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Characterization and Functional Studies of *PPM1D* in Breast Cancer

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ACADEMIC DISSERTATION
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List of original communications

This thesis is based on the following communications, which are referred to in the text by their Roman numerals:

I. **Pärssinen J**, Kuukasjärvi T, Karhu R, Kallioniemi A. High-level amplification at 17q23 leads to coordinated overexpression of multiple adjacent genes in breast cancer (2007). Br J Cancer 96:1258-1264.

II. **Rauta J**, Alarmo EL, Kauraniemi P, Karhu R, Kuukasjarvi T, Kallioniemi A. The serine-threonine protein phosphatase *PPM1D* is frequently activated through amplification in aggressive primary breast tumours (2006). Breast Cancer Res Treat 95:257-263.

III. **Pärssinen J**, Alarmo EL, Karhu R, Kallioniemi A. *PPM1D* silencing by RNAi inhibits proliferation and induces apoptosis in breast cancer cell lines with wild-type p53. Submitted for publication.

IV. **Pärssinen J**, Alarmo EL Khan S, Karhu R, Vihinen M, Kallioniemi A. Identification of differentially expressed genes after *PPM1D* silencing in breast cancer. Cancer Letters, in press.

Abbreviations

ABC1	amplified in breast cancer
ABL-BCR	v-abl Abelson murine leukemia viral oncogene homolog 1/ breakpoint cluster region –fusion protein
ACE	angiotensin-converting enzyme, somatic isoform precursor
APC	adenomatosis polyposis coli
APPBP2	amyloid beta precursor protein binding protein 2
ATM	ataxia-telangiectasia mutated
ATR	ataxia-telangiectasia and rad-3 related
BAC	bacterial artificial chromosome
BER	base excision repair
BFB	breakage-fusion-bridge
BCAS3	breast carcinoma amplified sequence 3
BCL2	B-cell CLL/lymphoma 2
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
BRIP1	BRCA1 interacting protein C-terminal helicase 1
CA4	carbonic anhydrase IV precursor
CCND1	cyclin D1
CDC25A	cell division cycle 25 homolog A
CDC25B	cell division cycle 25 homolog B
cDNA	complementary DNA
CGH	comparative genomic hybridization
CLTC	clathrin heavy chain 1
CYB561	cytochrome b561
DHX40	DEAH (Asp-Glu-Ala-His) box polypeptide 40
DNA-PK	protein kinase, DNA-activated, catalytic polypeptide
ERBB2	avian erythroblastic leukaemia viral oncogene homolog 2
FAM33A	family with sequence similarity 33, member A
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
GO	gene ontology
HMEC	human mammary epithelial cells
KCNH6	potassium voltage-gated channel, subfamily H, member 6
MDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein
MEF	mouse embryo fibroblast
miRNA	microRNA
MKK3	mitogen-activated protein kinase kinase 3
MKK4	mitogen-activated protein kinase kinase 4

MKK6	mitogen-activated protein kinase kinase 6
MMTV	mouse mammary tumor virus
MRC2	mannose receptor, C type 2
MYC	avian myelocutomatosis viral oncogene homolog
$p16^{INK4A}$	transcript of CDKN2A locus: cyclin-dependent kinase inhibitor 2A
$p19^{ARF}$	transcript of CDKN2A locus: cyclin-dependent kinase inhibitor 2A, isoform 4
p53	tumor protein p53
PALB2	partner and localizer of BRCA2
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PP2C	type 2C protein phosphatase
PPM1D	protein phosphatase 1D magnesium-dependent, delta isoform
PPM1E	protein phosphatase 1E
PRSS1	protease, serine, 1
PTRH2	peptidyl-tRNA hydrolase 2
qRT-PCR	quantitative real-time RT-PCR
RAD51C	DNA repair protein RAD51 homolog
RNAi	RNA interference
RB	retinoblastoma
RPS6KB1	ribosomal protein S6 kinase
Ser	serine
SEPT4	peanut-like protein 2
TBP	TATA box binding protein
TBX2	T-box transcription factor TBX2
TBX4	T-box transcription factor TBX4
TEX14	testis expressed sequence 14
Thr	threonine
THRAP1	thyroid hormone receptor-associated protein 1
TLK2	serine/threonine-protein kinase tousled-like 2
TMA	tissue microarray
TMEM49	transmembrane protein 49
TRIM37	tripartite motif-containing 37 protein
TUBD1	tubulin delta chain
USP32	ubiquitin carboxyl-terminal hydrolase 32
UNG2	uracil DNA glycosylase 2
YPEL2	yippee-like 2 protein
WRD68	WD-repeat protein 68

Yhteenveto

Geenimonistuma on yksi merkittävimmistä mekanismeista, jonka avulla syöpäsolut edistävät kasvainten kehittymiseen ja etenemiseen osallistuvien geenien ilmentymistä. Kromosomialue 17q23 on puolestaan yksi yleisemmin havaituista monistuneista alueista rintasyövässä ja tämän monistuman esiintyminen on myös osoitettu liittyvän kasvainten etenemiseen sekä potilaiden huonoon ennusteeseen. Tässä väitöskirjassa pyrittiin tarkemmin selvittämään 17q23 kromosomialueen monistuman vaikutuksia alueen geenien ilmentymiseen primaarisissa rintasyöpäkasvaimissa sekä määrittämään yhden tällä alueella sijaitsevan geenin, *PPM1D*:n, liiallisen ilmentymisen toiminnallista merkitystä rintasyövässä.

Uusia 17q23 alueen kohdegeenejä pyrittiin tunnistamaan tutkimalla systemaattisesti alueen geenien ilmentymistä kvantitatiivisen reaaliaikaisen käänteiskopiointipolymeraasiketjureaktion (qRT-PCR) avulla. Tutkimus-aineistona käytettiin primaareita rintasyöpäkasvaimia, joiden mahdollinen 17q23 monistuma-aste oli määritetty. Tässä tutkimuksessa kävi ilmi, että yhdentoista geenin ilmentymisessä oli tilastollisesti merkittävä ($p<0.01$) ero verrattaessa korkean monistuman omaavien kasvainten ryhmää niihin kasvaimiin, joissa monistumaa ei ollut. Samanlaista eroa ei huomattu kohtalaisesti monistuneiden sekä ei-monistuneiden ryhmien välillä. Lisäksi näiden yhdentoista geenin havaittiin sijaitsevan lähellä toisiaan 1.56 Mb:n suuruisella monistuman ydinalueella. Tällä alueella sijaitsevista geeneistä vain yhden ei havaittu ilmentyvän ylimäärin. Näiden tulosten perusteella voidaankin todeta, että rintasyövässä vain korkea-asteinen monistuma 17q23 alueen ydinosassa johtaa geenien kohonneeseen ilmentymiseen. Nämä tulokset edelleen korostavat sitä seikkaa, että 17q23 monistuma-alue sisältää useita kandidaattigeenejä. Tällainen geenien samanaikainen monistuminen ja yli-ilmeneminen syövässä on todennäköisesti yleisempi tapahtuma kuin aikaisemmin on uskottu.

PPM1D monistuman roolia rintasyövässä tutkittiin määrittämällä geenin kopiolukuja sekä primaarikasvainaineistossa että rintasyöpäsolulinjoissa. Analyysi paljasti *PPM1D* monistuman 11 %:ssa primaareita rintasyöpäkasvaimia. Kvantitatiivinen reaaliaikainen käänteiskopiointipolymeraasiketjureaktio puolestaan osoitti tilastollisesti merkittävän ($p=0.0148$) korrelaation näiden monistumien sekä lisääntyneen *PPM1D*:n ilmentymisen välillä. Lisäksi *PPM1D* monistuma havaittiin melkein yksinomaan kasvaimissa, joissa oli villityypin *TP53* kasvurajoitegeeni. *PPM1D* monistuman ja ERBB2 proteiinin ilmentymisen välillä havaittiin myös olevan merkittävä ($p=0.0001$) yhteys. Immunohistokemialliset analyysit eivät kuitenkaan osoittaneet, että kahden *PPM1D*-geenin kohdeproteiinin, CCND1:n

ja $p16^{INK4A}$:n, ilmentymisissä olisi eroja verrattaessa *PPM1D* monistuman omaavia kasvaimia sellaisiin, joissa tätä monistumaa ei ollut. Samankaltainen kopiolukuanalyysi paljasti, että *PPM1D* oli monistunut myös kuudessa (29 %) 21 rintasyöpäsolulinjasta. Northern-analyysi osoitti, että tämä monistuma johti *PPM1D*:n lisääntyneeseen ilmentymiseen viidessä niistä kuudesta rintasyöpäsolulinjasta, joissa oli *PPM1D* monistuma. Näiden tulosten perusteella voidaan olettaa, että *PPM1D* on ratkaiseva *TP53* kasvurajoiteproteiinin toiminnan säätelijä ja siten sillä on tärkeä rooli rintasyövässä. Lisäksi *PPM1D* monistuman ja ERBB2 ilmentymisen yhteys osoittaa *PPM1D* muutokset ovat merkki erityisen aggressiivisesta kasvaimesta.

PPM1D:n toiminnallista merkitystä tutkittaessa käytettiin hyväksi RNA interferenssi –tekniikkaa, jonka avulla voitiin estää *PPM1D*:n ilmentyminen rintasyöpäsolulinjoissa (BT-474, MCF7 ja ZR-75-1), joissa *PPM1D*:n tiedettiin olevan monistunut ja sen ilmentymisen olevan lisääntynyt. *PPM1D*:n ilmentymisen tehokas estäminen johti alentuneeseen solukasvuun MCF7 ja ZR-75-1 solulinjoissa, jotka omaavat normaalin *TP53* kasvurajoitegeenin, kun taas BT-474 soluissa, jotka kantavat mutatoitunutta *TP53* geeniä, tällaista vaikutusta ei havaittu. Lisäksi osoitettiin, että *PPM1D* geenin hiljentämisen aikaansaama hidastunut solukasvu johtuu ainakin osittain apoptoottisten solujen määrän kasvusta. Näiden löydösten perusteella voidaan päätellä, että *PPM1D* osallistuu solujen uudiskasvun säätelyyn rintasyövässä tavalla, joka on riippuvainen *TP53* kasvurajoitegeenistä. Lisääntyneen *PPM1D*:n ilmentymisen voidaan myös ajatella edistävän pahanlaatuista ilmiasua ylläpitämällä solujen kasvua sekä niiden elonjäämistä.

RNA-interferenssi –teknologiaa käytettiin myös tutkittaessa *PPM1D* geenin hiljentämisen vaikutuksia geenien ilmentymistasoihin sekä solujen signalointireitteihin BT-474, MCF7 ja ZR-75-1 rintasyöpäsolulinjoilla. *PPM1D* ilmentymisen estämisen jälkeen ihmisen genomin geenien ilmentymistasot analysoitiin oligonukleotidi-pohjaisia geenisiruja käyttäen. Näin tunnistettiin 1798 geeniä, joiden ilmentyminen oli muuttunut. Näiden geenien tiedetään liittyvän keskeisiin solunsisäisiin prosesseihin, kuten solusyklin säätelyyn, useiden solunsisäisten rakenteiden ja osatekijöiden kokoonpanoon sekä signalointi- ja aineenvaihduntareittien säätelyyn. Näiden tulosten perusteella voidaankin ajatella, että *PPM1D* geeni osallistuu rintasyövän syntyyn vaikuttamalla joko suorasti tai epäsuorasti useisiin tärkeisiin solunsisäisiin signalointireitteihin.

Tämän väitöskirjan tutkimukset osoittavat, että korkea-asteinen monistuma 17q23 kromosomialueella johtaa useiden geenien ilmentymisen kasvuun rintasyövässä. Tällainen samanaikainen aktivaatio voi puolestaan olla mukana rintasyövän syntyprosessissa. Lisäksi tulokset korostavat yhden tämän 17q23 alueen geenin, *PPM1D*:n, keskeistä roolia rintasyövän kehittymisessä sekä etenemisessä. Tästä syystä *PPM1D* geeniä voidaankin pitää yhtenä tärkeänä ehdokkaana kehitettäessä erilaisia uusia täsmäterapioita rintasyöpää vastaan.

Abstract

Gene amplification is one of the major mechanisms allowing cancer cells to promote the expression of genes that are involved in tumor development and progression. At the same time, 17q23 is one of the most commonly amplified chromosomal regions in breast cancer and this amplification has also been shown to be associated with tumor progression and poor prognosis. The main objectives of this study were to fully characterize the molecular consequences of the 17q23 amplification on gene expression levels in primary breast tumors and also to define the functional significance of overexpression of *PPM1D*, one the genes residing in this region in breast cancer.

To identify novel target genes at the 17q23 amplicon, a systematic gene expression survey using quantitative real-time RT-PCR (qRT-PCR) on primary breast tumors with known 17q23 amplification status was performed. This study revealed a statistically significant ($p<0.01$) difference between high-level and no amplification groups for a set of eleven genes. No difference in gene expression levels was observed between the moderate and the non-amplified tumor groups. Moreover, these eleven genes were found to be located adjacent to one another within a 1.56 Mb core region in which all except one of the genes were overexpressed. These data indicate that only high level amplification at the 17q23 amplicon core leads to elevated gene expression in breast cancer. Moreover, these results further underline the fact that 17q23 amplicon carries multiple candidate genes which may be a more common event in gene amplification than previously thought.

To explore the role of *PPM1D* aberrations in breast cancer, a copy number analysis was carried out both in primary tumor and cell line material. This analysis showed *PPM1D* amplification in 11% of the primary breast tumors, while qRT-PCR revealed a significant correlation ($p=0.0148$) between amplification and increased *PPM1D* expression. The data also demonstrated that *PPM1D* amplification occurs almost exclusively in tumors with wild-type p53. Furthermore, *PPM1D* amplification was associated with ERBB2 expression ($p=0.0001$). Immunohistochemical analyses, however, revealed no differences in the staining patterns of two downstream targets of *PPM1D,* CCND1 and p16 proteins in tumors with or without *PPM1D* aberrations. A similar copy number analysis also demonstrated *PPM1D* amplification also in six (29%) out of 21 breast cancer cell lines. Northern analysis further indicated that this amplification led to increased overexpression of *PPM1D* in five out of the six cell lines with amplification. Together, these data suggest that *PPM1D* acts as a critical regulator of p53 tumor suppressor function and has an important role in breast cancer. Moreover, the association of *PPM1D* amplification with ERBB2

expression implies an evident connection between *PPM1D* aberrations and aggressive disease.

To define the functional significance of *PPM1D* in breast cancer, RNA interference was used to inhibit *PPM1D* expression in BT-474, MCF7 and ZR-75-1 breast cancer cell lines harboring amplification and increased expression of *PPM1D*. Efficient downregulation of *PPM1D* resulted in significantly reduced cell proliferation in MCF7 and ZR-75-1 cells with wild-type p53 but not in BT-474 carrying mutant p53. Furthermore, the data indicated that the reduced cell growth observed after *PPM1D* silencing was at least partly due to increased apoptotic cell death. These findings suggest that *PPM1D* is involved in the regulation of cell proliferation in breast cancer in p53 dependent manner and that overexpression of *PPM1D* contributes to malignant phenotype by promoting sustained cell growth and cell survival.

To investigate the effects of *PPM1D* silencing on global gene expression patterns and signaling pathways, RNA interference (RNAi) was first utilized to downregulate *PPM1D* expression in BT-474, MCF7 and ZR-75-1 breast cancer cell lines and then oligonucleotide microarray analysis was performed. This way, altogether 1798 differentially expressed gene elements were identified. These genes were related to key cellular processes, such as regulation of cell cycle, assembly of various intracellular structures and components, and regulation of signaling pathways and metabolic cascades. These results suggest that *PPM1D* contributes to breast cancer associated phenotypic characteristics by directly or indirectly affecting several important cellular signaling pathways.

In conclusion, the findings of this study indicate that high level amplification of 17q23 in breast cancer leads to upregulation of multiple genes, suggesting that their concurrent activation may contribute to breast cancer. The results further emphasize the crucial role of one of the genes within the 17q23 amplicon, *PPM1D*, in the development and progression of breast cancer. Therefore, *PPM1D* may be considered as a candidate target for the development of specific molecular cancer therapies.

Introduction

The mammary gland is the organ in female mammals that produces milk for the offspring. The basic components of the mammary gland are the hollow cavities called alveoli that are lined with milk-secreting cells and surrounded by myoepithelial cells. Together the alveoli form groups known as lobules and each lobule has a lactiferous duct that drains into the openings in the nipple. The mammary glands are undeveloped until puberty, when, in response to ovarian hormones, they begin to maturate in females. Accordingly, hormones have a key role in the control of the development and growth of the mammary gland. If the cells of the mammary gland escape this normal growth control, cancer may develop. Typically, breast cancer arises from the epithelial cells of the terminal duct lobular unit and can be classified as either invasive or non-invasive (in situ) carcinoma based on its characteristic growth patterns (reviewed by Sainsbury et al., 2000). The commonly used classification further divides invasive carcinomas into ductal (80% of cases) and lobular types (10-15% of cases). Some relatively rare tumors with specific growth and morphological features are called invasive carcinomas of special type and include medullary, mucinous, papillary, tubular, and cribriform carcinomas.

Breast cancer is the most commonly diagnosed cancer in women worldwide, with constantly increasing incidence rates. Breast cancer affects about 10% of Finnish female population in their lifetime and the Finnish Cancer Registry estimates that about 4060 new cases in women will be reported for the year 2006. Breast cancer is also the leading cause of female cancer mortality, although around 88% of patients are still alive five years after diagnosis (Finnish Cancer Registry, 2006). The majority of breast carcinomas occur sporadically, due to both environmental and genetic factors. Several environmental or lifestyle factors, such as old age, early age at menarche, late age at first childbirth, late menopause, nulliparity, obesity, use of hormone replacement therapy or oral contraceptives, radiation exposure, and residence in western countries, have been associated with increased risk of developing breast cancer (reviewed by McPherson et al., 2000). On the other hand, most of the genetic variants that contribute to the risk of developing sporadic breast cancer are still unknown and are likely to further interact with environmental agents. Although hereditary predisposition accounts for only 5 to 10% of all breast cancer cases, positive family history of the disease is indeed a major risk factor for breast cancer. To date, most hereditary breast cancers have been associated with inherited mutations in two major breast cancer susceptibility genes, *BRCA1* and *BRCA2* (Miki et al., 1994; Wooster et al., 1995), even though several other genes (e.g. *CHK2*, *PALB2*) and loci have recently been implicated in breast cancer

predisposition (Nevanlinna et al., 2006; Easton et al., 2007; Erkko et al., 2007). *BRCA1* and *BRCA2* are considered high-penetrance genes, since inherited mutation in these genes is associated with a prominent family history of breast cancer and high cancer risk while *CHK2* and *PALB2* are associated with a smaller increase in the risk of breast cancer and a less prominent family history and are thus considered as low-penetrance cancer genes (Miki et al., 1994; Wooster et al., 1995, Nevanlinna et al., 2006; Easton et al., 2007; Erkko et al., 2007).

The primary treatment for breast cancer is surgery, either alone or combined with hormonal or cytotoxic adjuvant therapy and/or post-operative irradiation. Several features of breast cancer including tumor size, tumor differentiation, and nodal status provide clinically useful information for the assessment of patient prognosis and thus influence treatment decisions (reviewed by Bundred, 2001; reviewed by Cianfrocca et al., 2004). For example, poor histological grade may indicate a higher potential for response to chemotherapy. Steroid hormone receptors are also important, especially in guiding selection of hormone treatment. Estrogen (ER) and progesterone receptors (PR) are prime examples of indicators capable of identifying patients likely to respond to a particular form of therapy. Furthermore, additional prognostic indicators relating,for example, to proliferative rates of tumor cells and overexpression of the *ERBB2* oncogene are available. The overexpression of *ERBB2* has been found to act as a potential indicator of resistance to chemotherapy and hormone therapy (reviewed by Bundred, 2001; reviewed by Cianfrocca et al., 2004).

Like other cancers, breast cancer is a disease involving dynamic genetic changes, such as mutations, translocations, deletions, and amplifications (reviewed by Ponder, 2001; reviewed by Balmain et al., 2003). The multistep accumulation of these genetic alterations may then lead to inactivation of tumor suppressor genes or activation of oncogenes. The most common mechanism of oncogene activation in solid tumors including breast cancer is gene amplification. Usually, the amplified segment (amplicon) is considerably larger than the transcriptional unit of a gene (reviewed by Savelyeva et al., 2001), but the size and complexity of the amplicon may vary from one chromosomal location and tumor type to another. Therefore, several genes may be simultaneously amplified in a single amplicon, and hence play a part in tumor progression.

The purpose of this study was to evaluate the molecular consequences of 17q23 amplification in breast cancer with special emphasis on the functional significance of *PPM1D*.

Review of the literature

1. Molecular basis of cancer

Generally, cancer arises from single somatic cells. It is considered as a genetic disease, because it is triggered by accumulation of alterations in various genes controlling critical cellular functions. Development of cancer is a long process including complex succession of changes. Both genetic and epigenetic changes confer a clonal selective advantage to the cells in which they occur and as a result cancer cells can escape the normal growth control and proliferate without restraint (reviewed by Ponder, 2001; reviewed by Balmain et al., 2003). A model for the multistep genetic progression of cancer has been proposed by Fearon and Vogelstein based on their finding that specific mutations are associated with certain steps of colorectal tumorigenesis (reviewed by Kinzler et al., 1996). During tumor development, cancer cells must acquire a specific set of capabilities in order to achieve full malignant potential. These essential features include self-sufficiency in growth signals, insensitivity to antigrowth signals, escape from apoptosis, unlimited replication potential, angiogenesis, and tissue invasion and metastasis (reviewed by Hanahan et al., 2000).

The tumorigenic process is, of course, much more complicated that mere unchecked cell growth. Two categories of genes, oncogenes and tumor suppressor genes play major roles in triggering this process. Proto-oncogenes are normal cellular genes that when either mutated or expressed at abnormally high levels become oncogenes and contribute to converting a normal cell into a cancer cell. These genes typically function in the control of cell growth, differentiation, and development and include secreted growth factors (e.g. *PDGF*), cell surface receptors (*ERBB* gene family), intracellular signaling molecules (e.g. *ABL, RAS*), cell cycle regulators (e.g. cyclins, *MDM2*), transcription factors (e.g. *MYC*), and anti-apoptotic proteins (e.g. *BCL2*) (reviewed by Schwab, 1998; reviewed by Savelyeva et al., 2001; Roset et al., 2007). Oncogenes are dominant in their function and thus activation of a single allele of a gene is sufficient to give a growth advantage to the cell (reviewed by Todd et al., 1999). Several mechanisms, such as activating point mutations, gene amplification or translocation of the gene into the vicinity of a transcriptionally active area have been shown to take part in the activation of oncogenes and lead to the overexpression of the original protein product (Figure 1) (reviewed by Todd et al., 1999). On the other hand, oncogenes can also be activated through chromosomal rearrangements that can give rise to novel fusion proteins (e.g. ABL-BCR) and thus altered protein product (Figure 1). Interesting recent

findings have also suggested that alterations in microRNAs (miRNAs) can lead to the activation of oncogenes (reviewed by Esquela-Kerscher et al., 2006).

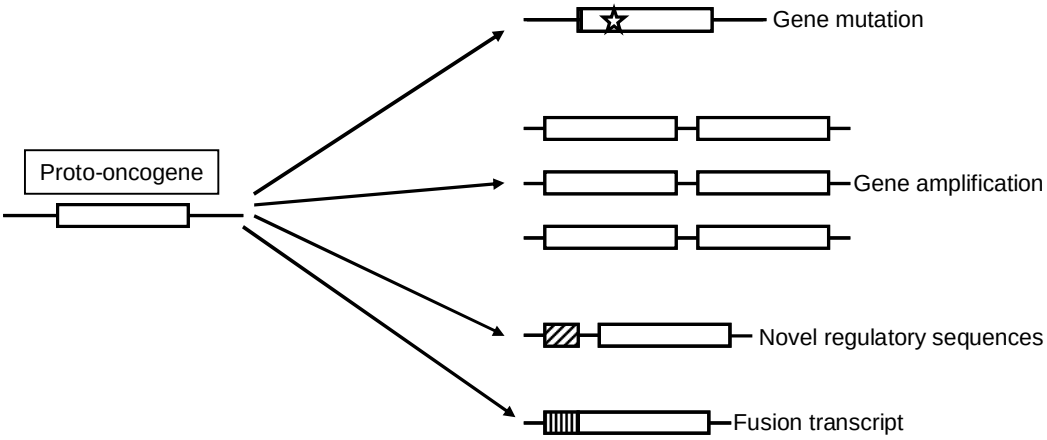

Figure 1. *Main mechanisms of oncogene activation. Point mutations can activate oncogenes by structurally altering the proteins they are encoding, which may lead to uncontrolled continuous activity of the mutated protein product. In the case of gene amplification, the copy number of the oncogene is increased, resulting in overexpression of the normal protein. Oncogene may also become activated when it is moved through chromosomal translocation to the vicinity of an active gene, where its expression becomes regulated by novel regulatory sequences. Chromosomal rearrangements, such as translocations as well as inversions, can also generate fusion transcripts giving rise to chimeric oncogenic proteins.*

To become cancerous, cells must also break free from the inhibitory mechanisms that normally counteract these growth-stimulating pathways. Somatic cell fusion and chromosome segregation experiments have demonstrated that transformed phenotypes can often be corrected *in vitro* by fusion of the transformed cell with a normal cell (reviewed by Harris et al., 1969; Harris, 1971). This provides evidence that tumorigenesis involves not only dominant activated oncogenes, but also recessive, loss-of-function mutations in other genes. In 1971, Knudson proposed a “two-hit” hypothesis according to which both alleles of a tumor suppressor gene have to be inactivated for tumor formation (Knudson, 1971; reviewed by Knudson, 2001). In general, tumor suppressor genes may be silenced by point mutations, deletions, loss of heterozygosity, or epigenetic changes, mainly methylation. In hereditary cancer cases, one defective allele is inherited and thus the likelihood for the inactivation of the remaining allele and in consequence loss of function of the entire tumor suppressor gene is much greater, thus explaining the increased cancer susceptibility. In some circumstances, one tumor suppressor allele is not sufficient to ensure normal function. This phenomenon is called haploinsufficiency, and can also contribute to cancer pathogenesis (reviewed by

Balmain et al., 2003). Like oncogenes, tumor suppressor genes also govern a wide range of normal cellular activities. They may participate in the regulation of the cell cycle, they may control cell proliferation or the integrity of the genome, or they may promote apoptosis (reviewed by Sherr, 2004). Most of the tumor suppressor genes belong to either gatekeeper or caretaker subclasses (Kinzler et al., 1997). Gatekeepers (e.g. *RB1, APC*) inhibit the proliferation of cancer cells by directly inhibiting growth or accelerating apoptosis, whereas caretakers (e.g. *BRCA1, BRCA2*) are guardians of the genome by preventing or repairing genomic damage. The third subclass, landscapers, includes genes (e.g. *SMAD4*) that act on the tumor microenvironment rather than the cancer cells (reviewed by Kinzler and Vogelstein, 1998).

miRNAs are a class of naturally occurring small noncoding RNA molecules that are also found to regulate gene expression by targeting mRNAs and triggering either translation repression or RNA degradation (reviewed by Bartel, 2004; Iorio et al., 2005). MiRNAs have been found to be abnormally expressed or mutated in several tumor types including breast cancer (Calin et al., 2002; Michael et al., 2003; Metzler et al., 2004; Takamizawa et al., 2004; Eis et al., 2005; Iorio et al., 2005; Porkka et al., 2007) and therefore they are likely to be important in the pathogenesis of human neoplasms. Still, only little is known about the specific target genes and pathways of each miRNA. Interestingly, recent studies have indicated that in one context, miRNAs may act as an oncogene, but in another, they may antagonize the effects of different oncogenes and thus have the role of a classic tumor suppressor gene (He et al., 2005; O'Donnell et al., 2005). This dual role of the miRNAs makes their function in human cancers even more complex.

2. Gene amplification in cancer

One of the major mechanisms of a tumor cell to upregulate and consequently activate cellular oncogenes during tumor development and cancer progression is gene amplification. It has been shown to play a crucial part in the pathogenesis of various human malignancies especially in solid tumors, such as breast, prostate, lung, ovarian, gastric, pancreatic, and colon cancers (reviewed by Knuutila et al., 1998). Nevertheless, it is not considered to be an early event in tumor progression but is restricted to genetically unstable cells (Otto et al., 1989; Wright et al., 1990).

The common molecular mechanisms through which different genes are amplified are not yet fully understood, however several different models have been proposed. Studies in rodent model systems have suggested that the mechanism underlying many of the early amplification events in mammalian cells is breakage-fusion-bridge (BFB) cycles (Toledo et al., 1992; Hellman et al., 2002). Cytogenetic analyses have also indicated that BFB cycles drive the amplification of oncogenes that are tightly linked to common fragile sites in human cancers (Coquelle et al., 1997; reviewed by Kuo et al., 1998; Coquelle et

al., 2002; Hellman et al., 2002). According to this model, an initial double strand break occurring within a chromosomal fragile site or at a dysfunctional telomere leads to the fusion of uncapped sister chromatids and thereby the formation of a dicentric chromosome. In subsequent cell division, the two centromeres may be pulled to the opposite ends of the dividing cell resulting in a bridge between the centromeres and finally breaking of the dicentric chromosome. In consequence, repeated break and fusion cycles lead to intrachromosomal amplification through unequal distribution of the genetic material (Hellman et al., 2002; reviewed by Murnane et al., 2004; reviewed by Albertson, 2006; reviewed by Myllykangas et al., 2006; Bignell, 2007). Recently, Chin and co-workers discovered that telomeres are progressively shortened in immortalized late-passage human mammary epithelial cell and this leads to crisis and telomere reactivation (Chin et al., 2004). At the same time, the frequence of cells with anaphase bridging increased considerably and several genetic changes were detected. These findings demonstrate that BFB cycles induced by telomere dysfunction have important roles in driving the genomic instability needed to transform benign tumors into malignant cancers (Chin et al., 2004; Depinho and Polyak, 2004).

Other proposed mechanisms of gene amplification include re-replication, unequal exchange, and episome excision. In the re-replication model, so-called onion skin structure is formed when DNA synthesis is initiated multiple times during one cell cycle (reviewed by Stark et al., 1984; reviewed by Windle et al., 1992; reviewed by Wintersberger, 1994; reviewed by Albertson, 2006; reviewed by Myllykangas et al., 2006; Bignell, 2007). Similarly, multiple gene copies can be generated by recombination events between homologous or non-homologous DNA sequences on two misaligned chromosomes or chromatids according to the unequal exchange model (Smith et al., 1990; reviewed by Windle et al., 1992; reviewed by Wintersberger, 1994; reviewed by Albertson, 2006; Bignell, 2007). Finally, in the episome excision model, extrachromosomal amplification is generated by the formation of small circular acentric molecules that can eventually form cytogenetically visible double minute chromosomes (DMs) (reviewed by Wahl, 1989; Windle et al., 1991; reviewed by Albertson, 2006; Bignell, 2007). However, the complex structures of the amplified sequences found in cancers are not always fully explained by these models. For example, a recent study has proposed two alternative models for oncogene amplification at the 17q21 locus that seem to be modifications of those previously reported mechanisms (Kuwahara et al., 2004). The first of these models suggests that tandem duplication occures after double strand break in a head-to-tail or head-to-head orientation in specific matrix attachment regions following DNA replication and integration into other chromosomes. In the other model, overreplication occurs in these same specific regions after double strand break and amplicon ends are joined in a site-specific manner resulting in episome formation and reintegration of these episomes into other chromosomes.

Several amplified oncogenes, including genes encoding growth factors, cell cycle regulators, and transcription factors, have been observed in breast cancer. Of these, the clinically most important amplified gene is the *ERBB2* oncogene (reviewed by Ross et al., 1999). Numerous studies have indicated the significant

prognostic value of *ERBB2* and also its role in predicting response to therapy (Baselga et al., 1999; Cobleigh et al., 1999; reviewed by Ross et al., 1999; Vogel et al., 2002; reviewed by Ross et al., 2003). Most importantly, *ERBB2* has been a target for new therapeutic approaches, such as Herceptin (trastuzumab), a recombinant antibody which is in a wide clinical use for *ERBB2* overexpressing breast cancer (Carter et al., 1992). Accordingly, other oncogenes that are activated through amplification in cancer may also be clinically useful and thus represent ideal targets for the development of novel anti-cancer therapies.

However, recent studies have demonstrated that oncogenes are rarely if ever amplified in isolation, but are rather present in large amplicons that contain multiple genes with altered copy numbers (reviewed by Albertson, 2003; reviewed by Ethier, 2003). For example, amplification of a minimal common region at 17q12 in breast cancer has been shown to lead to a simultaneous increase of expression levels of all genes within that genomic segment including *ERBB2* (Kauraniemi et al., 2003). Similar observations on overexpression of multiple genes within an amplicon have been reported on several occasions, for example at 8p11-p12 in breast cancer (Garcia et al., 2005; Gelsi-Boyer et al., 2005) and at the 11q13 locus in oral cancer (Huang et al., 2006) Although the amplicons contain multiple overexpressed genes, it is unlikely that all of them are essential for cancer progression. Some of the genes may be overexpressed simply due to their location within the amplicon with no functional relevance. Nevertheless, recent findings have indicated that amplicons can also be driven by a set of genes which are simultaneously overexpressed and thus provide a growth advantage to cancer cells (Huang et al., 2006; Kao et al., 2006).

2.1. The 17q23 amplicon

Amplification of the chromosomal region 17q23 was first discovered in breast cancer using comparative genomic hybridization (Kallioniemi et al., 1994). Since then, it has been shown to be one of the most frequently amplified chromosomal regions in breast cancer (Courjal et al., 1997; Tirkkonen et al., 1998). The 17q23 amplification has also been associated with tumor progression and poor prognosis in breast cancer (Isola et al., 1995; Bärlund et al., 2000a; Andersen et al., 2002). These data indicate that an increased dosage of one or more genes in this region is involved in the progression of this disease. In addition to breast cancer, gain and amplification of the 17q23 region has also been detected in tumors of brain, lung, ovary, pancreas, bladder, testis, and liver. Table 1 lists representative examples of studies demonstrating 17q23 gains in various tumor types. Furthermore, the association of 17q23 amplification with poor prognosis was also reported for ovarian clear cell adenocarcinoma, neuroblastoma, and acute myelogenous leukemia (Morerio et al., 2001; Hirasawa et al., 2003; Saito-Ohara et al., 2003), thus suggesting that the activated genes within this amplicon are likely to have an impact on disease pathogenesis and possibly the clinical management of cancer patients with various malignancies.

Table 1. Representative examples of studies demonstrating 17q23 gains and amplifications in various tumor types

Reference	Tumor type	Method	Frequency (%)
Morerio et al. 2001	Acute myelogenous leukemia	FISH	19
Sallinen et al. 1997	Astrocytoma	CGH	46
Voorter et al. 1995	Bladder cancer	CGH	14
Kallioniemi et al. 1994	Breast cancer	CGH	18
De Angelis et al. 1999	Colorectal carcinoma	CGH	16
Pedeutour et al. 1995	Dermatofibrosarcoma	CGH	57
Sonoda et al. 1997	Endometrial cancer	CGH	11
Pack et al. 1999	Esophageal squamous cell carcinoma	CGH	59
el-Rifai et al. 1996	Gastrointestinal stromal tumors	CGH	16
Marchio et al. 1997	Hepatocellular carcinoma	CGH	33
Ried et al. 1994	Lung cancer	CGH	23
Rao et al. 1998	Lymphoma	CGH	2
Kivipensas et al. 1996	Malignant mesothelioma	CGH	36
Lothe et al. 1996	Malignant peripheral nerve sheath tumors	CGH	50
Nicholson et al. 1998	Medulloblastoma	CGH	45
Weber et al. 1997	Meningioma	CGH	42
Brinkschmidt et al. 1997	Neuroblastoma	CGH	63
Terris et al. 1998	Neuroendocrine tumors of digestive system	CGH	55
Arnold et al. 1996	Ovarian cancer	CGH	11
Solinas-Toldo et al. 1996	Pancreatic carcinoma	CGH	19
Daniely et al. 1998	Pituitary tumors	CGH	17
Gronwald et al. 1997	Renal cancer	CGH	20
Weber-Hall et al. 1996	Rhabdomyosarcoma	CGH	30
Korn et al. 1996	Testicular cancer	CGH	36
Hemmer et al. 1999	Thyroid cancer	CGH	15

The 17q23 amplicon is very large in breast cancer and thereby several studies have been carried out to define its exact structure and limits (Couch et al., 1999; Bärlund et al., 2000b; Erson et al., 2001; Monni et al., 2001; Wu et al., 2001). Currently, the amplicon is considered to cover an approximately 5 Mb region at 17q23 and to include multiple independent peaks of amplification. The gene density of the entire chromosome 17 is one of the highest in the genome (16.2 genes per Mb) (Zody et al., 2006) and about 50 genes, including predicted genes as well as genes with known function, are located at the amplified region on 17q23 (reviewed by Sinclair et al., 2003, Figure 2). It is possible that such a high gene density predisposes to amplifications. However, in mice, the chromosomal region corresponding to human chromosome 17 also has high gene density but no amplifications. Therefore, gene density alone does not explain the frequency of amplifications in humans (Zody et al., 2006). Instead, Zody and co-workers

identified 20 rearrangement breakpoints in human chromosome 17, but only three in the corresponding mouse region. Therefore, the breakpoints near the amplified region may predispose to gene amplification in this locus (Zody et al., 2006).

In light of the overall amount of genes mapped into the 17q23 area, a large number of the genes within this amplicon may be regarded as putative candidate genes whose activation by amplification could play a role in breast cancer development and progression. Therefore, several studies have tried to identify these possible target genes at 17q23. For instance, *RPS6KB1* was one of the first genes to be identified as a potential oncogene in this region (Couch et al., 1999; Bärlund et al., 2000a). Subsequently numerous other target genes such as *APPBP2 (PAT1), RAD51C, TBX2, TRIM37 (MUL), THRAP1 (TRAP240), PPM1D*, and *BRIP1* have also been suggested (Bärlund et al., 2000b; Wu et al., 2000; Erson et al., 2001; Monni et al., 2001; Wu et al., 2001; Bulavin et al., 2002b; Li et al., 2002,). However, only a few of these genes (*RPS6KB1*, *TBX2*, and *PPM1D*) have also been demonstrated to exhibit clear oncogenic properties in functional analyses (Jacobs et al., 2000; Bulavin et al., 2002b; Choi et al., 2002; Bulavin et al., 2004). For the remaining genes merely a correlation between increased copy number and evevated expression has been established. In addition to the candidate genes described above, a number of other genes in 17q23 region reside in the amplified region, thereby making this amplicon attractive in the search for additional cancer associated genes.

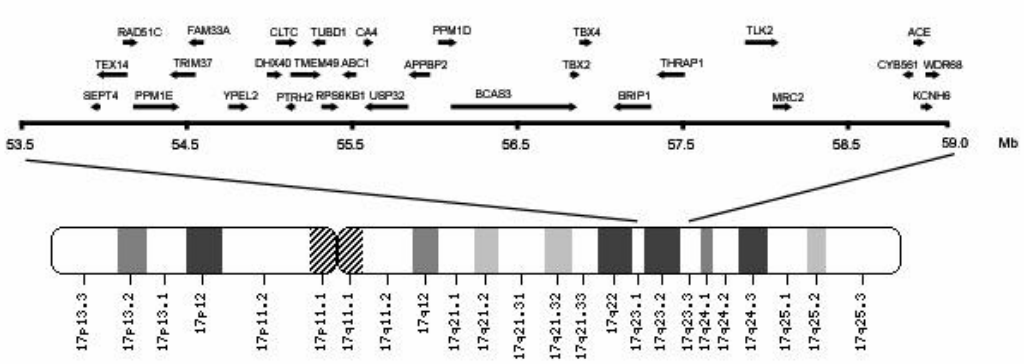

Figure 2*. Physical map of the 17q23 amplicon. The known genes mapping to the ~5Mb minimal region of amplification at 17q23 are represented using horizontal lines and their orientation is indicated with arrowheads. Chromosome 17 ideogram based on GeneMap99 (http://www.ncbi.nlm.nih.gov/projects/genome /genemap99/).*

3. *PPM1D*

PPM1D (also known as *Wip1)* is one of the genes lying within the 17q23 amplicon (Bulavin et al., 2002a; Li et al., 2002). It has frequently been observed

to be amplified and overexpressed in several human tumor types including breast cancers, neuroblastomas, ovarian clear cell adenocarcinomas, and medulloblastomas (Bulavin et al., 2002a; Li et al., 2002; Hirasawa et al., 2003; Saito-Ohara et al., 2003a; Mendrzyk et al., 2005). In breast cancer, *PPM1D* amplification has been reported to occur in 11-16% of primary tumors and amplification has also been shown to lead to elevated *PPM1D* mRNA expression (Bulavin et al., 2002a; Li et al., 2002). Moreover, *PPM1D* amplification and overexpression have been associated with poor clinical outcome (Hirasawa et al., 2003; Saito-Ohara et al., 2003a).

The *PPM1D* gene encodes a serine-threonine protein phosphatase and displays typical characteristics of evolutionary conserved type 2C protein phosphatases (PP2Cs) including magnesium dependence and relative insensitivity to okadaic acid (Fiscella et al., 1997). The PP2C-family members have previously been implicated in stress protection, sexual differentiation, and cell cycle regulation (Schweighofer et al., 2004). *PPM1D* itself was initially identified as a gene whose expression was rapidly and transiently induced by p53 tumor suppressor protein in response to ionizing radiation (Fiscella et al., 1997). Further experiments showed that it was transcriptionally upregulated in a p53-dependent manner also following other cellular stresses, such as ultraviolet radiation and anisomycin, H_2O_2, and methyl methane sulfonate treatments (Takekawa et al., 2000). This increase in *PPM1D* expression was found to correlate with the presence of wild-type p53 and no accumulation of *PPM1D* mRNA was seen in cells with disrupted *TP53* (Fiscella et al., 1997; Takekawa et al., 2000). However, Takekawa and co-workers also showed that in some cases, depending on the stress stimuli, transcription of *PPM1D* could also be regulated by p53-independent mechanisms (Takekawa et al., 2000).

3.1. PPM1D as an oncogene

Comparison of human *PPM1D* sequence with the corresponding mouse sequence revealed that this gene is evolutionarily highly conserved and also shares several regions of sequence similarity with PP2Cs from various other species (Choi et al., 2000). Thorough analysis of *Ppm1d* expression patterns in mice also specified that it is ubiquitously expressed in normal adult tissues, in most developmental stages, and throughout the whole embryo (Choi et al., 2000). Based on these results, mice were considered suitable model animals for further studies on *PPM1D* function.

The first evidence proposing that *PPM1D* might have oncogenic properties was derived from *in vitro* studies using primary mouse embryo fibroblasts (MEFs). These studies indicated that *Ppm1d* is able to complement several other oncogenes, such as *Ras*, *Myc*, and *Neu* for cellular transformation of MEFs (Bulavin et al., 2002a; Li et al., 2002). Overexpression of *Ppm1d* was also found to attenuate apoptosis induced by serum starvation (Li et al., 2002). These results were verified a few years later by two separate groups. Their data showed that suppression of *PPM1D* expression inhibited growth and induced apoptosis in

neuroblastoma cell lines and also reduced the proliferation rate of breast cancer cell lines (Saito-Ohara et al., 2003a; Belova et al., 2005).

After that, *Ppm1d*-deficient mice strain was generated to determine the normal biological function of Ppm1d in mammalian organisms (Choi et al., 2002). Studies showed that *Ppm1d*-deficient mice are viable but exhibit a variety of interesting phenotypes, including variable male runting, male reproductive organ athropy, reduced male fertility, reduced male longevity, and reduced male and female immune function (Choi et al., 2002; Nannenga et al., 2006; Schito et al., 2006). *Ppm1d*-deficient mice and cells derived from them have also been successfully used to examine and define the role and functional connections of *PPM1D* in cancer pathogenesis. First of all, *in vitro* studies have indicated that *Ppm1d*-null MEFs exhibit reduced proliferative capacity and that they suffer from premature onset of senescence (Choi et al., 2002). In addition, the *Ppm1d*-null MEFs were found to become completely resistant to transformation by *Ras*, *ErbB2*, and *Myc* oncogenes (Bulavin et al., 2004; reviewed by Harrison et al., 2004) and this resistance was correlated with increased p53 tumor suppressor protein levels (Bulavin et al., 2004; Nannenga et al., 2006).

The next question after the encouraging findings from the *in vitro* data was whether the ablation of *Ppm1d* would also inhibit oncogenesis *in vivo*. To study this question, mice deficient for *Ppm1d* were crossed with three different strains of mice, each engineered to overexpress a different oncogene in the epithelium of the mammary gland (Bulavin et al., 2004). These *in vivo* studies indicated that *Ppm1d*-null mice bearing mouse mammary tumor virus (MMTV) promoter-driven *ErbB2* or *Ras* oncogenes were indeed resistant to oncogene-induced mammary carcinogenesis. Conversely, the mammary gland tumorigenesis in mice expressing the *Wnt1* oncogene was not affected by the absence of *Ppm1d*.

Together these findings imply that *PPM1D* has oncogenic properties and also plays an important role in the regulation of cell growth and in the function of a number of adult organ systems. Nevertheless, *PPM1D* is considered to be a weak oncogene that can promote tumorigenesis only in conjunction with other oncogenes (Bulavin et al., 2002a; Li et al., 2002; Bulavin et al., 2004).

3.1.1. p38 MAPK-p53 pathway

Cells are constantly exposed to various environmental and endogenous mutagenic insults and stresses. They have consequently evolved a sophisticated array of damage sensors and repair systems. DNA damage, for example, activates checkpoint signaling pathways that may either arrest cell cycle to provide time for DNA repair, activate DNA repair processes or induce apoptosis (reviewed by Zhou et al., 2000). These mechanisms allow cells to maintain their genomic integrity.

Activation of the stress responsive p38 MAPK (mitogen-activated protein kinase) cascade is one prominent event in the early responses induced by DNA damage (reviewed by Kyriakis et al., 1996; Couch et al., 1999; reviewed by Kyriakis et al., 2001). The p38 MAPK is known to be activated by

environmental stresses such as ionizing and UV radiation, DNA damaging agents, osmotic shock, and oxidant stresses (reviewed by Kyriakis et al., 1996; reviewed by Waskiewicz et al., 1995; Ono et al., 2000; Bulavin et al., 2001; reviewed by Kyriakis et al., 2001).

Another important response to DNA damage is the activation of tumor suppressor p53 (reviewed by Ko et al., 1996; reviewed by Lacroix et al., 2006). The *TP53* gene has a crucial role in preserving genomic integrity by arresting cell cycle progression or inducing apoptosis after DNA damage and certain other cellular stresses (reviewed by Lacroix et al., 2006; reviewed by Levine, 1997). After DNA damage, p53 is stabilized and accumulates in the nucleus where it induces the transcription of several important target genes (reviewed by (Gottlieb et al., 1996). The function of p53 is regulated by post-transcriptional modifications such as phosphorylation and acetylation (Gu et al., 1997; reviewed by Giaccia et al., 1998). A number of protein kinases have been reported to phosphorylate p53 at several different serine and threonine residues. These include ATM, ATR, DNA-PK, cyclin dependent kinases, cdk-activating kinase, CHK1/CHK2 and also p38 MAPK (Bulavin et al., 1999; Huang et al., 1999; Keller et al., 1999; reviewed by Meek, 1998; reviewed by Prives et al., 1999; reviewed by Caspari, 2000).

Several reports have linked p38 MAPK and p53 pathways together by showing that p38 MAPK directly phosphorylates p53 on Ser33 and Ser46, which is crucial for the phosphorylation of other functionally important residues at the N-terminal part of p53 protein (Bulavin et al., 1999; Huang et al., 1999; Keller et al., 1999; Sanchez-Prieto et al., 2000). These results also imply that p38 MAPK has an important role in activating p53 in response to UV radiation and certain anti-cancer drugs. Furthermore, p38 MAPK and p53 cascades have also been shown to co-operate to induce apoptosis in cells exposed to UV radiation (Bulavin et al., 1999).

Since both p38 MAPK and p53 are activated by phosphorylation, protein phosphatases are likely to play a key role in the regulation of p38-p53 signaling. It is known that p38 MAPK is activated *in vitro* by three different protein kinases: MKK3, MKK4, and MKK6 (Brancho et al., 2003) whereas type 2C protein phosphatase alpha (PP2Cα) downregulates this stress-responsive MAPK (Takekawa et al., 1998; Fjeld et al., 1999). Recent studies have shown that PPM1D is also one of the PP2C family members regulating p38 MAPK pathway by specifically dephosphorylating its phosphothreonine 180 residue and thus inactivating p38 MAPK (Figure 3) (Takekawa et al., 2000). Transgenic mice have been generated to further address the significance of PPM1D in the p38 MAPK signaling under *in vivo* conditions (Demidov et al., 2007). In these animals, the expression of Ppm1d was targeted to the mammary gland epithelium using the MMTV-promoter. Data from this study indicated that these mice were prone to cancer when intercrossed with transgenic mice expressing *ErbB2*. However, this phenotype was fully eliminated by activating the Mkk6/p38 MAPK cascade, clearly indicating that Ppm1d operates within the Mkk6/p38 MAPK signaling pathway (Demidov et al., 2007).

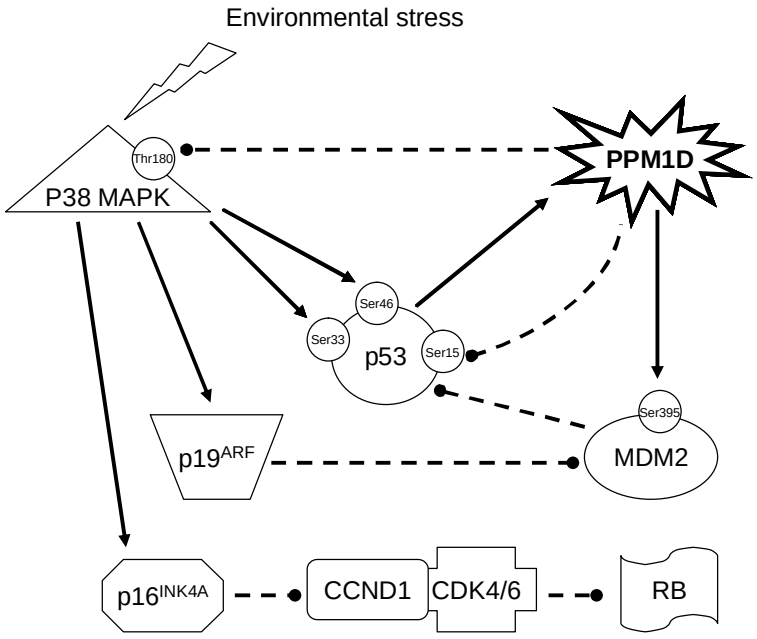

Figure 3. Role of PPM1D in p38 MAPK-p53 signaling and in other cell cycle and tumor suppressor pathways. The continuous line indicates activation and dash line inactivation.

Through the attenuation of p38 MAPK activity, PPM1D also negatively regulates p53 after cellular stresses (Takekawa et al., 2000). As mentioned above, the dephosphorylation of p38 MAPK by PPM1D leads to loss of kinase activity and thus p38 MAPK is not able to further phosphorylate p53 to trigger cell cycle arrest or apoptosis e.g. in response to DNA damage (Figure 3) (Takekawa et al., 2000). Since *PPM1D* itself is a p53-regulated gene (Fiscella et al., 1997), Takekawa and co-workers suggested that it mediates a negative feedback regulation on p38 MAPK-p53 signaling pathway leading to restoration of baseline p53 activity (Figure 3) (Takekawa et al., 2000). The great majority of primary breast tumors overexpressing *PPM1D* have a structurally intact *TP53* and thus PPM1D has been suggested to contribute to the functional inactivation of p53 in these tumors (Bulavin et al., 2002a; Li et al., 2002). In addition, Lu and co-workers have presented evidence that PPM1D is also capable of directly inactivating p53 by dephosphorylating its Ser15 residue (Figure 3) (Lu et al., 2005). The phosphorylation of this residue is known to be critical for the activation of p53 apoptotic function (Sluss et al., 2004). Recently, Ppm1d was also found to act as a gatekeeper in Mdm2-p53 regulatory loop by dephosphorylating Mdm2 (Lu et al., 2007). These findings provide an additional molecular mechanism for PPM1D oncogenicity by demonstrating that this dephosphorylation stabilizes MDM2, increases its affinity to p53, and thus facilitates ubiquitination and degradation of p53 protein. Taken together, overexpression of *PPM1D* is likely to contribute both directly and indirectly to the inactivation of p53 in cancer.

In addition to the role in the regulation of p53, p38 MAPK has also been implicated in the regulation of cell cycle progression by several other mechanisms. For instance, p38 MAPK inhibits the expression of D-type cyclins (Lavoie et al., 1996; Casanovas et al., 2000), phosphorylates and induces the degradation of the Ccd25A phosphatase (Goloudina et al., 2003), and inhibits the Cdc25B phosphatase (Bulavin et al., 2001). The cooperation of these p38 MAPK targets leads to the activation of cell cycle checkpoints. Therefore alterations in p38 MAPK regulation and function are likely to disturb cell cycle progression and lead to increased tumorigenesis. In light of these findings, PPM1D can be regarded as a negative regulator of both p53 and other crucial factors in cell cycle control.

3.1.2. Other cell cycle pathways

Recent studies have shown that *PPM1D* is also involved in other key cellular signaling networks. As mentioned earlier, MEFs from *Ppm1d*-null mice were resistant to transformation by *Ras*, *ErbB2*, and *Myc* oncogenes (Bulavin et al., 2004). Bulavin and co-workers further showed that not only elevated activity of p53 but also enhanced expression of two additional tumor suppressor genes, $p16^{Ink4a}$ and $p19^{Arf}$, contributed to this transformation resistant phenotype (Bulavin et al., 2004). The $p16^{INK4A}$ is a known inhibitor of the cyclin D-CDK4-CDK6 complex, which in turn is an upstream regulator of the retinoblastoma (RB) tumor suppressor protein (Figure 3) (Haller et al., 2005; reviewed by Macaluso et al., 2005). In turn, $p19^{ARF}$ is a known upstream regulator of p53 (Figure 2) (reviewed by Sherr, 1998). Both of these genes are encoded by the *CDKN2A* tumor suppressor locus (Quelle et al., 1995).

To further determine which of these pathways is more important in protecting *Ppm1d*-null MEFs from tumorigenesis, Bulavin and co-workers generated MEFs deficient in both *Ppm1d* and *Tp53*. As these *Ppm1d* and *Tp53* double-null MEFs were still resistant to transformation, it was suggested that the enhanced expression of $p16^{Ink4a}$ and $p19^{Arf}$ is indeed independent of p53 and an important factor in the transformation resistance phenomenon (Bulavin et al., 2004). In contrast, MEFs lacking *Ppm1d*, $p19^{Arf}$, and $p16^{Ink4a}$ were fully oncogenic when *Ras* or *Myc* oncogenes were introduced, while MEFs deficient for only *Ppm1d* and $p16^{Ink4a}$ had an intermediate phenotype. To verify these findings, the authors treated tumor resistant *Ppm1d*-null mice expressing *ErbB2* or *Ras* with p38 MAPK inhibitors and found that expression of $p16^{Ink4a}$ was repressed and these mice did indeed develop breast tumors (Bulavin et al., 2004). From these data, they draw the conclusion that the lack of *PPM1D* suppresses the transformation through activation of p38 MAPK followed by activation of p53 and $p16^{Ink4a}$ (Figure 3) and consequently prevents breast cancer induction. The authors also suggested that at least some of the effects of Ppm1d on p53 are probably mediated by its effect on $p19^{Arf}$ (Figure 3). However, few tumors did form in *Ppm1d*-deficient mice expressing *ErbB2* but they showed lower growth rates and higher apoptosis than tumors in wild-type mice. Interestingly, these tumors had

spontaneously suppressed $p16^{Ink4a}$ expression levels, indicating that still some other tumor resistance mechanism than elimination of $p16^{Ink4a}$were still functioning in Ppm1d-deficient mice (Bulavin et al., 2004; reviewed by Harrison et al., 2004). Bulavin and co-workers also showed that *Ppm1d*-deficiency did not block the tumor formation induced by *Wnt*. This indicates that not all breast tumors are dependent on Ppm1d (Bulavin et al., 2004). Nevertheless, the majority of human breast tumors might respond to *PPM1D* inhibition, because the inactivation of $p16^{INK4A}$ or $p19^{ARF}$ is relatively uncommon in this tumor type (reviewed by Harrison et al., 2004).

A substantial fraction of breast cancers are known to have increased expression of cyclin D1 (CCND1), one of the important regulators of G1 to S-phase transition and an upstream regulator of the retinoblastoma tumor suppressor (van Diest et al., 1997). CCND1 is required for the induction of breast cancer by certain oncogenes and its action is known to be inhibited by $p16^{INK4A}$ and p38 MAPK (Lavoie et al., 1996; Yu et al., 2001). Upon *PPM1D* overexpression, $p16^{INK4A}$ and p38 MAPK are likely to be downregulated leading to upregulation of CCND1 (Figure 3). Therefore it has been suggested that such tumors with activated *PPM1D* may benefit from the inhibition its expression (reviewed by (Bernards, 2004).

3.2. PPM1D in DNA damage repair

DNA damage repair is an essential part of the maintenance of genomic integrity. Two protein kinases, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), are also known to play important roles in DNA damage response pathways. ATM kinase is involved in transducing signals implicated in the regulation of cell cycle checkpoints, apoptosis, and DNA repair (reviewed by Kastan et al., 2004). The ATM-dependent pathway is activated soon after DNA damage and serves as a barrier that helps to delay or prevent cancer (Bartkova et al., 2005). This allows further activation of ATR following, for example, DNA double-strand breaks (Jazayeri et al., 2006). Regulation of the ATM/ATR dependent pathway is complex and involves several positive and negative regulators (reviewed by Kastan et al., 2004). Shreeram and co-workers have shown that inhibition of *PPM1D* results in ATM upregulation and suppression of tumorigenesis in B cells (Shreeram et al., 2006a; Shreeram et al., 2006b). In turn, they found that overexpression of PPM1D reduces activation of ATM-dependent signaling cascades after DNA damage. In light of these findings, the authors suggest that PPM1D dephosphorylates ATM at a critical site and is therefore implicated as a significant regulator at the ATM-dependent signaling pathway and tumor surveillance network

ATM/ATR kinases have been shown to directly phosphorylate CHK1 and CHK2 (reviewed by Shiloh, 2003). CHK1 is activated primarily by ATR in response to replicative stress whereas CHK2 is activated principally by ATM in response to DNA double-strand breaks (Oliva-Trastoy et al., 2007). Phosphorylated CHK1 and CHK2 are important effectors of the intra-S phase

and G_2/M checkpoints as well as activators of p53 and G_1 cell cycle arrest checkpoints (reviewed by Bartek et al., 2003; reviewed by Shiloh, 2003; reviewed by Bakkenist et al., 2004; reviewed by Sancar et al., 2004). The highly conserved phosphatase domain of PPM1D has been demonstrated to directly interact with CHK1 and dephosphorylate its ATM target site (Figure 4) (Lu et al., 2005). The authors hypothesize that PPM1D binding to CHK1 and dephosphorylation of its Ser345 may be a primary mechanism of the inhibition of this protein (Figure 4). Ser345 has been shown to be important for CHK1 activation in response to genotoxic stress and for nuclear retention after DNA damage (Lopez-Girona et al., 2001; Zhao et al., 2001; Jiang et al., 2003). Lu and co-workers further suggested that by dephosphorylating CHK1 PPM1D is able to return the cell to a homeostatic state following the completion of DNA repair (Lu et al., 2005).

CHK2 activation also requires its phosphorylation at Thr68 (Matsuoka et al., 2000; Melchionna et al., 2000). CHK2 further phosphorylates multiple substrates including BRCA1, p53, and CDC25A and thus indirectly promotes DNA repair, inhibits cell cycle progression in G_1, S, and G_2 phases, and stimulates apoptosis (reviewed by Bartek et al., 2003). Recently, PPM1D has also been reported to bind CHK2, dephosphorylate its Thr68 residue, and consequently oppose CHK2 activation by ATM (Figure 4) (Fujimoto et al., 2006; Fuku et al., 2007; Oliva-Trastoy et al., 2007;). Based on these studies, overexpression of *PPM1D* is expected to suppress the action of CHK2 in G2/M DNA damage checkpoint while inhibition of *PPM1D* is likely to increase the responsiveness of cells to DNA damage (Fujimoto et al., 2006; Fuku, 2007; Oliva-Trastoy et al., 2007).

Base excision repair (BER) pathway protects cells against DNA damage caused by endogenous cellular processes (reviewed by Hoeijmakers, 2001; Mitra et al., 2002). This pathway consists of several proteins including DNA glycosylases that recognize a specific type of damaged base and cleave the base from the deoxyribose (reviewed by Hang et al., 2003). One of these glycosylases is UNG2, which removes uracil residues from nuclear DNA (Kavli et al., 2002). Lu and co-workers found that PPM1D actually suppresses BER through dephosphorylation of this key BER effector, UNG2, at Thr6 residue (Lu et al., 2004a; Lu et al., 2004b). They also reported that point mutations that remove PPM1D phosphatase activity abrogate this BER suppression. In light of these results, the authors suggest that BER activity is controlled by PPM1D and that PPM1D has an important function in returning this DNA repair system to a deactivated homeostatic state (Lu et al., 2004a; Lu et al., 2004b).

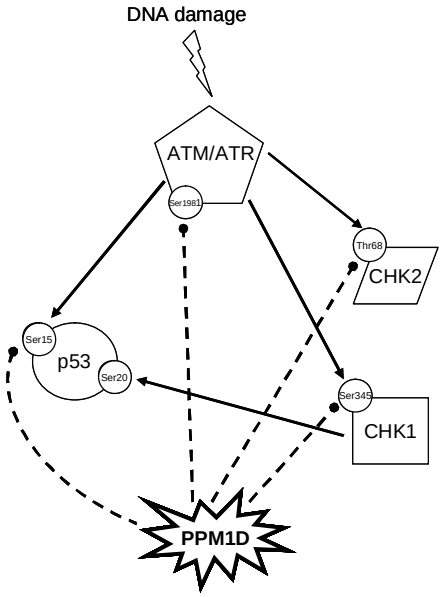

Figure 4. *Role of PPM1D in DNA damage repair pathways. The continuous line indicates activation and dash line inactivation.*

3.3. PPM1D and steroid receptor action

Steroid hormone receptors, ER (estrogen receptor) and PR (progesterone receptor), play a critical role in the development of breast cancer (reviewed by Cordera et al., 2006). Approximately 70% to 80% of all breast tumors express ER protein and these tumors tend to grow more slowly, are better differentiated, and are associated with a slightly better overall prognosis than ER-negative tumors (Arpino et al., 2005). Among ER-positive patients, the expression of PR has also proven to have clinical relevance. A lack of either ER or PR is associated with poor prognosis and a decrease in disease-free survival (Arpino et al., 2005). *PPM1D* is overexpressed and amplified in human breast cancers, which are hormone related tumors and thus questions have been raised about a possible connection between PPM1D and steroid receptors.

The action of steroid receptors is known to be controlled by the level of hormone as well as by the levels and activity of various coactivators (reviewed by (McKenna et al., 2002). Both steroid hormones and their coactivators are phosphoproteins, and thus their activity is suggested to be regulated by several candidate kinases (Zhang et al., 1997; Chen et al., 2000; Lange et al., 2000). However, less is known about the role of phosphatases in steroid receptor action. Proia and co-workers found that PPM1D stimulates the action of several nuclear receptors including PR (Proia et al., 2006). Nevertheless, their findings indicate that PR is not a direct target of PPM1D, but the stimulatory effect is achieved when PPM1D enhances the intrinsic activity of p160 coactivators and thus promotes their interaction with PR. In addition, PPM1D was also found to stimulate PR action partly through inhibition of p38 MAPK, one of the inhibitors of PR (Proia et al., 2006). The authors speculate that, although the capacity of

PPM1D to inhibit p53 function is likely a major factor in its action in cancer cells, PPM1D may also promote breast cancer also by enhancing steroid hormone receptor action.

3.4. The complex role of PPM1D in cancer

As the data reviewed above show, *PPM1D* has now been demonstrated to have a much wider role in cellular transformation and tumor pathogenesis than previously recognized. PPM1D is proposed to directly and indirectly regulate several essential cellular signaling pathways (Figures 3 and 4). Whether PPM1D has additional specific targets or is involved in other cellular processes remains to be discovered. Nevertheless, current knowledge clearly indicates that inactivation of *PPM1D* has a powerful effect in reducing cancer formation, although it is uncertain whether inhibition of PPM1D function would have any therapeutic effects on human tumors. Thus the real clinical value of PPM1D is currently open but in the future, PPM1D could perhaps be used as a diagnostic marker or exploited as a target for development of new therapy.

Aims of the study

1) To identify putative target genes at the 17q23 amplicon in breast cancer

2) To study the amplification and overexpression of *PPM1D* in breast cancer cell lines and primary breast tumors

3) To evaluate the functional effects of *PPM1D* overexpression in *PPM1D* amplified breast cancer cell lines

4) To identify genes and signaling pathways which are influenced by *PPM1D* overexpression in breast cancer

Materials and methods

1. Cell lines (I, II, III, IV)

A total of 21 established breast cancer cell lines was used in this study. Breast cancer cell lines BT-474, CAMA-1, DU4475, HCC38, HCC1419, HCC1954, MCF7, MDA-134, MDA-157, MDA-361, MDA-415, MDA-436, MDA-453, SK-BR-3, T-47D, UACC-732, UACC-812, UACC-893, UACC-3133, ZR-75-1, and ZR-75-30 were obtained from American Type Culture Collection (Manassas, VA). Normal human mammary epithelial cells (HMEC) were obtained from Clonetics (Walkersville, MD). Pancreatic cancer cell lines PANC-1 and HUPT3 and also prostate cancer cell lines DU145 and PC-3 were used as controls in RT-PCR analyses. HUPT3 cell line was obtained from the German Collection of Micro-organisms and Cell Cultures (Braunschweig, Germany) and DU145 and PC-3 from American Type Culture Collection (Manassas, VA). All cells were grown under recommended culture conditions.

2. Clinical tumor samples (I, II)

A tissue microarray containing 146 formalin-fixed, paraffin-embedded primary breast cancers from patients diagnosed between 2001-2003 was used in Studies I and II. The tumor collection was obtained from the Department of Pathology, Tampere University Hospital (Tampere, Finland). All samples were anonymous, archival tissue specimens with associated clinicopathological information. For expression analyses, 33 freshly frozen primary breast tumor specimens, a subset of the above-mentioned material, were obtained from the Department of Pathology, Tampere University Hospital. Tumors were selected according to their *PPM1D* amplification status to include both amplified and non-amplified cases. Study I included 26 tumors of which 8 had *PPM1D* amplification and 18 no *PPM1D* amplification whereas the 26 tumors in Study II consisted of 11 amplified and 15 non-amplified cases. Nineteen of these tumors were used in both studies. The use of these tissue specimens was approved by the Ethics Committee of the Pirkanmaa Hospital District and by the National Authority for Medicolegal Affairs of Finland.

3. Fluorescence in situ hybridization (FISH) (I,II,III)

3.1. FISH on tumor tissue microarray (TMA) (I,II)

Two *PPM1D*-specific bacterial artificial chromosome (BAC) clones (RP11-634F5 and RP11-1081E4) were identified by performing sequence similarity searches against the nonredundant and high throughput genomic sequence databases using the blastn program and the specificity of the probe was verified by PCR with gene specific primers. Two additional probe sets were used in Study I (RP11-579A4 and RP11-579O24; RP11-269G24 and CTD-2501B8). FISH to tissue microarray was performed as described (Andersen et al., 2001) using SpectrumOrange-dUTP labeled BAC probes and SpectrumGreen labeled chromosome 17 centromere probe as a reference. The formalin-fixed tissue microarray sections on microscope slides were deparaffinized and treated according to the Paraffin Pretreatment Reagent Kit protocol (Vysis). Briefly, the slides were fixed in 4% paraformaldehyde for 10 min, denatured at 94°C for 5 min in Tth-buffer (10mM Tris-HCl, pH 8.9, 0.1 M KCl, 1.5 mM MgCl, 50 µg/ml BSA, 0.05% Tween 20® (v/v)), treated with Proteinase K (10 µg/ml in PBS) at 37°C for 10 min, dehydrated, and air-dried. After an overnight hybridization, the slides were washed in 0.4xSSC/0.3%NP-40 at 72°C for 1 min, at RT 2XSSC for 2 min, and then counterstained with 4',6-diamidino-2-phenylindole in antifade solution. Hybridization signals were analyzed using an Olympus BX50 epifluorescence microscope (Tokyo, Japan) using an X60 objective (NA 1.4). The entire tissue core sample was screened, with a minimum of 50 cells analyzed for each case. Specimens containing a 3-fold or greater increase in the number of *PPM1D* probe signals, as compared with the chromosome 17 centromere signals, were considered to be amplified. Additionally, specimens containing a five-fold or greater increase in the number of *PPM1D* signals or tight clusters of signals were considered to be highly amplified.

3.2. Interphase FISH on breast cancer cell lines (III)

For Study III, the clone contig including RP11-634F5 and RP11-1081E4 BAC clones was used as a probe. Probes were labeled with SpectrumOrange-dUTP (Vysis, Downers Grove, IL) by random priming and SpectrumGreen labeled chromosome 17 centromeric probe (Vysis) was used as a reference. Metaphase and interphase cell preparations from the breast cancer cell lines and normal human lymphocytes were prepared as described (Kallioniemi et al., 1992). Control hybridizations to normal human metaphase chromosomes were done to confirm that the two clone contig recognized a single copy target at 17q23. Dual-color interphase FISH to breast cancer cell lines was done as described (Bärlund et al., 2000b). The hybridization signals were analyzed using an Olympus BX50 epifluorescence microscope (Tokyo, Japan) equipped with a ×60 objective (NA 1.4). Approximately 50 non-overlapping nuclei were scored to determine the

mean number of hybridization signals for both *PPM1D* and reference probes. Amplification was defined as a 3-fold or higher increase in the number of *PPM1D* probe signals, compared to the chromosome 17 centromere signals.

4. Analysis of *TP53* mutations (II)

Exons 4 to 10 of *TP53* were analyzed by direct sequencing from total genomic tumor DNA. Primer sequences are presented in Table 2. The PCR reactions for each exon contained 1 µl genomic DNA, 5 µl of 10x PCR Gold Buffer, 3 µl of 25µM $MgCl_2$, 1 µl of dNTPs, 0.9 µl each of 10 µM sense and antisense primers, 0.3 µl Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 40 µl of H_2O. The PCR program began with initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55º/58ºC for 1 min and extension at 72°C for 1 min, with final elongation at 72°C for 10 min. PCR products were purified using Qiagen QIAquick PCR Purification Kit according to the manufacturer's protocol (Qiagen Inc.). The cycle sequencing reactions were carried out using Big Dye Terminator chemistry (Applied Biosystems) according to the manufacturer's protocol and the sequencing was carried out using ABI 3100 sequencer (Applied Biosystems).

Table 2. Primer sequences used in *p53* sequencing.

p53 exon	Primer sequence (5'-3')
Exon 4	CTGAGGACCTGGTCCTCTGA
	CCGGTGTAGGAGCTGCTGGT
	AATGCCAGAGGCTGCTCCCC
	ACGGCCAGGCATTGAAGTCTC
Exon 5	TTCAACTCTGTCTCCTTCCT
	GCAATCAGTGAGGAATCAGA
Exon 6	TGGTTGCCCAGGGTCCCCAG
	CGGAGGGCCACTGACAACCA
Exon 7	AGGCGCACTGGCCTCATCTT
	AGGGGTCAGCGGCAAGCAGA
Exon 8	TTGGGAGTAGATGGAGCCT
	AGGCATAACTGCACCCTTGG
Exon 9	TCAGATTCACTTTTATCACC
	AGTGTTAGACTGGAAACTTT
Exon 10	GTACTGTGAATATACTTACTTCTC
	CCTATGGCTTTCCAACCTAGGAA

5. Immunohistochemistry (II)

Tissue microarray sections were stained using an automated TechMate 500 Plus immunostaining system (DakoCytomation, Glostrup, Denmark). First, the slides were de-waxed, then washed thoroughly with xylene and alcohol, and finally stained with antibodies against CCND1 and p16 using avidin-biotin enhanced immunoperoxidase technique (DAKO ChemMate EnVision Detection Kit, DakoCytomation). The following primary antibodies were used: CCND1 at 1:10 dilution (NovoCastra Laboratories, Newcastle, United Kingdom) and $p16^{INK4A}$ at 1:60 dilution (DakoCytomation). The results were evaluated by a pathologist using a light microscope equipped with 20X objective. Tumors were categorized either positive or negative on the basis of the presence or absence of distinct nuclear immunoreactivity.

6. Northern hybridization (III)

PPM1D expression was studied using northern hybridization. Total RNA from breast cancer cell lines was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA). Fifteen µg of total RNA was loaded on 1% formaldehyde gel, electroforesed, and transferred onto a Nytran SuperCharge membrane (Schleicher & Schuell BioScience, Dassel, Germany) by TurboBlotter (Schleicher & Schuell BioScience). A sequence–verified insert from *PPM1D* specific cDNA clone was labeled with ^{32}P-dCTP using random priming (*redi*prime II, Amersham Biosciences, Buckinghamshire, UK) and purified with Nuctrap Probe Purification columns by Push Column Beta Shield Device (Stratagene, Cedar Creek, TX). The membrane was prehybridized for one hour at 42°C in NorthernMax Prehybridization/Hybridization Buffer (Ambion, Inc., Austin, TX) together with 10 µg/ml denatured Herring Sperm DNA (Sigma Chemical Co., St. Louis, MO), and then hybridized with the labeled probe overnight in the prehybridization solution at 42°C. The membrane was washed twice with 2X SSC/0.1% SDS at room temperature and then twice in 0.1X SSC/0.1% SDS at 55°C. The hybridized probe was detected by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). After removal of the bound probe, the membrane was rehybridized with a glyceraldehyde-3-phosphate dehydrogenase probe (Clontech, Palo Alto, CA) to confirm equal loading of the samples.

7. RT-PCR (I)

For study I, regular RT-PCR was performed in BT-474 and MCF7 breast cancer cell lines as an initial screen of expression levels of genes located at the 17q23

amplicon. Gene specific primers for 29 genes from the 17q23 region were obtained from TIB MolBiol (Berlin, Germany). The PCR reactions contained 1 × PCR Gold buffer (Applied Biosystems, Foster city, CA, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs each, 0.2–0.4 mM gene specific primers, 1.5–2.5 units of Amplitaq Gold DNA polymerase (Applied Biosystems), and 1 µl cDNA template (BT-474 or MCF7) adjusted to 50 µl with sterile H_2O. The PCR program began with initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 60 s and elongation at 72°C for 60 s, with final elongation at 72°C for 10 min. The PCR products were run on a 1.5% agarose gel.

8. Quantitative real-time RT-PCR (qRT-PCR) (I,II,III,IV)

Gene expression analysis in breast cancer cell lines and in freshly frozen tumor tissue samples, and also verification of *PPM1D* silencing efficiency, and validation of microarray experiments were performed using qRT-PCR. Total RNA was isolated using RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). In order to avoid genomic contamination, tumor samples were additionally treated with RNase Free Dnase I (Epicentre, Madicon, WI) at 37°C for 30 min, followed by inactivation of the enzyme at 65°C from 15 min. First-strand cDNA synthesis was performed using SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA). DNA Hybridization Probe Sets for *PPM1D* (Studies I, II, III, IV), for other 23 genes of interest at 17q23 chromosomal region (Study I), for 6 differentially expressed genes (Study IV), and for the housekeeping gene TATA-box binding protein (*TBP*) were obtained from TIB MolBiol (Berlin, Germany).The primer and probe sequences are presented in Table 3. The PCR reactions were performed in the LightCycler apparatus using the LC FastStart DNA Hybridization Probes Kit according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). After 10 minutes of initial denaturation at 95°C, the cycling conditions (55 cycles) were as follows: denaturation at 95°C for 10 seconds, annealing at 55-58°C for 10-20 seconds, and elongation at 72°C for 6-12 seconds depending on the gene of interest. The fluorescence was measured in every cycle at the end of the annealing step. Quantitative analysis was performed using the LightCycler software according to manufacturer's instructions. First the proportional background adjustment was performed and then the fit point method was used to determine the crossing-point value, representing the cycle number where the fluorescence level for each sample was the same. A standard curve was generated by measuring the crossing point of serial dilutions of the same cell line cDNA and plotting them against the logarithmic value of concentrations. The concentrations of unknown samples were then calculated by setting their crossing points to the standard curve. The expression levels of the genes studies were normalized against the housekeeping gene *TBP*.

Table 3. Primer and probe sequences used in qRT-PCR.

Gene	Primer sequence (5'-3')	Hybridization probe sequences (5'-3')
ABC1	CCAGTCTTCTTGGTGGTGATGT	GGAGAGTTGGGTGTTCTGAATTCTGCA-FL
	AGTTGCAGCTTTCACTAAGCGA	GGCTCTGGGTAAAGGACCGTTCAGC
ACE	AGCGACGAGCATGACATCAAC	GTGATGCTTCCATCAAATACCCTCCAGC-FL
	GCCCTGGTACTTCAGCCTGAG	640-CCACTGATCGACGAGGTAGCTGAAGGGG
APPBP2	CAATTTATCAGGCAGCCCTT	AAGAGCTATTGGTATCATTACCCACATCCT-FL
	TGAAGCAGCCTCTGTTCAGTT	640-CCTGAAGATCATCTTCTTTTGGCTTCTTC
BCAS3	GACTCTGGTTAGAACCCCTCA	GCAGTACAGTATTATCAGTTCCTGCTTGCT-FL
	GGTCAGAAGCTAAACTGTCGTAAG	640-GCCTGGTTCCCCCTGGAAGT
BRIP1	CCTTTTCCAAATGTGAAAGATC	TTGAATTTCATACCACTGACGGCC-FL
	CCCCAATCATTTCTGTGTCTAAT	640-GGTAGAAGACCTCTCAATTTTGAATGGTGG
CLTC	ATGATGAATCATCCAACTGATGC	TTCAAGCCTCTGTTGTTAAATGATTTGCTG-FL
	GTGGTAGCTGTTTAACCTTGCTGA	640-TGGTGCTGTCTCCACGGTTGGATC
CYB561	ATCGCCCTGGTTGGCTTG	GTCCTGTACTTTGTGCAGTGGCTGGT-FL
	TGCCCACGGAAAGGAGG	640-GGCTTCAGCTTCTTCCTGTTCCCCG
DHX40	AAAAATGTAGCTCGAAGATCTGTTG	TGAAGTGCTGAGGAAGGATGAATGTGAAC-FL
	ATTGGGCATACAATTCTTGCG	640-GGGCTTCCACGACCATCCATTGTG
FAM33A	AGCAAACAGACCTGGAGCTGT	TCCAAGTCCATTTCTTCATAAATCTGGCA-FL
	TCATTATCCAGTCATTGAAGCCA	640-GTGAAATTTGAATTGCTCTGCCGCAGT
HIST1H2B0	GCTCTTTCACTCTCCTCCG	ATCTACGTGTACAAGGTGCTGAAGC-FL
	CCCTGGAGGTGATGGTC	640-AGTCCACCCCGACACCGG
NR4A1	CTGACCCCTGAGTTCATCAAG	CTGGTAGAGGAAGGTGTCAAACTCTCCTGTGT-FL
	GTACACCTGGAAGTCCTCGAA	640-GCCGTCCATGAAGGTGCTGAAGCT
PLK1	CCCTCATCCAGAAGATGCTTCA	AGTTCTTTACTTCTGGCTATATCCCTGCCC-FL
	GGCTTGGAGGCATTGACAC	640-TCTCCCCATCACCTGCCTGACCA
PPM1D	AGGACTTGGTGGGAGTGTAA	GTGAGTCGAGGTCGTTTCCAAACT-FL
	CAAATTCACCACTGAAGAAATCATAG	640-CACGATTCACCCCAGACTTGTTCA
PPM1E	AGCAGCCAGGGAGAGCTTAAG	GGCCACATGTAGCATGTTGCCTCTGATG-FL
	CCAGACTACGCAACCTCCAA	640-AAGTCACCACTCCTGTGGTCCCACA
PTRH2	GAAAGGAGGTACTGTAGATGCCCT	GGCACATGCCACAAGCAACTCCAAC-FL
	CCACTTTCCCTTTTCCCAT	640-GCCAAGCCGAGTGTACTGGGATGAGC
RAB25	AATCAGGTGTGGGGAAGA	GTGACCTCAGCCAGGCCC-FL
	CATTGTTTTCAGCGAACATT	640-GGAAGTGCCCACTGAGGAGGC
RAD51C	TCCGAGCTTAGCAAAGAAGTT	GTGTACAGCACTGGAACTTCTTGAGCAGG-FL
	CACCCCCAAGAATATCATCTAGT	640-GCATACCCAGGGCTTCATAATCACCTTCTG
RPS6KB1	AACACCTGTCAGCCCAGTCAA	CTTGTTTCCATTGGGTATTCCACAGGTG-FL
	CCGTTTGGAGATCATGGGA	640-CTGAGGATTTGCTGTGCTGGCCGAA
STC1	CAGGGAAAAGCATTCGTCAA	TCCACTTTCCAAAGGATGATTGCTGA-FL
	AGGCTTCGGACAAGTCTGTTATAGTA	640-GTGCAGGAAGAGTGCTACAGCAAGCT
TBP	GGAGAGTTCTGGGATTGTAC	GTGCAATGGTCTTTAGGTCAAGT-FL
	TGCCAGTCTGGACTGTT	640-TACAACCAAGATTCACTGTGGATACA
TBX2	ACGGCTTCACCATCCTAAACTC	TCCGCACCTACGTGTTCCCGG-FL
	CGGGTTGTTGTCGATCTTCAG	640-GACCGACTTCATCGCCGTCACTGCC
TBX4	ACAAACAACCACCTGGACC	CGGCTCCACATCGTTAAGGCTG-FL
	GGGTGATCTTGTGATTCTGGTAG	640-TGAGAACAATGCTTTCGGCTCCAAAA

THRAP1	TTCTTGCATCTTGCACAGATC	CGACGACTCAGCAAACAGCTCC-FL
	ATTCTACACATGTCTTTGAGCCT	640-TCTTTCAATTCTCCATGACCAATCCTTCCT
TLK2	CAATTTAAAGATCATCCAACGC	TGAGAAAAAGGAGAATTACCACAAGCAT-FL
	CCAGTGAAAAGTAATCATACAGCTTA	640-CATGTAGGGAATACCGGATTCATAAAGAGC
TMEM49	GCATCAACAGTATGTGCAACG	CCTTCAGTGCCCTCTTCATCTGGACAAA-FL
	GCTGTACCGATACCCCACATG	640-CTGATCAGGATAGGGTGGTTCGGGA
TRIM37	GCCCCTGAAGAAGGAATGAGT	TCGTGAAACCCGCCCACACTGG-FL
	GGGCCTATTTGTTCACCATCA	640-ATGCTCTTCCTGCTCCTCATTCTCAGTGTC
TUBD1	AAATCCACTAGGAGACTTAATGGAGC	ATTCCTCACATGTCTGAGAATTCATTGGCA-FL
	GCAATGGAAGTGTTAAAATGCAG	640-ACACCACATTTACTTGGGCTGGCC
ULK2	GTCTTTTTGGTGATGGAGTAT	CAAGCGAAAGGGACTCTCAGTG-FL
	TTCTGCGATTGGCATAG	640-AGACACGATCAGAGTGTTTCTGCATCA
USP32	TGTGCAGAAAGATGGGAACT	AGGCATTTCCAATGAAAGCTCTGTC-FL
	GCTCCACACTCTCATGCTCA	640-TCCCCACAATCAATTTTACAGCCTCT
WDR68	TGATCGCCCATGACAAAG	ATCTAGAACACAGCACCATCATTTACGA-FL
	GGAACCCGGACATCTAGAATC	640-GACCCACAGCATCACCCACTGC
YPEL2	GCAGTCGCAGACATTTACTGTG	GACAATGGCTGGGACTGATTGGACAGC-FL
	CACTCTCTCACGCTTGGGAA	640-CTACCCAACCCAGTGTCCACGTGAAC

FL, fluorescein; 640, Red640

9. Gene silencing (III, IV)

Three breast cancer cell lines were used in the siRNA experiments in Studies III and IV: BT-474, MCF7, and ZR-75-1. Scrambled nonsilencing control siRNA and three siRNAs against *PPM1D* were designed according to Elbashir and co-workers (Elbashir et al., 2001). The *PPM1D* specific siRNAs did not show significant sequence homology (not exceeding 14 contiguous base pairs) to any other human gene and did not contain possible interferon-inducing immunostimulatory sequences involved in the induction of interferon response (Hornung et al., 2005, Judge et al., 2005, Sioud et al., 2005). Cells were transfected with siRNA using the Oligofectamine (Invitrogen) protocol according to the manufacturer's instructions. Briefly, one day prior to transfection, either 75 000 cells (24-well plate) or 375 000 cells (6-well plate) were plated in medium containing 10% FBS and no antibiotics. The siRNA duplex mixed in OptiMEM I and also Oligofectamine plus mixed in OptiMEM I were incubated at room temperature for 5 minutes. Subsequently, the two mixtures were combined and incubated at room temperature for an additional 20 minutes. The siRNA:Oligofectamine complex was then added to the cells. Cells were incubated at 37°C for 4 hours followed by the addition of fresh culture medium (one third of the transfection volume) including 30% FBS. The final concentration of siRNA duplex was 100 nM. All experiments were performed in to three to six replicates and repeated at least twice.

10. Cell proliferation assay (III)

The effect of *PPM1D* silencing on proliferation of BT-474, MCF7, and ZR-75-1 cells in Study III was performed by determining the cell numbers following *PPM1D* siRNA and control siRNA transfections. A total of 75 000 cells (BT-474, MCF7, and ZR-75-1) per well were seeded into 24-well cell culture plates and transfected with siRNAs. Cells were harvested 24h, 48h, 72h and 96h post-transfection and the cell numbers counted using Z1™ Series COULTER COUNTER® Cell and Particle Counter (Beckman Coulter, Fullerton, CA).

11. Cell cycle analysis (III)

Analysis of cell cycle distribution after siRNA transfection in Study III was carried out using Coulter® Epics® XL-MCL™ Flow Cytometer (Beckman Coulter). A total of 375 000 cells (BT-474, MCF7, and ZR-75-1) per well were seeded into 6-well cell culture plates and, 24h, 48h, and 72h post-transfection with siRNAs, the cells were harvested and resuspended in 500 µl of Hypotonic Staining Buffer (0.1 mg/ml sodium citrate tribasic dehydrate, 0.03% Triton X-100, 50 µg/ml propidium iodide and 2 µg/ml Ribonuclease A in dH_2O). After 30 min incubation on ice, a total of 20 000 PI stained nuclei were analyzed using EXPO32 ADC version 1.2 analysis software (Beckman Coulter).

12. Apoptosis assay (III)

To explore the effect of siRNA treatment on cell death in Study III, the *PPM1D* and control siRNA transfected cells were subjected to apoptosis analysis. A total of 375 000 cells (BT-474, MCF7, and ZR-75-1) per well were seeded into 6-well cell culture plates and 24h, 48h, and 72h after siRNA treatment both floating and adherent cells were collected. The cell aliquots were combined, washed, resuspended in 1x binding buffer and stained using the Annexin V-Apoptosis Detection Kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. The percentage of apoptotic cells was quantified using Coulter® Epics® XL-MCL™ Flow Cytometer (Beckman Coulter). Annexin V-positive and PI-negative cells were considered to be in early-apoptotic phase and cells having positive staining both for Annexin V and PI were deemed to undergo late apoptosis or necrosis. Dead cells stained positive only for PI.

13. Statistical methods (I, II, III)

For each gene in Study I, two-tailed Mann-Whitney U-test was used to examine the possible statistical significance of differences in expression levels between the three tumor groups. Three independent comparisons (high vs. moderate, high vs. no, and moderate vs. no amplification tumor group) were performed.

Fisher's exact test or chi-square test was used to study the association between *PPM1D* amplification and clinicopathological parameters in Study II. Fisher's exact test was additionally used to evaluate the association between *PPM1D* alterations and immunohistochemical p16 and CCN1D staining. Comparison of the clinicopathological parameters and median *PPM1D* expression levels between the amplified and non-amplified tumor groups was done using Mann-Whitney U-test. All *P* values are two-tailed.

In Study III, the statistical differences between *PPM1D* targeted siRNA and control siRNA treatments in cell proliferation, cell cycle, and apoptosis were calculated using non-parametric, two-tailed Mann-Whitney U-test.

14. Global gene expression analyses (IV)

Oligomicroarray technology was used for the analysis of gene expression levels in BT-474, MCF7 and ZR-75-1 breast cancer cell lines in Study IV. Total RNA was harvested 24h and 48h after the transfection with *PPM1D* specific and control siRNA as described in *Quantitative real-time RT-PCR*- section (8). Six hundred nanograms of total RNA were used to generate fluorescent Cy-3 labeled cRNA (control siRNA treated cells) and Cy-5 labeled cRNA (*PPM1D* siRNA treated cells) using Agilent Low RNA Input Fluorescent Linear Amplification Kit protocol (Agilent Technologies, Palo Alto, CA, USA). A total amount of 750 nanograms of both Cy-3 and Cy-5 labeled cRNA were hybridized to the Agilent 44K Whole Human Genome oligo microarrays (Agilent Technologies) according to the manufacturer's protocol.

15. Microarray data analysis (IV)

Microarray slides were scanned (Agilent Microarray Scanner) after hybridization and data was extracted using Feature Extraction software, version A.7.5.1 (Agilent Technologies). For within array normalization between the two channels, LinearLowess normalization was performed. Then, GeneSpring GX 7.3.1 Expression Analysis software from Agilent Technologies was used to analyze the raw data from array scans. Agilent Feature Extractor Plug-In was used to import the files to the GeneSpring program. Only those features flagged present by the Feature Extraction software in at least one of the arrays were

included in the subsequent analyses, whereas the features with either marginal or absent call were excluded.

To define differentially expressed genes, absolute expression change values ≥2.0 and ≤0.5 were used as cut-offs. In that way, genes that showed ≥2.0 and ≤0.5 -fold change in their expression level when compared to the control were considered to be upregulated and downregulated respectively. Pearson‘s Correlation and unsupervised average linkage hierarchical clustering algorithm were used for hierarchical clustering of these differentially expressed genes.

Functional classification was performed using GeneSpring to determine the biological significance of the up and down regulated genes and thereby the genes were classified into relevant Gene Ontologies (Ashburner et al., 2000) dealing with molecular function, biological processes and cellular components. A p-value of 0.02 was used as a cut-off to indicate those functions, processes, or components that were significantly overrepresented among the differentially expressed genes.

Results

1. Copy number and expression analysis across the 17q23 amplicon in primary breast tumors (I)

To determine whether the amplification of 17q23 contributes to expression levels of genes within that region, a total of 26 tumors was selected for Study I based on their *PPM1D* copy number data. These tumor samples included eight cases with increased *PPM1D* copy number and 18 tumors with no copy number increase. To determine the 17q23 amplification status in this set of samples more precisely, additional probe pairs from both ends of the 5 Mb amplicon were used and their copy number levels were determined by FISH. The additional probe sets verified the distribution of the samples into amplified and non-amplified groups. Moreover, the eight amplified cases demonstrated more or less consistent copy number increase across the entire amplicon and could be classified into groups with high (n=4) and moderate (n=4) level of amplification.

Earlier studies and genome sequence information available at the time of the study restricted the 17q23 amplicon to a ~5 Mb region (53.95 - 59.02 Mb) and proposed it to contain 29 genes with known function. Initial screen by RT-PCR in MCF7 and BT-474 breast cancer cell lines, which are known to harbor high level 17q23 amplification, indicated that five (*SEPT4*, *TEX14*, *CA4*, *MRC2*, and *KCNH6*) of the 29 genes showed either very low or undetectable expression. These genes were thus excluded as putative targets of the amplification. Subsequently, the expression levels of the remaining 24 genes from the 17q23 amplicon were measured in the 26 primary breast tumors using qRT-PCR. Two genes, *PTRH2* and *ACE*, were observed to have considerably higher expression levels than any other gene within this region. Nevertheless, *ACE* was also expressed at a similar level in normal human mammary gland (HMG). However, the expression level of *PPM1E* was very low across all 26 tumor samples and, additionally, *PPM1E* as well as *TBX4* showed highest expression levels in non-amplified tumor samples.

The expression levels of the 24 genes were then compared between the three tumor groups. The high level and no amplification tumor groups showed a statistically significant ($p<0.01$) difference in expression levels for a set of 11 genes (*FAM33A*, *DHX40*, *CLTC*, *PTRH2*, *TMEM49*, *TUBD1*, *RPS6KB1*, *ABC1*, *USP32*, *APPBP2*, and *PPM1D*). These genes, with the exception of *FAM33A* and *USP32*, also showed a significant difference ($p<0.05$) in expression levels between the high level and moderate level amplification groups. No difference between the moderate and no amplification tumor groups was observed.

Furthermore, these eleven genes were found to be located adjacent to each other within a 1.56 Mb region (54.54 Mb to 56.10 Mb) at the centre of the 17q23 amplicon. Only one gene, *YPEL2*, within this region did not show association between amplification and increased mRNA expression.

2. *PPM1D* amplification and overexpression in breast cancer (II, III)

In order to determine the frequency and levels of amplification *PPM1D*, 146 primary breast tumors and 21 breast cancer cell lines were analyzed. In total, 80% of the tumor samples could be evaluated using FISH on TMA and 11% of them showed threefold or higher *PPM1D* amplification relative to the chromosome 17 centromere. Moreover, 4.2% of the tumors had high-level amplification (>5-fold or tight clusters of signals). Similarly, interphase FISH analysis revealed threefold or higher *PPM1D* amplification in six (BT-474, MCF7, UACC-732, UACC-893, ZR-75-1, and ZR-75-30) out of the 21 breast cancer cell lines.

To determine the *PPM1D* expression levels in primary breast tumors, a set of 26 freshly-frozen tumors consisting of 11 amplified and 15 non-amplified cases were analyzed using qRT-PCR. The tumors with *PPM1D* amplification showed significantly ($p = 0.0148$) higher median expression level than those without amplification. Furthermore, only a single tumor in the amplified group had expression level below the median of the non-amplified group, whereas three tumors in the non-amplified group showed expression levels that were higher than the median of the amplified group.

Expression analysis of *PPM1D* was carried out in 21 breast cancer cell lines using Northern analysis. Five of the six cell lines with *PPM1D* amplification showed increased *PPM1D* expression levels compared to normal human mammary epithelial cells. Conversely, the UACC-893 cells with 3.5-fold amplification did not show elevated *PPM1D* mRNA levels.

3. Clinical and biological significance of *PPM1D* amplification in primary breast tumors (II)

To study a possible association of *PPM1D* amplification with other important features of breast cancer, the clinicopathological characteristics of the primary breast tumors with *PPM1D* amplification were examined. A routine immunohistochemistry revealed a highly significant association ($p=0.0001$) between *PPM1D* amplification and positive staining for the *ERBB2* oncogene. No significant associations to other tumor characteristics, for instance grade and nodal status, were observed.

To explore the possible relationship between *PPM1D* aberrations and p53 tumor suppressor status of the tumors, exons 4 through 10 of the *TP53* gene were sequenced from a subset of 16 tumors. These included ten tumors with *PPM1D* amplification and six with no *PPM1D* copy number increase. Altogether four *TP53* mutations were identified. A truncating mutation at exon 5 (W146X) occurred in a tumor with high-level *PPM1D* amplification and increased mRNA expression, whereas the remaining mutations were detected in tumors with no *PPM1D* alterations. These included two missense mutations (R175H in exon 5, V272M in exon 8) as well as a nonsense mutation (R213X in exon 6). All of these mutations had been previously observed in various tumor types (http://p53.curie.fr/). Furthermore, a sequence alteration in exon 4 (R72P) that had previously been classified as a polymorphism (http://p53.curie.fr/) was found in four separate tumor samples.

To further evaluate the downstream effects of *PPM1D* alterations, the protein expression levels of two possible downstream targets of PPM1D, p16 and CCND1, were determined using immunohistochemistry on TMA. No p16 expression was observed in 83% of the tumors (104/126), whereas positive CCND1 expression was seen in 31% (39/125). In addition, neither CCND1 nor p16 protein expression was associated with *PPM1D* amplification or the level of *PPM1D* mRNA expression.

4. *PPM1D* silencing in breast cancer cell lines (III)

Based on the *PPM1D* copy number analysis, BT-474, MCF7, and ZR-75-1 cell lines with varying levels of *PPM1D* amplification and with elevated mRNA expression were selected for functional experiments. Three synthetic siRNA molecules (siRNA-1, siRNA-2, and siRNA-3) directed against different regions of the *PPM1D* gene were tested for their capacity to reduce *PPM1D* expression in BT-474, MCF7, and ZR-75-1 cells. All three siRNAs efficiently downregulated the level of the *PPM1D* transcript, but the siRNA-3 had maximal and the most consistent inhibitory effect from 24h to 96h. The inhibition efficacy in BT-474 and ZR-75-1 cells was up to 85% and 80% respectively, whereas in MCF7 cells 74% downregulation was observed.

The effect of *PPM1D* silencing on proliferation of BT-474, MCF7, and ZR-75-1 cells was studied by counting the cell numbers using Z1™ Series COULTER COUNTER® Cell and Particle Counter (Beckman Coulter, Fullerton, CA) at 24h, 48h, 72h, and 96h following *PPM1D* and control siRNA transfections. A statistically significant ($p<0.01$) reduction in cell growth was seen in both MCF7 and ZR-75-1 cell lines after *PPM1D* silencing. Two days post-transfection, the inhibition was already noticeable in ZR-75-1 cells, while in MCF7 cells the growth reduction became visible at 72h. The difference in cell growth was still present at day four ($p<0.01$) for both cell lines. By contrast, no difference in cell growth was observed between *PPM1D* and control siRNA transfected BT-474 cells.

The *PPM1D* and control siRNA transfected cells were subjected to cell cycle and apoptosis analyses to ascertain whether the reduction in cell number was caused by reduced cell proliferation or increased cell death. Silencing of *PPM1D* was not observed to induce any changes in cell cycle distribution or the amount of apoptotic cells in BT-474 and MCF7 cell lines. No effects in cell cycle parameters for ZR-75-1 were observed at 24h and 48h, nevertheless, a statistically significant ($p<0.01$) increase of cells in S and G2 phase as well as a concomitant decrease of cells in G1 phase was detected 72h after transfection. The amount of apoptotic (including early-apoptotic and apoptotic/necrotic cells) ZR-75-1 cells was also significantly increased ($p<0.01$) at 48h and 72h. Furthermore, *PPM1D* silencing significantly ($p<0.01$) increased the amount of dead ZR-75-1 cells at 72h when compared to control siRNA treated cells.

5. Effects of *PPM1D* silencing on global gene expression levels in breast cancer cell lines (IV)

To explore the effects of *PPM1D* silencing on global gene expression levels in breast cancer, RNA interference was applied to three cell lines with *PPM1D* amplification and overexpression (BT-474, MCF7, and ZR-75-1). Two of these, MCF7 and ZR-75-1, carry wild-type *TP53* gene, whereas BT-474 harbors *TP53* mutation (Runnebaum et al., 1991; Concin et al., 2003). *PPM1D* specific (siRNA-3) and control siRNA treated cells were collected 24h and 48h after transfection and the silencing efficiency was verified by qRT-PCR before performing microarray hybridizations. The reduction of *PPM1D* mRNA levels was observed at time point 24h (68% downregulation as compared to control siRNA treated cells for BT-474, 79% for MCF7, 64% for ZR-75-1) and still remained at 48h after transfection (73% for BT-474, 61% for MCF7, 65% for ZR-75-1).

To identify genes influenced by *PPM1D* silencing in the breast cancer cell lines (BT-474, MCF7, ZR-75-1), Agilent 44K Whole Human Genome oligonucleotide microarrays were used. A total of 1798 gene elements representing 1579 individual genes were identified as differentially expressed after *PPM1D* silencing in at least one of the cell lines and at one time point. Most of these genes showed moderate increase (up to 4-fold) or decrease (≤0.2-0.5-fold) in expression, even though there were also a few genes with greatly elevated (over 10-fold) expression levels. Moreover, more genes were observed to have increased than decreased expression level. Altogether 764 gene elements were differentially expressed in BT-474 cell line, of which 634 were upregulated and 130 downregulated. In the other two cell lines, MCF7 and ZR-75-1, a total of 361 and 590 gene elements was recognized as overexpressed, while 202 and 102 gene elements were underexpressed.

To further evaluate the overall expression changes caused by *PPM1D* silencing, the 1798 differentially expressed gene elements were hierarchically clustered according to their expression profile using GeneSpring software. The

hierarchical clustering dendrogram revealed that for the ZR-75-1 cell line the two different time points cluster together whereas the BT-474 24h and MCF7 48h time points clustered together. In addition, these 1798 gene elements were classified according to the gene ontology (GO) information to understand the biological relevance of their differential expression. The ontology data was available for 58% of the differentially expressed genes. In general, GOs are grouped into three classes namely biological process, molecular function, and cellular component. Almost half of the genes altered after *PPM1D* silencing were found to be related to binding activities within the molecular function category. Additionally, several biological processes, such as development, behavior, reproduction, and growth were commonly observed.

Finally, those GO categories that were significantly ($p<0.02$) enriched after *PPM1D* silencing compared to the overall distribution of GO terms across the entire microarray were identified. A set of 31 biological process categories associated with *PPM1D* silencing was revealed, whereas the corresponding numbers for enriched cellular component and molecular function terms were 4 and 17. For example, GO categories related to regulation of cell cycle, particularly in G1 phase, assembly of various intracellular structures and components, such as nucleosomes and chromatin, and regulation of general cell signaling processes and metabolic cascades were among the greatly enriched categories. To study the properties of the ontologies further, the levels of the GOs were determined. If there were several levels, the shortest path was chosen. Ontologies form a nested tree structure in which the general terms are in the root and most detailed ontologies in the leaves. The root term is the most common classifier and it was given a value of 0. Most of the ontologies in molecular function were found to appear on levels 4 to 5, while ontologies in the biological process were located on much more specific levels. In general, cellular components are paradoxical to analyze because they do appear on high ontology levels, but due to their nature they are usually poor in information. Moreover, different processes, functions and components have different depths, and therefore it was not possible to directly compare the depths for different properties.

Next the list of 1798 differentially expressed gene elements was examined more closely. For this purpose, consistent expression was defined as ratio values ≥ 1.75 and ≤ 0.7 for upregulated and downregulated genes respectively. Then the analyses were focused on those genes whose expression patterns were consistent at both time points for a particular cell line or that showed consistent expression patterns in at least two of the three cell lines. With this approach, a subset of 266 gene elements representing 264 individual genes that were differentially expressed after *PPM1D* silencing was extracted. Unsurprisingly, the expression of *PPM1D* was downregulated in all three breast cancer cell lines and thus *PPM1D* was included in the list of 266 gene elements. The list also included multiple genes with DNA binding activities. For example, two zinc finger proteins were upregulated (*ZNF212* in MCF7 and *ZNF278* in all cell lines), whereas *RAB25* (a member of the RAS oncogene family), *NSBP1* (nucleosomal binding protein 1), and *ESR1* (estrogen receptor 1) showed decreased expression.

RAB25 was downregulated in all three cell lines, while *ESR1* expression decreased in BT-474 and ZR-75-1, and *NSBP1* expression only in ZR-75-1. Moreover, four different human histone coding genes, *HIST1H2AC*, *HIST1H2BF*, *HIST1H2BO*, and *HIST1H1D*, were found to be overexpressed. Interestingly, *HIST1H2AC* was upregulated only in MCF7 cell line, *HIST1H2BF*, *HIST1H2BO* in BT-474, *HIST1H1D* in ZR-75-1, and thus none of these genes showed increased expression throughout all cell lines. In the same way, several differentially expressed genes were discovered to be associated with the hormone related activities of the cell. In addition to *ESR1*, these included *NR4A1* (nuclear receptor subfamily 4, group A, member 1) and *STC1* (stanniocalcin 1). *NR4A1* was consistently downregulated after *PPM1D* silencing, whereas *STC1* showed increased expression in all cell lines. In the enriched GO category of protein-tyrosine kinase activity, five genes with changed expression levels were observed. Three of them, *PLK1* (polo-like kinase 1), *NEK4* (NIMA-related kinase 4), and *WIF1* (WNT inhibitory factor 1), showed decreased expression, whereas two, *PRKY* (protein kinase, Y-linked), and *ULK2* (unc-51-like kinase 2), were upregulated. The expression of *WIF1* was changed only in MCF7 and ZR-75-1 cell lines, unlike the other four genes.

Discussion

1. Putative target genes of the 17q23 amplicon (I)

Amplification is a common mechanism for the activation of oncogenes whose deregulated expression promotes growth of cancer cells and thus plays an important role in tumorigenesis. The amplified regions of the genome are typically large and contain multiple genes thereby making the identification of the specific driver gene(s) quite complicated. One of the frequently occurring amplicons in breast cancer is located at 17q23. Several studies have reported recurrent amplification of this region in multiple human tumor types and the association of amplification with poor clinical outcome (Isola et al., 1995; Bärlund et al., 2000b; Morerio et al., 2001; Andersen et al., 2002; Hirasawa et al., 2003; Saito-Ohara et al., 2003). In breast cancer, various genes from this amplicon have been proposed to be important for cancer development and progression and thus represent promising targets for diagnostic, prognostic, and therapeutic approaches (Couch et al., 1999; Bärlund et al., 2000a; Bärlund et al., 2000b; Wu et al., Erson et al., 2001; Monni et al., 2001; 2000, Wu et al., 2001; Bulavin et al., 2002b; Li et al., 2002). Nevertheless, none of these earlier studies had performed a systematic characterization of all genes within the 17q23 region in primary tumor material, even though it would clearly be important for a complete understanding of the underlying biology contributing to the phenotype of tumors with 17q23 amplification.

Amplification is expected to lead to elevated expression of the putative target genes. Thus, the first step in the identification of novel amplification target genes is to study their expression levels. Accordingly, in order to assess the putative role of the 29 known genes located within the common amplified segment at 17q23, their expression levels were examined using qRT-PCR in 26 primary breast tumors. Results showed that the expression levels of eleven genes (*FAM33A*, *DHX40*, *CLTC*, *PTRH2*, *TMEM49*, *TUBD1*, *RPS6KB1*, *ABC1*, *USP32*, *APPBP2*, *PPM1D*) were significantly ($p<0.01$) higher in primary breast tumors with high level 17q23 amplification than in tumors without amplification. On the other hand, the tumor group with moderate amplification showed expression levels similar to those seen in the non-amplified tumors, indicating that low level copy number increases at this region did not have a significant effect on gene expression levels. Three of the genes (*RPS6KB1*, *APPBP2*, and *PPM1D)* have earlier been proposed as putative target genes of the 17q23 amplification (Couch et al., 1999; Bärlund et al., 2000a; Bärlund et al., 2000b; Wu et al., 2001; Bulavin et al., 2002b; Li et al., 2002). On the contrary, the

expression levels of previously implicated target genes *RAD51C*, *TRIM37*, *TBX2*, *THRAP1*, and *BRIP1* (Bärlund et al., 2000a; Wu et al., 2000; Erson et al., 2001; Monni et al., 2001; Wu et al., 2001) did not show any correlation with 17q23 amplification in Study I. Only primary tumor material was used in Study I, and therefore the above-mentioned results may differ from the findings of these earlier studies that were done mainly using established breast cancer cell lines. Novel upregulated genes identified in Study I included *FAM33A*, *DHX40*, *CLTC*, *PTRH2*, *TMEM49*, *TUBD1*, *ABC1*, and *USP32*. The expression of these genes was also clearly associated with the amplification status in primary breast tumors. These results concur with recent findings implicating gene copy number alterations as significant determinants of gene expression patterns (Hyman et al., 2002; Pollack et al., 2002; Wolf et al., 2004; Fridlyand et al., 2006).

Another interesting finding of Study I was the fact that all the above-mentioned eleven overexpressed genes were located at a 1.56 Mb region which appears to represent the amplicon core at 17q23. The expression level of only one gene, *YPEL2*, at this core region did not show any correlation with the amplification status. The reason for this result is currently unclear because only very little is known about *YPEL2* and its function. Similar observations of overexpression of multiple genes within an amplicon have previously been reported in few occasions. For example, a 1 Mb segment of common amplification at 8p11-p12 has been identified in breast cancer and demonstrated to contain at least 14 candidate genes showing a strong correlation between amplification and overexpression (Garcia et al., 2005; Gelsi-Boyer et al., 2005). However, the actual target oncogene(s) that drive this aberration have not been identified with certainty. Likewise, most of the genes in a 3.6 Mb core region at the frequently observed 11q13 amplicon were overexpressed in amplified tumors and cell lines (Huang et al., 2006). In addition, the amplification of a 280 kb minimal common region at *ERBB2* amplicon at 17q12 has been shown by Kauraniemi and co-workers to lead to simultaneous overexpression of all genes within that genomic region in breast cancer (Kauraniemi et al., 2001). Taken together, the above-mentioned data imply that in general the vast majority of the genes within amplicons are overexpressed and therefore the expression level alone cannot be used to identify putative target genes.

In conclusion, the data in Study I demonstrate that high level amplification at 17q23 in breast cancer leads to concomitant overexpression of practically all genes within the 1.56 Mb amplicon core. Furthermore, a set of genes not previously implicated as possible targets was identified and might contribute to the tumor phenotype. However, due to the close association between amplification and elevated gene expression, it is difficult to determine which of the genes within 17q23 amplicon actually contribute to tumor pathogenesis. It is possible that, instead of a single target gene, amplifications are driven by a series of genes whose coordinated overexpression provides cancer cells with growth advantage (Huang et al., 2006; Kao et al., 2006). On the other hand, it is also conceivable that not all of the overexpressed genes are necessary for cancer progression but are overexpressed simply due to their location within the amplicon. Functional studies exploring the potential contribution of these genes

to cancer cell phenotype are thus needed to elucidate their possible role in cancer pathogenesis.

2. *PPM1D* is frequently activated by amplification in breast cancer (II, III)

To evaluate the involvement of *PPM1D* aberrations in breast cancer, copy number analysis was performed in a large number of both primary breast tumors and established breast cancer cell lines. First of all, in FISH analysis *PPM1D* amplification was found in 11% of the primary tumor samples. This finding concurs nicely with earlier studies indicating that the 17q23 locus or *PPM1D* itself is amplified in about 10% of primary tumor cases (Monni et al., 2001; Bulavin et al., 2002a; Sinclair et al., 2003). In addition, the copy number analysis also demonstrated that 29% of the breast cancer cell lines had amplification of *PPM1D*.

Study II also revealed that *PPM1D* amplification was highly significantly associated (p=0.0001) with ERBB2 overexpression, a well-known indicator of poor prognosis in primary breast tumors. This indicates that *PPM1D* amplification occurs in more advanced cases even though it was not found to be linked to other markers of tumor aggressiveness, such as high histological grade and positive nodal status. Such association had not been previously reported, nevertheless, it corroborates the findings of an earlier study that concomitant amplification of the 17q23 region and *ERBB2* indicates an even poorer prognosis than amplification of either locus alone (Bärlund et al., 2000b). Furthermore, this finding substantiates the data from another study on *Ppm1d*-null mice showing that the presence of *PPM1D* is essential for *ERBB2* induced mammary tumorigenesis (Bulavin et al., 2004). Even though *PPM1D* and *ERBB2* are located in the same arm of chromosome 17, they are positioned in separate amplicons (Bärlund et al., 2000b; Hyman et al., 2002; Orsetti et al., 2004). Therefore, the activation of *ERBB2* and *PPM1D* should be considered as two separate events that cooperate in breast cancer development although amplification of the *PPM1D* region at 17q23 has also been found to have independent prognostic value in breast cancer (Bärlund et al., 2000b; Rennstam et al., 2003). Together these data imply that *PPM1D* amplification in breast cancer is obviously associated with aggressive disease.

Next, the association of *PPM1D* amplification and mRNA expression was studied in a set of 26 primary breast tumors, representing both amplified and non-amplified tumors, using qRT-PCR. The tumors with amplification did indeed express significantly (p = 0.0148) more *PPM1D* than the tumors without amplification. Similarly, Northern analysis showed that five of the six breast cancer cell lines with *PPM1D* amplification had elevated *PPM1D* expression levels. These results concur with earlier studies where the association between *PPM1D* amplification and overexpression has been demonstrated using smaller sample materials (Bulavin et al., 2002a; Li et al., 2002). Interestingly, Study II

also revealed that a few of the tumors showed high *PPM1D* expression levels without amplification, thus suggesting that also mechanisms other than increased copy number are also involved in the regulation of *PPM1D* expression.What such alternative mechanisms are in this case is not known, but these observed increases in gene expression could, for example, result from point mutation, or epigenetic regulation, activation of upstream signaling molecules. Even though amplification seems to be the most prevalent mechanism for upregulation of *PPM1D*, the involvement of additional mechanisms for regulation of its expression levels further strengthens the hypothesis that *PPM1D* indeed has a critical role in breast cancer pathogenesis.

One of the main functions of *PPM1D* is believed to be the regulation of p53 tumor suppressor activity, hence overexpression of *PPM1D* is considered to represent an additional mechanism for inactivation of p53 in cancer (Takekawa et al., 2000). To explore whether this really is the case in primary breast tumors, the mutation status of *TP53* was studied in a subset of tumors by direct sequencing. The fact that *TP53* mutations were predominantly found in tumors without *PPM1D* amplification implies that these two events are mutually exclusive. This result is consistent with earlier findings (Bulavin et al., 2002a; Lu et al., 2007) and indeed supports the role of *PPM1D* as an important regulator of p53 function in breast cancer.

Based on data derived from *Ppm1d* knock-out mouse studies, CCND1 and p16 are downstream targets of PPM1D and could be expected to be upregulated and downregulated respectively in tumors with intensified PPM1D activity (Bulavin et al., 2004). In order to investigate whether such a connection exists in primary breast tumors, the protein expression levels of CCND1 and p16 were determined. However, the expression levels of these proteins showed no differences between the *PPM1D* amplified and non-amplified tumors, thus not supporting the previous data obtained earlier from the mouse model. Yet the mouse model was designed to investigate the absence of *Ppm1d* and the opposite effect, i.e. *PPM1D* overexpression, may have downstream consequences not exactly the opposite to those obtained when knocking-out *Ppm1d*. Furthermore, CCND1 and $p16^{INK4A}$ are likely to be regulated in a complex manner involving multiple players in addition to PPM1D.

3. *PPM1D* regulates growth of breast cancer cells (III)

Study III aimed to further investigate the functional consequences of *PPM1D* overexpression in breast cancer. RNA interference (RNAi) technology was applied to specifically downregulate *PPM1D* mRNA levels in BT-474, MCF7, and ZR-75-1 breast cancer cell lines with amplification and overexpression of this gene. The analysis showed that *PPM1D* silencing had a strong antiproliferative effect in ZR-75-1 breast cancer cells, whereas less noticeable reduction in cell growth was observed in MCF7 cells. No change in cell growth rate was observed for BT-474 cells. All three cell lines used in Study III are

known to express high levels of *PPM1D* and therefore the discovered differences in their behavior following *PPM1D* silencing were somewhat unexpected. These cell lines showed comparable levels of *PPM1D* downregulation following siRNA transfection and hence this variation between cell lines could not be explained by differences in the efficiency of *PPM1D* silencing. Since ZR-75-1 and MCF7 are known to carry wild-type p53, whereas BT-474 harbors mutant p53 (Runnebaum et al., 1991), the p53 status of the cells could explain these diverse responses. As stated above, one of the main effects of *PPM1D* activation is the inactivation of p53 tumor suppressor (Takekawa et al., 2000) and thus *PPM1D* downregulation could be assumed to have an impact only on cells with wild-type p53 and not on such p53 deficient cells as BT-474. Consequently, Study III suggests that the effect of PPM1D on cell growth is dependent on the p53 status of the cells. Nevertheless, the possible contribution of other factors to the differential response of *PPM1D* silencing in these cell lines should not be ignored. For example, BT-474 cells may be dependent on other proliferation and survival pathways than those involving *PPM1D*.

Next, Study III investigated whether the reduced growth in MCF7 and ZR-75-1 cells after *PPM1D* silencing might be due to changes in cell cycle. The cell cycle analysis did not reveal any major differences in cell cycle distribution between the *PPM1D* siRNA and control siRNA treatments. A larger portion of *PPM1D* siRNA transfected ZR-75-1 cells were in the S and G_2 phases of the cell cycle 72h after transfection, but this was most probably caused by contact inhibition in the fully confluent control siRNA cultures rather than real change in *PPM1D* deficient cells. Due to their slower growth rate, the *PPM1D* siRNA transfected cells had not yet reached confluency.

Because the cell cycle analysis did not explain the reduced growth in MCF7 and ZR-75-1 cells after *PPM1D* silencing, analysis of apoptosis was performed. The findings suggested that *PPM1D* downregulation leads to increased apoptosis in ZR-75-1, but not in MCF7 cells. Accordingly, the reduced growth of ZR-75-1 cells could be explained by induction of programmed cell death, whereas the growth reduction observed in MCF7 cells remains unexplained. In any case, these results are consistent with an earlier report showing that *PPM1D* silencing suppresses cell growth and induces apoptosis in neuroblastoma cell lines (Saito-Ohara et al., 2003a). Very recently Rayter et al. also reported that PPM1D inhibition selectively reduces the viability of human breast tumor cells overexpressing *PPM1D* (Rayter et al., 2007). *PPM1D* knockdown has also been previously found to augment apoptosis after DNA damage and E2F1 induction (Fujimoto et al., 2006; Hershko et al., 2006). In addition, the findings of Study III also concur with data demonstrating that MEFs from *Ppm1d*-null mice show decreased proliferative capacity and premature senescence (Choi et al., 2002; Bulavin et al., 2004). Moreover, small molecule inhibitors of PPM1D have been suggested to be capable of reducing tumor cell proliferation and viability both *in vitro* and in *vivo* (Belova et al., 2005; Rayter et al., 2007).

All in all, Study III confirmed the TP53 dependent role of *PPM1D* in the regulation of cell proliferation and further corroborated its involvement in the regulation of essential prosurvival pathways in breast cancer. Inhibition of

positive regulators of cell proliferation and induction of apoptosis are crucial approaches for intervention in modern cancer therapy, and thus *PPM1D* appears to be an ideal therapeutic target.

4. Genome-wide gene expression changes following *PPM1D* silencing in breast cancer (IV)

In Study IV, the contribution of *PPM1D* overexpression to breast cancer tumorigenesis was further investigated. Genome-wide oliconucleotide microarrays were used to carry out a comprehensive gene expression screen in order to identify genes whose expression levels were affected following RNAi mediated *PPM1D* silencing. These analyses were carried out in BT-474, MCF7, and ZR-75-1 breast cancer cell lines with known *PPM1D* amplification and overexpression. Study IV identified a total of 1798 gene elements representing 1579 individual genes that were differentially expressed after *PPM1D* silencing. PPM1D is known to directly regulate several important genes in various signaling pathways, for example the p38 MAPK signaling cascade (Takekawa et al., 2000; Bulavin et al., 2004; Lu et al., 2005), and this may at least partly explain the large number of differentially expressed genes. On the other hand, the majority of the observed gene expression changes may represent downstream signaling events of only a few direct PPM1D target genes.

Gene ontology analysis was performed to assess the overall function of these differentially expressed genes. The data illustrated that a large fraction of them are associated with molecular binding activities and, at the same time, are included in several different biological process categories. Further investigation of these genes revealed that they are commonly involved, for example, in cell cycle regulation and thus play a part in the control of cell proliferation. These findings are consistent with previous observations indicating that PPM1D is a regulator of a set of important cell cycle checkpoint effectors (Lu et al., 2005; Fujimoto et al., 2006; Shreeram et al., 2006a; Shreeram et al., 2006b; Oliva-Trastoy et al., 2007), and that its silencing leads to the inhibition of cell proliferation (Choi et al., 2002; Saito-Ohara et al., 2003a; Bulavin et al., 2004; Belova et al., 2005) (Study III). In addition, many of the differentially expressed genes were implicated in the construction of new cellular structures and components and also in the regulation of general cell signaling processes and metabolic cascades. The majority of these genes were also found to be located either in nucleosome and nucleoplasm or in different kinds of chromosomal structures and therefore may have a role in the packaging of DNA into chromosomes and/or the control of gene expression.

Next, the functions of the most commonly affected genes were investigated more thoroughly. First of all, three genes, known to be involved in the control of apoptosis, were found to be differentially expressed in all cell lines following *PPM1D* silencing. *NR4A1* has previously been shown to be involved in caspase-independent cell death, (Kim et al., 2003; Li et al., 2006) whereas *RAB25*

signaling pathway has been found to play a role in the regulation of cell proliferation and apoptosis in ovarian cancer cells (Cheng et al., 2004; Fan et al., 2006). Furthermore, *PLK1* reduction is known to induce apoptosis in cervical cancer, prostate cancer, and breast cancer cells (Liu et al., 2003a; Reagan-Shaw et al., 2005; Spankuch et al., 2007). These observations are consistent with previous findings demonstrating increased apoptosis following *PPM1D* knockdown (Saito-Ohara et al., 2003; Fujimoto et al., 2006; Hershko et al., 2006,) (Study III). Another function of *PLK1* is its involvement in the regulation of cell cycle progression, cell division, and DNA damage repair pathways (Nigg, 1998; Liu et al., 2003b). It has also been shown to possess protein tyrosine kinase activity which plays a pivotal role in many cell regulatory processes (Nigg, 1998, Robinson et al., 2000). Moreover, other kinases, such as *NEK4*, *PRKY*, *ULK2*, and *WIF1* were also found to be differentially expressed after *PPM1D* silencing.

Study IV also indicated a set of genes with hormone related activities, *NR4A1*, *ESR1*, and *STC1*, to be differentially expressed. Interestingly, all these genes have also previously been associated with potential and interesting cancer associated cellular features. For example, *ESR1*, a ligand-activated transcription factor, is known to be involved in the regulation of gene transcription, growth control, breast cancer development, and the origin of hormone resistance (Speirs et al., 2007). Furthermore, *ESR1* as well as *RAB25*, *PLK1*, and *STC1* have been found to be amplified and to have altered expression levels in several tumor types (Chang et al., 2003; Cheng et al., 2004; Takai et al., 2005; Holst et al., 2007).

Data from Study IV also identified four histone coding genes (*HIST1H2AC*, *HIST1H2BF*, *HIST1H2BO*, and *HIST1H1D*) that were upregulated following *PPM1D* silencing. In general, histones are responsible for the nucleosome structure in eukaryotes. Moreover, their overexpression has been shown to block transcription *in vitro* by triggering chromatin aggregation and to increase the incidence of mitotic chromosome loss (Meeks-Wagner et al., 1986; Steger et al., 1999). Accordingly, an appropriate balance of histone proteins is required for the accurate assembly of chromatin, likewise for proper cell division and growth. In light of this data, the observed overexpression of the histone coding genes might contribute to the inhibition of cell growth following *PPM1D* silencing.

In conclusion, Study IV was the first systematic survey to investigate the effects of *PPM1D* silencing on global gene expression patterns and signaling pathways in breast cancer. The study identified a large set of differentially expressed genes in response to *PPM1D* downregulation. Furthermore, many of these genes are indicated in several essential cell cycle, cell growth, and cell survival pathways. Given these results, *PPM1D* is suggested to directly and indirectly regulate complex cellular transcriptional programs and thus have widespread effects on breast cancer biology.

5. Specificity of RNA interference methodology (III, IV)

Since recent reports have raised some questions concerning the specificity of the RNA interference approach, the experimental design and the sequences of siRNAs used in Study III and IV were thoroughly planned. The two main issues raised have been the possible off-target effects and the induction of an immunological response that may in turn lead to global inhibition of protein synthesis (Hornung et al., 2005; Judge et al., 2005; Sioud, 2005). Therefore, the sequences of the *PPM1D* specific siRNAs were first analysed and no significant homologies to any other human gene were found. This indicates that these siRNAs are unlikely to silence other known genes. Morever, control siRNAs likewise showed no homology to any human gene. Nor were the sequences found to contain any immunostimulatory sequences (Hornung et al., 2005; Judge et al., 2005; Sioud, 2005). In addition, the induction of immunological response is not considered a major problem when using such a short siRNA oligos, because they are detected by toll-like receptor TLR7, which is not expressed by non-immune cells, including the breast cancer cells used in these studies (Schlee et al., 2006). The synthetic siRNAs are generally known to induce weaker immune response than vector based siRNAs (Schlee et al., 2006). Together, the findings suggest that the methodology used in Studies III and IV most likely resulted in specific silencing of *PPM1D*.

6. *PPM1D* as a drug target

It has become obvious that *PPM1D* is activated in a number of human cancers and that this activation contributes to tumor pathogenesis through multiple different mechanisms. *PPM1D* should thus be considered a promising drug target and downregulation of *PPM1D* activity could open up new options in cancer therapy. However, before such therapies can be developed, the possibility of negative side effects of *PPM1D* inhibition should be taken into consideration. Although the *Ppm1d*-null mice developed normally, they did indeed suffer from defects in immune function (Choi et al., 2002). For example, most neuroblastoma patients are young infants with a developing immune system and treatment inhibiting PPM1D may have immunological complications (Schito et al., 2006). These authors also suggested that the severity and extent of *PPM1D* inhibition could affect thymic development in these young patients. Therefore appropriate precautions must be taken to identify any long-term immunological risks possibly associated with inhibiting *PPM1D* in pediatric patients. However, the fact remains that inactivation of *PPM1D* could be beneficial for treating certain types of human cancers and therefore efforts to develop such a therapy should be continued.

Modulation of kinase activities has been used relatively successfully in treating several types of malignancies (Kelland, 1999; Sudbeck et al., 1999;

reviewed by Pearson et al., 2004; reviewed by Strumberg et al., 2005). Conversely, a similar strategy is much less utilized for protein phosphatases, even though phosphatases are, in general, susceptible to targeting by drugs (reviewed by McCluskey et al., 2002). Despite several studies indicating that PPM1D has an important and complex role in controlling cell cycle checkpoints and DNA damage repair pathways in response to cellular stresses, only recently have specific inhibitors of PPM1D been reported (Belova et al., 2005; Yamaguchi et al., 2006). The main obstacle has been the discovery of a specific chemical inhibitor for this particular phosphatase due to the high degree of structural homology among the PP2C family members (reviewed by Cohen, 1989). Another difficulty has been the location of PP2C catalytic site, which is not ideal for binding to organic small molecules (Das et al., 1996). However, Belova and co-workers have reported several PPM1D inhibitor compounds with anticancer properties both *in vitro* and *in vivo* (Belova et al., 2005). They showed that the inactivation of PPM1D decreases the proliferation of breast cancer cell lines, xenograph tumors, and tumors developed in MMTV-c-Neu transgenic mice. In this study, one additional and important finding was that PPM1D inhibitor enhances the growth inhibition achieved by chemotherapeutic agents and thus may allow the use of smaller doses of drugs. With a similar goal in mind, Yamaguchi and co-workers have designed specific phosphopeptide inhibitors for the catalytic site of PPM1D, which could be useful in the further development of potential anti-cancer drugs (Yamaguchi et al., 2006). In addition, a small molecule that is selective in killing *PPM1D* overexpressing cells has recently been identified (Rayter et al., 2007). This further confirms that selective therapeutic agents targeting PPM1D could be developed and used for the treatment of many types of human tumors.

In addition to specific small molecule inhibitors, several other strategies for the downregulation of PPM1D activity can be envisaged. For example, targeted antibody-based therapy against ERBB2, called Herceptin, is already in clinical use (Carter et al., 1992) and similarly, monoclonal PPM1D antibodies could be utilized as a method for the inhibition of PPM1D activity. Such antibodies may also be used in diagnostic procedures whereby patients are tested for abnormal levels of PPM1D protein. Nevertheless, such antibodies against PPM1D have not yet been developed. Another alternative method that has already been used for *PPM1D* inhibition in cell lines is liposome-based transfection of antisense oligonucleotides. These have been shown to downregulate *PPM1D* and remarkably suppress the growth of neuroblastoma cell lines (Saito-Ohara et al., 2003a). Furthermore, the use of RNAi could also be an attractive option for silencing of *PPM1D* even though it appears to have some downsides. So far, siRNAs have not been shown to be effective at reducing target gene expression in murine tissue systems (Song et al., 2003). For both antisense oliconucleotide and siRNA based therapies, the delivery of the drug to the target tumor tissue will be a major problem.

7. Future prospects

In this study, several new genes possibly involved in breast cancer tumorigenesis were identified and shown to be located at the amplicon core of 17q23. Since overexpression alone is not sufficient to underline the putative target genes at this chromosomal region, further functional analyses are required to ascertain the possible biological and clinical significance of these new candidate genes. Such overexpressed genes could also be promising targets for developing diagnostic, prognostic, and therapeutic approaches and should be taken into consideration in the development of novel anticancer therapies. Furthermore, it is important to continue developing technologies that will enable difficult proteins such as PPM1D to be more successfully targeted.

This study also identified a large set of genes affected by *PPM1D* silencing. Further characterization of the molecular interplay between PPM1D and these differentially expressed genes is needed to fully understand their role and the role of PPM1D in tumorigenesis. On the other hand, further characterization of these signaling networks on protein level will provide a better understanding of the cellular targets and pathways affected by *PPM1D* alterations in cancer. It should also be noted that targeting genes downstream of PPM1D rather than PPM1D itself may offer a more specific and druggable target for the development of anti-cancer therapies.

Conclusions

The present study was conducted to identify novel amplified and overexpressed genes at 17q23 in breast cancer and to explore the functional and transcriptional effects of *PPM1D* overexpression in breast cancer cell lines.

The major findings of the study were:

1. Eleven genes (*FAM33A, DHX40, CLTC, PTRH2, TMEM49, TUBD1, RPS6KB1, ABC1, USP32, APPBP2, PPM1D*) showed significantly higher expression levels in primary breast tumors with high level 17q23 amplification compared to tumors without amplification. In consequence, high level amplification at the 17q23 leads to concomitant overexpression of virtually all genes located at a 1.56 Mb central region of the amplicon.

2. *PPM1D* amplification was observed in a substantial proportion of primary breast tumors and was demonstrated to lead to *PPM1D* overexpression. Additionally, *PPM1D* amplification was associated with ERBB2 expression, thus implying that *PPM1D* aberrations occur in tumors with poor prognosis. *PPM1D* amplification was also found to occur almost exclusively in tumors with wild-type p53, thus confirming the role of *PPM1D* as a negative regulator of p53 in breast cancer.

3. *PPM1D* was demonstrated to be involved in the regulation of cell proliferation in breast cancer in p53 dependent manner. Moreover, overexpression of *PPM1D* was shown to contribute to malignant phenotype by promoting sustained cell growth and cell survival. Based on these results, *PPM1D* could be considered as an ideal target for developing potential therapeutic strategies.

4. *PPM1D* amplification and overexpression was illustrated to contribute to breast cancer development by directly or indirectly affecting several important cell cycle, cell growth, and cell survival pathways that are involved in multiple cellular functions.

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References

Albertson DG (2006): Gene amplification in cancer. Trends Genet 22:447-455.

Albertson DG (2003): Profiling breast cancer by array CGH. Breast Cancer Res Treat 78:289-298.

Andersen CL, Hostetter G, Grigoryan A, Sauter G and Kallioniemi A (2001): Improved procedure for fluorescence in situ hybridization on tissue microarrays. Cytometry 45:83-86.

Andersen CL, Monni O, Wagner U, Kononen J, Bärlund M, Bucher C, Haas P, Nocito A, Bissig H, Sauter G and Kallioniemi A (2002): High-throughput copy number analysis of 17q23 in 3520 tissue specimens by fluorescence in situ hybridization to tissue microarrays. Am J Pathol 161:73-79.

Arnold N, Hagele L, Walz L, Schempp W, Pfisterer J, Bauknecht T and Kiechle M (1996): Overrepresentation of 3q and 8q material and loss of 18q material are recurrent findings in advanced human ovarian cancer. Genes Chromosomes Cancer 16:46-54.

Arpino G, Weiss H, Lee AV, Schiff R, De Placido S, Osborne CK and Elledge RM (2005): Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. J Natl Cancer Inst 97:1254-1261.

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM and Sherlock G (2000): Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25:25-29.

Bakkenist CJ and Kastan MB (2004): Initiating cellular stress responses. Cell 118:9-17.

Balmain A, Gray J and Ponder B (2003): The genetics and genomics of cancer. Nat Genet 33 Suppl:238-244.

Bartek J and Lukas J (2003): Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell 3:421-429.

Bartel DP (2004): MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281-297.

Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Orntoft T, Lukas J and Bartek J (2005): DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 434:864-870.

Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC and Norton L (1999): Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. Semin Oncol 26:78-83.

Belova GI, Demidov ON, Fornace AJ,Jr and Bulavin DV (2005): Chemical inhibition of Wip1 phosphatase contributes to suppression of tumorigenesis. Cancer Biol Ther 4:1154-1158.

Bernards R (2004): Wip-ing out cancer. Nat Genet 36:319-320.

Bignell GR, Santarius T, Pole JC, Butler AP, Perry J, Pleasance E, Greenman C, Menzies A, Taylor SE, Edkins S, Campbell P, Quail M, Plumb B, Matthews L, McLay K, Edwards PA, Rogers J, Wooster R, Futreal PA and Stratton MR (2007): Architectures of somatic genomic rearrangement in human cancer amplicons at sequence-level resolution. Genome Res (Epub ahead of print).

Brancho D, Tanaka N, Jaeschke A, Ventura JJ, Kelkar N, Tanaka Y, Kyuuma M, Takeshita T, Flavell RA and Davis RJ (2003): Mechanism of p38 MAP kinase activation in vivo. Genes Dev 17:1969-1978.

Brinkschmidt C, Christiansen H, Terpe HJ, Simon R, Boecker W, Lampert F and Stoerkel S (1997): Comparative genomic hybridization (CGH) analysis of neuroblastomas--an important methodological approach in paediatric tumour pathology. J Pathol 181:394-400.

Bulavin DV, Higashimoto Y, Popoff IJ, Gaarde WA, Basrur V, Potapova O, Appella E and Fornace AJ,Jr (2001): Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. Nature 411:102-107.

Bulavin DV, Demidov ON, Saito S, Kauraniemi P, Phillips C, Amundson SA, Ambrosino C, Sauter G, Nebreda AR, Anderson CW, Kallioniemi A, Fornace AJ,Jr and Appella E (2002a): Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity. Nat Genet 31:210-215.

Bulavin DV, Demidov ON, Saito S, Kauraniemi P, Phillips C, Amundson SA, Ambrosino C, Sauter G, Nebreda AR, Anderson CW, Kallioniemi A, Fornace AJ,Jr and Appella E (2002b): Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity. Nat Genet 31:210-215.

Bulavin DV, Phillips C, Nannenga B, Timofeev O, Donehower LA, Anderson CW, Appella E and Fornace AJ,Jr (2004): Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK-mediated activation of the p16(Ink4a)-p19(Arf) pathway. Nat Genet 36:343-350.

Bulavin DV, Saito S, Hollander MC, Sakaguchi K, Anderson CW, Appella E and Fornace AJ,Jr (1999): Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J 18:6845-6854.

Bundred NJ (2001): Prognostic and predictive factors in breast cancer. Cancer Treat Rev 27:137-142.

Bärlund M, Forozan F, Kononen J, Bubendorf L, Chen Y, Bittner ML, Torhorst J, Haas P, Bucher C, Sauter G, Kallioniemi OP and Kallioniemi A (2000a): Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. J Natl Cancer Inst 92:1252-1259.

Bärlund M, Monni O, Kononen J, Cornelison R, Torhorst J, Sauter G, Kallioniemi OP and Kallioniemi A (2000b): Multiple genes at 17q23 undergo amplification and overexpression in breast cancer. Cancer Res 60:5340-5344.

Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F and Croce CM (2002): Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 99:15524-15529.

Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME and Shepard HM (1992): Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci U S A 89:4285-4289.

Casanovas O, Miro F, Estanyol JM, Itarte E, Agell N and Bachs O (2000): Osmotic stress regulates the stability of cyclin D1 in a p38SAPK2-dependent manner. J Biol Chem 275:35091-35097.

Caspari T (2000): How to activate p53. Curr Biol 10:315-317.

Chang AC, Jellinek DA and Reddel RR (2003): Mammalian stanniocalcins and cancer. Endocr Relat Cancer 10:359-373.

Chen D, Riedl T, Washbrook E, Pace PE, Coombes RC, Egly JM and Ali S (2000): Activation of estrogen receptor alpha by S118 phosphorylation involves a ligand-dependent interaction with TFIIH and participation of CDK7. Mol Cell 6:127-137.

Cheng KW, Lahad JP, Kuo WL, Lapuk A, Yamada K, Auersperg N, Liu J, Smith-McCune K, Lu KH, Fishman D, Gray JW and Mills GB (2004): The RAB25 small GTPase determines aggressiveness of ovarian and breast cancers. Nat Med 10:1251-1256.

Chin K, de Solorzano CO, Knowles D, Jones A, Chou W, Rodriguez EG, Kuo WL, Ljung BM, Chew K, Myambo K, Miranda M, Krig S, Garbe J, Stampfer M, Yaswen P, Gray JW and Lockett SJ (2004): In situ analyses of genome instability in breast cancer. Nat Genet 36:984-988.

Choi J, Appella E and Donehower LA (2000): The structure and expression of the murine wildtype p53-induced phosphatase 1 (Wip1) gene. Genomics 64:298-306.

Choi J, Nannenga B, Demidov ON, Bulavin DV, Cooney A, Brayton C, Zhang Y, Mbawuike IN, Bradley A, Appella E and Donehower LA (2002): Mice deficient for the wild-type p53-induced phosphatase gene (Wip1) exhibit defects in reproductive organs, immune function, and cell cycle control. Mol Cell Biol 22:1094-1105.

Cianfrocca M and Goldstein LJ (2004): Prognostic and predictive factors in early-stage breast cancer. Oncologist 9:606-616.

Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, Paton V, Shak S, Lieberman G and Slamon DJ (1999): Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 17:2639-2648.

Cohen P (1989): The structure and regulation of protein phosphatases. Annu Rev Biochem 58:453-508.

Concin N, Zeillinger C, Tong D, Stimpfl M, König M, Printz D, Stonek F, Schneeberger C, Hefler L, Kainz C, Leodolter S, Haas OA and Zeillinger R (2003): Comparison of p53 mutational status with mRNA and protein expression in a panel of 24 human breast carcinoma cell lines. Breast Cancer Res Treat 79:37-46.

Coquelle A, Pipiras E, Toledo F, Buttin G and Debatisse M (1997): Expression of fragile sites triggers intrachromosomal mammalian gene amplification and sets boundaries to early amplicons. Cell 89:215-225.

Coquelle A, Rozier L, Dutrillaux B and Debatisse M (2002): Induction of multiple double-strand breaks within an hsr by meganucleaseI-SceI expression or fragile site activation leads to formation of double minutes and other chromosomal rearrangements. Oncogene 21:7671-7679.

Cordera F and Jordan VC (2006): Steroid receptors and their role in the biology and control of breast cancer growth. Semin Oncol 33:631-641.

Couch FJ, Wang XY, Wu GJ, Qian J, Jenkins RB and James CD (1999): Localization of PS6K to chromosomal region 17q23 and determination of its amplification in breast cancer. Cancer Res 59:1408-1411.

Courjal F and Theillet C (1997): Comparative genomic hybridization analysis of breast tumors with predetermined profiles of DNA amplification. Cancer Res 57:4368-4377.

Daniely M, Aviram A, Adams EF, Buchfelder M, Barkai G, Fahlbusch R, Goldman B and Friedman E (1998): Comparative genomic hybridization analysis of nonfunctioning pituitary tumors. J Clin Endocrinol Metab 83:1801-1805.

Das AK, Helps NR, Cohen PT and Barford D (1996): Crystal structure of the protein serine/threonine phosphatase 2C at 2.0 A resolution. EMBO J 15:6798-6809.

De Angelis PM, Clausen OP, Schjolberg A and Stokke T (1999): Chromosomal gains and losses in primary colorectal carcinomas detected by CGH and their associations with tumour DNA ploidy, genotypes and phenotypes. Br J Cancer 80:526-535.

Demidov ON, Kek C, Shreeram S, Timofeev O, Fornace AJ, Appella E and Bulavin DV (2007): The role of the MKK6/p38 MAPK pathway in Wip1-dependent regulation of ErbB2-driven mammary gland tumorigenesis. Oncogene 12:2502-2506.

DePinho RA and Polyak K (2004): Cancer chromosomes in crisis. Nat Genet 36:932-934.

Easton DF, Pooley KA, Dunning AM, Pharoah PD, Thompson D, Ballinger DG, Struewing JP, Morrison J, Field H, Luben R, Wareham N, Ahmed S, Healey CS, Bowman R, SEARCH collaborators, Meyer KB, Haiman CA, Kolonel LK, Henderson BE, Le Marchand L, Brennan P, Sangrajrang S, Gaborieau V, Odefrey F, Shen CY, Wu PE, Wang HC, Eccles D, Evans DG, Peto J, Fletcher O, Johnson N, Seal S, Stratton MR, Rahman N, Chenevix-Trench G, Bojesen SE, Nordestgaard BG, Axelsson CK, Garcia-Closas M, Brinton L, Chanock S, Lissowska J, Peplonska B, Nevanlinna H, Fagerholm R, Eerola H, Kang D, Yoo KY, Noh DY, Ahn SH, Hunter DJ, Hankinson SE, Cox DG, Hall P, Wedren S, Liu J, Low YL, Bogdanova N, Schurmann P, Dork T, Tollenaar RA, Jacobi CE, Devilee P, Klijn JG, Sigurdson AJ, Doody MM, Alexander BH, Zhang J, Cox A, Brock IW, MacPherson G, Reed MW, Couch FJ, Goode EL, Olson JE, Meijers-Heijboer H, van den Ouweland A, Uitterlinden A, Rivadeneira F, Milne RL, Ribas G, Gonzalez-Neira A, Benitez J, Hopper JL, McCredie M, Southey M, Giles GG, Schroen C, Justenhoven C, Brauch H, Hamann U, Ko YD, Spurdle AB, Beesley J, Chen X, kConFab, AOCS Management Group, Mannermaa A, Kosma VM, Kataja V, Hartikainen J, Day NE, Cox DR and Ponder BA (2007): Genome-wide association study identifies novel breast cancer susceptibility loci. Nature 447:1087-1093.

Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, Lund E and Dahlberg JE (2005): Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci U S A 102:3627-3632.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K and Tuschl T (2001): Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:494-498.

el-Rifai W, Sarlomo-Rikala M, Miettinen M, Knuutila S and Andersson LC (1996): DNA copy number losses in chromosome 14: an early change in gastrointestinal stromal tumors. Cancer Res 56:3230-3233.

Erkko H, Xia B, Nikkila J, Schleutker J, Syrjakoski K, Mannermaa A, Kallioniemi A, Pylkas K, Karppinen SM, Rapakko K, Miron A, Sheng Q, Li G, Mattila H, Bell DW, Haber DA, Grip M, Reiman M, Jukkola-Vuorinen A, Mustonen A, Kere J, Aaltonen LA, Kosma VM, Kataja V, Soini Y, Drapkin RI, Livingston DM and Winqvist R (2007): A recurrent mutation in PALB2 in Finnish cancer families. Nature 446:316-319.

Erson AE, Niell BL, DeMers SK, Rouillard JM, Hanash SM and Petty EM (2001): Overexpressed genes/ESTs and characterization of distinct amplicons on 17q23 in breast cancer cells. Neoplasia 3:521-526.

Esquela-Kerscher A and Slack FJ (2006): Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6:259-269.

Ethier SP (2003): Identifying and validating causal genetic alterations in human breast cancer. Breast Cancer Res Treat 78:285-287.

Fan Y, Xin XY, Chen BL and Ma X (2006): Knockdown of RAB25 expression by RNAi inhibits growth of human epithelial ovarian cancer cells in vitro and in vivo. Pathology 38:561-567.

Fiscella M, Zhang H, Fan S, Sakaguchi K, Shen S, Mercer WE, Vande Woude GF, O'Connor PM and Appella E (1997): Wip1, a novel human protein phosphatase that is induced in response to ionizing radiation in a p53-dependent manner. Proc Natl Acad Sci U S A 94:6048-6053.

Fjeld CC and Denu JM (1999): Kinetic analysis of human serine/threonine protein phosphatase 2Calpha. J Biol Chem 274:20336-20343.

Fridlyand J, Snijders AM, Ylstra B, Li H, Olshen A, Segraves R, Dairkee S, Tokuyasu T, Ljung BM, Jain AN, McLennan J, Ziegler J, Chin K, Devries S, Feiler H, Gray JW, Waldman F, Pinkel D and Albertson DG (2006): Breast tumor copy number aberration phenotypes and genomic instability. BMC Cancer 6:96.

Fuku T, Semba S Yutori H, Yokozaki H (2007): Increased wild-type p53-induced phosphatase 1 (Wip1 or PPM1D) expression correlated with downregulation of checkpoint kinase 2 in human gastric carcinoma. Pathol Int 57:566-571.

Fujimoto H, Onishi N, Kato N, Takekawa M, Xu XZ, Kosugi A, Kondo T, Imamura M, Oishi I, Yoda A and Minami Y (2006): Regulation of the antioncogenic Chk2 kinase by the oncogenic Wip1 phosphatase. Cell Death Differ 13:1170-1180.

Garcia MJ, Pole JC, Chin SF, Teschendorff A, Naderi A, Ozdag H, Vias M, Kranjac T, Subkhankulova T, Paish C, Ellis I, Brenton JD, Edwards PA and Caldas C (2005): A 1 Mb minimal amplicon at 8p11-12 in breast cancer identifies new candidate oncogenes. Oncogene 24:5235-5245.

Gelsi-Boyer V, Orsetti B, Cervera N, Finetti P, Sircoulomb F, Rouge C, Lasorsa L, Letessier A, Ginestier C, Monville F, Esteyries S, Adelaide J, Esterni B, Henry C, Ethier SP, Bibeau F, Mozziconacci MJ, Charafe-Jauffret E, Jacquemier J, Bertucci F, Birnbaum D, Theillet C and Chaffanet M (2005): Comprehensive profiling of 8p11-12 amplification in breast cancer. Mol Cancer Res 3:655-667.

Giaccia AJ and Kastan MB (1998): The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev 12:2973-2983.

Goloudina A, Yamaguchi H, Chervyakova DB, Appella E, Fornace AJ,Jr and Bulavin DV (2003): Regulation of human Cdc25A stability by Serine 75 phosphorylation is not sufficient to activate a S phase checkpoint. Cell Cycle 2:473-478.

Gottlieb TM and Oren M (1996): P53 in Growth Control and Neoplasia. Biochim Biophys Acta 1287:77-102.

Gronwald J, Storkel S, Holtgreve-Grez H, Hadaczek P, Brinkschmidt C, Jauch A, Lubinski J and Cremer T (1997): Comparison of DNA gains and losses in primary renal clear cell carcinomas and metastatic sites: importance of 1q and 3p copy number changes in metastatic events. Cancer Res 57:481-487.

Gu W and Roeder RG (1997): Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell 90:595-606.

Haller F, Gunawan B, von Heydebreck A, Schwager S, Schulten HJ, Wolf-Salgo J, Langer C, Ramadori G, Sultmann H and Fuzesi L (2005): Prognostic role of E2F1 and members of the CDKN2A network in gastrointestinal stromal tumors. Clin Cancer Res 11:6589-6597.

Hanahan D and Weinberg RA (2000): The hallmarks of cancer. Cell 100:57-70.

Hang B and Singer B (2003): Protein-protein interactions involving DNA glycosylases. Chem Res Toxicol 16:1181-1195.

Harris H (1971): Cell fusion and the analysis of malignancy. Proc R Soc Lond B Biol Sci 179:1-20.

Harris H, Miller OJ, Klein G, Worst P and Tachibana T (1969): Suppression of malignancy by cell fusion. Nature 223:363-368.

Harrison M, Li J, Degenhardt Y, Hoey T and Powers S (2004): Wip1-deficient mice are resistant to common cancer genes. Trends Mol Med 10:359-361.

He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ and Hammond SM (2005): A microRNA polycistron as a potential human oncogene. Nature 435:828-833.

Hellman A, Zlotorynski E, Scherer SW, Cheung J, Vincent JB, Smith DI, Trakhtenbrot L and Kerem B (2002): A role for common fragile site induction in amplification of human oncogenes. Cancer Cell 1:89-97.

Hershko T, Korotayev K, Polager S and Ginsberg D (2006): E2F1 modulates p38 MAPK phosphorylation via transcriptional regulation of ASK1 and Wip1. J Biol Chem 281:31309-31316.

Hirasawa A, Saito-Ohara F, Inoue J, Aoki D, Susumu N, Yokoyama T, Nozawa S, Inazawa J and Imoto I (2003): Association of 17q21-q24 gain in ovarian clear cell adenocarcinomas with poor prognosis and identification of PPM1D and APPBP2 as likely amplification targets. Clin Cancer Res 9:1995-2004.

Hoeijmakers JH (2001): Genome maintenance mechanisms for preventing cancer. Nature 411:366-374.

Holst F, Stahl PR, Ruiz C, Hellwinkel O, Jehan Z, Wendland M, Lebeau A, Terracciano L, Al-Kuraya K, Janicke F, Sauter G and Simon R (2007): Estrogen receptor alpha (ESR1) gene amplification is frequent in breast cancer. Nat Genet 39:655-660.

Hornung V, Guenthner-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S, Noronha A, Manoharan M, Akira S, de Fougerolles A, Endres S and Hartmann G (2005): Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med 11:263-270.

Huang C, Ma WY, Maxiner A, Sun Y and Dong Z (1999): p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. J Biol Chem 274:12229-12235.

Huang X, Godfrey TE, Gooding WE, McCarty KS,Jr and Gollin SM (2006): Comprehensive genome and transcriptome analysis of the 11q13 amplicon in human oral cancer and synteny to the 7F5 amplicon in murine oral carcinoma. Genes Chromosomes Cancer 45:1058-1069.

Hyman E, Kauraniemi P, Hautaniemi S, Wolf M, Mousses S, Rozenblum E, Ringner M, Sauter G, Monni O, Elkahloun A, Kallioniemi OP and Kallioniemi A (2002): Impact of DNA amplification on gene expression patterns in breast cancer. Cancer Res 62:6240-6245.

Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Menard S, Palazzo JP, Rosenberg A, Musiani P, Volinia S, Nenci I, Calin GA, Querzoli P, Negrini M and Croce CM (2005): MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65:7065-7070.

Isola JJ, Kallioniemi OP, Chu LW, Fuqua SA, Hilsenbeck SG, Osborne CK and Waldman FM (1995): Genetic aberrations detected by comparative genomic hybridization predict outcome in node-negative breast cancer. Am J Pathol 147:905-911.

Jacobs JJ, Keblusek P, Robanus-Maandag E, Kristel P, Lingbeek M, Nederlof PM, van Welsem T, van de Vijver MJ, Koh EY, Daley GQ and van Lohuizen M (2000): Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. Nat Genet 26:291-299.

Jazayeri A, Falck J, Lukas C, Bartek J, Smith GC, Lukas J and Jackson SP (2006): ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat Cell Biol 8:37-45.

Jiang K, Pereira E, Maxfield M, Russell B, Goudelock DM and Sanchez Y (2003): Regulation of Chk1 includes chromatin association and 14-3-3 binding following phosphorylation on Ser-345. J Biol Chem 278:25207-25217.

Judge AD, Sood V, Shaw JR, Fang D, McClintock K and MacLachlan I (2005): Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotechnol 23:457-462.

Kallioniemi A, Kallioniemi OP, Piper J, Tanner M, Stokke T, Chen L, Smith HS, Pinkel D, Gray JW and Waldman FM (1994): Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. Proc Natl Acad Sci U S A 91:2156-2160.

Kao J and Pollack JR (2006): RNA interference-based functional dissection of the 17q12 amplicon in breast cancer reveals contribution of coamplified genes. Genes Chromosomes Cancer 45:761-769.

Kastan MB and Bartek J (2004): Cell-cycle checkpoints and cancer. Nature 432:316-323.

Kauraniemi P, Bärlund M, Monni O and Kallioniemi A (2001): New amplified and highly expressed genes discovered in the ERBB2 amplicon in breast cancer by cDNA microarrays. Cancer Res 61:8235-8240.

Kauraniemi P, Kuukasjarvi T, Sauter G and Kallioniemi A (2003): Amplification of a 280-kilobase core region at the ERBB2 locus leads to activation of two hypothetical proteins in breast cancer. Am J Pathol 163:1979-1984.

Kavli B, Sundheim O, Akbari M, Otterlei M, Nilsen H, Skorpen F, Aas PA, Hagen L, Krokan HE and Slupphaug G (2002): hUNG2 is the major repair enzyme for removal of uracil from U:A matches, U:G mismatches, and U in single-stranded DNA, with hSMUG1 as a broad specificity backup. J Biol Chem 277:39926-39936.

Kelland LR (1999): Small molecule anticancer drugs. IDrugs 2:550-552.

Keller D, Zeng X, Li X, Kapoor M, Iordanov MS, Taya Y, Lozano G, Magun B and Lu H (1999): The p38MAPK inhibitor SB203580 alleviates ultraviolet-induced phosphorylation

at serine 389 but not serine 15 and activation of p53. Biochem Biophys Res Commun 261:464-471.

Kim SO, Ono K, Tobias PS and Han J (2003): Orphan nuclear receptor Nur77 is involved in caspase-independent macrophage cell death. J Exp Med 197:1441-1452.

Kinzler KW and Vogelstein B (1996): Lessons from hereditary colorectal cancer. Cell 87:159-170.

Kinzler KW and Vogelstein B (1997): Cancer-susceptibility genes. Gatekeepers and caretakers. Nature 386:761-763.

Kinzler KW and Vogelstein B (1998): Landscaping the cancer terrain. Science 280:1036-1037.

Kivipensas P, Bjorkqvist AM, Karhu R, Pelin K, Linnainmaa K, Tammilehto L, Mattson K, Kallioniemi QP and Knuutila S (1996): Gains and losses of DNA sequences in malignant mesothelioma by comparative genomic hybridization. Cancer Genet Cytogenet 89:7-13.

Knudson AG (2001): Two genetic hits (more or less) to cancer. Nat Rev Cancer 1:157-162.

Knudson AG,Jr (1971): Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 68:820-823.

Knuutila S, Bjorkqvist AM, Autio K, Tarkkanen M, Wolf M, Monni O, Szymanska J, Larramendy ML, Tapper J, Pere H, El-Rifai W, Hemmer S, Wasenius VM, Vidgren V and Zhu Y (1998): DNA copy number amplifications in human neoplasms: review of comparative genomic hybridization studies. Am J Pathol 152:1107-1123.

Ko LJ and Prives C (1996): P53: Puzzle and Paradigm. Genes Dev 10:1054-1072.

Korn WM, Oide Weghuis DE, Suijkerbuijk RF, Schmidt U, Otto T, du Manoir S, Geurts van Kessel A, Harstrick A, Seeber S and Becher R (1996): Detection of chromosomal DNA gains and losses in testicular germ cell tumors by comparative genomic hybridization. Genes Chromosomes Cancer 17:78-87.

Kuo MT, Sen S, Hittelman WN and Hsu TC (1998): Chromosomal fragile sites and DNA amplification in drug-resistant cells. Biochem Pharmacol 56:7-13.

Kuwahara Y, Tanabe C, Ikeuchi T, Aoyagi K, Nishigaki M, Sakamoto H, Hoshinaga K, Yoshida T, Sasaki H and Terada M (2004): Alternative mechanisms of gene amplification in human cancers. Genes Chromosomes Cancer 41:125-132.

Kyriakis JM and Avruch J (2001): Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 81:807-869.

Kyriakis JM and Avruch J (1996): Sounding the alarm: protein kinase cascades activated by stress and inflammation. J Biol Chem 271:24313-24316.

Lacroix M, Toillon RA and Leclercq G (2006): P53 and Breast Cancer, an Update. Endocr Relat Cancer 13:293-325.

Lange CA, Shen T and Horwitz KB (2000): Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci U S A 97:1032-1037.

Lavoie JN, L'Allemain G, Brunet A, Muller R and Pouyssegur J (1996): Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J Biol Chem 271:20608-20616.

Levine AJ (1997): P53, the Cellular Gatekeeper for Growth and Division. Cell 88:323-331.

Li J, Yang Y, Peng Y, Austin RJ, van Eyndhoven WG, Nguyen KC, Gabriele T, McCurrach ME, Marks JR, Hoey T, Lowe SW and Powers S (2002): Oncogenic properties of PPM1D located within a breast cancer amplification epicenter at 17q23. Nat Genet 31:133-134.

Li QX, Ke N, Sundaram R and Wong-Staal F (2006): NR4A1, 2, 3--an orphan nuclear hormone receptor family involved in cell apoptosis and carcinogenesis. Histol Histopathol 21:533-540.

Liu X and Erikson RL (2003a): Polo-like kinase (Plk)1 depletion induces apoptosis in cancer cells. Proc Natl Acad Sci U S A 100:5789-5794.

Liu X and Erikson RL (2003b): Polo-like kinase 1 in the life and death of cancer cells. Cell Cycle 2:424-425.

Lopez-Girona A, Tanaka K, Chen XB, Baber BA, McGowan CH and Russell P (2001): Serine-345 is required for Rad3-dependent phosphorylation and function of checkpoint kinase Chk1 in fission yeast. Proc Natl Acad Sci U S A 98:11289-11294.:

Lothe RA, Karhu R, Mandahl N, Mertens F, Saeter G, Heim S, Borresen-Dale AL and Kallioniemi OP (1996): Gain of 17q24-qter detected by comparative genomic hybridization in malignant tumors from patients with von Recklinghausen's neurofibromatosis. Cancer Res 56:4778-4781.

Lu X, Bocangel D, Nannenga B, Yamaguchi H, Appella E and Donehower LA (2004): The p53-induced oncogenic phosphatase PPM1D interacts with uracil DNA glycosylase and suppresses base excision repair. Mol Cell 15:621-634.

Lu X, Nguyen TA, Appella E and Donehower LA (2004): Homeostatic regulation of base excision repair by a p53-induced phosphatase: linking stress response pathways with DNA repair proteins. Cell Cycle 3:1363-1366.

Lu X, Nannenga B and Donehower LA (2005): PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints. Genes Dev 19:1162-1174.

Lu X, Ma O, Nguyen TA, Jones SN, Oren M and Donehover LA (2007): The Wip1 phosphatase acts as a gatekeeper in the p53-Mdm2 autoregulatory loop. Cancer Cell 12:342-354.

Macaluso M, Montanari M, Cinti C and Giordano A (2005): Modulation of cell cycle components by epigenetic and genetic events. Semin Oncol 32:452-457.

Marchio A, Meddeb M, Pineau P, Danglot G, Tiollais P, Bernheim A and Dejean A (1997): Recurrent chromosomal abnormalities in hepatocellular carcinoma detected by comparative genomic hybridization. Genes Chromosomes Cancer 18:59-65.

Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K and Elledge SJ (2000): Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc Natl Acad Sci U S A 97:10389-10394.

McCluskey A, Sim AT and Sakoff JA (2002): Serine-threonine protein phosphatase inhibitors: development of potential therapeutic strategies. J Med Chem 45:1151-1175.

McKenna NJ and O'Malley BW (2002): Minireview: nuclear receptor coactivators--an update. Endocrinology 143:2461-2465.

McPherson K, Steel CM and Dixon JM (2000): ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. BMJ 321:624-628.

Meek DW (1998): New developments in the multi-site phosphorylation and integration of stress signalling at p53. Int J Radiat Biol 74:729-737.

Meeks-Wagner D and Hartwell LH (1986): Normal stoichiometry of histone dimer sets is necessary for high fidelity of mitotic chromosome transmission. Cell 44:43-52.

Melchionna R, Chen XB, Blasina A and McGowan CH (2000): Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. Nat Cell Biol 2:762-765.

Mendrzyk F, Radlwimmer B, Joos S, Kokocinski F, Benner A, Stange DE, Neben K, Fiegler H, Carter NP, Reifenberger G, Korshunov A and Lichter P (2005): Genomic and protein expression profiling identifies CDK6 as novel independent prognostic marker in medulloblastoma. J Clin Oncol 23:8853-8862.

Metzler M, Wilda M, Busch K, Viehmann S and Borkhardt A (2004): High expression of precursor microRNA-155/BIC RNA in children with Burkitt lymphoma. Genes Chromosomes Cancer 39:167-169.

Michael MZ, O'Connor SM, van Holst Pellekaan NG, Young GP and James RJ (2003): Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res 1:882-891.

Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM and Ding W (1994): A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266:66-71.

Mitra S, Izumi T, Boldogh I, Bhakat KK, Hill JW and Hazra TK (2002): Choreography of oxidative damage repair in mammalian genomes. Free Radic Biol Med 33:15-28.

Monni O, Bärlund M, Mousses S, Kononen J, Sauter G, Heiskanen M, Paavola P, Avela K, Chen Y, Bittner ML and Kallioniemi A (2001a): Comprehensive copy number and gene expression profiling of the 17q23 amplicon in human breast cancer. Proc Natl Acad Sci U S A 98:5711-5716.

Morerio C, Russo I, Rosanda C, Rapella A, Leszl A, Basso G, Maserati E, Pasquali F and Panarello C (2001): 17q21-qter trisomy is an indicator of poor prognosis in acute myelogenous leukemia. Cancer Genet Cytogenet 124:12-15.

Murnane JP and Sabatier L (2004): Chromosome rearrangements resulting from telomere dysfunction and their role in cancer. Bioessays 26:1164-1174.

Myllykangas S and Knuutila S (2006): Manifestation, mechanisms and mysteries of gene amplifications. Cancer Lett 232:79-89.

Nannenga B, Lu X, Dumble M, Van Maanen M, Nguyen TA, Sutton R, Kumar TR and Donehower LA (2006): Augmented cancer resistance and DNA damage response phenotypes in PPM1D null mice. Mol Carcinog 45:594-604.

Nevanlinna H and Bartek J (2006): The CHEK2 gene and inherited breast cancer susceptibility. Oncogene 25:5912-5919.

Nicholson JC, Ross FM, Kohler JA and Ellison DW (1999): Comparative genomic hybridization and histological variation in primitive neuroectodermal tumours. Br J Cancer 80:1322-1331.

Nigg EA (1998): Polo-like kinases: positive regulators of cell division from start to finish. Curr Opin Cell Biol 10:776-783.

O'Donnell KA, Wentzel EA, Zeller KI, Dang CV and Mendell JT (2005): c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435:839-843.

Oliva-Trastoy M, Berthonaud V, Chevalier A, Ducrot C, Marsolier-Kergoat MC, Mann C and Leteurtre F (2007): The Wip1 phosphatase (PPM1D) antagonizes activation of the Chk2 tumour suppressor kinase. Oncogene 26:1449-1458.

Ono K and Han J (2000): The p38 signal transduction pathway: activation and function. Cell Signal 12:1-13.

Orsetti B, Nugoli M, Cervera N, Lasorsa L, Chuchana P, Ursule L, Nguyen C, Redon R, du Manoir S, Rodriguez C and Theillet C (2004): Genomic and expression profiling of chromosome 17 in breast cancer reveals complex patterns of alterations and novel candidate genes. Cancer Res 64:6453-6460.

Otto E, McCord S and Tlsty TD (1989): Increased incidence of CAD gene amplification in tumorigenic rat lines as an indicator of genomic instability of neoplastic cells. J Biol Chem 264:3390-3396.

Pack SD, Karkera JD, Zhuang Z, Pak ED, Balan KV, Hwu P, Park WS, Pham T, Ault DO, Glaser M, Liotta L, Detera-Wadleigh SD and Wadleigh RG (1999): Molecular cytogenetic fingerprinting of esophageal squamous cell carcinoma by comparative genomic hybridization reveals a consistent pattern of chromosomal alterations.Genes Chromosomes Cancer 25:160-168.

Pearson MA and Fabbro D (2004): Targeting protein kinases in cancer therapy: a success? Expert Rev Anticancer Ther 4:1113-1124.

Pedeutour F, Simon MP, Minoletti F, Sozzi G, Pierotti MA, Hecht F and Turc-Carel C (1995): Ring 22 chromosomes in dermatofibrosarcoma protuberans are low-level amplifiers of chromosome 17 and 22 sequences. Cancer Res 55:2400-2403.

Pollack JR, Sorlie T, Perou CM, Rees CA, Jeffrey SS, Lonning PE, Tibshirani R, Botstein D, Borresen-Dale AL and Brown PO (2002): Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. Proc Natl Acad Sci U S A 99:12963-12968.

Ponder BA (2001): Cancer genetics. Nature 411:336-341.

Porkka KP, Pfeiffer MJ, Waltering KK, Vessella RL, Tammela TL and Visakorpi T (2007): MicroRNA expression profiling in prostate cancer. Cancer Res 67:6130-6135.

Prives C and Hall PA (1999): The p53 pathway. J Pathol 187:112-126.

Proia DA, Nannenga BW, Donehower LA and Weigel NL (2006): Dual roles for the phosphatase PPM1D in regulating progesterone receptor function. J Biol Chem 281:7089-7101.

Quelle DE, Zindy F, Ashmun RA and Sherr CJ (1995): Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell 83:993-1000.

Rao PH, Houldsworth J, Dyomina K, Parsa NZ, Cigudosa JC, Louie DC, Popplewell L, Offit K, Jhanwar SC and Chaganti RS (1998): Chromosomal and gene amplification in diffuse large B-cell lymphoma. Blood 92:234-240.

Rayter S, Elliott R, Travers J, Rowlands MG, Richardson TB, Boxall K, Jones K, Linardopoulos S, Workman P, Aherne W, Lord CJ and Ashworth A (2007): A chemical inhibitor of PPM1D that selectively kills cells overexpressing PPM1D. Oncogene (Epub ahead of print).

Reagan-Shaw S and Ahmad N (2005): Silencing of polo-like kinase (Plk) 1 via siRNA causes induction of apoptosis and impairment of mitosis machinery in human prostate cancer cells: implications for the treatment of prostate cancer. FASEB J 19:611-613.

Rennstam K, Ahlstedt-Soini M, Baldetorp B, Bendahl PO, Borg A, Karhu R, Tanner M, Tirkkonen M and Isola J (2003): Patterns of chromosomal imbalances defines subgroups of breast cancer with distinct clinical features and prognosis. A study of 305 tumors by comparative genomic hybridization. Cancer Res 63:8861-8868.

Ried T, Petersen I, Holtgreve-Grez H, Speicher MR, Schrock E, du Manoir S and Cremer T (1994): Mapping of multiple DNA gains and losses in primary small cell lung carcinomas by comparative genomic hybridization. Cancer Res 54:1801-1806.

Robinson DR, Wu YM and Lin SF (2000): The protein tyrosine kinase family of the human genome. Oncogene 55:5548-5557.

Roset R, Ortet L and Gil-Gomez G (2007): Role of Bcl-2 family members on apoptosis: what we have learned from knock-out mice. Front Biosci 12:4722-4730.

Ross JS and Fletcher JA (1999): The HER-2/neu oncogene: prognostic factor, predictive factor and target for therapy. Semin Cancer Biol 9:125-138.

Ross JS, Fletcher JA, Linette GP, Stec J, Clark E, Ayers M, Symmans WF, Pusztai L and Bloom KJ (2003): The Her-2/neu gene and protein in breast cancer 2003: biomarker and target of therapy. Oncologist 8:307-325.

Runnebaum IB, Nagarajan M, Bowman M, Soto D and Sukumar S (1991): Mutations in p53 as potential molecular markers for human breast cancer. Proc Natl Acad Sci U S A 88:10657-10661.

Sainsbury JR, Anderson TJ and Morgan DA (2000): ABC of breast diseases: breast cancer. BMJ 321:745-750.

Saito-Ohara F, Imoto I, Inoue J, Hosoi H, Nakagawara A, Sugimoto T and Inazawa J (2003a): PPM1D is a potential target for 17q gain in neuroblastoma. Cancer Res 63:1876-1883.

Sallinen SL, Sallinen P, Haapasalo H, Kononen J, Karhu R, Helen P and Isola J (1997): Accumulation of genetic changes is associated with poor prognosis in grade II astrocytomas. Am J Pathol 151:1799-1807.

Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K and Linn S (2004): Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 73:39-85.

Sanchez-Prieto R, Rojas JM, Taya Y and Gutkind JS (2000): A role for the p38 mitogen-acitvated protein kinase pathway in the transcriptional activation of p53 on genotoxic stress by chemotherapeutic agents. Cancer Res 60:2464-2472.

Savelyeva L and Schwab M (2001): Amplification of oncogenes revisited: from expression profiling to clinical application. Cancer Lett 167:115-123.

Schito ML, Demidov ON, Saito S, Ashwell JD and Appella E (2006): Wip1 phosphatase-deficient mice exhibit defective T cell maturation due to sustained p53 activation. J Immunol 176:4818-4825.

Schlee M, Hornung V and Hartmann G (2006): siRNA and isRNA: two edges of one sword. Mol Ther 14:463-470.

Schwab M (1998): Amplification of oncogenes in human cancer cells. Bioessays 20:473-479.

Schweighofer A, Hirt H and Meskiene I (2004): Plant PP2C phosphatases: emerging functions in stress signaling. Trends Plant Sci 9:236-243.

Sherr CJ (2004): Principles of tumor suppression. Cell 116:235-246.

Sherr CJ (1998): Tumor surveillance via the ARF-p53 pathway. Genes Dev 12:2984-2991.

Shiloh Y (2003): ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer 3:155-168.

Shreeram S, Demidov ON, Hee WK, Yamaguchi H, Onishi N, Kek C, Timofeev ON, Dudgeon C, Fornace AJ, Anderson CW, Minami Y, Appella E and Bulavin DV (2006a): Wip1 phosphatase modulates ATM-dependent signaling pathways. Mol Cell 23:757-764.

Shreeram S, Hee WK, Demidov ON, Kek C, Yamaguchi H, Fornace AJ,Jr, Anderson CW, Appella E and Bulavin DV (2006b): Regulation of ATM/p53-dependent suppression of myc-induced lymphomas by Wip1 phosphatase. J Exp Med 203:2793-2799.

Sinclair CS, Rowley M, Naderi A and Couch FJ (2003): The 17q23 amplicon and breast cancer. Breast Cancer Res Treat 78:313-322.

Sioud M (2005): Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. J Mol Biol 348:1079-1090.

Sluss HK, Armata H, Gallant J and Jones SN (2004): Phosphorylation of serine 18 regulates distinct p53 functions in mice. Mol Cell Biol 24:976-984.

Smith KA, Gorman PA, Stark MB, Groves RP and Stark GR (1990): Distinctive chromosomal structures are formed very early in the amplification of CAD genes in Syrian hamster cells. Cell 63:1219-1227.

Solinas-Toldo S, Wallrapp C, Muller-Pillasch F, Bentz M, Gress T and Lichter P (1996): Mapping of chromosomal imbalances in pancreatic carcinoma by comparative genomic hybridization. Cancer Res 56:3803-3807.

Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P and Lieberman J (2003): RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med 9:347-351.

Sonoda G, du Manoir S, Godwin AK, Bell DW, Liu Z, Hogan M, Yakushiji M and Testa JR (1997): Detection of DNA gains and losses in primary endometrial carcinomas by comparative genomic hybridization. Genes Chromosomes Cancer 18:115-125.

Spankuch B, Kurunci-Csacsko E, Kaufmann M and Strebhardt K (2007): Rational combinations of siRNAs targeting Plk1 with breast cancer drugs. Oncogene [Epub ahead of print].

Speirs V and Walker RA (2007): New perspectives into the biological and clinical relevance of oestrogen receptors in the human breast. J Pathol 211:499-506.

Stark GR and Wahl GM (1984): Gene amplification. Annu Rev Biochem 53:447-491.

Steger DJ and Workman JL (1999): Transcriptional analysis of purified histone acetyltransferase complexes. Methods 19:410-416.

Strumberg D and Seeber S (2005): Raf kinase inhibitors in oncology. Onkologie 28:101-107.

Sudbeck EA and Uckun FM (1999): Recent advances in JAK3 kinase inhibitors. IDrugs 2:1026-1030.

Takai N, Hamanaka R, Yoshimatsu J and Miyakawa I (2005): Polo-like kinases (Plks) and cancer. Oncogene 24:287-291.

Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T and Takahashi T (2004): Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 64:3753-3756.

Takekawa M, Adachi M, Nakahata A, Nakayama I, Itoh F, Tsukuda H, Taya Y and Imai K (2000): p53-inducible wip1 phosphatase mediates a negative feedback regulation of p38 MAPK-p53 signaling in response to UV radiation. EMBO J 19:6517-6526.

Takekawa M, Maeda T and Saito H (1998): Protein phosphatase 2Calpha inhibits the human stress-responsive p38 and JNK MAPK pathways. EMBO J 17:4744-4752.

Terris B, Meddeb M, Marchio A, Danglot G, Flejou JF, Belghiti J, Ruszniewski P and Bernheim A (1998): Comparative genomic hybridization analysis of sporadic neuroendocrine tumors of the digestive system. Genes Chromosomes Cancer 22:50-56.

Tirkkonen M, Tanner M, Karhu R, Kallioniemi A, Isola J and Kallioniemi OP (1998): Molecular cytogenetics of primary breast cancer by CGH. Genes Chromosomes Cancer 21:177-184.

Todd R and Wong DT (1999): Oncogenes. Anticancer Res 19:4729-4746.

Toledo F, Le Roscouet D, Buttin G and Debatisse M (1992): Co-amplified markers alternate in megabase long chromosomal inverted repeats and cluster independently in interphase nuclei at early steps of mammalian gene amplification. EMBO J 11:2665-2673.

van Diest PJ, Michalides RJ, Jannink L, van der Valk P, Peterse HL, de Jong JS, Meijer CJ and Baak JP (1997): Cyclin D1 expression in invasive breast cancer. Correlations and prognostic value. Am J Pathol 150:705-711.

Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ and Press M (2002): Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol 20:719-726.

Voorter C, Joos S, Bringuier PP, Vallinga M, Poddighe P, Schalken J, du Manoir S, Ramaekers F, Lichter P and Hopman A (1995): Detection of chromosomal imbalances in transitional cell carcinoma of the bladder by comparative genomic hybridization. Am J Pathol 146:1341-1354.

Wahl GM (1989): The importance of circular DNA in mammalian gene amplification. Cancer Res 49:1333-1340.

Waskiewicz AJ and Cooper JA (1995): Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. Curr Opin Cell Biol 7:798-805.

Weber RG, Bostrom J, Wolter M, Baudis M, Collins VP, Reifenberger G and Lichter P (1997): Analysis of genomic alterations in benign, atypical, and anaplastic meningiomas: toward a genetic model of meningioma progression. Proc Natl Acad Sci U S A 94:14719-14724.

Weber-Hall S, Anderson J, McManus A, Abe S, Nojima T, Pinkerton R, Pritchard-Jones K and Shipley J (1996): Gains, losses, and amplification of genomic material in rhabdomyosarcoma analyzed by comparative genomic hybridization. Cancer Res 56):3220-3224.

Windle B, Draper BW, Yin YX, O'Gorman S and Wahl GM (1991): A central role for chromosome breakage in gene amplification, deletion formation, and amplicon integration. Genes Dev 5:160-174.

Windle BE and Wahl GM (1992): Molecular dissection of mammalian gene amplification: new mechanistic insights revealed by analyses of very early events. Mutat Res 276:199-224.

Wintersberger E (1994): DNA amplification: new insights into its mechanism. Chromosoma 103:73-81.

Wolf M, Mousses S, Hautaniemi S, Karhu R, Huusko P, Allinen M, Elkahloun A, Monni O, Chen Y, Kallioniemi A and Kallioniemi OP (2004): High-resolution analysis of gene copy number alterations in human prostate cancer using CGH on cDNA microarrays: impact of copy number on gene expression. Neoplasia 6:240-247.

Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C and Micklem G (1995): Identification of the breast cancer susceptibility gene BRCA2. Nature 378:789-792.

Wright JA, Smith HS, Watt FM, Hancock MC, Hudson DL and Stark GR (1990): DNA amplification is rare in normal human cells. Proc Natl Acad Sci U S A 87:1791-1795.

Wu G, Sinclair C, Hinson S, Ingle JN, Roche PC and Couch FJ (2001): Structural analysis of the 17q22-23 amplicon identifies several independent targets of amplification in breast cancer cell lines and tumors. Cancer Res 61:4951-4955.

Wu GJ, Sinclair CS, Paape J, Ingle JN, Roche PC, James CD and Couch FJ (2000): 17q23 amplifications in breast cancer involve the PAT1, RAD51C, PS6K, and SIGma1B genes. Cancer Res 60:5371-5375.

Yamaguchi H, Durell SR, Feng H, Bai Y, Anderson CW and Appella E (2006): Development of a substrate-based cyclic phosphopeptide inhibitor of protein phosphatase 2Cdelta, Wip1. Biochemistry 45:13193-13202.

Yu Q, Geng Y and Sicinski P (2001): Specific protection against breast cancers by cyclin D1 ablation. Nature 411:1017-1021.

Zhang Y, Beck CA, Poletti A, Clement JP,4th, Prendergast P, Yip TT, Hutchens TW, Edwards DP and Weigel NL (1997): Phosphorylation of human progesterone receptor by cyclin-dependent kinase 2 on three sites that are authentic basal phosphorylation sites in vivo. Mol Endocrinol 11:823-832.

Zhao H and Piwnica-Worms H (2001): ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol 21:4129-4139.

Zhou BB and Elledge SJ (2000): The DNA damage response: putting checkpoints in perspective. Nature 408:433-439.

Zody MC, Garber M, Adams DJ, Sharpe T, Harrow J, Lupski JR, Nicholson C, Searle SM, Wilming L, Young SK, Abouelleil A, Allen NR, Bi W, Bloom T, Borowsky ML, Bugalter BE, Butler J, Chang JL, Chen CK, Cook A, Corum B, Cuomo CA, de Jong PJ, DeCaprio D, Dewar K, FitzGerald M, Gilbert J, Gibson R, Gnerre S, Goldstein S, Grafham DV, Grocock R, Hafez N, Hagopian DS, Hart E, Norman CH, Humphray S, Jaffe DB, Jones M, Kamal M, Khodiyar VK, LaButti K, Laird G, Lehoczky J, Liu X, Lokyitsang T, Loveland J, Lui A, Macdonald P, Major JE, Matthews L, Mauceli E, McCarroll SA, Mihalev AH, Mudge J, Nguyen C, Nicol R, O'Leary SB, Osoegawa K, Schwartz DC, Shaw-Smith C, Stankiewicz P, Steward C, Swarbreck D, Venkataraman V, Whittaker CA, Yang X, Zimmer AR, Bradley A, Hubbard T, Birren BW, Rogers J, Lander ES and Nusbaum C (2006): DNA

sequence of human chromosome 17 and analysis of rearrangement in the human lineage. Nature 440:1045-1049.

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High-level amplification at 17q23 leads to coordinated overexpression of multiple adjacent genes in breast cancer

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Increased copy numbers of 17q23 chromosomal region have been shown to occur in different tumour types and to be associated with tumour progression and with poor prognosis. Several genes have earlier been proposed as potential oncogenes at this region largely on the grounds of cell lines studies. In this study, we performed a systematic gene expression survey on 26 primary breast tumours with known 17q23 amplification status by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The 17q23 amplicon is restricted to an ~5 MB region in breast cancer and contains 29 known genes. Our survey revealed a statistically significant ($P<0.01$) difference between the high level and no amplification groups in a set of eleven genes whereas no difference between the moderate and the non-amplified tumour groups were observed. Interestingly, these 11 genes were located adjacent to one another within a 1.56 Mb core region in which all except one of the genes were overexpressed. These data suggest that only high-level amplification at the 17q23 amplicon core leads to elevated gene expression in breast cancer. Moreover, our results highlight the fact that 17q23 amplicon carries multiple candidate genes and that this may be a more common event in gene amplification than previously thought.
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Gene amplification is one of the major mechanisms that allow cancer cells to promote expression of genes that are involved in tumour development and progression. Oncogene overexpression as a result of gene amplification has been shown to play a crucial part in the pathogenesis of various human malignancies, especially in solid tumours such as breast, prostate, lung, ovarian, gastric, pancreatic, and colon cancers (Knuutila *et al*, 1998). The first illustration on the clinical significance of amplified oncogenes in human cancer was the discovery of an association between amplification of the *MYC* oncogene and more aggressive neuroblastoma variants (Schwab, 1993). To date, one of the clinically most important amplified gene is the *ERBB2* oncogene, which is well known for its central role as a prognostic and predictive factor in breast cancer as well as a therapeutic target (Baselga *et al*, 1999; Cobleigh *et al*, 1999; Ross and Fletcher, 1999; Vogel *et al*, 2002). These examples illustrate that genes that are altered by amplification in cancer are likely to have an impact on both disease pathogenesis and the clinical management of cancer patients.

Amplification of the chromosomal region 17q23 was first discovered in breast cancer (Kallioniemi *et al*, 1994). After that, numerous studies have reported increased copy numbers of 17q23 in tumours of brain, lung, ovary, pancreas, bladder, testis, and liver (Muleris *et al*, 1994; Ried *et al*, 1994; Korn *et al*, 1996; Solinas-Toldo *et al*, 1996; Brinkschmidt *et al*, 1997; Marchio *et al*, 1997; Richter *et al*, 1997; Schwendel *et al*, 1997; Sonoda *et al*, 1997; Weber *et al*, 1997; Vandesompele *et al*, 1998; Clark *et al*, 2002; Willis *et al*, 2003). We recently performed an extensive survey on the distribution and frequency of the 17q23 copy number increases in 3520 tumours representing 166 different tumour types (Andersen *et al*, 2002). The results confirmed previous data and indicated that increased 17q23 copy number occurs most commonly in brain, lung, breast, ovarian, urinary bladder, and soft tissue tumours, although high-level amplification was observed exclusively in breast cancer. Importantly, the increased copy number of 17q23 region has also been associated with tumour progression (Andersen *et al*, 2002) and with poor prognosis in breast cancer (Isola *et al*, 1995; Barlund *et al*, 2000a), ovarian clear cell adenocarcinoma (Hirasawa *et al*, 2003), neuroblastoma (Saito-Ohara *et al*, 2003), and acute myelogenous leukaemia (Morerio *et al*, 2001). Taken together, these data suggest that the genes affected by the 17q23 amplification contribute to cancer pathogenesis.

In breast cancer, several studies have been performed to define the limits of the 17q23 amplicon (Barlund *et al*, 1997, 2000b; Couch *et al*, 1999; Erson *et al*, 2001; Monni *et al*, 2001; Wu *et al*, 2001). Based on the combination of data from these studies and the current information available in the human genome databases (http://www.ncbi.nlm.nih.gov/mapview/ and http://www.ensembl.org/Homo_sapiens/index.html), the amplicon is considerably large covering an approximately 5 Mb region at 17q23. A number of studies have also aimed to uncover the possible target genes for this amplification, mainly by looking at correlation between amplification and high-level mRNA expression. One of the first

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genes to be identified as a potential oncogene in this region was the ribosomal protein S6 kinase, *RPS6KB1* (Couch *et al*, 1999; Barlund *et al*, 2000a). Thereafter several other putative target genes have been proposed including *APPBP2* (also known as *PAT1*), *RAD51C*, *TBX2*, *TRIM37* (*MUL*), *THRAP1* (*TRAP240*), *PPM1D*, and *BRIP1* (Barlund *et al*, 2000b; Wu *et al*, 2000, 2001; Erson *et al*, 2001; Monni *et al*, 2001; Bulavin *et al*, 2002; Li *et al*, 2002). However, these studies were carried out at a time when the genome sequence of this region was incomplete and thus all genes within this region had not been identified. In addition, these previous studies mainly concentrated on the analysis of established breast cancer cell lines and the expression levels of only five genes from this region have been evaluated in a small number of primary breast tumours (Couch *et al*, 1999; Barlund *et al*, 2000b; Li *et al*, 2002).

In the present study, we aimed to further characterise the molecular consequences of the 17q23 amplification on gene expression levels in primary breast tumours. To this end, all known genes localised within the 5 Mb amplicon at 17q23 were obtained from publicly available databases and their expression levels were measured in 26 primary breast tumours using quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). Our data revealed that high-level amplification at 17q23 in primary breast cancer leads to coordinated overexpression of 11 adjacent genes located at a 1.56 Mb central region of the amplicon.

MATERIALS AND METHODS

Primary tumours and breast cancer cell lines

Freshly frozen primary breast tumour specimens and corresponding paraffin-embedded tissue samples from 26 breast cancer patients were acquired from the Department of Pathology, Tampere University Hospital. These samples represent a subset of a larger material that has been described in detail previously (Rauta *et al*, 2006). The clinicopathological characteristics of these 26 tumour samples are presented in Table 1. The mean age of the patients at diagnosis was 62 years (range 38–84). The patients had not received any therapy before tumour removal. The use of these specimens in this study was approved by the Ethics Committee of the Pirkanmaa Hospital District and by the National Authority for Medicolegal Affairs in Finland.

Two breast cancer cell lines BT-474 and MCF7 were included in this study. Pancreatic cancer cell lines PANC-1 and HUPT3 as well as prostate cancer cell lines DU145 and PC-3 were used as controls in RT-PCR analyses. HUPT3 cell line came from the German Collection of Micro-organisms and Cell Cultures (Braunschweig, Germany), all other cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were cultured under recommended conditions. Normal testis and pancreas cDNA (Ambion, Foster City, CA, USA) were also used as controls in RT-PCR analyses. Human Mammary Gland (HMG) cDNA was from BD Biosciences Clontech (Palo Alto, CA, USA).

Copy number analysis by fluorescence *in situ* hybridisation

Three pairs of bacterial artificial chromosome (BAC) clones corresponding to the 17q23 chromosome region (probe set A: RP11-579A4 and RP11-579O24; probe set B: RP11-634F5 and RP11-1081E4; probe set C: RP11-269G24 and CTD-2501B8) were identified from public databases (http://www.ncbi.nlm.nih.gov/mapview/ and http://www.ensembl.org). BAC DNA was labelled with SpectrumOrange-dUTP (Vysis, Downers Grove, IL, USA) by random priming and a SpectrumGreen labelled chromosome 17 probe (Vysis) was used as a reference. Fluorescence in situ hybridisation (FISH) to normal metaphase chromosomes was performed to confirm that the clone contigs recognised a single copy target at 17q23. Fluorescence in situ hybridisation on paraffin-embedded breast tumour samples was performed using a modified Paraffin Pretreatment Reagent kit protocol (Vysis) as described previously (Rauta *et al*, 2006). Hybridisation signals were analysed using an Olympus BX50 epifluorescence microscope (Tokyo, Japan) using an $\times 60$ objective (NA 1.4). Specimens containing a three to fivefold increase in the number of gene specific probe signals, as compared with the chromosome 17 centromere signals, were considered to be moderately amplified. In addition, specimens containing a fivefold or higher increase in the number of *PPM1D* signals or tight clusters of signals were considered to be highly amplified. The results using the RP11-634F5 and RP11-1081E4 BAC contigs were included in a previous publication (Rauta *et al*, 2006).

Table 1 Clinicopathological characteristics of 26 primary breast tumours

Variable	*n*	%
Tumour type		
Infiltrating ductal	22	84.6
Infiltrating lobular	4	15.4
Tumour size		
<20 mm	12	46.2
$\geqslant 20$ mm	14	53.8
Histological grade[a]		
I	0	0.0
II	8	36.4
III	14	63.6
Stage		
T1	11	42.3
T2	5	19.2
T3	4	15.4
T4	6	23.1
Nodal status		
Negative	12	46.2
Positive	11	42.3
Not available	3	11.5
ER		
Positive	21	81.8
Negative	5	19.2
PR		
Positive	13	50.0
Negative	13	50.0
ERBB2		
Negative	21	80.8
Positive	5	19.2

[a]Histological grade was determined only from the infiltrating ductal carcinomas.

RNA isolation and cDNA synthesis

Total RNA was extracted from cancer cell lines using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. For primary tumour samples, a representative tumour area was selected from freshly frozen tumour specimen based on haematoxylin–eosin stained tissue section and a core-biopsy ($\varnothing$ 2 mm) was obtained for RNA isolation. Tumour tissues were homogenised with a syringe and a needle (20G, $\varnothing$ 0.9 mm) and total RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). First-strand cDNA synthesis for both cell line and primary tumour RNAs was performed using SuperScript II reverse transcriptase and random hexamer primers (Invitrogen).

Expression screen by RT-PCR

Gene specific primers for 29 genes from the 17q23 region (primer sequences are available on request) were obtained from TIB MolBiol (Berlin, Germany). The PCR contained 1 × PCR Gold buffer (Applied Biosystems, Foster city, CA, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs each, 0.2–0.4 mM gene specific primers, 1.5–2.5 units of Amplitaq Gold DNA polymerase (Applied Biosystems), and 1 μl cDNA template (MCF7 or BT474) adjusted to 50 μl with sterile H_2O. The PCR programme began with initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 60 s and elongation at 72°C for 60 s, with final elongation at 72°C for 10 min. The PCR products were run on a 1.5% agarose gel. For those genes with no detectable expression in MCF7 and BT-474 cells, the functionality of the PCR primers was verified using cDNA from PANC-1, HUPT3, DU145, PC-3, normal testis or normal pancreas as a template.

Quantitative real-time RT-PCR

DNA Hybridisation Probe Sets for 24 genes at 17q23 and the housekeeping gene TATA-box-binding protein (*TBP*) were obtained from TIB MolBiol (Berlin, Germany). The PCR reactions were performed in the LightCycler apparatus using the LC FastStart DNA Hybridisation Probes Kit according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Briefly, the PCR contained 1 × LightCycler FastStart DNA Master HybProbe mix (Roche Applied Science, Mannheim, Germany), 4 mM $MgCl_2$, 0.2–0.4 μM gene specific probes, 0.8 μM gene specific primers, and 1.5 μl cDNA template adjusted to 20 μl with sterile H_2O. After 10 min of initial denaturation at 95°C, the cycling conditions (55 cycles) were as follows: denaturation at 95°C for 10 s, annealing at 55–58°C for 10–20 s, and elongation at 72°C for 6–12 s depending on the gene of interest. Quantitative analysis was performed using the LightCycler software according to the fit-point method as described earlier (Kauraniemi *et al*, 2003). The expression levels were normalised against the housekeeping gene *TBP*.

Statistical analysis

For each gene, two-tailed Mann–Whitney *U*-test was used to examine the possible statistical significance of differences in expression levels between the three tumour groups. Three separate comparisons (high *vs* moderate, high *vs* no, and moderate *vs* no amplification tumour group) were made.

RESULTS

Primary tumour selection and copy number analysis

We had previously determined the DNA copy number levels within the centre of the 17q23 amplicon (at the *PPM1D* gene locus) in a set of 146 primary breast tumours by using formalin-fixed, paraffin-embedded samples (Rauta *et al*, 2006). On the basis of these data, a total of 26 tumours, including eight cases with increased copy number and 18 tumours with no copy number increase, were selected for this study owing to the availability of freshly frozen tumour material required for the mRNA expression analyses. To determine further the 17q23 amplification status in this set of samples, we used additional probe pairs from both ends of the 5 Mb amplicon and determined their copy number levels by FISH (Table 2). As might be expected, the 18 non-amplified tumours did not show copy number increase with the additional probe sets either (data not shown). The eight amplified cases demonstrated more or less consistent copy number changes across the entire amplicon and could now be classified into groups with high ($n=4$) and moderate ($n=4$) level of amplification (Table 2).

Transcript identification

On the basis of previous studies (Barlund *et al*, 1997, 2000b; Couch *et al*, 1999; Erson *et al*, 2001; Monni *et al*, 2001; Wu *et al*, 2001) and the current genome sequence information (http://www.ncbi.nlm.nih.gov/mapview/ and http://www.ensembl.org/Homo_sapiens/index.html), the 17q23 amplicon is restricted to an ~5 Mb region (53.95–59.02 Mb) and contains 29 genes with known function (Figure 1, Table 3). First, the expression levels of these 29 genes were screened by regular RT-PCR in MCF7 and BT-474 breast cancer cell lines that are known to harbour high level 17q23 amplification (Couch *et al*, 1999; Barlund *et al*, 2000a; Monni *et al*, 2001). Five (*SEPT4, TEX14, CA4, MRC2,* and *KCNH6*) of the 29 genes had either very low or undetectable expression in both MCF7 and BT-474 cells (data not shown) and were thus excluded from further analyses based on the fact that a potential amplification target gene is expected to show elevated mRNA expression.

Expression screen in primary breast tumours

The expression levels of the remaining 24 genes from the 17q23 amplicon were then measured in the 26 primary breast tumours using qRT-PCR. Two genes, *PTRH2* and *ACE*, showed considerably higher expression levels than any other gene within this region

Table 2 Copy number levels at the 17q23 amplicon relative to chromosome 17 centromere in eight primary breast tumours by FISH

	Probe sets[a]		
Tumour number	**A[b]**	**B[c]**	**C[d]**
High			
133	1.5	Amp[e]	3
206	Amp[e]	Amp[e]	2.5
209	Amp[e]	Amp[e]	Amp[e]
236	1	>5	1
Moderate			
249	1.5	3.5	1
251	3	3.5	3.5
255	2	3	2
256	3.5	3.5	3

FISH = fluorescence in situ hybridisation. [a]See Materials and Methods. [b]Includes *RAD51C-PPM1E genes*. [c]Includes *PPM1D gene*. [d]Includes *CYB561-ACE-KCNH6-WDR68* genes. [e]Tight cluster of signals indicative of amplification.

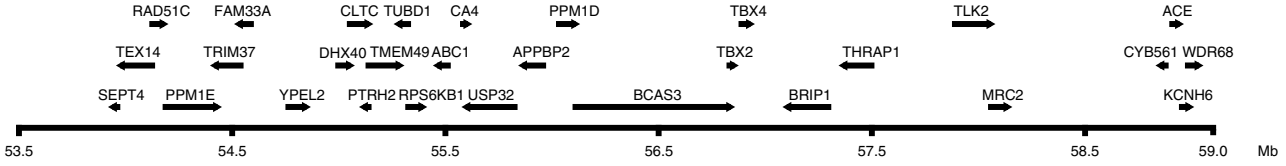

Figure 1 Physical map of the 17q23 amplicon. The known genes mapping to the ~5 Mb minimal region of amplification at 17q23 are represented using horizontal lines and their orientation is indicated with arrowheads.

Table 3 List of the 29 known genes located in the 5 Mb minimal region of amplification at 17q23 and their minimum, maximum and median expression levels in 26 primary breast tumours

Gene	Description	Min	Max	Median
SEPT4	Peanut-like protein 2	ND	ND	ND
TEX14	Testis expressed sequence 14	ND	ND	ND
RAD51C	DNA repair protein RAD51 homolog	0.0	1.9	0.3
PPM1E	Protein phosphatase 1E	0.0	1.7	0.0
TRIM37	Tripartite motif-containing 37 protein	0.0	1.1	0.2
FAM33A	Family with sequence similarity 33, member A	0.0	3.3	0.3
YPEL2	Yippee-like 2 protein	0.1	1.0	0.3
DHX40	DEAH (Asp-Glu-Ala-His) box polypeptide 40	0.0	4.5	0.2
CLTC	Clathrin heavy chain 1	0.0	3.7	0.4
PTRH2	Peptidyl-tRNA hydrolase 2	0.1	14.8	1.4
TMEM49	Transmembrane protein 49	0.0	1.2	0.2
TUBD1	Tubulin δ chain	0.0	2.4	0.3
RPS6KB1	Ribosomal protein S6 kinase	0.0	1.0	0.2
ABC1	Amplified in breast cancer	0.0	1.7	0.3
CA4	Carbonic anhydrase IV precursor	ND	ND	ND
USP32	Ubiquitin C-terminal hydrolase 32	0.0	1.5	0.3
APPBP2	Amyloid β precursor protein binding protein 2	0.0	1.6	0.1
PPM1D	Protein phosphatase 2C δ isoform, magnesium-dependent	0.0	2.8	0.2
BCAS3	Breast carcinoma amplified sequence 3	0.1	2.7	0.3
TBX2	T-box transcription factor TBX2	0.0	0.7	0.1
TBX4	T-box transcription factor TBX4	0.0	9.0	0.3
BRIP1	BRCA1 interacting protein C-terminal helicase 1	0.0	2.1	0.2
THRAP1	Thyroid hormone receptor-associated protein	0.0	1.1	0.2
TLK2	Serine/threonine-protein kinase tousled-like 2	0.1	1.6	0.3
MRC2	Mannose receptor, C type 2	ND	ND	ND
CYB561	Cytochrome b561	0.3	9.2	1.3
ACE	Angiotensin-converting enzyme, somatic isoform precursor	4.0	233.0	32.1
KCNH6	Potassium voltage-gated channel, subfamily H, member 6	ND	ND	ND
WRD68	WD-repeat protein 68	0.1	1.8	0.8

ND = not determined (low or now expression in breast cancer cell lines MCF7 and BT-474 with high-level amplification).

(Table 3). However, *ACE* was also expressed at a similar level in normal HMG, and therefore does not appear to be interesting as putative target gene for 17q23 amplification. The expression levels of *PPM1E* were very low across all 26 tumour samples and, additionally, both *PPM1E* and *TBX4* showed highest expression levels in non-amplified tumour samples (Table 3, Figure 2).

Finally, the expression levels of the 24 genes within the 17q23 amplicon were compared between the three tumour groups. A statistically significant ($P<0.01$) difference between the high level and no amplification groups were observed for a set of 11 genes (*FAM33A*, *DHX40*, *CLTC*, *PTRH2*, *TMEM49*, *TUBD1*, *RPS6KB1*, *ABC1*, *USP32*, *APPBP2*, and *PPM1D*) (Figure 3). All of these, except *FAM33A* and *USP32*, also showed significant difference ($P<0.05$) in expression levels between the high-and moderate-level amplification groups suggesting that these genes are activated by high-level copy number increases (Figure 3). No difference between the moderate and the no amplification tumour groups were observed. Interestingly, these 11 genes are located adjacent to each other within a 1.56 Mb region (54.54 – 56.10 Mb) at the centre of the 17q23 amplicon (Figures 1 and 2). It was even more intriguing that only a single gene, *YPEL2*, within this region did not demonstrate an association between amplification and increased mRNA expression.

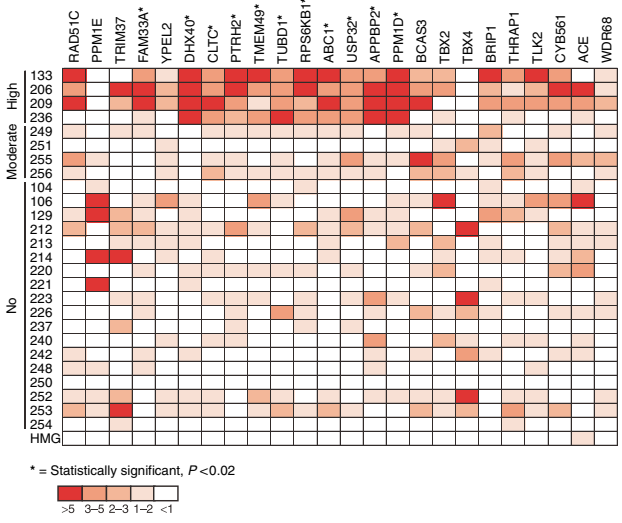

Figure 2 Schematic representation of expression levels of 24 known genes within the 17q23 chromosomal region in 26 primary breast tumours and normal HMG. Primary tumours are arranged according to their 17q23 amplification status and genes are organised based on their physical order at the 17q23 region from centromere to telomere. Expression levels were determined using qRT-PCR and were normalised against a housekeeping gene *TBP*. The relative expression values of each gene were median-corrected and displayed using a colour code (shown at the bottom).

DISCUSSION

Several studies have illustrated recurrent amplification of 17q23 in various human tumours and its association to poor clinical outcome (Isola *et al*, 1995; Barlund *et al*, 2000a; Morerio *et al*, 2001; Andersen *et al*, 2002; Hirasawa *et al*, 2003; Saito-Ohara *et al*, 2003). These studies have proposed multiple genes from this region to be important for cancer development and progression and thus promising targets for diagnostic, prognostic, and therapeutic approaches. However, no systematic survey of all genes within the 17q23 region has earlier been performed in primary tumour material. Here 29 known genes located within the

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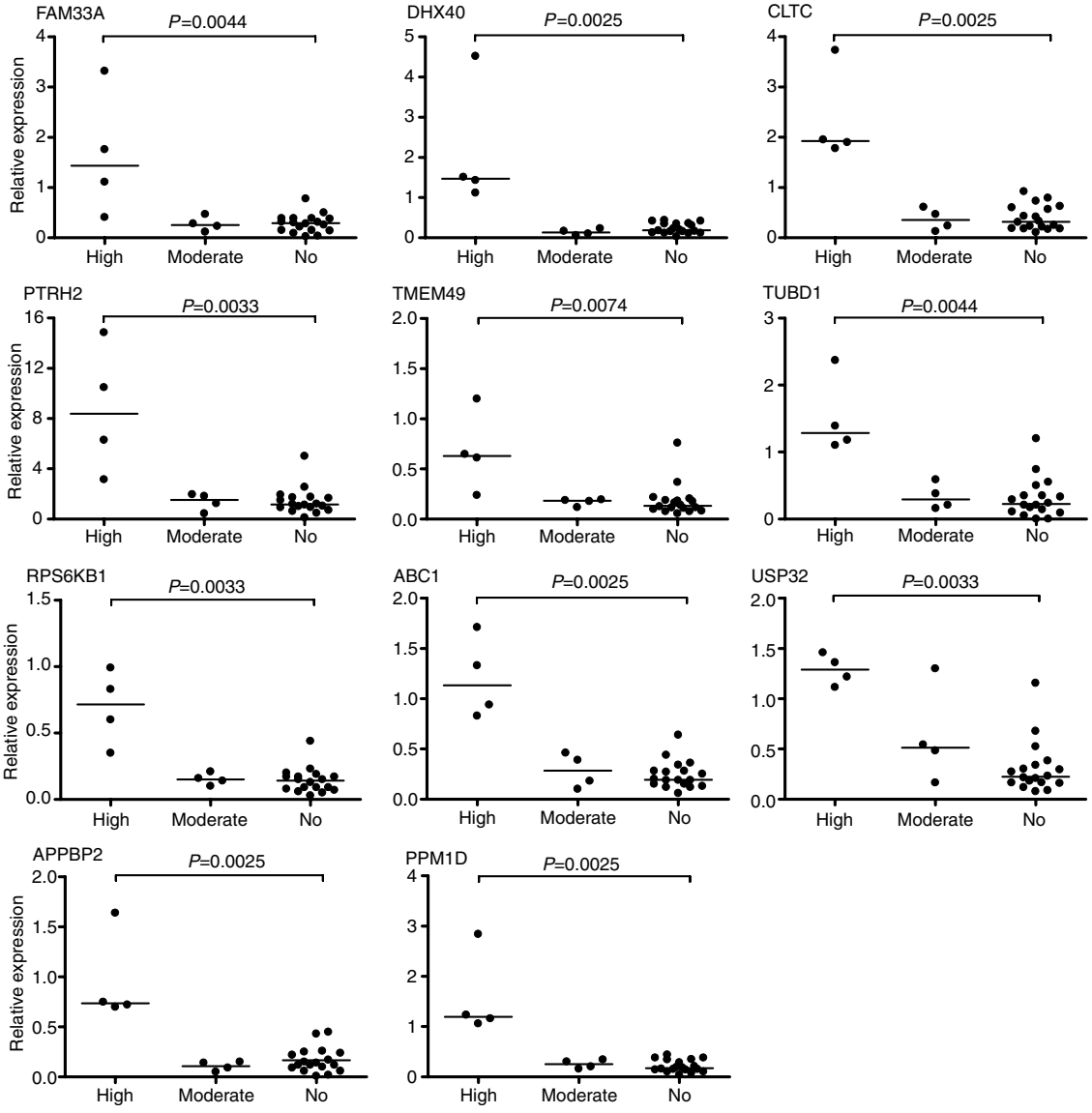

Figure 3 Comparison of *FAM33A, DHX40, CLTC, PTRH2, TMEM49, TUBD1, RPS6KB1, ABC1, USP32, APPBP2,* and *PPM1D* expression in highly, moderately, and no amplification tumour groups by qRT-PCR. Median value of expression is indicated by a horizontal line. Two-tailed Mann–Whitney *U*-test was used to assess the statistical significance of differences in expression levels between tumour groups. *P*-values between highly non-amplified groups are shown.

common amplified segment at 17q23 were defined. Five of these were excluded from further analysis, because they showed very low or undetectable expression in cell lines with high level amplification. The expression levels of the remaining 24 genes were then examined using qRT-PCR in 26 primary breast tumours to assess their role as putative target genes for 17q23 amplification in breast cancer.

Our systematic study revealed eleven genes (*FAM33A*, *DHX40*, *CLTC*, *PTRH2*, *TMEM49*, *TUBD1*, *RPS6KB1*, *ABC1*, *USP32*, *APPBP2*, *PPM1D*) that showed significantly ($P<0.01$) higher expression levels in primary breast tumours with high level 17q23 amplification compared to tumours without amplification. Interestingly, these eleven genes are all located within a 1.56 Mb region at the centre of the 17q23 amplicon. The amplicon core also includes one additional gene, *YPEL2*, but for unknown reasons it does not demonstrate copy number dependent overexpression. As only very little is known about the function of this gene, it is not possible to speculate on the basis of this phenomenon. Overall, our findings are in good agreement with previous studies that have implicated gene copy number alterations as significant determinants of gene expression patterns (Hyman *et al*, 2002; Pollack *et al*, 2002; Wolf *et al*, 2004; Fridlyand *et al*, 2006). However, it has to be noted that the tumour group with moderate amplification showed expression levels similar to those seen in the non-amplified tumours, indicating that low level copy number increases at this region did not have a significant effect on gene expression levels. This finding is supported by recent studies demonstrating that the high-level amplifications but not copy number gains are associated with poor prognosis in various tumour types (Sticht *et al*, 2005; Staebler *et al*, 2006). In summary, the qRT-PCR screen demonstrated that high level amplification at 17q23 in breast cancer leads to concomitant overexpression of virtually all genes within the amplicon core.

Several genes have earlier been proposed as putative target genes of the 17q23 amplification. These include *RPS6KB1*, *APPBP2,* and *PPM1D* (Couch *et al*, 1999; Barlund *et al*, 2000a, b; Wu *et al*, 2001; Bulavin *et al*, 2002; Li *et al*, 2002) that also showed strong association between amplification and increased expression in our

primary tumour material. Earlier studies have also implicated *RAD51C, TRIM37, TBX2, THRAP1*, and *BRIP1* as genes whose expression correlates with amplification (Barlund *et al*, 2000b; Wu *et al*, 2000, 2001; Erson *et al*, 2001; Monni *et al*, 2001). In our tumour series, these genes did also show elevated expression in some samples with high level amplification, but the difference in expression levels between the high and no amplification tumour groups, did not reach statistical significance. This finding might reflect the fact that we focussed solely on the analysis of primary tumour material instead of established breast cancer cell lines that were mainly used in these previous studies. Finally, our data revealed seven other protein coding genes (*FAM33A*, *DHX40*, *CLTC*, *PTRH2*, *TMEM49*, *TUBD1*, *ABC1*, and *USP32*) that have not been implicated previously in 17q23 studies, but whose expression was clearly associated with copy number status in primary breast tumours. Taken together, our results highlight a set of new genes that are overexpressed because of the 17q23 amplification in breast cancer and thus might contribute to the tumour phenotype.

Similar observations on overexpression of multiple genes within an amplicon have been reported previously in several occasions. For example, recent studies identified a 1 Mb segment of common amplification at 8p11–12 in breast cancer and demonstrated that it contains at least 14 candidate genes showing a strong correlation between amplification and overexpression (Garcia *et al*, 2005; Gelsi-Boyer *et al*, 2005). At 11q13, which is amplified in a wide variety of tumours (Saunders *et al*, 2000; Gollin, 2001, 2004), Huang *et al* (2006) constructed a detailed 3.6 Mb map of the amplicon core and showed that most of the genes in that region are overexpressed in amplified tumours and cell lines. Similarly, we have demonstrated that amplification of a 280 kb minimal common region at 17q12 in breast cancer leads to simultaneous increase of expression levels of all genes within that genomic segment (Kauraniemi *et al*, 2001). In general, these data implicate that despite the size of the affected region, the vast majority of the genes within amplicons are overexpressed and hence amplification events typically lead to overexpression of multiple genes. Owing to this tight association between amplification and elevated gene expression, it is difficult to determine which of the genes within particular amplicon actually contribute to tumour pathogenesis. It is likely that all of the overexpressed genes are not critical for cancer progression but are overexpressed merely because of their location within the amplicon. However, it is also possible that, instead of a single target gene, amplicons are driven by a set of genes whose simultaneous overexpression provides growth advantage to cancer cells (Huang *et al*, 2006; Kao and Pollack, 2006).

Traditionally, the search for amplification target genes has been based on two primary schemes; namely location within the maximal amplitude of the amplicon and overexpression of the putative target gene. Our results demonstrate that a whole set of genes, 11 of the 12 known genes within the 1.56 Mb core region, is overexpressed in primary breast tumours with high level amplification at 17q23. Owing to such coordinated overexpression of genes in the amplification core, overexpression alone is not sufficient to highlight putative target genes but functional studies are required to uncover the possible biological and clinical significance of candidate genes.

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REFERENCES

Andersen CL, Monni O, Wagner U, Kononen J, Barlund M, Bucher C, Haas P, Nocito A, Bissig H, Sauter G, Kallioniemi A (2002) High-throughput copy number analysis of 17q23 in 3520 tissue specimens by fluorescence *in situ* hybridization to tissue microarrays. *Am J Pathol* **161:** 73–79

Barlund M, Forozan F, Kononen J, Bubendorf L, Chen Y, Bittner ML, Torhorst J, Haas P, Bucher C, Sauter G, Kallioniemi OP, Kallioniemi A (2000a) Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. *J Natl Cancer Inst* **92:** 1252–1259

Barlund M, Monni O, Kononen J, Cornelison R, Torhorst J, Sauter G, Kallioniemi O, Kallioniemi A (2000b) Multiple genes at 17q23 undergo amplification and overexpression in breast cancer. *Cancer Res* **60:** 5340–5344

Barlund M, Tirkkonen M, Forozan F, Tanner MM, Kallioniemi O, Kallioniemi A (1997) Increased copy number at 17q22–q24 by CGH in breast cancer is due to high-level amplification of two separate regions. *Genes Chromosomes Cancer* **20:** 372–376

Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, Norton L (1999) Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. *Semin Oncol* **26:** 78–83

Brinkschmidt C, Christiansen H, Terpe HJ, Simon R, Boecker W, Lampert F, Stoerkel S (1997) Comparative genomic hybridization (CGH) analysis of neuroblastomas–an important methodological approach in paediatric tumour pathology. *J Pathol* **181:** 394–400, doi:10.1002/(SICI)1096-9896(199704)181:4〈394::AID-PATH800〉3.0.CO;2-1

Bulavin DV, Demidov ON, Saito S, Kauraniemi P, Phillips C, Amundson SA, Ambrosino C, Sauter G, Nebreda AR, Anderson CW, Kallioniemi A, Fornace Jr AJ, Appella E (2002) Amplification of PPM1D in human tumours abrogates p53 tumour-suppressor activity. *Nat Genet* **31:** 210–215, doi:10.1038/ng894

Clark J, Edwards S, John M, Flohr P, Gordon T, Maillard K, Giddings I, Brown C, Bagherzadeh A, Campbell C, Shipley J, Wooster R, Cooper CS (2002) Identification of amplified and expressed genes in breast cancer by comparative hybridization onto microarrays of randomly selected cDNA clones. *Genes Chromosomes Cancer* **34:** 104–114, 10.1002/gcc.10039

Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, Paton V, Shak S, Lieberman G, Slamon DJ (1999) Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* **17:** 2639–2648

Couch FJ, Wang XY, Wu GJ, Qian J, Jenkins RB, James CD (1999) Localization of PS6K to chromosomal region 17q23 and determination of its amplification in breast cancer. *Cancer Res* **59:** 1408–1411

Erson AE, Niell BL, DeMers SK, Rouillard JM, Hanash SM, Petty EM (2001) Overexpressed genes/ESTs and characterization of distinct amplicons on 17q23 in breast cancer cells. *Neoplasia* **3:** 521–526, doi:10.1038/sj/neo/7900187

Fridlyand J, Snijders AM, Ylstra B, Li H, Olshen A, Segraves R, Dairkee S, Tokuyasu T, Ljung BM, Jain AN, McLennan J, Ziegler J, Chin K, Devries S, Feiler H, Gray JW, Waldman F, Pinkel D, Albertson DG (2006) Breast tumour copy number aberration phenotypes and genomic instability. *BMC Cancer* **6:** 96, doi:10.1186/1471-2407-6-96

Garcia MJ, Pole JC, Chin SF, Teschendorff A, Naderi A, Ozdag H, Vias M, Kranjac T, Subkhankulova T, Paish C, Ellis I, Brenton JD, Edwards PA, Caldas C (2005) A 1 Mb minimal amplicon at 8p11–12 in breast cancer identifies new candidate oncogenes. *Oncogene* **24:** 5235–5245, doi:10.1038/sj.onc.1208741

Gelsi-Boyer V, Orsetti B, Cervera N, Finetti P, Sircoulomb F, Rouge C, Lasorsa L, Letessier A, Ginestier C, Monville F, Esteyries S, Adelaide J, Esterni B, Henry C, Ethier SP, Bibeau F, Mozziconacci MJ, Charafe-Jauffret E, Jacquemier J, Bertucci F, Birnbaum D, Theillet C, Chaffanet M (2005) Comprehensive profiling of 8p11–12 amplification in breast cancer. *Mol Cancer Res* **3:** 655–667, doi:10.1158/1541–7786.MCR-05-0128

Gollin SM (2001) Chromosomal alterations in squamous cell carcinomas of the head and neck: window to the biology of disease. *Head Neck* **23:** 238 – 253

Gollin SM (2004) Chromosomal instability. *Curr Opin Oncol* **16:** 25 – 31

Hirasawa A, Saito-Ohara F, Inoue J, Aoki D, Susumu N, Yokoyama T, Nozawa S, Inazawa J, Imoto I (2003) Association of 17q21 – q24 gain in ovarian clear cell adenocarcinomas with poor prognosis and identification of PPM1D and APPBP2 as likely amplification targets. *Clin Cancer Res* **9:** 1995 – 2004

Huang X, Godfrey TE, Gooding WE, McCarty Jr KS, Gollin SM (2006) Comprehensive genome and transcriptome analysis of the 11q13 amplicon in human oral cancer and synteny to the 7F5 amplicon in murine oral carcinoma. *Genes Chromosomes Cancer* **45:** 1058 – 1069, doi:10.1002/gcc.20371

Hyman E, Kauraniemi P, Hautaniemi S, Wolf M, Mousses S, Rozenblum E, Ringner M, Sauter G, Monni O, Elkahloun A, Kallioniemi OP, Kallioniemi A (2002) Impact of DNA amplification on gene expression patterns in breast cancer. *Cancer Res* **62:** 6240 – 6245

Isola JJ, Kallioniemi OP, Chu LW, Fuqua SA, Hilsenbeck SG, Osborne CK, Waldman FM (1995) Genetic aberrations detected by comparative genomic hybridization predict outcome in node-negative breast cancer. *Am J Pathol* **147:** 905 – 911

Kallioniemi A, Kallioniemi OP, Piper J, Tanner M, Stokke T, Chen L, Smith HS, Pinkel D, Gray JW, Waldman FM (1994) Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proc Natl Acad Sci USA* **91:** 2156 – 2160

Kao J, Pollack JR (2006) RNA interference-based functional dissection of the 17q12 amplicon in breast cancer reveals contribution of coamplified genes. *Genes Chromosomes Cancer* **45:** 761 – 769, doi:10.1002/gcc.20339

Kauraniemi P, Barlund M, Monni O, Kallioniemi A (2001) New amplified and highly expressed genes discovered in the ERBB2 amplicon in breast cancer by cDNA microarrays. *Cancer Res* **61:** 8235 – 8240

Kauraniemi P, Kuukasjarvi T, Sauter G, Kallioniemi A (2003) Amplification of a 280-kilobase core region at the ERBB2 locus leads to activation of two hypothetical proteins in breast cancer. *Am J Pathol* **163:** 1979 – 1984

Knuutila S, Bjorkqvist AM, Autio K, Tarkkanen M, Wolf M, Monni O, Szymanska J, Larramendy ML, Tapper J, Pere H, El-Rifai W, Hemmer S, Wasenius VM, Vidgren V, Zhu Y (1998) DNA copy number amplifications in human neoplasms: review of comparative genomic hybridization studies. *Am J Pathol* **152:** 1107 – 1123

Korn WM, Oide Weghuis DE, Suijkerbuijk RF, Schmidt U, Otto T, du Manoir S, Geurts van Kessel A, Harstrick A, Seeber S, Becher R (1996) Detection of chromosomal DNA gains and losses in testicular germ cell tumours by comparative genomic hybridization. *Genes Chromosomes Cancer* **17:** 78 – 87, doi:10.1002/(SICI)1098-2264(199610)17:2〈78::AID-GCC2〉3.0.CO;2-Y

Li J, Yang Y, Peng Y, Austin RJ, van Eyndhoven WG, Nguyen KC, Gabriele T, McCurrach ME, Marks JR, Hoey T, Lowe SW, Powers S (2002) Oncogenic properties of PPM1D located within a breast cancer amplification epicenter at 17q23. *Nat Genet* **31:** 133 – 134, doi:10.1038/ng888

Marchio A, Meddeb M, Pineau P, Danglot G, Tiollais P, Bernheim A, Dejean A (1997) Recurrent chromosomal abnormalities in hepatocellular carcinoma detected by comparative genomic hybridization. *Genes Chromosomes Cancer* **18:** 59 – 65

Monni O, Barlund M, Mousses S, Kononen J, Sauter G, Heiskanen M, Paavola P, Avela K, Chen Y, Bittner ML, Kallioniemi A (2001) Comprehensive copy number and gene expression profiling of the 17q23 amplicon in human breast cancer. *Proc Natl Acad Sci USA* **98:** 5711 – 5716, doi:10.1073/pnas.091582298

Morerio C, Russo I, Rosanda C, Rapella A, Leszl A, Basso G, Maserati E, Pasquali F, Panarello C (2001) 17q21-qter trisomy is an indicator of poor prognosis in acute myelogenous leukemia. *Cancer Genet Cytogenet* **124:** 12 – 15

Muleris M, Almeida A, Gerbault-Seureau M, Malfoy B, Dutrillaux B (1994) Detection of DNA amplification in 17 primary breast carcinomas with homogeneously staining regions by a modified comparative genomic hybridization technique. *Genes Chromosomes Cancer* **10:** 160 – 170

Pollack JR, Sorlie T, Perou CM, Rees CA, Jeffrey SS, Lonning PE, Tibshirani R, Botstein D, Borresen-Dale AL, Brown PO (2002) Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumours. *Proc Natl Acad Sci USA* **99:** 12963 – 12968, doi:10.1073/pnas.162471999

Rauta J, Alarmo EL, Kauraniemi P, Karhu R, Kuukasjarvi T, Kallioniemi A (2006) The serine-threonine protein phosphatase PPM1D is frequently activated through amplification in aggressive primary breast tumours. *Breast Cancer Res Treat* **95:** 257 – 263, doi:10.1007/s10549-005-9017-7

Richter J, Jiang F, Gorog JP, Sartorius G, Egenter C, Gasser TC, Moch H, Mihatsch MJ, Sauter G (1997) Marked genetic differences between stage pTa and stage pT1 papillary bladder cancer detected by comparative genomic hybridization. *Cancer Res* **57:** 2860 – 2864

Ried T, Petersen I, Holtgreve-Grez H, Speicher MR, Schrock E, du Manoir S, Cremer T (1994) Mapping of multiple DNA gains and losses in primary small cell lung carcinomas by comparative genomic hybridization. *Cancer Res* **54:** 1801 – 1806

Ross JS, Fletcher JA (1999) The HER-2/neu oncogene: prognostic factor, predictive factor and target for therapy. *Semin Cancer Biol* **9:** 125 – 138, doi:10.1006/scbi.1998.0083

Saito-Ohara F, Imoto I, Inoue J, Hosoi H, Nakagawara A, Sugimoto T, Inazawa J (2003) PPM1D is a potential target for 17q gain in neuroblastoma. *Cancer Res* **63:** 1876 – 1883

Saunders WS, Shuster M, Huang X, Gharaibeh B, Enyenihi AH, Petersen I, Gollin SM (2000) Chromosomal instability and cytoskeletal defects in oral cancer cells. *Proc Natl Acad Sci USA* **97:** 303 – 308

Schwab M (1993) Amplification of N-myc as a prognostic marker for patients with neuroblastoma. *Semin Cancer Biol* **4:** 13 – 18

Schwendel A, Langreck H, Reichel M, Schrock E, Ried T, Dietel M, Petersen I (1997) Primary small-cell lung carcinomas and their metastases are characterized by a recurrent pattern of genetic alterations. *Int J Cancer* **74:** 86 – 93

Solinas-Toldo S, Wallrapp C, Muller-Pillasch F, Bentz M, Gress T, Lichter P (1996) Mapping of chromosomal imbalances in pancreatic carcinoma by comparative genomic hybridization. *Cancer Res* **56:** 3803 – 3807

Sonoda G, Palazzo J, du Manoir S, Godwin AK, Feder M, Yakushiji M, Testa JR (1997) Comparative genomic hybridization detects frequent overrepresentation of chromosomal material from 3q26, 8q24, and 20q13 in human ovarian carcinomas. *Genes Chromosomes Cancer* **20:** 320 – 328

Staebler A, Karberg B, Behm J, Kuhlmann P, Neubert U, Schmidt H, Korsching E, Burger H, Lelle R, Kiesel L, Bocker W, Shih IEM, Buchweitz O (2006) Chromosomal losses of regions on 5q and lack of high-level amplifications at 8q24 are associated with favorable prognosis for ovarian serous carcinoma. *Genes Chromosomes Cancer* **45:** 905 – 917, doi:10.1002/gcc.20356

Sticht C, Hofele C, Flechtenmacher C, Bosch FX, Freier K, Lichter P, Joos S (2005) Amplification of Cyclin L1 is associated with lymph node metastases in head and neck squamous cell carcinoma (HNSCC). *Br J Cancer* **92:** 770 – 774, doi: 10.1038/sj.bjc.6602400

Vandesompele J, Van Roy N, Van Gele M, Laureys G, Ambros P, Heimann P, Devalck C, Schuuring E, Brock P, Otten J, Gyselinck J, De Paepe A, Speleman F (1998) Genetic heterogeneity of neuroblastoma studied by comparative genomic hybridization. *Genes Chromosomes Cancer* **23:** 141 – 152

Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ, Press M (2002) Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol* **20:** 719 – 726

Weber RG, Bostrom J, Wolter M, Baudis M, Collins VP, Reifenberger G, Lichter P (1997) Analysis of genomic alterations in benign, atypical, and anaplastic meningiomas: toward a genetic model of meningioma progression. *Proc Natl Acad Sci USA* **94:** 14719 – 14724

Willis S, Hutchins AM, Hammet F, Ciciulla J, Soo WK, White D, van der Spek P, Henderson MA, Gish K, Venter DJ, Armes JE (2003) Detailed gene copy number and RNA expression analysis of the 17q12 – 23 region in primary breast cancers. *Genes Chromosomes Cancer* **36:** 382 – 392, doi:10.1002/gcc.10138

Wolf M, Mousses S, Hautaniemi S, Karhu R, Huusko P, Allinen M, Elkahloun A, Monni O, Chen Y, Kallioniemi A, Kallioniemi OP (2004) High-resolution analysis of gene copy number alterations in human prostate cancer using CGH on cDNA microarrays: impact of copy number on gene expression. *Neoplasia* **6:** 240 – 247, doi:10.1593/neo.3439

Wu G, Sinclair C, Hinson S, Ingle JN, Roche PC, Couch FJ (2001) Structural analysis of the 17q22 – 23 amplicon identifies several independent targets of amplification in breast cancer cell lines and tumours. *Cancer Res* **61:** 4951 – 4955

Wu GJ, Sinclair CS, Paape J, Ingle JN, Roche PC, James CD, Couch FJ (2000) 17q23 amplifications in breast cancer involve the PAT1, RAD51C, PS6K, and SIGma1B genes. *Cancer Res* **60:** 5371 – 5375

The serine-threonine protein phosphatase *PPM1D* is frequently activated through amplification in aggressive primary breast tumours

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Running head: *PPM1D* amplification in primary breast cancer

Article type: Report

Summary

The serine-threonine protein phosphatase *PPM1D* is likely to play an important role in tumorigenesis. Through inactivation of p38 MAPK, *PPM1D* acts as a negative feedback regulator of p53 tumour suppressor gene and controls the expression of other cell cycle regulatory proteins, such as CCND1. In addition, recent knock-out mouse studies implicated *PPM1D* in the regulation of p16 expression and the RB tumour suppressor pathway. Here we explored the role of *PPM1D* aberrations in primary breast cancer. *PPM1D* copy number analysis showed amplification in 11% (13/117) of the tumours and quantitative real-time RT-PCR revealed a significant correlation (p=0.0148) between *PPM1D* amplification and increased expression. *PPM1D* amplification occurred almost exclusively in tumours with wild-type p53 suggesting that these events are mutually exclusive and further confirming the role of *PPM1D* as a negative regulator of p53. Interestingly, *PPM1D* amplification was associated with ERBB2 expression (p=0.0001) thus implying that *PPM1D* aberrations occurs in tumours with poor prognosis. We also explored the expression levels of two possible downstream targets of *PPM1D*. However, immunohistochemical analyses revealed no differences in the staining patterns of CCND1 and p16 proteins in tumours with or without *PPM1D* aberrations, thus suggesting that previous data from animal model experiments is not directly transferable to primary human tumours. On the other hand, these key cellular proteins are likely to be regulated through a complex fashion in breast cancer and apparently *PPM1D* represents only one of these mechanisms. Taken together, our findings substantiate an important role for *PPM1D* in breast cancer.

Key words: breast cancer, gene amplification, p53, *PPM1D*, protein phosphatase

Abbreviations: TMA, tissue microarray; FISH, fluorescence *in situ* hybridization; RT-PCR, reverse transcription-PCR

Introduction

The serine-threonine protein phosphatase *PPM1D* (also known as *Wip1*) was initially identified as a gene whose expression is induced by p53 in response to gamma or UV radiation [1]. After radiation exposure or other environmental stresses, p38 MAPK activates the p53 tumour suppressor gene signalling pathway which results in either cell cycle arrest or apoptosis depending on the strength of the initial event. The p53 activation also leads to subsequent activation of PPM1D, which in turn directly inactivates p38 MAPK thereby creating a negative feedback-loop that efficiently restores basal p53 activity [2]. The p38 MAPK is involved in cell cycle control also through other mechanisms than regulation of p53 activity. For example, p38 MAPK inhibits cell cycle stimulatory proteins, such as CCND1 and CDC25 phosphatases, and thereby prevents cell cycle progression [3,4]. Based on the functional characteristics of p38 MAPK and the fact that PPM1D can directly inactivate p38 MAPK, it is evident that PPM1D itself is a critical negative regulator of p53 and a central factor in cell cycle control.

Recent studies have provided new evidence on the role of *PPM1D* in various cellular processes. First of all, *PPM1D* induces cell proliferation and prevents apoptosis [5,6], whereas downregulation of *PPM1D* expression results in growth inhibition that is at least partially caused by induction of apoptotic cell death [7]. Overexpression of *PPM1D* was shown to complement several oncogenes for cell transformation *in vitro*, indicating that *PPM1D* possesses obvious oncogenic properties [5,6]. Recently, *PPM1D* was also implicated in DNA repair and overexpression of this gene was suggested to lead to increased genomic instability and thereby increased mutation frequency [8].

Generation of *Ppmd1* knockout mice has provided further evidence on the function of *PPM1D* [9,10]. Mouse embryonic fibroblasts (MEFs) from *Ppm1d*-null mice showed reduced proliferative potential and premature onset of senescence. This phenotype could be explained by

elevated activity of p53 and its downstream targets, that is a consequence of the missing negative feed-back regulation by Ppm1d [10]. In addition, the *Ppm1d*-null MEFs were resistant to transformation by *Ras*, *Myc* and *Erbb2* oncogenes. Interestingly, this characteristic was not linked to p53 function but was caused by high-level activity of p19 (Arf) and p16 (Ink4a), two well-known upstream regulators of the RB pathway [10]. So the lack of Ppm1d resulted in increased p19 and p16 activity, an effect that was also shown to be mediated by a p38 MAPK dependent mechanism [10].

The data presented above indicates that the lack of PPM1D leads to increased activity of both the p53 and RB tumour suppressor pathways and thereby influences cell cycle control and impairs tumorigenesis. In contrast, inappropriate *PPM1D* activation is thus likely to lead to inactivation of p53 and RB pathways and stimulation of cell cycle and tumorigenesis. *PPM1D* is located at 17q23, a chromosomal region that is frequently amplified in various human tumours [11,12], and represents a putative target gene that is activated by amplification in cancer [5,6,7,13]. *PPM1D* amplification has been previously reported to occur in about 11% of primary breast tumours, but the *PPM1D* mRNA expression levels have only been explored in a very small number of cases where amplification was shown to lead to elevated expression levels [5,6]. Nevertheless, the clinical characteristics of breast tumours with *PPM1D* aberrations have not been explored at all. In this study, we evaluated *PPM1D* amplification and expression levels in primary breast tumours and examined the clinical characteristics of tumours with *PPM1D* aberrations. Furthermore, we explored the expression levels of CCND1 and p16, two downstream targets of *PPM1D*, to further characterize the consequences of PPM1D alterations in breast cancer.

Materials and methods

Primary tumours and tissue microarray construction

A total of 146 formalin-fixed, paraffin-embedded primary breast cancers from the years 2001-2003 were obtained from Tampere University Hospital (Tampere, Finland). These included 71% infiltrating ductal carcinomas, 21% infiltrating lobular carcinomas, 4% mucinous carcinomas, and 1% ductal carcinomas *in situ*. Histological grade was determined only from the infiltrating ductal carcinomas and was 13% grade 1, 40% grade 2, and 47% grade 3. The pT stage was Tis in 1% of patients, pT1 in 48%, pT2 in 25%, pT3 in 9%, and pT4 in 17%. The tumour samples included 85% ER positive, 15% ER negative, 65% PR positive and 35% PR negative carcinomas. The median age of the patients was 58 years (range 26-88); 51% of patients had node-negative disease and 41% were node-positive. A tissue microarray (TMA) containing a single cylindrical tissue biopsy (with a diameter of 0.6 mm) from each tumour specimen was constructed as described [14]. In addition, freshly frozen tumour specimens were obtained from 26 patients. The use of all tumour specimens was approved by the Ethics Committee of the University of Tampere and by the National Authority for Medicolegal Affairs of Finland.

Fluorescence in situ hybridisation (FISH)

Two genomic clones corresponding to *PPM1D* (RP11-634F5 and RP11-1081E4) were identified through the NCBI Map Viewer (www.ncbi.nlm.nih.gov/mapview). The clone identity was verified by PCR and clone DNA was labelled with SpectrumOrange-dUTP (Vysis, Downers Grove, IL) by random priming. A SpectrumGreen labelled chromosome 17 centromeric probe (Vysis) was used as a reference. FISH on TMA was performed using a modified Paraffin Pretreatment Reagent kit

protocol (Vysis) as described previously [15]. Hybridisation signals were analysed using an Olympus BX50 epifluorescence microscope (Tokyo, Japan) using an X60 objective (NA 1.4). The entire tissue core sample was screened, with a minimum of 50 cells analysed for each case. Specimens containing a threefold or higher increase in the number of *PPM1D* probe signals, as compared with the chromosome 17 centromere signals, were considered to be amplified. In addition, specimens containing a fivefold or higher increase in the number of *PPM1D* signals or tight clusters of signals, were considered to be highly amplified.

Quantitative real-time RT-PCR

A core biopsy (with a diameter of 2 mm) was obtained from histologically representative areas of freshly frozen tumour specimens and total RNA was isolated using Qiagen RNeasy MiniKit (Qiagen Inc., Valencia, CA). First-strand cDNA synthesis was performed using SuperScript II reverse transcriptase and random hexamer primers (Invitrogen, San Diego, CA). DNA Hybridisation Probe Sets for *PPM1D* and the housekeeping gene *TBP* (TATA-box binding protein) were obtained from TIB MolBiol (Berlin, Germany). The PCR reactions were performed in the LichtCycler apparatus using the LC FastStart DNA Hybridisation Probes Kit according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) and the data was analysed using the fit point method (Roche Diagnostics). The *PPM1D* expression levels were presented relative to those of *TBP*.

Analysis of p53 mutations

p53 mutations (exons 4-10) were analysed by direct sequencing from total genomic tumour DNA. The primer sequences are available on request. For each exon, the PCR reaction was performed

using 1 µl genomic DNA, 5 µl of 10x PCR Gold Buffer, 3 µl of 25µM $MgCl_2$, 1 µl of dNTPs, 0.9 µl each of 10 µM sense and antisense primers, 0.3 µl Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 40 µl of H_2O. The reaction was subjected to 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 55º/58ºC for 1 minute, and extension at 72º for 1 minute. PCR products were purified using Qiagen QIAquick PCR Purification Kit according to the manufacturer's protocol (Qiagen Inc.). The cycle sequencing reactions were carried out using Big Dye Terminator chemistry (Applied Biosystems) according to the manufacturer's protocol and the sequencing was carried out using ABI 3100 sequencer (Applied Biosystems).

Immunohistochemistry

Sections from the TMA described above were stained using an automated TechMate 500 Plus immunostaining system (DakoCytomation, Glostrup, Denmark). Slides were de-waxed, washed thoroughly with xylene and alcohol, and stained with antibodies against CCND1 and p16 using avidin-biotin enhanced immunoperoxidase technique (DAKO ChemMate EnVision Detection Kit, DakoCytomation). The following primary antibodies were used: CCND1 at 1:10 dilution (NovoCastra Laboratories, Newcastle, United Kingdom) and p16 INK4a at 1:60 dilution (DakoCytomation). Evaluation of results was done using a light microscope equipped with 20X objective. For both CCND1 and p16 staining, tumours were categorized either positive or negative on the basis of the presence or absence of distinct nuclear immunoreactivity.

Statistical analyses

Fisher's exact test (two rows, two columns) or chi-square test (larger contingency tables) were used to study the association between *PPM1D* amplification and clinicopathological parameters. Fisher's

exact test was also used to evaluate the association between *PPM1D* alterations and immunohistochemical p16 and CCN1D staining. Comparison of the clinicopathological parameters and median *PPM1D* expression levels between the amplified and non-amplified tumour groups were done using Mann-Whitney U-test. All *P* values are two-tailed.

Results

PPM1D is amplified in a large portion of primary breast tumours

To evaluate the involvement of *PPM1D* aberrations in breast cancer, we first explored *PPM1D* copy number levels in 146 primary breast tumours by FISH to a TMA. A total of 117 tumour samples (80%) could be evaluated. In the rest of the cases, the analysis failed either due to loss of the sample during processing or lack of hybridisation signals. *PPM1D* amplification (≥ 3-fold amplification relative to the chromosome 17 centromere) was observed in 11% of the tumours (Figure 1). High-level amplification (>5-fold or tight clusters of signals) was found in 4.2% of the samples. Next we examined the clinicopathological characteristics of the tumours with *PPM1D* amplification. Interestingly, a highly significant association (p=0.0001) was found between *PPM1D* amplification and positive staining for the ERBB2 oncogene as determined by routine immunohistochemistry (Table 1). No significant associations to other tumour characteristics, such as grade and nodal status, were observed.

PPM1D amplification leads to mRNA overexpression

We next measured the *PPM1D* mRNA levels in primary breast cancer using real-time RT-PCR. A set of 26 tumours were selected for the analysis based on their *PPM1D* copy number status. Frozen

tumour specimens from the same patients were used for the expression analysis and the amplified (n=11) and non-amplified (n=15) tumour groups were matched with respect to standard clinicopathological features. The highest *PPM1D* expression levels were observed in the amplified tumour group (Figure 2). Furthermore, the median level of expression was clearly higher in the amplified than in the non-amplified tumour group (33.6 vs. 11.3) and this difference was statistically significant (p=0.0148). Only a single tumour in the amplified group had expression level below the median of the non-amplified group. Conversely, three tumours in the non-amplified group showed expression levels that were higher than the median of the amplified group (Figure 2).

PPM1D amplification occurs predominantly in tumours with wild type p53

Because p53 is one of the targets for the oncogenic function of *PPM1D*, we next explored the possible relationship between *PPM1D* aberrations and the p53 status of the tumours. To this end, we sequenced exons 4 through 10 of the *p53* gene from a subset of 16 tumours. These included ten tumours with *PPM1D* amplification and six tumours with no *PPM1D* copy number increase. A total of four p53 mutations were identified (Figure 3), all of which have been previously observed in various tumour types (http://p53.curie.fr/). One of the mutations, a truncating mutation at exon 5 (W146X), occurred in a tumour with high-level *PPM1D* amplification and increased mRNA expression (relative expression level 74), whereas the rest were detected in tumours with no *PPM1D* alterations. These included two missense mutations (R175H in exon 5, V272M in exon 8) as well as a nonsense mutation (R213X in exon 6). Furthermore, we found in four separate samples a sequence alteration in exon 4 (R72P) that has been previously classified as a polymorphism (http://p53.curie.fr/).

PPM1D aberrations do not alter p16 and CCND1 expression levels

Based on the information obtained from the *Ppm1d* null-mice, p16 and CCND1 represent downstream targets of *PPM1D* that are likely to be downregulated or upregulated, respectively, upon *PPM1D* overexpression. To test this hypothesis in vivo, we determined the p16 and CCND1 protein expression levels in primary breast tumours using immunohistochemistry on the TMA described above. The majority of the tumours (83%, 104/126) showed no p16 expression whereas positive CCND1 expression was seen in 31% (39/125) (Figure 4). The protein expression levels were then evaluated in relation to *PPM1D* status. However, neither CCND1 nor p16 expression was associated with *PPM1D* amplification or the level of mRNA expression.

Discussion

The serine-threonine protein phosphatase *PPM1D* has been demonstrated to have clear oncogenic properties and to play an important role in tumorigenesis [5,6]. *PPM1D* is involved in the regulation of several key cellular processes, such as cell cycle and apoptosis, that are vital for tumour development and progression. The tumorigenic effects of *PPM1D* are largely mediated by its ability to regulate the activity of two distinct tumour suppressor pathways, the p53 and RB pathways [10]. Inappropriate activation of *PPM1D* in cancer is believed to inactivate p53 and RB pathways and therefore results in stimulation of cell cycle and tumorigenesis.

Here we evaluated the role of *PPM1D* in human primary breast cancer. The tissue microarray technology was utilized to study the *PPM1D* gene copy number levels in primary breast tumours and amplification was observed in 11% of the cases. This finding confirms previous studies where amplification of the *PPM1D* locus at 17q23 or *PPM1D* itself has been observed in

about 10% of the cases [5,12,16]. In addition, our results also revealed a novel highly significant association (p=0.0001) between *PPM1D* amplification and ERBB2 overexpression suggesting that *PPM1D* amplification occurs in more aggressive disease. However, it has to be noted that *PPM1D* amplification was not associated with other markers of tumour aggressiveness, such as high histological grade and positive nodal status. The *ERBB2* oncogene is a well-known prognostic indicator in breast cancer whose amplification and overexpression correlates with poor patient outcome [17]. *ERBB2* is located at 17q21, the same chromosome arm than *PPM1D*, and therefore it could be argued that the association observed here is simply caused by co-amplification events, owing to the physical location of these two genes. However, there is ample evidence that multiple separate amplicons are present along the q-arm of chromosome 17 in breast cancer and that the *ERBB2* and the *PPM1D* amplicons are indeed distinct [18,19,20]. Furthermore, a recent study on Ppm1d-null mice demonstrated that the presence of PPM1D is essential for ERBB2 induced mammary tumorigenesis [10]. Our observation on the association between *PPM1D* amplification and ERBB2 overexpression in human primary breast cancer validates this data and further substantiates that activation of *ERBB2* and *PPM1D* are two separate events that act in concert in mammary tumour development. Previous studies have further illustrated that amplification of the *PPM1D* region at 17q23 has independent prognostic value in breast cancer [18,21]. In particular, the study by Bärlund et al. (2000) demonstrated that co-amplification of the 17q23 region and *ERBB2* indicated an even poorer prognosis than amplification of either one alone [18]. Taken together, it seems obvious that *PPM1D* amplification in breast cancer is associated with aggressive disease. Similar results have recently been reported in neuroblastomas and ovarian clear cell adenocarcinomas where high *PPM1D* expression was associated with poor patient survival [7,13].

To explore the connection between *PPM1D* amplification and expression, we quantitated the relative level of *PPM1D* mRNA from 26 tumour samples, representing both amplified and non-amplified tumours, using real-time quantitative RT-PCR. The tumours with amplification showed

significantly ($p = 0.0148$) higher median expression level than those without amplification thus indicating that increased *PPM1D* copy number leads to elevated mRNA expression levels in breast cancer. This result is in agreement with recent microarray based analyses that have implicated gene copy number as an important regulator of gene expression level [19,22]. In addition, our results also validate the previous study by Bulavin et al. and Li et al. in where they showed the association between *PPM1D* amplification and expression in 11 and 5 tumour samples [5,6]. However, in contrast to these studies, our somewhat larger series of tumours included more samples also without *PPM1D* amplification. In addition, it has to be noted that a few of these nonamplified samples had high *PPM1D* expression levels. It is therefore obvious that mechanisms other than increased copy number are involved in the regulation of *PPM1D* expression, thus confirming the hypothesis that *PPM1D* has a critical role in cancer. Nevertheless, according to our data, amplification seems to be the most prevalent mechanism for upregulation of *PPM1D* in breast cancer.

The *PPM1D* activation represents one of the mechanisms leading to inactivation of p53 in cancer and therefore it is not likely to occur in tumours where p53 is inactivated by other means. To investigate this issue in primary breast tumours, we determined the p53 mutation status in a subset of 16 tumours by direct sequencing of exons 4 through 10, the most frequent locations of p53 mutations (http://p53.curie.fr/). As expected, *PPM1D* amplification occurred predominantly in tumours with wild-type p53. Only a single tumour with *PPM1D* amplification also had a p53 mutation, although the general frequency of these mutations in breast cancer is reported to reach 40% [23]. This result is consistent with the previous work by Bulavin et al. in where they sequenced 11 tumour samples and the notion that *PPM1D* amplification and p53 mutation are mutually exclusive events that on the cellular level lead to the same end-result, inactivation of p53 [5].

To further evaluate the downstream effects of *PPM1D* alterations, we determined the expression levels of CCND1 and p16. According to the data from *Ppm1d* knock-out mouse models, CCND1 is expected to be upregulated and p16 downregulated in tumours with *PPM1D* activation

[10]. However, the immunohistochemical analyses showed no differences in CCND1 and p16 expression levels between the *PPM1D* amplified and non-amplified tumours. Nor was there any difference when only *PPM1D* expression levels were considered. These results seem to suggest that the data obtained from the mouse model is not directly transferable to primary human tumours. However, it has to be remembered that the mouse model was investigating the lack of *Ppm1d* and it is of course possible that the opposite effect, i.e. *PPM1D* activation, does not generate exactly the opposite downstream consequences. It is also likely that key cellular proteins, such as CCND1 and p16, are regulated in a complex fashion via multiple different mechanisms and *PPM1D* activity represents only one of those mechanisms.

In conclusion, we observed *PPM1D* amplification in a considerable portion of primary breast tumours and demonstrated that amplification leads to *PPM1D* overexpression. We also provide evidence for a highly significant association between *PPM1D* amplification and ERBB2 overexpression in breast cancer and suggest an evident connection between *PPM1D* aberrations and aggressive disease. In addition, mutations of the tumour suppressor gene p53 were primarily found in tumours without *PPM1D* amplification, supporting the role of *PPM1D* as an important regulator of p53 function in breast cancer.

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References

1. Fiscella M, Zhang H, Fan S, Sakaguchi K, Shen S, Mercer WE, Vande Woude GF, O'Connor PM, Appella E: Wip1, a novel human protein phosphatase that is induced in response to ionizing radiation in a p53-dependent manner. Proc Natl Acad Sci USA 94: 6048-6053, 1997

2. Takekawa M, Adachi M, Nakahata A, Nakayama I, Itoh F, Tsukuda H, Taya Y, Imai K: p53-inducible wip1 phosphatase mediates a negative feedback regulation of p38 MAPK-p53 signaling in response to UV radiation. EMBO J 19: 6517-6526, 2000

3. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J: Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J Biol Chem 271: 20608-20616, 1996

4. Bulavin DV, Higashimoto Y, Popoff IJ, Gaarde WA, Basrur V, Potapova O, Appella E, Fornace AJ Jr: Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. Nature 411: 102-107, 2001

5. Bulavin DV, Demidov ON, Saito S, Kauraniemi P, Phillips C, Amundson SA, Ambrosino C, Sauter G, Nebreda AR, Anderson CW, Kallioniemi A, Fornace AJ,Jr, Appella E: Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity. Nat Genet 31: 210-215, 2002

6. Li J, Yang Y, Peng Y, Austin RJ, van Eyndhoven WG, Nguyen KC, Gabriele T, McCurrach ME, Marks JR, Hoey T, Lowe SW, Powers S: Oncogenic properties of PPM1D located within a breast cancer amplification epicenter at 17q23. Nat Genet 31: 133-134, 2002

7. Saito-Ohara F, Imoto I, Inoue J, Hosoi H, Nakagawara A, Sugimoto T, Inazawa J: PPM1D is a potential target for 17q gain in neuroblastoma. Cancer Res 63: 1876-1883, 2003

8. Lu X, Nguyen TA, Appella E, Donehower LA: Homeostatic regulation of base excision repair by a p53-induced phosphatase: Linking stress response pathways with DNA repair proteins. Cell Cycle 3: 1363-1366, 2004

9. Choi J, Nannenga B, Demidov ON, Bulavin DV, Cooney A, Brayton C, Zhang Y, Mbawuike IN, Bradley A, Appella E, Donehower LA: Mice deficient for the wild-type p53-induced phosphatase gene (Wip1) exhibit defects in reproductive organs, immune function, and cell cycle control. Mol Cell Biol 22: 1094-1105, 2002

10. Bulavin DV, Phillips C, Nannenga B, Timofeev O, Donehower LA, Anderson CW, Appella E, Fornace AJ Jr: Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK-mediated activation of the p16(Ink4a)-p19(arf) pathway. Nat Genet 36: 343-350, 2004

11. Andersen CL, Monni O, Wagner U, Kononen J, Barlund M, Bucher C, Haas P, Nocito A, Bissig H, Sauter G, Kallioniemi A: High-throughput copy number analysis of 17q23 in 3520 tissue

specimens by fluorescence in situ hybridization to tissue microarrays. Am J Pathol 161: 73-79, 2002

12. Sinclair CS, Rowley M, Naderi A, Couch FJ: The 17q23 amplicon and breast cancer. Breast Cancer Res Treat 78: 313-322, 2003

13. Hirasawa A, Saito-Ohara F, Inoue J, Aoki D, Susumu N, Yokoyama T, Nozawa S, Inazawa J, Imoto I: Association of 17q21-q24 gain in ovarian clear cell adenocarcinomas with poor prognosis and identification of PPM1D and APPBP2 as likely amplification targets. Clin Cancer Res 9: 1995-2004, 2003

14. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP: Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 4: 844-847, 1998

15. Andersen CL, Hostetter G, Grigoryan A, Sauter G, Kallioniemi A: Improved procedure for fluorescence in situ hybridization on tissue microarrays. Cytometry 45: 83-86, 2001

16. Monni O, Barlund M, Mousses S, Kononen J, Sauter G, Heiskanen M, Paavola P, Avela K, Chen Y, Bittner ML, Kallioniemi A: Comprehensive copy number and gene expression profiling of the 17q23 amplicon in human breast cancer. Proc Natl Acad Sci USA 98: 5711-5716, 2001

17. Ross JS, Fletcher JA: The HER-2/neu oncogene: Prognostic factor, predictive factor and target for therapy. Semin Cancer Biol 9: 125-138, 1999

18. Bärlund M, Monni O, Kononen J, Cornelison R, Torhorst J, Sauter G, Kallioniemi OP, Kallioniemi A: Multiple genes at 17q23 undergo amplification and overexpression in breast cancer. Cancer Res 60: 5340-5344, 2000

19. Hyman E, Kauraniemi P, Hautaniemi S, Wolf M, Mousses S, Rozenblum E, Ringner M, Sauter G, Monni O, Elkahloun A, Kallioniemi OP, Kallioniemi A: Impact of DNA amplification on gene expression patterns in breast cancer. Cancer Res 62: 6240-6245, 2002

20. Orsetti B, Nugoli M, Cervera N, Lasorsa L, Chuchana P, Ursule L, Nguyen C, Redon R, du Manoir S, Rodriguez C, Theillet C: Genomic and expression profiling of chromosome 17 in breast cancer reveals complex patterns of alterations and novel candidate genes. Cancer Res 64: 6453-6460, 2004

21. Rennstam K, Ahlstedt-Soini M, Baldetorp B, Bendahl PO, Borg A, Karhu R, Tanner M, Tirkkonen M, Isola J: Patterns of chromosomal imbalances defines subgroups of breast cancer with distinct clinical features and prognosis. A study of 305 tumors by comparative genomic hybridization. Cancer Res 63: 8861-8868, 2003

22. Pollack JR, Sorlie T, Perou CM, Rees CA, Jeffrey SS, Lonning PE, Tibshirani R, Botstein D, Borresen-Dale AL, Brown PO: Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. Proc Natl Acad Sci USA 99: 12963-12968, 2002

23. Borresen-Dale AL: TP53 and breast cancer. Hum Mutat 21: 292-300, 2003

Figure legends

Figure 1. *PPM1D* is amplified in a large portion of primary breast tumours. Copy number levels were explored by FISH to a tissue microarray containing 146 primary breast cancer specimens. An example of a tumour sample with high-level *PPM1D* amplification is presented. The *PPM1D* specific hybridisation signals are shown in red, chromosome 17 centromere signals in green, and the nuclei were counterstained with DAPI (blue).

Figure 2. *PPM1D* amplification leads to mRNA overexpression in breast cancer. *PPM1D* expression levels were determined in 26 primary breast tumours by quantitative real-time RT-PCR and normalized against the housekeeping gene *TBP*. The horizontal lines indicate median expression levels which differ significantly between the amplified (n=11) and non-amplified (n=15) tumour groups.

Figure 3. *PPM1D* amplification occurs predominantly in tumours with wild type *p53*. Four p53 mutations found in primary breast tumours are presented. Mutation is specified on the top of the picture and the mutated nucleotide is indicated with an arrow.

Figure 4. *PPM1D* aberrations do not alter p16 and CCND1 expression levels. Examples of immunohistochemical detection of CCND1 and p16 proteins in primary breast tumours are shown: (a) CCND1 negative, (b) CCND1 positive, (c) p16 negative, and (d) p16 positive tumours.

Table 1. **Correlation between *PPM1D* amplification and clinicopathological parameters in 117 primary breast tumours.**

Variable	Amplified	Nonamplified	p-value
All tumours	11	106	
Grade[a]			
I	0	11	
II	3	29	
III	6	33	0.7954
n.a.[b]	2	33	
Tumour size			
<2 cm	6	45	
≥2 cm	5	61	0.5297
Lymph nodes			
positive	5	44	
negative	5	62	0.7408
n.a.[b]	1		
ER			
positive	8	94	
negative	3	12	0.1492
PR			
positive	6	71	
negative	5	35	0.5071
ERBB2			
negative	4	95	
positive	7	11	0.0001[c]
Proliferation			
Ki67<45%	6	83	
Ki67≥45%	5	21	0.1210
n.a.[b]		2	

[a]Histological grade is determined only from infiltrating ductal carcinomas
[b]not available
[c]statistically significant

Figure 1.

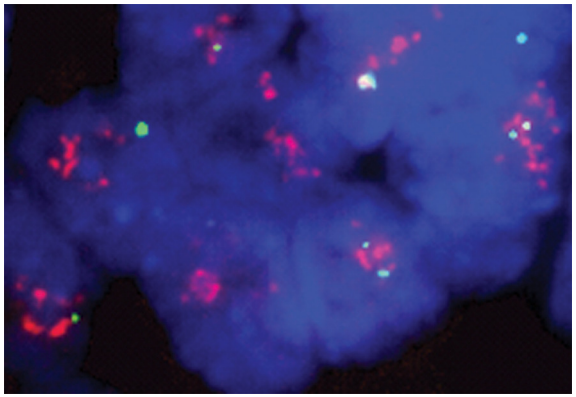
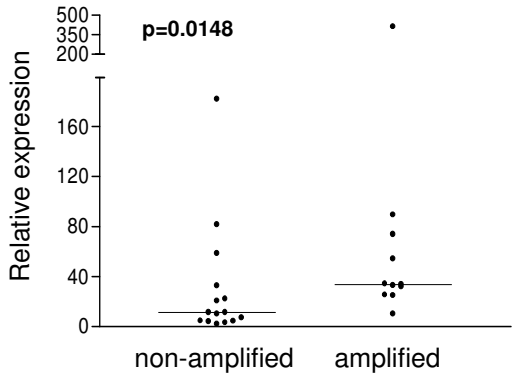


Figure 2.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Relative expression
500
350
200
160
120
80
40
0
non-amplified
amplified

Figure 3.

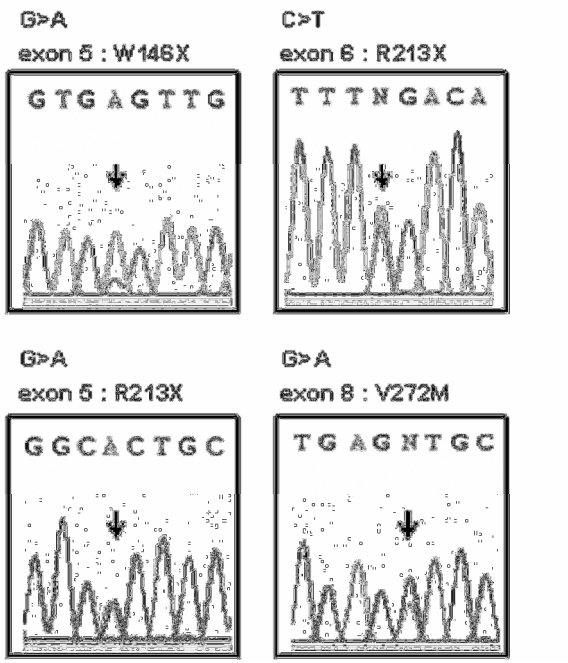
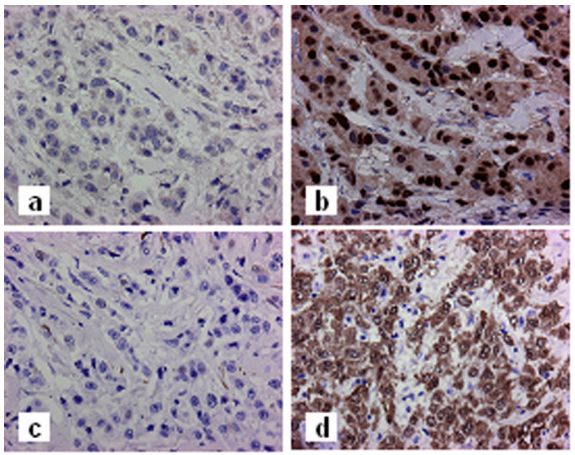

G>A
exon 5 : W146X
G T G A G T T G
C>T
exon 6 : R213X
T T T N G A C A
G>A
exon 5 : R213X
G G C A C T G C
G>A
exon 8 : V272M
T G A G N T G C

Figure 4.



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Identification of differentially expressed genes after *PPM1D* silencing in breast cancer

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Abstract

Amplification and overexpression of *PPM1D* (protein phosphatase magnesium-dependent 1 delta) has been observed in various cancer cell lines and primary tumors and has also been associated with cancers of poor prognosis. In addition to the negative feedback regulation of p38–p53 signaling, PPM1D inhibits other tumor suppressor activities and is involved in the control of DNA damage and repair pathways. To elucidate the functional significance of *PPM1D* in breast cancer, we employed RNA interference to downregulate *PPM1D* expression in BT-474, MCF7, and ZR-75-1 breast cancer cell lines and then investigated the effects of *PPM1D* silencing on global gene expression patterns and signaling pathways using oligonucleotide microarrays. We identified 1798 differentially expressed (at least a two-fold change) gene elements with functions related to key cellular processes, such as regulation of cell cycle, assembly of various intracellular structures and components, and regulation of signaling pathways and metabolic cascades. For instance, genes involved in apoptosis (*NR4A1, RAB25, PLK1*), formation of nucleosome structure (*HIST1H2AC*, *HIST1H2BF*, *HIST1H2BO*, *HIST1H1D*), and hormone related activities (*NR4A1*, *ESR1, STC1*) were among the differentially expressed genes. Overall, our findings suggest that PPM1D contributes to breast cancer associated phenotypic characteristics by directly or indirectly affecting several important cellular signaling pathways.

Keywords: PPM1D; Breast cancer; RNA interference; Oligonucleotide microarray; Gene expression

1. Introduction

Type 2C protein phosphatase (PP2C) family is a group of evolutionarily conserved protein phosphatases that have been implicated in the regulation of a wide range of physiological functions including cellular stress response signaling, apoptosis, sexual differentiation, and cell cycle [1]. One of the members of this family is the protein phosphatase magnesium-dependent 1 delta (PPM1D) which is ubiquitously expressed both in adult and embryonic tissues and is shown to be induced by

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exposure to ionizing or ultraviolet radiation in a p53 dependent manner [2–4]. Mice genetically deficient in *Ppm1d* are viable despite the fact that they show defects in growth, organ structure, fertility, and lymphoid function [5,6]. Interestingly, *Ppm1d* null mice are considerably more resistant to mammary tumor formation induced by *Erbb2* or *Hras1* oncogenes than mice with wild-type intact *Ppm1d* [7–9].

There is increasing evidence suggesting the involvement of *PPM1D* in tumorigenesis. First of all, the gene is located at chromosome 17q23, a hotspot for gene amplification found in multiple types of human cancers [10–16]. *PPM1D* is also shown to be overexpressed in human primary breast, ovarian and neuroblastoma tumors [17–21]. Most importantly, *PPM1D* can complement several oncogenes, such as *Ras*, *Myc*, and *Neu,* for cellular transformation both *in vitro* and *in vivo* [17,18,22]. The oncogenic properties of PPM1D were originally thought to stem from its ability to inactivate the p53 tumor suppressor gene either through direct dephosphorylation or dephosphorylation p38 MAPK, an upstream regulator of p53 [4,7,23]. However, recent studies have shown that PPM1D targets also other key stress response kinases, such as ATM, Chk1, and Chk2, as well as UNG2 which functions in DNA damage response and repair [23–28]. PPM1D has also been suggested to inhibit $p16^{INK4A}$ and $p19^{ARF}$ tumor suppressor activities [7]. Together these findings indicate that PPM1D is involved in the regulation of several essential signaling pathways that are implicated in cancer pathogenesis.

To better understand the role of *PPM1D* in breast cancer, we previously studied the functional effects of *PPM1D* silencing in breast cancer cell lines with *PPM1D* amplification and overexpression using RNA interference. We found that decrease in *PPM1D* levels led to significantly reduced cell growth in breast cancer cell lines carrying wild-type p53 but not in a cell line with mutant p53 [29]. Our results also showed that this reduction in cell growth was at least partly due to increase in apoptosis. However, the molecular mechanisms that underlie the regulation of cell growth and cell survival by PPM1D are still poorly understood. Herein, we used RNA interference and oligonucleotide microarray technologies to identify genes and signaling pathways that are influenced by *PPM1D* silencing in breast cancer cells.

2. Materials and methods

2.1. Cell lines and cell culture

Three breast cancer cell lines BT-474, MCF7, and ZR-75-1 were used in this study. These cell lines were obtained from American Type Culture Collection (Manassas, VA) and were grown under recommended culture conditions.

2.2. PPM1D silencing

Scrambled non-silencing control siRNA and siRNA targeted against *PPM1D* mRNA (siRNA-3) were designed according to Elbashir et al. [30] and were described previously [29]. The siRNA sequences were 5′-ACACTGTCCCCACCATAACTT-3′ (scrambled control) and 5′-GGACCATATACCTGCCCTGTT-3′ (*PPM1D*). The *PPM1D* siRNA did not show significant sequence homology (not exceeding 14 contiguous base pairs) to any other human gene and neither siRNA contained immunostimulatory sequences involved in induction of interferon response [31–33]. One day prior to transfection, 75,000 cells per well in 24-well plate were plated in medium containing 10% FBS without antibiotics. The cells were transfected with *PPM1D* or control siRNA as described [29] using 100 nM as the final concentration of siRNA duplex. The experiment was performed in three replicates.

2.3. Quantitative real-time RT-PCR (qRT-PCR)

The *PPM1D* silencing efficiency was verified at 24 and 48 h using quantitative real-time RT-PCR (qRT-PCR). Similarly, for validation of microarray data, expression levels of a set of six differentially expressed genes were analyzed. The qRT-PCR was performed as described [21]. Briefly, total RNA was isolated using RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA), the mRNA quality was assessed using 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA), and was reverse-transcribed using SuperScript™ First-Strand Synthesis System (Invitrogen). DNA Hybridization Probe Sets for *PPM1D,* six differentially expressed genes, and the housekeeping gene TATA-box binding protein (*TBP*) were obtained from TIB MolBiol (Berlin, Germany). The expression levels were normalized against the housekeeping gene *TBP*.

2.4. Microarray based gene expression analysis

Total RNA was harvested 24 and 48 h after the transfection with *PPM1D* and control siRNA as described above. For each cell line, the RNAs from three individual replicates were pooled together. Six hundred nanograms of pooled total RNA was used to generate fluorescent Cy-3 labeled cRNA (control siRNA treated cells) and Cy-5 labeled cRNA (siRNA-3 treated cells) by using Agi-

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lent Low RNA Input Fluorescent Linear Amplification Kit protocol (Agilent Technologies). Total amount of 750 ng of both Cy-3 and Cy-5 labeled cRNA were hybridized to the Agilent 44K Whole Human Genome oligonucleotide microarrays (Agilent Technologies) containing over 33,000 known and novel human genes (~41,000 human genes and transcripts) according to the manufacturer's instructions. A total of six arrays were hybridized, one for every cell line and time point.

2.5. Data analysis

Following hybridization, microarray slides were scanned (Agilent Microarray Scanner) and data extracted using the Feature Extraction software, version A.7.5.1. (Agilent Technologies). LinearLowess normalization was performed for within array normalization between the two channels. After that, the raw data from array scans were analyzed with GeneSpring GX 7.3.1 Expression Analysis software (Agilent Technologies). In order to import the files to the GeneSpring program Agilent Feature Extractor Plug-In was used. The features that were flagged either marginal or absent by the Feature Extraction software were excluded and only those features with a present call in at least one of the arrays were included in the subsequent analyses.

Absolute expression change values $\geqslant 2.0$ and $\leqslant 0.5$ were used as cut-offs to define differentially expressed genes. Thereby genes that showed $\geqslant 2.0$- and $\leqslant 0.5$-fold change in their expression level when compared to control were regarded to be upregulated and downregulated, respectively. For hierarchical clustering of these differentially expressed genes, we used Pearson Correlation as similarity measure and unsupervised average linkage hierarchical clustering algorithm.

To determine the biological significance of the up and down regulated genes, functional classification was performed using GeneSpring. This classifies the genes into relevant Gene Ontologies [34] dealing with molecular function, biological processes, and cellular components. The p-value 0.02 was used as a cut-off to indicate those functions/processes/components that were significantly overrepresented among the differentially expressed genes.

3. Results

In order to study the effects of *PPM1D* silencing on global gene expression levels in breast cancer, we applied RNA interference to three cell lines, BT-474, MCF7, and ZR-75-1, that are known to harbor *PPM1D* amplification and overexpression [18,23]. Two of these, MCF7 and ZR-75-1, have wild-type p53 gene, whereas BT-474 harbors p53 mutation [35,36]. The cells were treated for 24 and 48 h with either *PPM1D* specific siRNA (siRNA-3) or control siRNA. To verify the silencing efficiency, *PPM1D* mRNA levels were quantified by qRT-PCR before performing microarray hybridizations. siRNA-3 significantly decreased the levels of *PPM1D* transcript in all three breast cancer cell lines. The reduction was already present at time point 24 h (68% downregulation as compared to control siRNA treated cells for BT-474, 79% for MCF7, 64% for ZR-75-1) and still persisted at 48 h after transfection (73% for BT-474, 61% for MCF7, 65% for ZR-75-1).

Agilent 44K Whole Human Genome oligonucleotide microarrays were used to identify genes influenced by *PPM1D* downregulation in the three breast cancer cell lines at 24 and 48 h after the siRNA transfection. We recognized a total of 1798 gene elements representing 1579 individual genes as differentially expressed after *PPM1D* silencing in at least one of the cell lines and at one time point (Table S1). The majority of these showed moderate increase (up to fourfold) or decrease ($\leqslant$0.2- to 0.5-fold) in expression, although there were also a few genes with highly elevated (over 10-fold) expression levels. Interestingly, there were more genes with increased than decreased expression level. In BT-474 cell line, a total of 764 gene elements were differentially expressed, of these 634 were upregulated and 130 downregulated. In the other two cell lines, MCF7 and ZR-75-1, altogether 361 and 590 gene elements were identified as overexpressed whereas 202 and 102 gene elements were underexpressed, respectively. The exact number of up and downregulated gene elements for each time point and for each cell line is presented in Table 2. To validate the microarray results, the expression levels of a set of six genes were measured using qRT-PCR. A good correlation ($R^2 = 0.4885$) was detected between the two methods.

These 1798 differentially expressed gene elements were hierarchically clustered according to their expression profile using GeneSpring software to evaluate the overall expression changes caused by *PPM1D* silencing. The hierarchical clustering dendrogram revealed that for ZR-75-1 cell line the two different time points cluster together whereas the BT-474 24-h and MCF7 48-h time points clustered together, indicating that each cell line showed a unique and time dependent response to *PPM1D* silencing (Fig. 1). To understand the biological relevance of the differential expression of these 1798 gene elements, we classified them according to the gene ontology (GO) information. The ontology data were available for 58% of the differentially expressed genes (Fig. 2). GOs are grouped to three classes namely biological process, molecular function and cellular component, and within the molecular function category, almost half of the genes altered after *PPM1D* silencing were found to be related to binding activities (Fig. 2). At the same time, several biological processes, such as development, behavior, reproduction, and growth, were commonly represented.

Next, we wanted to distinguish those GO categories that were significantly ($p < 0.02$) enriched after *PPM1D* silencing as compared to the overall distribution of GO terms across the entire microarray. This analysis revealed

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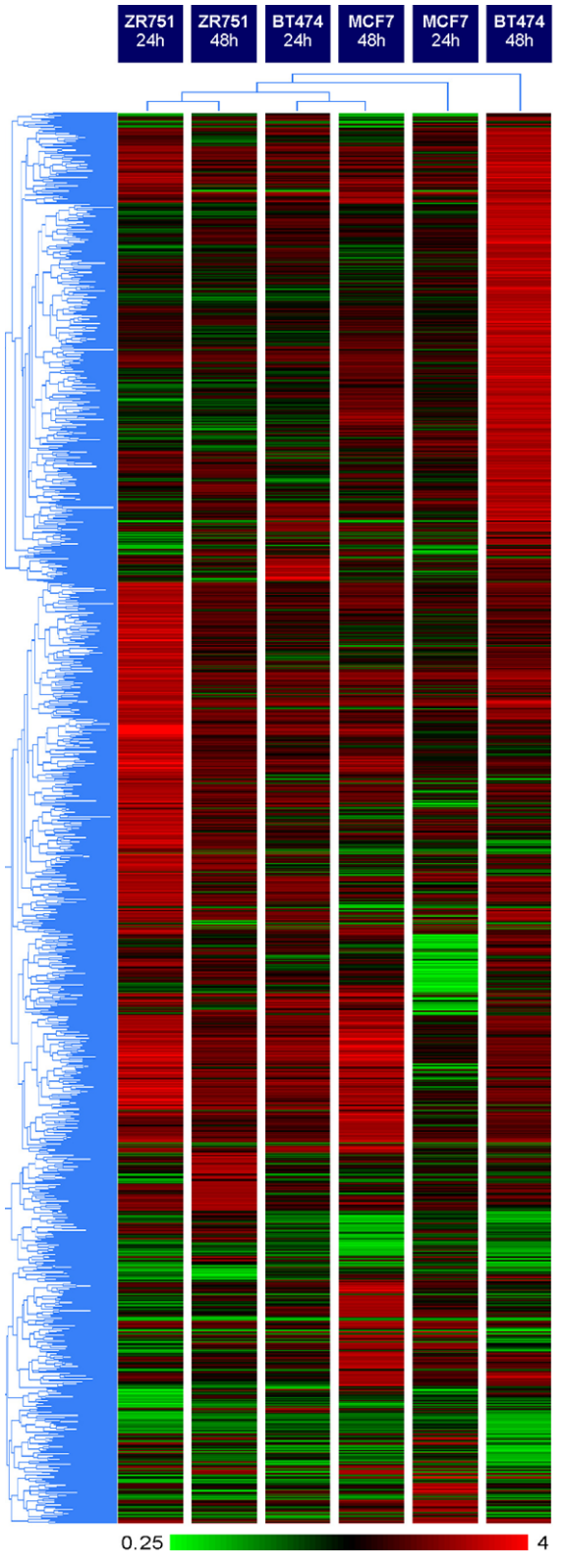

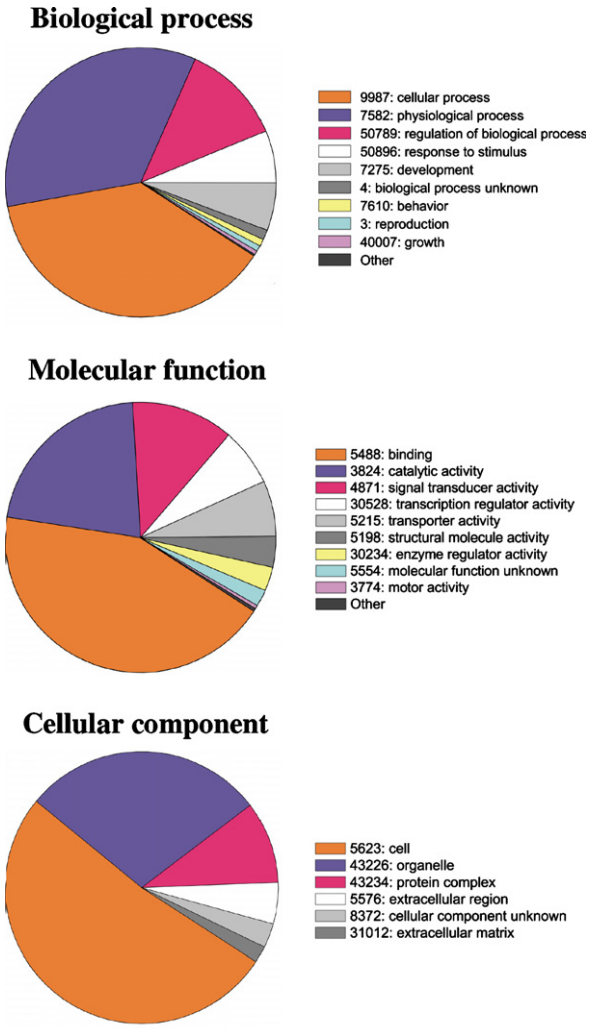

Fig. 2. Pie charts presenting classification of the 1798 gene elements according to the gene ontology information within the biological process, cellular component, and molecular function category.

a set of 31 biological process categories associated with *PPM1D* silencing whereas the same numbers for enriched cellular component and molecular function terms were 4 and 17, respectively (Table 1). The highly enriched GO categories included for example those related to regulation of cell cycle, particularly in G1 phase, assembly of various intracellular structures and components, such as

Fig. 1. Hierarchical clustering of the 1798 differentially expressed gene elements representing 1579 individual transcripts. Clustering was performed using Pearson correlation. In the two-dimensional dendrogram, genes are represented by each row and experimental samples by each column. Changes in the expression levels are illustrated using color coding, the key to the color code is shown at the bottom.

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Table 1
Enriched gene ontology categories for differentially expressed genes

Category	Total number of genes	%	Number of differentially expressed genes	%	*p*-Value[a]	Depth
Biological function						
GO:6334: nucleosome assembly	134	0.7	19	2.0	0.000	6
GO:31497: chromatin assembly	148	0.7	19	2.0	0.000	5
GO:7165: signal transduction	4286	21.6	247	26.3	0.000	4
GO:80: G1 phase of mitotic cell cycle	22	0.1	6	0.6	0.000	7
GO:7154: cell communication	5342	26.9	297	31.6	0.001	3
GO:51318: G1 phase	24	0.1	6	0.6	0.001	6
GO:6333: chromatin assembly or disassembly	219	1.1	22	2.3	0.001	8
GO:6461: protein complex assembly	408	2.1	33	3.5	0.002	6
GO:7242: intracellular signaling cascade	1771	8.9	108	11.5	0.004	5
GO:9725: response to hormone stimulus	16	0.1	4	0.4	0.006	4
GO:30319: di-, tri-valent inorganic anion homeostasis	3	0.0	2	0.2	0.007	8
GO:9755: hormone-mediated signaling	9	0.0	3	0.3	0.007	6
GO:6098: pentose-phosphate shunt	17	0.1	4	0.4	0.007	9
GO:6740: NADPH regeneration	17	0.1	4	0.4	0.007	10
GO:7595: lactation	10	0.1	3	0.3	0.010	5
GO:6325: establishment and/or maintenance of chromatin architecture	407	2.1	30	3.2	0.011	7
GO:6357: regulation of transcription from RNA polymerase II promoter	425	2.1	31	3.3	0.012	9
GO:42159: lipoprotein catabolism	4	0.0	2	0.2	0.013	7
GO:45192: low-density lipoprotein catabolism	4	0.0	2	0.2	0.013	8
GO:6063: uronic acid metabolism	4	0.0	2	0.2	0.013	7
GO:19585: glucuronate metabolism	4	0.0	2	0.2	0.013	8
GO:46398: UDP-glucuronate metabolism	4	0.0	2	0.2	0.013	9
GO:30002: anion homeostasis	4	0.0	2	0.2	0.013	7
GO:6259: DNA metabolism	946	4.8	60	6.4	0.013	5
GO:30073: insulin secretion	11	0.1	3	0.3	0.013	8
GO:6366: transcription from RNA polymerase II promoter	764	3.9	50	5.3	0.013	8
GO:6739: NADP metabolism	21	0.1	4	0.4	0.016	9
GO:6323: DNA packaging	419	2.1	30	3.2	0.017	6
GO:6733: oxidoreduction coenzyme metabolism	44	0.2	6	0.6	0.017	6
GO:6944: membrane fusion	45	0.2	6	0.6	0.019	5
GO:51276: chromosome organization and biogenesis	494	2.5	34	3.6	0.019	5
Cellular component						
GO:786: nucleosome	109	0.6	16	1.8	0.000	11
GO:785: chromatin	243	1.3	24	2.7	0.000	10
GO:5694: chromosome	431	2.2	31	3.4	0.011	8
GO:5654: nucleoplasm	407	2.1	29	3.2	0.015	11
Molecular function						
GO:5179: hormone activity	134	0.6	16	1.5	0.001	5
GO:5102: receptor binding	868	4.0	62	6.0	0.001	4
GO:15085: calcium ion transporter activity	26	0.1	6	0.6	0.001	6
GO:16563: transcriptional activator activity	379	1.7	32	3.1	0.001	3
GO:15082: di-, tri-valent inorganic cation transporter activity	61	0.3	9	0.9	0.002	5
GO:4871: signal transducer activity	3552	16.2	202	19.5	0.002	3
GO:3707: steroid hormone receptor activity	79	0.4	10	1.0	0.004	6
GO:3713: transcription coactivator activity	255	1.2	22	2.1	0.005	6
GO:4879: ligand-dependent nuclear receptor activity	83	0.4	10	1.0	0.006	5
GO:30156: benzodiazepine receptor binding	3	0.0	2	0.2	0.006	5
GO:3677: DNA binding	3010	13.7	169	16.3	0.009	4
GO:8889: glycerophosphodiester phosphodiesterase activity	10	0.0	3	0.3	0.010	7

(*continued on next page*)

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Table 1 (*continued*)

Category	Total number of genes	%	Number of differentially expressed genes	%	*p*-Value[a]	Depth
GO:3684: damaged DNA binding	64	0.3	8	0.8	0.010	5
GO:4713: protein-tyrosine kinase activity	494	2.3	35	3.4	0.012	7
GO:30023: extracellular matrix constituent conferring elasticity	4	0.0	2	0.2	0.013	4
GO:30528: transcription regulator activity	1966	9.0	114	11.0	0.013	2
GO:46873: metal ion transporter activity	152	0.7	14	1.4	0.013	4

[a] *p*-Value 0.02 was used as a cut-off.

Table 2
The number of differentially expressed genes in different cell lines and time points

Differentially expressed genes	BT-474		MCF7		ZR-75-1	
	24 h	48 h	24 h	48 h	24 h	48 h
Number of upregulated genes	79	569	72	303	515	89
Number of downregulated genes	23	118	133	82	72	41
Total	102	687	205	385	587	130

nucleosomes and chromatin, and regulation of general cell signaling processes and metabolic cascades (Table 1). Ontologies form a nested tree structure in which the general terms are in the root and most detailed ontologies in the leaves. To study the properties of the ontologies in Table 1, the levels of the GOs were determined. In case several levels exist, the shortest path was chosen. The root term is the most common classifier and it was given value 0. Most of the ontologies in molecular function appear on levels 4–5 while ontologies in biolocical process are located on much more specific levels. Cellular components are paradoxical to analyze since they do appear on high ontology levels, but due to their nature they usually are information poor. Different processes function and components have different depths, therefore it is not possible to directly compare the depths for different properties.

Finally, we examined more closely the list of 1798 differentially expressed gene elements and focussed our analyses on those genes whose expression patterns were consistent at both time points for a particular cell line or that showed consistent expression patterns in at least two of the three cell lines. For this purpose, consistent expression was defined as ratio values $\geqslant 1.75$ or $\leqslant 0.7$ for upregulated and downregulated genes, respectively. Through this approach, we extracted a subset of 266 gene elements representing 264 individual genes (Table S2) that were differentially expressed after *PPM1D* silencing. As expected, *PPM1D* was in the list and its expression was downregulated in all three breast cancer cell lines. The list included multiple genes with DNA binding activities. For example, two zinc finger proteins were upregulated (*ZNF212* in MCF7 and *ZNF278* in all cell lines), whereas *RAB25* (member of RAS oncogene family), *NSBP1* (nucleosomal binding protein 1), and *ESR1* (estrogen receptor 1) showed decreased expression. *RAB25* was downregulated in all cell lines, while *ESR1* expression decreased in BT-474 and ZR-75-1, and *NSBP1* expression only in ZR-75-1. Furthermore, four different human histone coding genes, *HIST1H2AC*, *HIST1H2BF*, *HIST1H2BO*, and *HIST1H1D*, were found to be overexpressed. Interestingly, *HIST1H2AC* was upregulated only in MCF7 cell line, *HIST1H2BF*, *HIST1H2BO* in BT-474, *HIST1H1D* in ZR-75-1, and thus none of these genes showed increased expression throughout all cell lines. Similarly, we discovered several differentially expressed genes associated with hormone related activities of the cell. In addition to the above mentioned *ESR1*, these included *NR4A1* (nuclear receptor subfamily 4, group A, member 1) and *STC1* (stanniocalcin 1). *NR4A1* was consistently downregulated after *PPM1D* silencing whereas *STC1* showed increased expression in all cell lines. In the enriched GO category of protein-tyrosine kinase activity, we noticed five genes with changed expression levels. Three of them, *PLK1* (polo-like kinase 1), *NEK4* (NIMA-related kinase 4), and *WIF1* (WNT inhibitory factor 1), showed decreased expression, whereas two, *PRKY* (protein kinase, Y-linked), and *ULK2* (unc-51-like kinase 2), were upregulated. Unlike the other four genes, expression of *WIF1* was changed only in MCF7 and ZR-75-1 cell lines.

4. Discussion

In this study, we aimed to investigate how the previously reported *PPM1D* overexpression contributes to breast cancer tumorigenesis. To this end, we used RNA interference to silence *PPM1D* expression in three breast cancer cell lines (BT-474, MCF7, and ZR-75-1) with *PPM1D* amplifica-

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tion and overexpression and subsequently performed a large scale gene expression screen to explore the molecular consequences of *PPM1D* downregulation on global gene expression patterns. The microarray data indicated that the three breast cancer cell lines showed different gene expression changes following *PPM1D* silencing despite the fact that they all overexpress *PPM1D*. ZR-75-1 and MCF7 cells carry wt p53 whereas BT-474 has mutant p53 [35,36] and thus the p53 status of the cells might explain these results. However, hierarchical clustering indicated that the wt p53 MCF7 cells and mutant p53 BT-474 cells clustered together whereas wt p53 ZR-75-1 formed its own branch in the dendogram. Taken together, the p53 status alone does not seem to determine the consequences of *PPM1D* inhibition on gene expression patterns.

Our analysis yielded a total of 1798 differentially expressed gene elements representing 1579 individual genes following *PPM1D* silencing. This surprisingly large number of affected genes might reflect the fact that PPM1D directly regulates several important genes in various signaling pathways. For example, PPM1D is known to modulate the activity of p38 MAPK signaling cascade and thereby its ability to control the p53 tumor suppressor protein function [4,7,23]. However, it is also possible that the majority of the observed gene expression changes represent downstream signaling events of only few direct PPM1D target genes. In any case, these results point towards the importance of PPM1D in the regulation of key cellular functions in breast cancer.

We noticed that a large fraction of the differentially expressed genes are associated with molecular binding activity whereas, at the same time, they fall into several different biological process categories (Fig. 2). This indicates that *PPM1D* contributes to a wide range of cellular mechanisms and pathways. Closer examination of genes affected by *PPM1D* silencing revealed that these molecules are commonly involved for example in cell cycle regulation (Table 1) and thus participate in the control of cell proliferation. This is consistent with previous observations that PPM1D regulates a set of key cell cycle checkpoint effectors, such as p53, ATM, Chk1, and Chk2 [23,25–28]. Accordingly, we and others have previously shown that *PPM1D* silencing leads to inhibition of cell proliferation [5,7,20,29,37]. A large part of the differentially expressed genes were also implicated in construction of new cellular structures and components and the regulation of general cell signaling processes and metabolic cascades. Additionally, most of these genes were found to be located either in nucleosome and nucleoplasm or in different kinds of chromosomal structures (Table 1). This cellular localization implies that these genes have a role in packaging of DNA into chromosomes and/or the control of gene expression. Together these results suggest that *PPM1D* has a role in a variety of cellular functions and thus may have widespread effects on breast cancer biology.

Three genes involved in the control of apoptosis were differentially expressed in all cell lines with downregulation of *PPM1D*. *NR4A1* has previously been shown to be involved in caspase-independent cell death [38,39], *RAB25* signaling pathway is found to play a role in the regulation of cell proliferation and apoptosis in ovarian cancer cells [40,41], and *PLK1* reduction induces apoptosis in cervical cancer, prostate cancer, and breast cancer cells [42–44]. These findings are consistent with the previous data from us and others demonstrating increased apoptosis after *PPM1D* knockdown [20,25,29,45]. *PLK1* is also believed to be involved in the regulation of the progression of the cell cycle, cell division, and DNA damage repair pathways [46,47]. Additionally, it possesses protein tyrosine kinase activity which plays a crucial role in many cell regulatory processes [46]. Disregulated kinase activity is frequently observed in cancer where various kinases regulate, e.g. cell growth, movement, and death [48]. Other kinases, such as *NEK4*, *PRKY*, *ULK2*, and *WIF1*, were also differentially expressed after *PPM1D* silencing. Together with previous studies our findings indicate that *PPM1D* is likely to have an important influence on several essential cell cycle, cell growth, and cell survival pathways during breast cancer pathogenesis.

Another interesting set of genes implicated in our study, *NR4A1*, *ESR1*, and *STC1*, is associated with hormone related activities of the cell. For example, *ESR1* is a ligand-activated transcription factor with important roles in regulation of gene transcription, growth control, breast cancer development, and evolvement of hormone resistance [49]. In addition, *ESR1* as well as *RAB25*, *PLK1*, and *STC1* are found to be amplified and to have altered expression levels in several tumor types [38,50–52]. Consequently, all the abovementioned genes have been shown to be associated with potential and interesting cancer associated cellular features.

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Histones are responsible for the nucleosome structure in eukaryotes and their overexpression has been shown to block transcription *in vitro* by triggering chromatin aggregation and to increase the incidence of mitotic chromosome loss [53,54]. This implies that appropriate balance of histone proteins is required for the accurate assembly of chromatin as well as for the proper cell division and growth. Interestingly four histone coding genes (*HIST1H2AC*, *HIST1H2BF*, *HIST1H2BO*, and *HIST1H1D*) were upregulated in our analyses and thus their overexpression might contribute to the inhibition of cell growth following *PPM1D* silencing.

Recent reports have raised some questions concerning the specificity of RNA interference methodology, the two main issues being possible off-target effects and induction of an immunological response that may in turn lead to global inhibition of protein synthesis [31–33]. Sequence analysis of our *PPM1D* siRNA did not reveal significant homologies to any other human gene indicating that it is unlikely to silence other known genes. The induction of immunological response is typically not a major problem when using short siRNA oligos. Such short siRNAs are detected by toll-like receptor TLR7 which is not expressed by non-immune cells, including the breast cancer cells used in this study [55]. We also verified that our siRNAs did not contain immunostimulatory sequences [31–33]. Moreover, we used synthetic siRNAs because they are known to induce a weaker immune response than vector based siRNAs [55]. The above mentioned issues indicate that the approach used in this study most likely resulted in specific silencing of *PPM1D*.

In summary, this study is the first to systematically investigate the underlying mechanisms of *PPM1D* amplification and overexpression on breast cancer development by analyzing global gene expression responses to *PPM1D* silencing. Through this approach, we were able to identify several differentially expressed genes that are involved in multiple cellular functions, thus highlighting a complex transcriptional program regulated directly and indirectly by PPM1D.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2007.09.019.

References

[1] A. Schweighofer, H. Hirt, I. Meskiene, Plant PP2C phosphatases: emerging functions in stress signaling, Trends. Plant. Sci. 9 (2004) 236–243.
[2] M. Fiscella, H. Zhang, S. Fan, K. Sakaguchi, S. Shen, W.E. Mercer, et al., Wip1, a novel human protein phosphatase that is induced in response to ionizing radiation in a p53-dependent manner, Proc. Natl. Acad. Sci. USA 94 (1997) 6048–6053.
[3] J. Choi, E. Appella, L.A. Donehower, The structure and expression of the murine wildtype p53-induced phosphatase 1 (Wip1) gene, Genomics 64 (2000) 298–306.
[4] M. Takekawa, M. Adachi, A. Nakahata, I. Nakayama, F. Itoh, H. Tsukuda, et al., p53-inducible wip1 phosphatase mediates a negative feedback regulation of p38 MAPK-p53 signaling in response to UV radiation, EMBO J. 19 (2000) 6517–6526.
[5] J. Choi, B. Nannenga, O.N. Demidov, D.V. Bulavin, A. Cooney, C. Brayton, et al., Mice deficient for the wild-type p53-induced phosphatase gene (Wip1) exhibit defects in reproductive organs, immune function, and cell cycle control, Mol. Cell. Biol. 22 (2002) 1094–1105.
[6] M.L. Schito, O.N. Demidov, S. Saito, J.D. Ashwell, E. Appella, Wip1 phosphatase-deficient mice exhibit defective T cell maturation due to sustained p53 activation, J. Immunol. 176 (2006) 4818–4825.
[7] D.V. Bulavin, C. Phillips, B. Nannenga, O. Timofeev, L.A. Donehower, C.W. Anderson, et al., Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK-mediated activation of the p16(Ink4a)-p19(Arf) pathway, Nat. Genet. 36 (2004) 343–350.
[8] R. Bernards, Wip-ing out cancer, Nat. Genet. 36 (2004) 319–320.
[9] M. Harrison, J. Li, Y. Degenhardt, T. Hoey, S. Powers, Wip1-deficient mice are resistant to common cancer genes, Trends. Mol. Med. 10 (2004) 359–361.
[10] M. Barlund, O. Monni, J. Kononen, R. Cornelison, J. Torhorst, G. Sauter, et al., Multiple genes at 17q23 undergo amplification and overexpression in breast cancer, Cancer Res. 69 (2000) 5340–5344.
[11] G.J. Wu, C.S. Sinclair, J. Paape, J.N. Ingle, P.C. Roche, C.D. James, et al., 17q23 amplifications in breast cancer involve the PAT1, RAD51C, PS6K, and SIGma1B genes, Cancer Res. 60 (2000) 5371–5375.
[12] A.E. Erson, B.L. Niell, S.K. DeMers, J.M. Rouillard, S.M. Hanash, E.M. Petty, Overexpressed genes/ESTs and characterization of distinct amplicons on 17q23 in breast cancer cells, Neoplasia 3 (2001) 521–526.
[13] O. Monni, M. Barlund, S. Mousses, J. Kononen, G. Sauter, M. Heiskanen, et al., Comprehensive copy number and gene

expression profiling of the 17q23 amplicon in human breast cancer, Proc. Natl. Acad. Sci. USA 98 (2001) 5711–5716.
[14] G. Wu, C. Sinclair, S. Hinson, J.N. Ingle, P.C. Roche, F.J. Couch, Structural analysis of the 17q22-23 amplicon identifies several independent targets of amplification in breast cancer cell lines and tumors, Cancer Res. 61 (2001) 4951–4955.
[15] C.L. Andersen, O. Monni, U. Wagner, J. Kononen, M. Barlund, C. Bucher, et al., High-throughput copy number analysis of 17q23 in 3520 tissue specimens by fluorescence in situ hybridization to tissue microarrays, Am. J. Pathol. 161 (2002) 73–79.
[16] C.S. Sinclair, M. Rowley, A. Naderi, F.J. Couch, The 17q23 amplicon and breast cancer, Breast Cancer Res. Treat. 78 (2003) 313–322.
[17] D.V. Bulavin, O.N. Demidov, S. Saito, P. Kauraniemi, C. Phillips, S.A. Amundson, et al., Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity, Nat. Genet. 31 (2002) 210–215.
[18] J. Li, Y. Yang, Y. Peng, R.J. Austin, W.G. van Eyndhoven, K.C. Nguyen, et al., Oncogenic properties of PPM1D located within a breast cancer amplification epicenter at 17q23, Nat. Genet. 31 (2002) 133–134.
[19] A. Hirasawa, F. Saito-Ohara, J. Inoue, D. Aoki, N. Susumu, T. Yokoyama, et al., Association of 17q21-q24 gain in ovarian clear cell adenocarcinomas with poor prognosis and identification of PPM1D and APPBP2 as likely amplification targets, Clin. Cancer. Res. 9 (2003) 1995–2004.
[20] F. Saito-Ohara, I. Imoto, J. Inoue, H. Hosoi, A. Nakagawara, T. Sugimoto, et al., PPM1D is a potential target for 17q gain in neuroblastoma, Cancer Res. 63 (2003) 1876–1883.
[21] J. Rauta, E-L. Alarmo, P. Kauraniemi, R. Karhu, T. Kuukasjarvi, A. Kallioniemi, The serine-threonine protein phosphatase PPM1D is frequently activated through amplification in aggressive primary breast tumours, Breast Cancer Res. Treat. 95 (2006) 257–263.
[22] O.N. Demidov, C. Kek, S. Shreeram, O. Timofeev, A.J. Fornace, E. Appella, The role of the MKK6/p38 MAPK pathway in Wip1-dependent regulation of ErbB2-driven mammary gland tumorigenesis, Oncogene 26 (2007) 2502–2506.
[23] X. Lu, B. Nannenga, L.A. Donehower, PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints, Genes Dev. 19 (2005) 1162–1174.
[24] X. Lu, D. Bocangel, B. Nannenga, H. Yamaguchi, E. Appella, L.A. Donehower LA, The p53-induced oncogenic phosphatase PPM1D interacts with uracil DNA glycosylase and suppresses base excision repair, Mol. Cell. 15 (2004) 621–634.
[25] H. Fujimoto, N. Onishi, N. Kato, M. Takekawa, X.Z. Xu, A. Kosugi, et al., Regulation of the antioncogenic Chk2 kinase by the oncogenic Wip1 phosphatase, Cell. Death. Differ. 13 (2006) 1170–1180.
[26] M. Oliva-Trastoy, V. Berthonaud, A. Chevalier, C. Ducrot, M.C. Marsolier-Kergoat, C. Mann, et al., The Wip1 phosphatase (PPM1D) antagonizes activation of the Chk2 tumour suppressor kinase, Oncogene 26 (2006) 1449–1458.
[27] S. Shreeram, O.N. Demidov, W.K. Hee, H. Yamaguchi, N. Onishi, C. Kek, et al., Wip1 phosphatase modulates ATM-dependent signaling pathways, Mol. Cell. 23 (2006) 757–764.
[28] S. Shreeram, W.K. Hee, O.N. Demidov, C. Kek, H. Yamaguchi, A.J. Fornace Jr., et al., Regulation of ATM/p53-dependent suppression of myc-induced lymphomas by Wip1 phosphatase, J. Exp. Med. 203 (2006) 793–2799.
[29] J. Pärssinen, E-L. Alarmo, R. Karhu, A. Kallioniemi, PPM1D silencing by RNAi inhibits proliferation and induces apoptosis in breast cancer cell lines with wild-type p53, submitted for publication.
[30] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.
[31] V. Hornung, M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, et al., Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7, Nat. Med. 11 (2005) 263–270.
[32] A.D. Judge, V. Sood, J.R. Shaw, D. Fang, K. McClintock, I. MacLachlan, Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA, Nat. Biotechnol. 23 (2005) 457–462.
[33] M. Sioud, Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization, J. Mol. Biol. 348 (2005) 1079–1090.
[34] M. Ashburner, C.A. Ball, J.A. Blake, D. Botstein, H. Butler, J.M. Cherry, et al., Nat. Genet. 25 (2000) 25–29.
[35] I.B. Runnebaum, M. Nagarajan, M. Bowman, D. Soto, S. Sukumar, Mutations in p53 as potential molecular markers for human breast cancer, Proc. Natl. Acad. Sci. USA 88 (1991) 10657–10661.
[36] N. Concin, C. Zeillinger, D. Tong, M. Stimpfl, M. König, D. Printz, et al., Comparison of p53 mutational status with mRNA and protein expression in a panel of 24 human breast carcinoma cell lines, Breast Cancer Res. Treat. 79 (2003) 37–46.
[37] G.I. Belova, O.N. Demidov, A.J. Jr Fornace, D.V. Bulavin, Chemical inhibition of Wip1 phosphatase contributes to suppression of tumorigenesis, Cancer Biol. Ther. 4 (2005) 1154–1158.
[38] S.O. Kim, K. Ono, P.S. Tobias, J. Han, Orphan nuclear receptor Nur77 is involved in caspase-independent macrophage cell death, J. Exp. Med. 197 (2003) 1441–1452.
[39] C.X. Li, N. Ke, R. Sundaman, F. Wong-Staal, NR4A1, 2, 3 – an orphan nuclear hormone receptor family involved in cell apoptosis and carcinogenesis, Histopathol. 21 (2006) 533–540.
[40] Y. Fan, X.Y. Xin, B.L. Chen, X. Ma, Knockdown of RAB25 expression by RNAi inhibits growth of human epithelial ovarian cancer cells in vitro and in vivo, Pathology 38 (2006) 561–567.
[41] K.W. Cheng, J.P. Lahad, W.L. Kuo, A. Lapuk, K. Yamada, N. Auersperg, et al., The RAB25 small GTPase determines aggressiveness of ovarian and breast cancers, Nat. Med. 10 (2004) 1251–1256.
[42] X. Liu, R.L. Erikson, Polo-like kinase (Plk)1 depletion induces apoptosis in cancer cells, Proc. Natl. Acad. Sci. USA 100 (2003) 5789–5794.
[43] S. Reagan-Shaw, N. Ahmad, Silencing of polo-like kinase (Plk) 1 via siRNA causes induction of apoptosis and impairment of mitosis machinery in human prostate cancer cells: implications for the treatment of prostate cancer, FASEB J. 19 (2005) 611–613.
[44] B. Spänkuch, E. Kurunci-Csacsko, M. Kaufmann, K. Strebhardt, Rational combinations of siRNAs targeting

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Plk1 with breast cancer drugs, Oncogene (2007) [Epub ahead of print].
[45] T. Hershko, K. Korotayev, S. Polager, D. Ginsberg, E2F1 modulates p38 MAPK phosphorylation via transcriptional regulation of ASK1 and Wip1, J. Biol. Chem. 281 (2006) 31309–31316.
[46] E.A. Nigg, Polo-like kinases: positive regulators of cell division from start to finish, Curr. Opin. Cell. Biol. 10 (1998) 776–783.
[47] X. Liu, R.L. Erikson, Polo-like kinase 1 in the life and death of cancer cells, Cell Cycle 2 (2003) 424–425.
[48] D.R. Robinson, Y.M. Wu, S.F. Lin, The protein tyrosine kinase family of the human genome, Oncogene 55 (2000) 5548–5557.
[49] V. Speirs, R.A. Walker, New perspectives into the biological and clinical relevance of oestrogen receptors in the human breast, J. Pathol. 211 (2007) 499–506.
[50] N. Takai, R. Hamanaka, J. Yoshimatsu, I. Miyakawa, Polo-like kinases (Plks) and cancer, Oncogene 24 (2005) 287–291.
[51] F. Holst, P.R. Stahl, C. Ruiz, O. Hellwinkel, Z. Jehan, M. Wendland, et al., Estrogen receptor alpha (ESR1) gene amplification is frequent in breast cancer, Nat. Genet. 39 (2007) 655–660.
[52] A.C. Chang, D.A. Jellinek, R.R. Reddel, Mammalian stanniocalcins and cancer, Endocr. Relat. Cancer. 10 (2003) 359–373.
[53] D. Meeks-Wagner, L.H. Hartwell, Normal stoichiometry of histone dimer sets is necessary for high fidelity of mitotic chromosome transmission, Cell 44 (1986) 43–52.
[54] D.J. Steger, J.L. Workman, Transcriptional analysis of purified histone acetyltransferase complexes, Methods 19 (1999) 410–416.
[55] M. Schlee, V. Hornung, G. Hartmann, siRNA and isRNA: two edges of one sword, Mol. Ther. 14 (2006) 463–470.

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