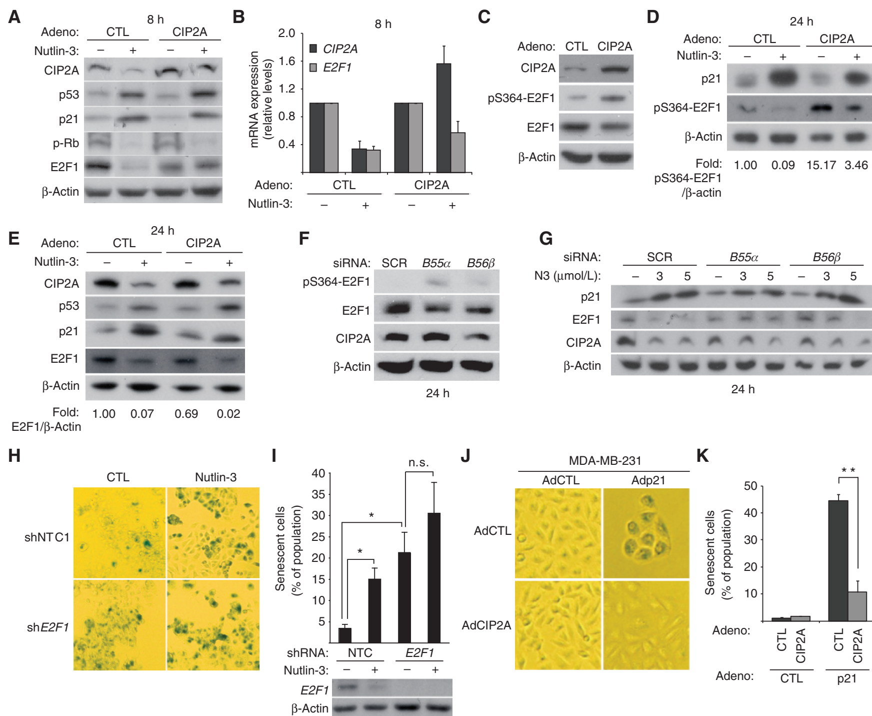


Figure 4. Positive feedback loop between CIP2A and E2F1 functions as a barrier for p21-mediated senescence induction. **A,** Western blot analysis of CIP2A, p53, p21, phosphorylated (serine 807/serine 811) Rb (ppRb) and E2F1 expression in either control (AdCTL) or CIP2A (AdCIP2A) adenovirus-transduced (MOI = 40) MCF-7 cells treated with Nutlin-3 (3 μmol/L) for 8 hours. Irrelevant data have been removed from the original graph. **B,** RT-PCR analysis of mRNA expression of *CIP2A* and *E2F1* from either control (AdCTL) or CIP2A (AdCIP2A) adenovirus-transduced (MOI = 40) MCF-7 cells (MOI = 40) treated with Nutlin-3 (3 μmol/L) for 8 hours. Shown is mean ± SEM of 2 independent experiments. **C,** Western blot analysis of phosphorylated serine 364 (pS364)–E2F1, E2F1, and CIP2A expression in either control (AdCTL) or CIP2A (AdCIP2A) adenovirus-transduced (MOI = 40) MCF-7 cells. **D,** Western blot analysis of CIP2A, p21, and pS364-E2F1 expression in AdCTL- or AdCIP2A-transduced (MOI = 40) MCF-7 cells treated with Nutlin-3 for 24 hours. **E,** Western blot analysis of p21, CIP2A, and E2F1 expression in either control (AdCTL) or CIP2A (AdCIP2A) adenovirus-transduced (MOI = 40) MCF-7 cells treated with Nutlin-3 (3 μmol/L) for 24 hours. Irrelevant data have been removed from the original graph. **F,** Western blot analysis of B55α, pS364-E2F1, E2F1, and CIP2A expression in scrambled (SCR), B55α, or B56β siRNA-transfected MCF-7 cells. **G,** Western blot analysis of p21, E2F1, and CIP2A expression in SCR, B55α, or B56β siRNA-transfected MCF-7 cells treated with Nutlin-3 (3 μM) for 24 hours. **H,** SA-β-gal staining of either nontargeting shRNA (shNTC) or E2F1shRNA (shE2F1) stable-expressing MCF-7 cells treated with Nutlin-3 (2 μmol/L) for 3 days. **I,** percentage of SA-β-gal–stained and morphologically flattened cells in either shNTC or shE2F1-expressing MCF-7 cells treated with Nutlin-3 (2 μmol/L) for 3 days. Shown is mean of replicates ± SEM from one representative experiment. **, (shNTC control vs. shNTC N3) $P = 0.0019$; **, (shNTC control vs. shE2F1 control) $P = 0.0032$; and n.s. $P = 0.1358$ by Student *t* test. **J,** SA-β-gal staining of MDA-MB-231 cells 3 days after transduction with combination of indicated adenoviruses. AdCIP2A and AdCTL were transduced at MOI = 80 and Adp21 and AdCTL at MOI = 150. **K,** percentage of SA-β-gal–positive and morphologically flattened MDA-MB-231 cells 3 days after transduction with combination of indicated adenoviruses (**I**). Shown is mean ± SEM from a representative experiment. **, $P = 0.0021$ by Student *t* test. **A, C, D–H,** and **J,** representative result of at least 2 independent experiments with similar results.

Downregulation of E2F1 has been reported to induce senescence in a p53-independent manner and to prevent tumorigenesis (19–21). To show that loss of E2F1 results in induction of the senescent phenotype in the cell type studied, E2F1 expression was downregulated in MCF-7 cells by short hairpin RNA (shRNA; shE2F1). *E2F1* depletion significantly increased the number of SA-β-gal–positive cells as compared with control cells expressing nontargeted shRNA (shNTC1) (Fig. 4H and I). Moreover, *E2F1* downregulation either by Nutlin-3, or by E2F1 shRNA, mirrored their effectiveness in inducing the senescent phenotype, but Nutlin-3 could not further increase SA-β-gal positivity in E2F1-depleted cells (Fig. 4H and I). These results indicate that E2F1 downregulation is critical for senescence induction by Nutlin-3–elicited p53 reactivation.

Recent studies have shown that cellular senescence can also be triggered by either p21 induction or E2F1 inhibition in cells carrying mutant p53 (4, 19, 20, 43). In contrast, we show here that p21 overexpression downregulates E2F1 and CIP2A expression in p53-mutant MDA-MB-231 cells, in which CIP2A depletion provokes senescence induction (Figs. 2F and 3C). To study whether CIP2A downregulation is required for senescence induced by p21, CIP2A adenovirus-infected MDA-MB-231 cells were reinfected with either control or p21-expressing adenovirus. As shown in Fig. 4J and K, stable expression of CIP2A rescued the senescence phenotype induced by p21 overexpression. Moreover, inhibition of Rb had no effect on CIP2A depletion–induced senescence in MCF-7 cells (Supplementary Fig. S3B and S3C), further indicating that CIP2A regulates senescence downstream of the p53–p21–Rb pathway.

Taken together, these results reveal the E2F1-CIP2A positive feedback loop and its role in determining cellular senescence induction in breast cancer cell lines. Interestingly, our results suggest that even transient stabilization of E2F1 upon p53 reactivation is sufficient to prevent initiation of senescence. Importantly, the functional role of this newly identified feedback loop is not restricted to p53-induced senescence, but contributes also to senescence induction by p21 in p53-mutant cells.

CIP2A Inactivation Induces Senescence and Growth Arrest and Restricts Tumorigenesis in a Breast Cancer Mouse Model

We have recently generated a *CIP2A* hypomorphic mouse model (*CIP2A*^{HOZ}) using gene trap technology (44). Despite efficient inhibition of CIP2A expression in all examined tissues, *CIP2A*^{HOZ} mice do not show obvious developmental or growth defects (Supplementary Fig. S4A–S4G; ref. 44). However, consistent with the senescence phenotype observed in CIP2A-depleted cancer cells (Fig. 3A–C), MEFs isolated from *CIP2A*^{HOZ} mouse embryos (Fig. 5A) underwent growth arrest after only a few passages (Fig. 5B), and displayed increased SA-β-gal staining and flattened cell morphology (Fig. 5C and D). Importantly, Nutlin-3 treatment of WT MEFs induced a level of senescence equal to that observed in *CIP2A*^{HOZ} cells spontaneously, but Nutlin-3 could not further increase senescence in *CIP2A*^{HOZ} cells (Fig. 5D). Moreover, overexpression of CIP2A also rescued Nutlin-3–induced downregulation of E2F1 also in MEFs, indicating that CIP2A-mediated E2F1

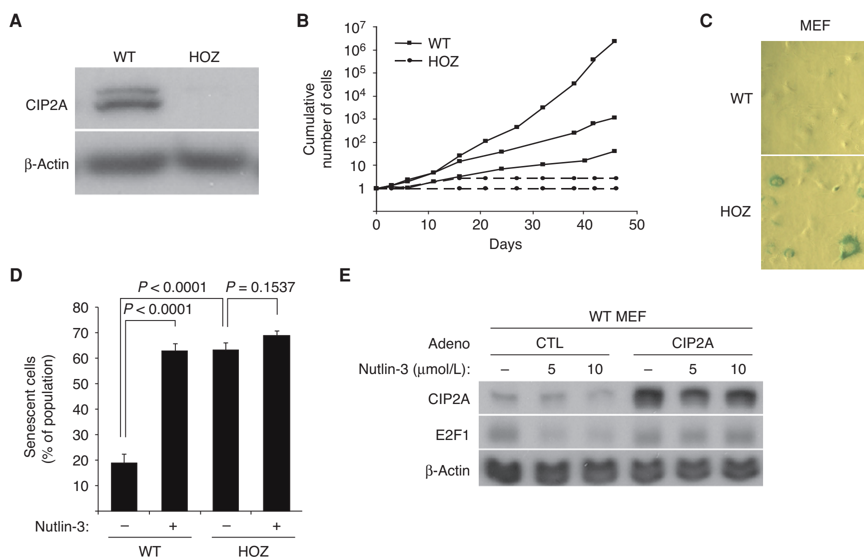


Figure 5. Inhibition of CIP2A inhibits growth and induces senescence in MEFs. **A,** Western blot analysis of CIP2A expression in MEFs isolated from WT and *CIP2A* genetrap hypomorph (*CIP2A*^{HOZ}) mouse embryos. **B,** growth curve presenting proliferation capacity of WT and *CIP2A*^{HOZ} MEFs. MEFs from 3 different WT or *CIP2A*^{HOZ} embryos were cultured for 46 days. Two *CIP2A*^{HOZ} MEF colonies ceased to proliferate after first passage, and therefore their flat curves overlap in the graph. **C,** SA-β-gal staining of WT and *CIP2A*^{HOZ} MEFs at passage 4. Shown is a representative of 2 independent experiments. **D,** percentage of SA-β-gal–stained WT and *CIP2A*^{HOZ} MEFs treated with Nutlin-3 (10 μmol/L) for 3 days. Shown is mean ± SEM of 2 independent experiments. *P* values by Student *t* test. **E,** Western blot analysis of CIP2A and E2F1 expression in either control (AdCTL) or CIP2A (AdCIP2A) adenovirus-transduced (MOI = 50) WT MEFs. Shown is a representative result of 2 independent experiments.

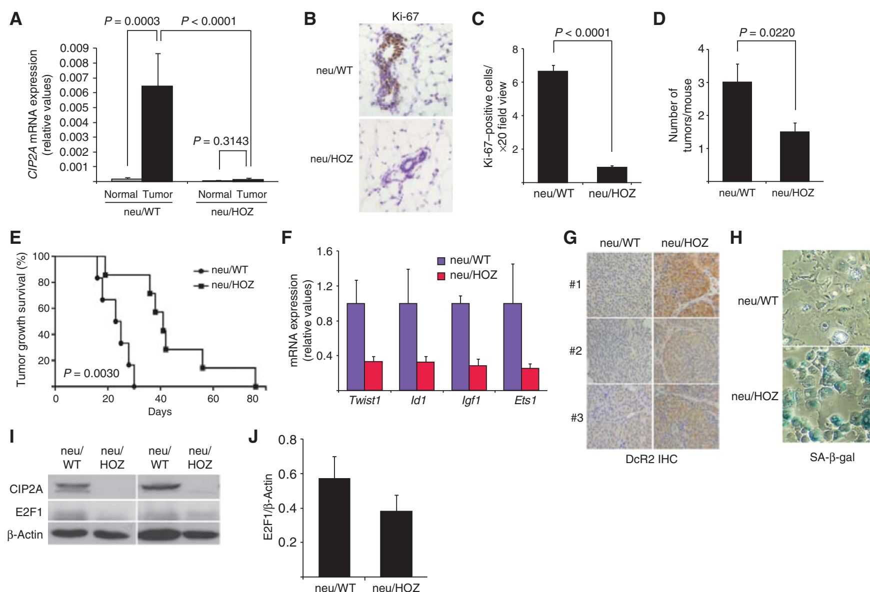


Figure 6. CIP2A inactivation induces senescence and growth arrest, and restricts tumorigenesis in a breast cancer mouse model. **A,** RT-PCR analysis of *CIP2A* mRNA expression from parental $MMTV^{neu}$ (neu/WT) and $MMTV^{neu} \times CIP2A^{HOZ}$ (neu/HOZ) normal mammary glands and tumors. Shown is mean ± SEM of mammary glands from 6 *neu/WT* and 8 *neu/HOZ* mice and 25 tumors from 9 *neu/WT* and 14 tumors from 10 *neu/HOZ* mice. *P* values by Mann–Whitney test. **B,** representative Ki-67 immunohistochemistry staining from 5 *neu/WT* and 4 *neu/HOZ* macroscopic tumor-free mouse mammary glands at the time of tumor appearance. **C,** quantitation of Ki-67 staining in **B**. Shown is mean ± SEM of Ki-67–positive cells in a field at 20× magnification. $P < 0.0001$ by Mann-Whitney test. **D,** number of mammary gland tumors per mouse in *neu/WT* and *neu/HOZ* mice. Tumors were counted when mice were sacrificed owing to 20-mm size of the largest tumor. Shown is mean ± SEM in 9 *neu/WT* and 10 *neu/HOZ* mouse. $P = 0.0220$ by Student *t* test. **E,** tumor growth was followed from the day of tumor appearance to the day when the mice had to be sacrificed owing to 20-mm size of the largest tumor. Shown is the tumor growth (days) of 6 *neu/WT* and 7 *neu/HOZ* mice. $P = 0.0030$ by log-rank test. **F,** RT-PCR analysis of senescence markers from *neu/WT* and *neu/HOZ* mammary gland tumors at the time of tumor appearance. Shown is mean ± SEM from 2 *neu/WT* and 2 *neu/HOZ* tumors. **G,** representative DcR2 immunohistochemistry staining from 7 *neu/WT* and 3 *neu/HOZ* mammary gland tumors at the time of tumor appearance. **H,** representative SA-β-gal staining from isolated *neu/WT* and *neu/HOZ* mammary gland tumor cells after 3 days in culture. Experiment was carried out twice with cells isolated from 2 different *neu/WT* and 2 different *neu/HOZ* mammary gland tumors with similar results. **I,** Representative Western blot analysis of CIP2A and E2F1 expression in *neu/WT* and *neu/HOZ* mammary gland tumors isolated at the time of tumor appearance. **J,** quantitation of E2F1 protein levels from **I**. E2F1 protein expression normalized to β-actin. Shown is mean ± SEM of 9 *neu/WT* and 6 *neu/HOZ* tumor lysates.

stabilization is a conserved mechanism between humans and rodents (Fig. 5E).

To study whether, in addition to p53 activation (10), the loss of CIP2A also suppresses tumorigenesis, we analyzed mammary tumor initiation and progression in the $MMTV^{neu}$ breast cancer mouse model crossed with $CIP2A^{HOZ}$ mice. Notably, 35% of $MMTV^{neu}$ tumors are known to harbor mutations in the p53 DNA binding domain, a frequency relatively similar to that seen in unselected human breast cancer material (45). In accordance with results from human samples (22, 23), normal mouse mammary glands expressed very low levels of *CIP2A* (Fig. 6A). However, *CIP2A* mRNA expression was greatly increased in $MMTV^{neu} \times CIP2A^{WT}$ (neu/WT) tumors ($P = 0.003$; Fig. 6A), and efficient inhibition of *CIP2A* expression in $MMTV^{neu} \times CIP2A^{HOZ}$ *(neu/HOZ)* tumors was confirmed by reverse transcriptase PCR (RT-PCR) analysis (Fig. 6A). Interestingly, as compared with *neu/WT* mice, *neu/HOZ* mice had fewer Ki-67–positive epithelial cells in macroscopically tumor-free mammary glands (Fig. 6B and C and Supplementary Fig. S4H). In line with these observations, the average number of mammary tumors per mouse was significantly reduced in *neu/HOZ* mice ($P = 0.0220$; Fig. 6D). Furthermore, follow-up of the tumors that developed in mice with either of the genotypes showed that the time for tumor growth, from the day of tumor appearance to the day when the mice had to be sacrificed because the 20-mm maximum size of the largest tumor allowed was reached, was significantly delayed in *neu/HOZ* mice ($P = 0.0030$; Fig. 6E).

In concert with the *in vitro* results shown above, mammary tumors in CIP2A-deficient mice displayed gene expression changes indicative of senescence induction (Fig. 6F). Of

these senescence-inhibiting genes downregulated in *neu/HOZ* tumors (6, 7, 46–48), *Twist1* and *Id1* are particularly interesting, as they have both recently been shown to block oncogene-driven senescence in breast cancer cells (46, 48). Importantly, expression of the p53-induced senescence marker DcR2 (11) was also increased in CIP2A-deficient *neu/HOZ* tumors at the protein level (Fig. 6H). Moreover, we observed spontaneous induction of SA-β-gal expression in cultured cells isolated from *neu/HOZ* tumors (Fig. 6G and H). Together, these results validate the senescence phenotype of CIP2A-deficient breast cancer cells *in vivo*.

To confirm the *in vivo* role for CIP2A in the inhibition of senescence in another setting, and without potentially confounding effects of mouse strain crossings, the effect of CIP2A expression in dimethylbenzanthracene (DMBA) treatment–induced senescence in mouse skin (5) was examined. As hypothesized, we detected significantly more SA-β-gal staining in DMBA-treated *CIP2A*^{HOZ} mouse skin as compared with WT mouse skin (Supplementary Fig. S4I and S4J). Together, these results validate induction of senescence as a plausible cause for decreased mammary tumorigenesis in CIP2A-deficient mice.

To examine whether the above-described role for CIP2A in promoting E2F1 expression would also be observed in an *in vivo* setting, we conducted Western blot analysis of tumor lysates. Indeed, E2F1 expression was decreased in *neu/HOZ* tumors as compared with *neu/WT* tumors (Fig. 6I and J). In addition, mRNA expression of direct E2F1 target genes, *Rbl1* and *Id1*, was decreased in *neu/HOZ* tumors (Supplementary Fig. S4K).

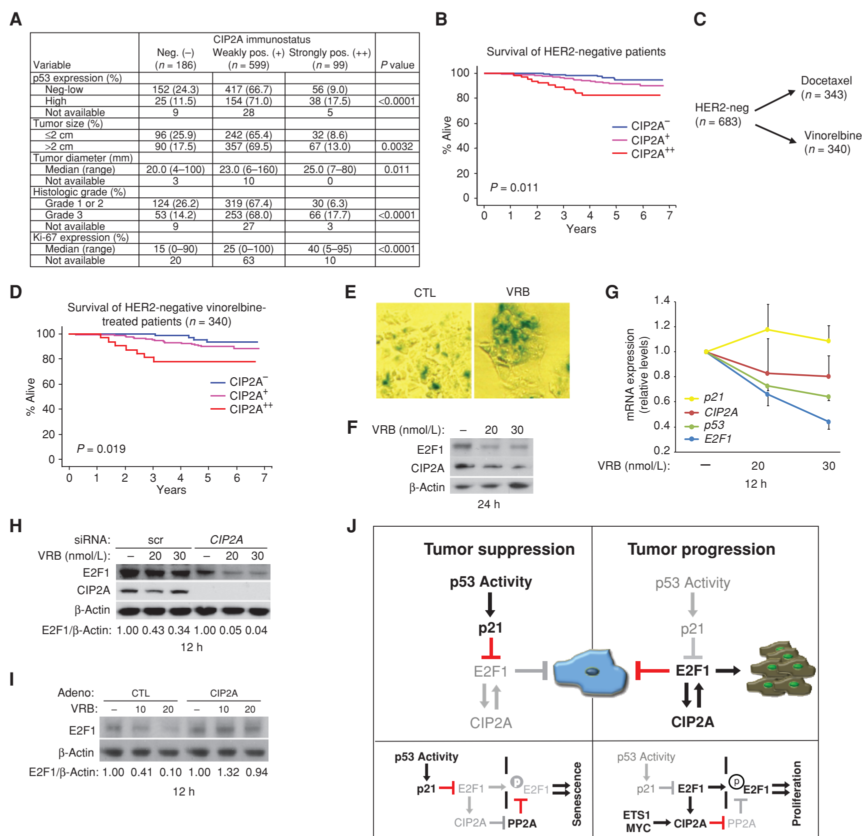
Taken together, these results provide the first genetic evidence for the requirement of CIP2A for tumor formation and growth. Moreover, these findings validate CIP2A's functional role as an *in vivo* inhibitor of senescence induction in breast cancer (Fig. 6B–H).

CIP2A Confers Resistance of Human Breast Tumors to Senescence-Inducing Chemotherapy

Our results thus far have shown that CIP2A expression determines cellular senescence induction in response to p53 and p21 activation. To study the clinical relevance of these findings, the expression levels of, and the prognostic role for, CIP2A were studied in a cohort of breast cancer tumor samples from patients with advanced disease (n = 1,010; ref. 49). Interestingly, CIP2A was overexpressed in 79% of the breast cancers in this population of women (Fig. 7A), of whom 89% had axillary node–positive breast cancer and the rest had high-risk node-negative cancer (49). This frequency is far greater than the frequency of CIP2A overexpression in unselected human breast cancers (approximately 40%; Fig. 1B; ref. 22). Also in this cohort, CIP2A expression is significantly associated with high p53 immunopositivity (Fig. 7A) and with several features linked with aggressive disease (Fig. 7A). The difference in overall survival of patients with low or high CIP2A expression did not quite reach statistical significance in the entire patient population ($P = 0.073$; Supplementary Fig. S5A). However, in HER2-negative breast cancers, representing the great majority (77%) of the studied patient material (45), high tumor CIP2A expression was significantly associated with poor overall survival ($P = 0.011$; Fig. 7B) and distant recurrence or death ($P = 0.024$; Supplementary Fig. S5B). In multivariate analysis, assessing the independent role for CIP2A as a prognostic factor in HER2-negative breast cancers, tumor CIP2A expression tended to be associated with poor outcome [$P = 0.058$; for CIP2A^{++} vs. CIP2A^{-}, HR = 4.26; 95% confidence interval (CI), 1.29–14.08; $P = 0.017$; for CIP2A^{++} vs. CIP2A^{+}, HR = 1.54; 95% CI, 0.75–3.15; $P = 0.241$], whereas tumor size (>2.0 cm vs. ≤2.0 cm), axillary nodal status (positive vs. negative), histologic grade (poorly vs. moderately vs. well differentiated), and p53 expression (positive vs. negative) were not associated with survival ($P \geq 0.10$ for each). However, absent estrogen receptor expression was independently associated with poor survival in HER2-negative breast cancer (HR = 2.18; 95% CI, 1.12–4.23; $P = 0.022$). We speculate that CIP2A does not have prognostic value in HER2-positive cancers ($P = 0.687$; Supplementary Fig. S5C), even though it supports mammary tumorigenesis in the HER2-driven mouse model (Fig. 6E), because human cancers have a more complex pattern of oncogenically active proteins, the combined activity of which masks CIP2A's prognostic effect.

To study the role of tumor CIP2A in the response of HER2-negative cancers to adjuvant therapy, the association of CIP2A expression with survival of patients was studied in patient groups stratified by the type of chemotherapy administered (Fig. 7C). In these groups, patients were randomly assigned to receive either single-agent docetaxel or vinorelbine (3 cycles) followed (in both groups) by 3 cycles of fluorouracil, epirubicin, and cyclophosphamide (FEC; ref. 49). Notably, CIP2A overexpression significantly correlated with poor overall survival in the subgroup of patients who were assigned to receive vinorelbine followed by FEC ($P = 0.019$; Fig. 7D), whereas CIP2A expression was not significantly associated with survival of patients assigned to docetaxel followed by FEC ($P = 0.373$; Supplementary Fig. S5D).

Vinorelbine is a semisynthetic vinca alkaloid used to treat several kinds of human cancer, including non–small cell lung cancer and advanced breast cancer (50, 51). Interestingly, another vinca alkaloid, vincristine, has been shown to induce senescence in MCF-7 cells (52). On the basis of this information, and the novel role for the E2F1-CIP2A feedback loop in preventing chemotherapy-induced senescence, we hypothesized that the favorable survival of the patients with CIP2A-negative cancer in the vinorelbine group could be linked with sensitivity of these cancers to vinorelbine-induced inhibition of E2F1. Indeed, vinorelbine-treated MCF-7 cells mimicked the E2F1 and CIP2A inhibition-associated phenotype by displaying increased SA-β-gal positivity and flattened cellular morphology (Fig. 7E). Importantly, induction of a senescence phenotype by vinorelbine was preceded by inhibition of both E2F1 and CIP2A protein expression at the 24-hour time point (Fig. 7F). Interestingly, vinorelbine-induced E2F1 downregulation was not accompanied by either p53 or p21 induction (Fig. 7G and Supplementary Fig. S5E and S5F), but similarly to Nutlin-3 treatment, it was associated with inhibition of *E2F1* mRNA expression (Fig. 7G). To study whether CIP2A-deficient breast cancer cells are indeed more sensitive to vinorelbine-elicited E2F1 inhibition, MCF-7 cells transfected with either scrambled or *CIP2A* siRNA were treated with vinorelbine for 12 hours, at which time point, vinorelbine did not yet inhibit CIP2A expression in parental cells (Fig. 7H).



Variable	CIP2A immunostatus Neg. (–) (n = 186)	Weakly pos. (+) (n = 599)	Strongly pos. (++) (n = 99)	P value
p53 expression (%)				
Neg-low	152 (24.3)	417 (66.7)	56 (9.0)	
High	25 (11.5)	154 (71.0)	38 (17.5)	<0.0001
Not available	9	28	5	
Tumor size (%)				
≤2 cm	96 (25.9)	242 (65.4)	32 (8.6)	
>2 cm	90 (17.5)	357 (69.5)	67 (13.0)	0.0032
Tumor diameter (mm)				
Median (range)	20.0 (4–100)	23.0 (6–160)	25.0 (7–80)	0.011
Not available	3	10	0	
Histologic grade (%)				
Grade 1 or 2	124 (26.2)	319 (67.4)	30 (6.3)	
Grade 3	53 (14.2)	253 (68.0)	66 (17.7)	<0.0001
Not available	9	27	3	
Ki-67 expression (%)				
Median (range)	15 (0–90)	25 (0–100)	40 (5–95)	<0.0001
Not available	20	63	10	

Figure 7. CIP2A confers resistance of human breast tumors to senescence-inducing chemotherapy. **A,** CIP2A expression in human breast cancer tumors in FinHer study. CIP2A is expressed in 79% of breast tumors and correlates with high p53 immunopositivity and with other poor prognostic factors. *P* values by χ^2 test, except for Ki-67 and tumor diameter the Kruskal–Wallis test was used. **B,** CIP2A expression significantly correlates with survival of patients with HER2-negative tumors. CIP2A⁻, CIP2A-negative tumor; CIP2A⁺, moderately CIP2A-positive tumor; CIP2A⁺⁺, high CIP2A-expressing tumor. *P* = 0.011 by log-rank test. **C,** stratification scheme of patients with HER2-negative tumors to receive therapies including either vinorelbine followed by FEC (*n* = 340) or docetaxel followed by FEC (*n* = 343). **D,** CIP2A overexpression is significantly associated with poor survival of vinorelbine + FEC–treated HER2-negative patients. *P* = 0.019 by log-rank test. **E,** SA-β-gal staining of MCF-7 cells treated with vinorelbine (VRB; 30 nmol/L) for 5 days. **F,** Western blot analysis of E2F1 and CIP2A expression in MCF-7 cells treated with VRB (20 and 30 nmol/L) for 24 hours. **G,** RT-PCR analysis of *p53, p21, E2F1,* and *CIP2A* mRNA expression in MCF-7 cells treated with VRB (20 and 30 nmol/L). Shown is mean ± SEM of 2 independent experiments. **H,** Western blot analysis of E2F1 and CIP2A expression in either scrambled (scr) or *CIP2A* siRNA-transfected MCF-7 cells treated with VRB (20 and 30 nmol/L) for 12 hours. Quantitation of E2F1 expression normalized to β-actin expression is shown below the graph. **I,** Western blot analysis of E2F1 in either control (AdCTL) or CIP2A (AdCIP2A) adenovirus-transduced (MOI = 40) MCF-7 cells treated with VRB (10 and 20 nmol/L) for 12 hours. Quantitation of E2F1 expression normalized to β-actin expression is shown below the graph. **E, F, H,** and **I,** representative result of at least 2 experiments with identical results. **J,** schematic of the positive feedback loop between E2F1 and CIP2A in regulation of cellular senescence sensitivity downstream of p53. Inactive molecules and functions are shown in gray. In nontransformed cells (left), either oncogene- or chemotherapy-induced p53 activity inhibits E2F1 expression, resulting in subsequent inhibition of CIP2A expression. CIP2A inhibition further inhibits E2F1 protein expression by a posttranslational mechanism involving PP2A. Loss of E2F1-CIP2A positive feedback activity provokes cellular senescence and hence tumor suppression. In tumorigenic cells (right), in which p53 activity is inhibited either by mutations or by enhanced proteolytic degradation, the E2F1-CIP2A positive feedback loop is active, resulting in inhibition of senescence induction and hence tumor progression. Importantly, in addition to p53 inactivation, activity of E2F1–CIP2A feedback may be stimulated by ETS1 and MYC, which enhance CIP2A expression.

As expected, *CIP2A* siRNA inhibited E2F1 protein expression in nontreated cells, and, importantly, CIP2A deficiency dramatically potentiated E2F1 downregulation in vinorelbine-treated cells (Fig. 7H). Furthermore, exogenous CIP2A expression totally prevented E2F1 downregulation in vinorelbine-treated MCF-7 cells (Fig. 7I).

These results show clinical relevance for CIP2A in the progression and chemotherapy response of human breast cancers. Importantly, these findings imply that CIP2A could be a useful predictive marker for selecting patients with HER2-negative breast cancer, which currently lacks efficient targeted therapy options, for vinca alkaloid-containing chemotherapies. Moreover, these results indicate that the E2F1-CIP2A feedback mechanism is involved in chemotherapy resistance toward compounds that inhibit E2F1 expression independently of p53 or p21 activation.

DISCUSSION

Mounting evidence indicates that the tumor suppression function of p53 relies on its capacity to induce senescence (1, 8–10, 53). In this study, we identify inhibition of CIP2A expression as a previously unrecognized mechanism required for senescence induction by activated p53 and p21 (Fig. 7J). CIP2A's role as a functional p53 target is supported strongly by both unbiased bioinformatics analysis of the transcriptome in CIP2A-depleted cells (Fig. 1K) and by senescence experiments (Figs. 3A, C, E, and I and 5C and D). Importantly, CIP2A is positively regulated by p53 inactivation regardless of whether p53 activity is inhibited by Mdm2 (Fig. 1D and E), by mutations (Fig. 1H), or by RNAi (Fig. 1C). In addition to *in vitro* conditions, CIP2A expression correlates with p53 mutation in human breast cancer (Figs. 1A and B and 7A), and *in vivo* reactivation of p53 in transgenic lymphomas expressing p53ER fusion protein potently inhibits CIP2A protein expression (Fig. 1I and J). Furthermore, we show that loss of CIP2A restricts mammary carcinogenesis in a mouse model known to harbor p53 mutations (Fig. 6E; ref. 45). Moreover, a recent study showed that in human gastric cancer, CIP2A has the most significant prognostic role in p53-immunopositive tumors (24). These findings together validate the *in vivo* relevance of CIP2A as a novel p53 target protein. Of note, *CIP2A* is not a direct p53 target gene, but is regulated via the p21-E2F1 axis (Fig. 2), albeit its expression is not sensitive to cell-cycle inhibition (24). Moreover, we show that CIP2A inhibition is required for p21-induced senescence in p53-mutated cancer cells (Fig. 4J and K). These results provide a novel mechanistic explanation for the recently shown *in vivo* function of p21 in inducing senescence and delaying tumor onset (4, 54). The results of this study strongly indicate that inhibition of CIP2A oncoprotein expression is a novel tumor suppression mechanism driven by the p53–p21 pathway (Fig. 7J). Moreover, these results explain how inactivation of the p53–p21 pathway promotes senescence resistance in cancer.

Inhibition of E2F transcriptional activity provokes senescence in human tumor cells and inhibits tumor growth (19–21). Nevertheless, E2F1 target genes involved in preventing senescence induction in cancer cells have been elusive. Our results show that activation of the p53–p21 pathway by Nutlin-3 simultaneously induces dephosphorylation of Rb, and transcriptional inhibition of *E2F1* gene expression (Fig. 4A and B). We postulate that transcriptional inhibition of *E2F1* by both Nutlin-3 and vinorelbine (Figs. 4B and 7G) explains consequent inhibition of CIP2A expression and triggers inhibition of a positive feedback loop between E2F1 and CIP2A (Fig. 7J). Our data indicate that CIP2A supports E2F1 protein expression at the posttranslational level in both human and mouse cells. In addition to overexpression data, we also confirmed that CIP2A depletion caused inhibition of E2F1 protein expression (Fig. 7H). In search of a mechanistic explanation for CIP2A-mediated stabilization of E2F1 protein expression, we observed that CIP2A promotes E2F1 serine 364 phosphorylation, and this phosphorylation has been previously shown in another context to be associated with increased stability of E2F1 (17, 41). Moreover, we observed that inhibition of a regulatory subunit of PP2A, B55α, increases E2F1 serine 364 phosphorylation and reverses Nutlin-3–induced downregulation of E2F1 (Fig. 4F and G). Previously, we showed that inhibition of the *PPP2R2A* gene, encoding B55α, reverses CIP2A depletion–induced antiproliferative and gene expression effects (34). Interestingly, deletion of the B55α gene was recently identified as a potential driver mutation specifically in the luminal B type of breast cancer (55). These results indicate that the B55α-containing PP2A tumor suppressor complex needs to be inhibited during breast cancer progression either by genetic mutations or via overexpression of CIP2A. Importantly, our data also indicate that mechanisms other than p53 inactivation–induced E2F1 expression may drive high CIP2A expression in human breast cancer (Figs. 1B and 7A). We postulate that in these cases ETS-1– and MYC-mediated CIP2A expression (24, 30) supports E2F1 expression and thereby confers on these cells resistance to senescence induction (see Fig. 7J for schematic presentation).

Although CIP2A expression has been shown to predict for poor patient survival in many different human cancer types (refs. 24, 28; Supplementary Table S1), such evidence has thus far been lacking for breast cancer. In this study, we show that CIP2A has a prognostic role in HER2-negative breast cancer, for which novel therapy targets are in high demand. Interestingly, low *E2F1* mRNA expression levels were found specifically in HER2-negative breast tumors (56). Therefore, it can be envisioned that the prognostic value of CIP2A becomes more apparent in HER2-negative cancers in which CIP2A-mediated posttranslational increase of E2F1 protein becomes critical for tumor progression. Moreover, the observation that the E2F1 response to senescence-inducing vinorelbine chemotherapy is dependent on CIP2A status provides a plausible mechanistic explanation for the favorable survival of patients who have CIP2A/HER2-negative breast cancer and who were treated with vinorelbine before FEC (Fig. 7D).

Prosenescence therapies are emerging as an alternative approach for cancer treatment (6, 7). However, the majority of the strategies suggested thus far for therapeutic senescence induction rely on activation of p53 and other cellular checkpoint mechanisms (6, 7). Although hypothetically reasonable, these strategies suffer from serious shortcomings because in the majority of human cancers several checkpoint mechanisms are functionally impaired. Therefore, identification of the E2F1–CIP2A–positive feedback

loop as a novel prosenescence therapeutic target mechanism that functions downstream of inactivated p53, in which inhibition induces senescence independently of p53 activation, is a fundamentally important finding. As an example of the *in vivo* importance of the p53-independent senescence-inducing mechanisms, Lin and colleagues (4) recently showed a role for p21-induced senescence in tumor suppression. In that regard, our data indicate that CIP2A expression not only inhibits p53-induced senescence (Fig. 3E, F, I, and J) but also p21-induced senescence in p53-mutant breast cancer cells (Fig. 4J and K). As p53 inhibition promotes CIP2A expression (Figs. 1 and 2), these results together indicate that senescence resistance in p53-mutant tumors is caused by a combined effect of impaired p53 checkpoint activity and increased activity of the E2F1-CIP2A feedback loop. Therefore, CIP2A deregulation could be considered a novel gain-of-function for mutant p53 in cancer (13). Importantly, the feasibility of targeting the identified E2F1-CIP2A positive feedback loop for prosenescence therapy is supported by the lack of any obvious developmental defects in the CIP2A knockdown mouse used in this study (Supplementary Fig. S4; ref. 44). Moreover, as CIP2A is overexpressed at an exceptionally high frequency in 65% to 90% of tumor samples of most major human cancer types (Supplementary Table S1), its inhibition could serve as a general strategy to sensitize cancer cells to prosenescence therapies. These conclusions are supported by a previously reported increase in SA-β-gal activity in a CIP2A-depleted gastric cancer cell line (57).

In sum, this study identifies a hitherto unrecognized oncogenic mechanism downstream of the inactivated p53–p21 pathway. Our results show that although E2F1 stimulates CIP2A expression in cells with an inactive p53–p21 pathway, inhibition of the E2F1-CIP2A feedback loop is essential for senescence induction (Fig. 7J). Moreover, as inhibition of the E2F1-CIP2A feedback loop also induces senescence in p53-mutant cells, and pRb is not needed for CIP2A inhibition–induced senescence (Supplementary Fig. S3), these results indicate that inhibition of E2F1 and CIP2A can induce senescence in cancer cells without activation of the upstream p53–p21 pathway. In general, these findings suggest that senescence induction in cancer cells is determined by the activity of this newly identified feedback mechanism between E2F1 and CIP2A, rather than simply by the strength of the senescence-inducing stimuli (Fig. 7J). Finally, results of this study should encourage development of approaches both to target E2F1-CIP2A feedback mechanism and to stratify patients to senescence-inducing cancer therapies based on tumor CIP2A status.

METHODS

Cell Culture and Drug Treatments

MCF-7, MDA-MB-231, HeLa, and SAOS-2 cell lines were obtained from American Type Culture Collection. HCT116 and its clonal p53 (*p53*$^{-/-}$) and p21 (*p21*$^{-/-}$) deletion mutants were kindly provided by Prof. B. Vogelstein (Johns Hopkins University, Baltimore, MD). Cells were tested twice a year for negativity for mycoplasmas and acholeplasmas with Mycoplasma Detection Kit (Roche). Cells were exposed to the indicated concentrations of Nutlin-3 (Cayman Chemicals), doxorubicin (Sigma), vinorelbine (Sigma), or RITA (Cayman Chemicals).

Antibodies

For immunoblotting, the following antibodies were used: CIP2A: rabbit polyclonal (57) and mouse monoclonal 2G10-3B5 (Santa Cruz); p21: rabbit polyclonal C-19 (Santa Cruz); p53: mouse monoclonal DO-1 (Santa Cruz) and rabbit polyclonal CM5 (Vector Laboratories); β-actin: mouse monoclonal (Sigma); Rb: rabbit polyclonal C-15 (Santa Cruz); B55α: mouse monoclonal 2G9 (Cell Signaling); Ser 807/811 phosphorylated Rb: rabbit polyclonal (Santa Cruz); E2F1 KH95: mouse monoclonal (Santa Cruz); serine 364 phosphorylated E2F1: rabbit polyclonal (Abcam); and DcR2: rabbit polyclonal (Abcam).

Immunohistochemical and Statistical Analysis of Human Breast Cancer Patient Samples

CIP2A immunostaining in both FinProg and FinHer breast cancer patient cohorts was conducted with polyclonal rabbit antibody (58). CIP2A was immunostained and analyzed from both cohorts of human breast cancer patient tumor samples (FinProg and FinHer studies), as described previously (34). In the FinProg cohort of patients with breast cancer, p53 and Ki-67 immunostaining of breast tumor samples and analysis of tumor size and tumor grades were conducted as previously described (59). In the FinHer cohort of patients with breast cancer, HER2 and Ki-67 immunostaining; analysis of tumor diameter, tumor size, and tumor grade; and statistical analysis of total and cumulative survival and percentage of alive patients in different subgroups were conducted similarly as before (49). The p53 immunostaining from the FinHer cohort was done following same protocol as published for the FinProg study (59). An ethics committee at Helsinki University Hospital (Helsinki, Finland) approved the FinHer study (HUCH 426/E6/00). Regarding FinProg material, permission to use formalin-fixed, paraffin-embedded tissues for research purposes was provided by the Ministry of Social Affairs and Health, Finland (permission 123/08/97).

Animal Experiments

MMTV^{neu} mice (60) expressing oncogenic HER2 under the control of the mouse mammary tumor virus promoter specifically in the mouse mammary gland were purchased from The Jackson Laboratory and crossed with *CIP2A* heterozygous genetrap hypomorphic mutant mice (*CIP2A*^{HEZ}; ref. 44). *MMTV*^{neu}/*CIP2A*^{HEZ} mice were intercrossed to produce *MMTV*^{neu}/*CIP2A*^{WT}, *MMTV*^{neu}/*CIP2A*^{HEZ}, and *MMTV*^{neu}/*CIP2A*^{HOZ} mice. Mice were genotyped by PCR analysis of genomic DNA for *MMTV*^{neu} transgene according to The Jackson Laboratory's protocol and for *CIP2A* genetrap, as previously described (44). *CIP2A* genotyping results were confirmed with mRNA analysis by RT-PCR. Mice were checked for tumor appearance twice a week. Formed tumors were palpated twice a week, and mice were sacrificed when tumor diameter reached 20 mm. Tumor size was measured by palpating and by weighing after preparation of the tumor from sacrificed mice. Immunohistochemical staining for Ki-67 and DcR2 and hematoxylin and eosin (H&E) staining were conducted as previously described (41). Tumor cells were isolated by forcing cells through a 70-μm pore filter (BD Biosciences). Cells were cultured with Dulbecco modified Eagle medium (DMEM)/F12 Ham medium containing 10% serum, insulin, hydrocortisone, and mouse EGF. MEFs were isolated from WT and *CIP2A*^{HOZ} embryos at 13.5 days of gestation, and cultured in DMEM containing 15% serum.

In DMBA treatment, the dorsal skin of WT and *CIP2A*^{HOZ} mice was treated with DMBA (20 μg in 200 μL of acetone) 3 times a week for 2 weeks. A day before the first treatment, mouse backs were shaved, and mice were sacrificed 24 hours after the last treatment. Lymphoma lysates from *EμMyc:p53ER* mice systemically treated with either tamoxifen or peanut oil were prepared as described previously (33). All animal work protocols were approved by the Regional State Administrative Agency for Southern Finland (ESLH-2007-08517, ESLH-2009-00515/Ym-23).

Proliferation Assay and SA-β-gal Staining

Proliferation capacity of MEFs was studied by calculating cell numbers of MEFs from 3 different WT and *CIP2A*[HOZ] embryos seeded to 14,000 cells/cm^2 and divided when 70% to 80% confluent. Cells were cultured for 46 days. To detect senescent cells, cells and mouse skin sections were fixed and stained for SA-β-gal at pH 6.0 (Sigma) according to the manufacturer's protocol. Senescent cells in *in vitro* assays were quantified under the microscope by counting morphologically flattened and SA-β-gal–positive cells. SA-β-gal staining in mouse skin was quantitated by counting positively stained areas from 2 to 3 sections per mouse.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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