Phosphatases in Cancer

shifting the balance

Elmer Hoekstra

Colophon

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verschuiving van het evenwicht

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Chapter 1

General introduction and outline of the thesis

Cancer

Cancer was first described in 400 BC by the Greek physician and "father of Medicine" Hippocrates. He found masses all over the body with a finger- like structure, which he called 'carcinos', referring to the Greek word for crab. It is thought that the typical structure of the lesions reminded him of the shape of this animal. Later, the Roman physician Celcus (28-50 BC) translated the Greek term into the Latin word for crab; cancer.

Nowadays, cancer is the generic term for a group of over a hundred diseases, affecting any part of our body. Although there are many different kinds of cancer, they all have one thing in common, which is that cells acquire abnormal properties and lose their intrinsic proliferation inhibition. In normal cells, cell proliferation is a very well-orchestrated process, with cell growth and cell death occurring in a tightly balanced manner. Cancer cells tend to escape from regulation and instead of dying off, they continue to divide more rapidly resulting in increasing numbers of abnormal cells which all together form a mass of cells; a tumor. Furthermore, tumors can grow outside the usual boundaries to which the tissues they are derived from are limited, and invade adjacent parts of the body. Moreover, tumor cells can travel to other parts of the body where they can seed, grow and form new tumors that crowd out normal tissue. This usually occurs when cancer cells get into the body's bloodstream or lymph vessels, which facilitate their migration to other locations. The process of cancer spreading, i.e. metastasis, is the major cause of cancer mortality.

Cancer is emerging as one of the leading causes of morbidity and mortality worldwide, with approximately 14 million newly diagnosed patients and 8.2 million cancer related deaths in 2012 [1]. Amongst men the most common sites for tumorigenesis are lung, prostate, colorectum, stomach, and liver cancer, while in women breast, colorectum, lung, cervix, and stomach cancer are most prevalent [2]. This thesis will focus mainly on colorectal cancer, however in chapter 3 we will also discuss prostate cancer.

Colorectal cancer

Colorectal cancer (CRC) is one of the most common types of cancer, and includes tumors in the large intestine, rectum, and appendix. Worldwide, approximately 1.2 million people are diagnosed with CRC each year, accounting for almost 10% of all cancer cases [3]. There are environmental and genetic factors that contribute to the development of CRC [4]. Although inherited susceptibility results in the most striking increase in risk, the majority of CRCs are sporadic rather than familial [5]. Familial adenomatous polyposis (FAP) is a relatively common form of familial colon

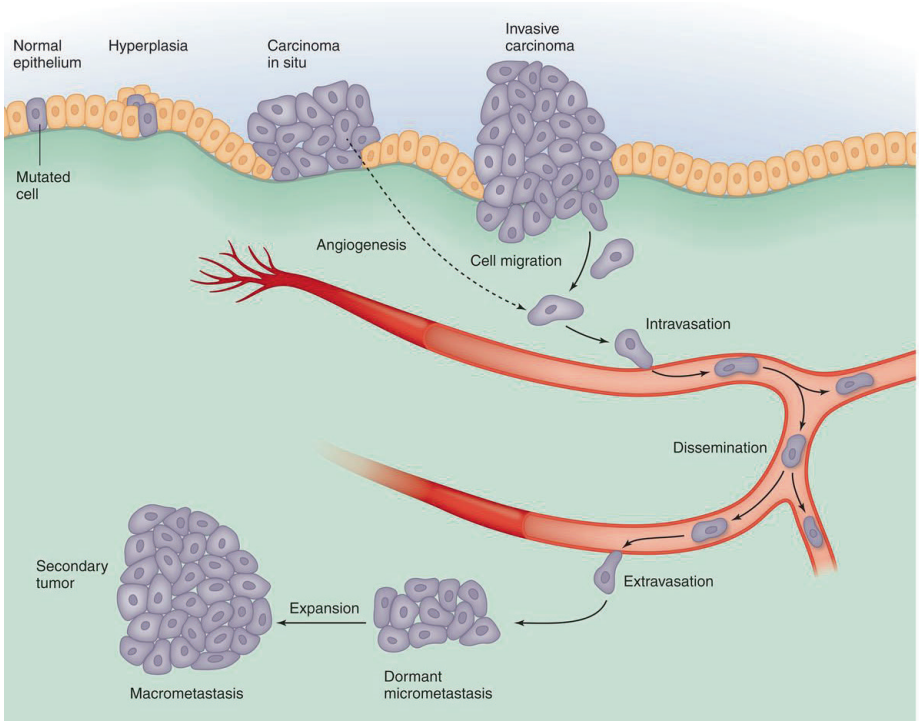

Figure 1. Schematic overview of cancer development. Normal epithelial cells gain mutations which results in the formation of hyperplasia and eventually invasive carcinoma. Carcinoma cells migrate away from the primary tumor via the blood stream to form a secondary tumor or metastasis. (figure adapted from CSH perspectives.)

cancer syndrome, but accounts for less than 1 percent of the total CRC cases. Environmental factors that are involved in the pathogenesis of CRC include low physical activity, obesity, smoking, and diet [6].

CRC is typically starts with the growth of a non-cancerous polyp, which in time progresses from precursor lesions (adenoma) to a malignant cancer. Removal of adenomas could reduce the risk of developing full blown cancer [7] and as a result screening strategies in order to identify such lesions before they turn malignant have made their appearance in the clinic [8].

Typical symptoms associated with CRC include fecal blood loss or melena, abdominal pain, and/or a change in bowel habits. Most patients however, present with an otherwise unexplained iron deficiency anemia due to the fecal occult blood loss, and do not have any specific complaints [9]. When detected in a non-metastatic state, patients can be treated curatively by removal of the primary cancer lesion [10]. Unfortunately, around 20 percent of the patients are diagnosed after spreading of the cancer has already occurred, therefore removal of the primary tumor will not be sufficient to cure these patients [3].

Several therapeutic options have been developed to treat patients with metastasized CRC. Chemotherapy was the first additional kind of therapy, and is still in use in the clinic today [11]. These chemical substances are cytotoxic, interfering in processes that govern DNA synthesis and selectively attacking rapidly dividing cells. Since this is one of the hallmarks of cancer cells, these therapies have been quite successful. However, chemotherapy also targets cells that under normal circumstances divide quickly, resulting in several side-effects, such as hair loss and decreased production of blood cells [12]. Even though the two traditional cancer treatments, chemotherapy and radiation, were not specifically designed to target tumor cells alone, the enhanced sensitivity to either DNA damage or cell cycle arrest due to the inherent replication stress in cancer cells has been exploited, seeking an optimal dose and schedule to kill tumor cells while minimizing the damage to normal cells. However, the ideal anticancer strategy would be the one that selectively kills tumor cells while sparing normal cells. This is the goal of 'targeted cancer therapies', which made their appearance in the clinic over the last decades [13,14]. These 'targeted therapies' are designed to block specific molecules that play a role in the signaling pathways involved in the growth, spreading, or progression of cancer cells. The limitation of these therapies is however, that cancer cells can become resistant to them [15]. The tumors will find a new pathway to achieve tumor growth that does not depend on the target of the therapy. Therefore these types of targeted therapies will probably work best in combination.

CRC as heterogeneous disease

All carcinomas found in the large intestine are referred to as 'colorectal cancer', suggesting that they are all similar. However, this is actually an overall term for a large heterogeneous group of diseases which all differ in terms of molecular mechanism, regional distribution, pathology of the lesions, therapy response, and natural history [16]. There are at least three major molecular mechanisms that give rise to colorectal cancer. The predominant chromosomal instability (CIN) pathway [17], the CpG island methylator phenotype (CIMP) pathway [18], and the microsatellite instability (MSI) pathway which is characterized by a (germline) mutation in DNA mismatch repair (MMR) genes [19]. Regardless of the underlying pathway, for a cancer to develop, multiple and sequential genetic alterations must occur. These alterations occur in tumor suppressor genes and oncogenes. In their normal state, tumor suppressor genes inhibit cell proliferation. This growth inhibition is lost when both alleles are inactivated by mutation and/or epigenetic changes, such as promoter methylation. Tumor suppressor genes broadly conform to Knudson's classic two-hit hypothesis, where inactivation of both alleles is required for tumor suppressor genes to lose their normal function [20]. In contrast, oncogenes act by promoting cell proliferation. Mutation of these genes leads to abnormal overexpression or

increased activity of the protein, leading to increased cell proliferation [21]. Each genetic perturbation provides the cell with a relative proliferative advantage, together resulting in uncontrolled cancer growth.

Chromosomal instability (CIN) pathway

Up to 85% of the CRC cases develop via the "classical" CIN-pathway. This pathway is associated with a loss of function mutation in the *APC* (Adenomatous Polyposis Coli) tumor suppressor gene [17,22]. This is the most critical gene in CRC development. Somatic mutations in both alleles are present in 80 percent of sporadic CRCs, and a single germline mutation in this gene is responsible for the hereditary polyposis syndrome FAP [23] – a second hit in the remaining *APC* allele (loss of heterozygosity, LOH) is required for these patients to develop CRC. The *APC* gene encodes a 312 kDa multidomain protein that participates in several cellular processes, including cell cycle regulation, apoptosis, cell adhesion and cell migration [24–26]. The most important role of APC however, is its tumor suppressing function through its capacity to regulate intracellular β-catenin (*CTNNB1*) levels [27–29]. As part of the canonical Wnt signaling pathway, the APC protein binds to β-catenin and, together with glycogen synthase kinase 3β (GSK3β) and axin/conductin, promotes β-catenin degradation, thereby preventing its signaling activity to the nucleus. When *APC* is mutated, its ability to downregulate β-catenin expression is lost, resulting in an accumulates of cytoplasmic β-catenin, which subsequently translocates to the nucleus where it forms a complex with T-cell factor (TCF) and lymphoid enhancer factor (LEF), activating transcription of Wnt target genes involved in proliferation and apoptosis [30,31]. Usually a loss of function mutation in *APC* is the first "hit" in the adenoma-to-carcinoma sequence of the CIN-pathway, resulting in an early "tumor" lesion called aberrant crypt foci [32].

In the transition from an early to intermediate lesion, *APC* mutations are followed by activating mutations in the oncogenes *KRAS* and *BRAF* in approximately 50% and 10% of cases, respectively [33,34]. The proteins encoded by both these genes are involved in the transduction of cell division signals as part of the RAS/RAF/ERK pathway [35]. In response to extracellular engagement by growth factors, cytokines or hormones of their respective cell surface receptors, Ras is activated, resulting in activation of the mitogen-activated protein kinase cascade. Herein, Ras activates the protein kinase activity of RAF kinase, which in turn phosphorylates and activates the kinase MEK, which subsequently activates the ERK kinase, resulting in ERK-dependent gene transcription. Mutations in *KRAS* and *BRAF* constitutively activate this pathway resulting in increased proliferation [35].

The next step in the adenoma-to-carcinoma sequence is loss of the SMAD proteins, SMAD2 and SMAD4. They are located at 18q21.1 and allelic loss at this site is found in up to 60% of CRCs [36]. SMADs are essential regulators of the transform-

ing growth factor (TGF)-β signaling pathway, which is important in regulation of cell growth, cell differentiation, apoptosis, and cellular homeostasis [37]. Loss of SMAD4 protein function appears to be directly correlated to metastatic potential of CRC [38].

Finally, impairment of tumor suppressor gene *TP53* through allelic loss of 17p chromosomal arm, is often the latest event in the traditional CIN-pathway resulting in the transition from adenoma to adenocarcinoma [36]. *TP53* mutations or LOH increase relative to the increasing histological stage of the lesion. Only 4–26% of the adenomas have impaired function of the TP53 protein product p53, while it is observed in 50% of adenomas with invasive foci, and 50–75% of CRCs respectively [34]. The normal function of p53 is to slow down the cell cycle, providing ample time for DNA repair. However, when the sustained DNA damage is too great to repair, p53 induces pro-apoptotic genes, sending the cell into programmed cell death [39]. Thus, loss of p53 function allows tumor cells to rapidly divide, while acquiring new mutations.

The CIN pathway and the corresponding adenoma-to-carcinoma sequence has provided the basis for the molecular classification of CRC, however it is now clear that this is not the only pathway by which CRC can develop.

Microsatellite instability (MSI) pathway

Microsatellites are short repeat nucleotide sequences that are prone to errors during replication due to their repetitive nature. Usually, when errors in these repeats occur during DNA replication, the DNA mismatch repair (MMR) system recognizes these errors and repairs the mismatches. Microsatellite instability refers to a discrepancy in the number of repeats found in the microsatellite regions of tumor versus germline DNA. Therefore this 'instability of microsatellites' is a reflection of the inability of the MMR-genes to correct these errors in the microsatellite areas [40,41]. The affected cell thus acquires thousands of mutations, and while microsatellite loss in itself is not causative of cancer, when they occur inn cancer-related genes, such DNA replication errors may result in unbridled cell proliferation. In around 15% of sporadic CRCs somatic mutations or hypermethylation-based silencing of MMR genes occurs [42]. Several members of the MMR gene family have been identified such as *MSH2*, *MLH1*, *MSH6*, *PMS2*, *MLH3*, *MSH3*, and *PMS1*. The pure form of MSI-H tumors are due to an inherited autosomal dominant syndrome with a germline defect in one of the mismatch repair (MMR) proteins, called hereditary nonpolyposis CRC (HNPCC) or Lynch syndrome [43]. However, sporadic mutations in these genes also occur, resulting in tumors with a similar phenotype as the inherited form [44].

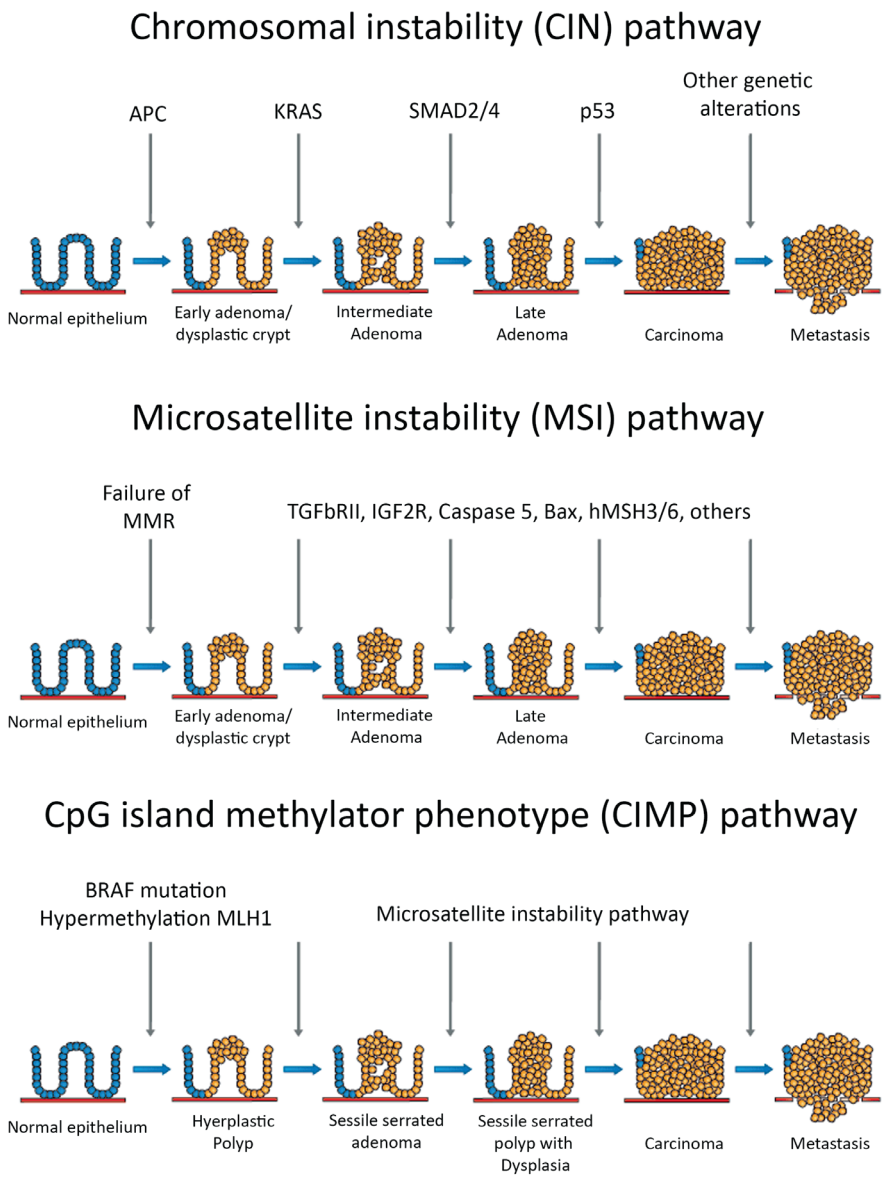

Figure 2. Schematic representation of the progression from normal epithelium to colorectal cancer for the three main CRC pathways; CIN, MSI, and CIMP. Progression from normal epithelium through adenoma to colorectal carcinoma is characterized by accumulated abnormalities of particular genes. In the CIN-pathway, the loss of one normal copy of the tumor suppressor gatekeeper gene APC is the first event. Mutations of the oncogene K-RAS seem to occur next. Additional mutations or losses of heterozygosity of SMAD2/4 and inactivation of the tumor suppressor gene p53. MSI-high tumors are characterized by a mutation in one of the mismatch repair (MMR) genes, resulting in DNA replication errors. CIMP-positive tumors are associated with BRAF-mutations, and are characterized by a hypermethylation of CpG-islands. This is phenotypically associated with sessile serrated adenomas. (Adapted from R. Justin Davies, Richard Miller & Nicholas Coleman, Nature Reviews Cancer 2005)

The CpG Island Methylator Phenotype (CIMP) Pathway

The CpG Island Methylator Phenotype pathway is the second most common pathway for sporadic CRCs and accounts for approximately 15% of sporadic cases [45,46]. The CIMP pathway is characterized by a widespread hypermethylation of promoter CpG islands, which results in so called epigenetic gene-silencing of target genes (46). Via this hypermethylation, the expression of tumor suppressor genes such as *MLH1* can be decreased, leading to the development of CIMP-positive CRCs. This CIMP-positive status is based on the hypermethylation analysis of a panel of CIMP-genes, however, there is no universal consensus on which panel is the golden standard for this diagnosis. Clinically, CIMP-positive CRCs are characterized by proximal location, female gender, and older age. The phenotype is dependent on the genes that are silenced, especially if there is a concomitant presence or absence of microsatellite instability due to methylation-induced transcriptional silencing of *MLH1* [48]. Classically, CIMP-positive CRCs that are also MSI-H, have similar clinical characteristics to the MSI-H, such as the relative good prognosis. However, in the absence of MSI-H, the CIMP-positive phenotype tumors are characterized by a worse prognosis and more advanced pathology. However the most important difference concerns the precursor lesion - CIMP-positive cancer arises from sessile serrated adenomas, while CRCs developing via the CIN and MSI pathway, originate from adenomatous polyps [49,50].

Kinases and phosphatases in cancer signaling pathways

What all the pathways described above have in common, is that due to mutations or epigenetic alterations, cellular homeostasis is disturbed, resulting in uncontrolled cell growth. Every cell in the body has a tightly regulated system that dictates when it needs to divide, grow, mature and eventually die off. This system is based on a network of signaling pathways, cooperating in maintaining a normal cellular homeostasis. Regulation of these signaling pathways is heavily dependent on a balanced equilibrium between two seemingly opposing enzymes; kinases and phosphatases. While kinases add phosphate groups to their substrates, phosphatases remove these groups by hydrolyzing the phosphoester bonds by which they are attached to the substrate [51]. Protein phosphorylation typically occurs on either serine, threonine or tyrosine residues, with an estimated relative distribution within the human proteome of 79.3%, 16.9% and 3.8% respectively [52], with approximately 17.000 proteins that possess at least one of these residues [53]. The human genome encodes 518 protein kinases of which the majority is known or predicted to phosphorylate serine and/or threonine residues [54,55]. In contrast, there are only approximately 200 phosphatases encoded in the human genome, targeting either phosphorylated proteins or lipids. Based on structure, phosphatases are subdivided into six distinct functional and structural superfamilies: protein tyrosine phosphatases (PTPs, 108 members), metal-dependent protein phosphatases (PPMs, 13

members), phosphoprotein protein phosphatases (PPPs, 15 members), lipid phosphatases (LPs, 37 members), haloacid dehalogenases (HADs, 21 members) and nucleoside-diphosphate-linked moiety X (NUDT,five members) [56]. This thesis will focus on several phosphatases, mostly concerning the protein tyrosine (PTP) family, and the lipid phosphatase family.

Phosphorylation is an efficient means to control cell response to internal and external cues: it is rapid, sometimes taking as little as a few seconds, it does not require new proteins to be synthesized or degraded and can be easily reverted. It is well-established that aberrant activation of kinases due to mutations or upregulation often occurs in cancer [57]. As a result of these alterations, cancer cells tend to be characterized by a shift the balance between the kinases and phosphatases, leading to uncontrolled cell growth and increased capability to invade surrounding tissue. Therefore, these crucial signal transduction molecules represent attractive targets for cancer therapy. Much of the research in past years has focused on the potential role of kinases in cancer development, since it is generally assumed that phosphorylation of substrates by kinases results in activation of signaling and that this class of enzymes may therefor present as a valuable target for treatment. As such, kinase inhibitors have been developed with some promising success [58]. Phosphatases however, have received far less attention. The current dogma states that phosphatases act as tumor suppressors by damping growth factor-induced signaling pathways.

Furthermore, due to their highly conserved structure and close similarity, phosphatases have the reputation to be ‘undruggable’. It was deemed to be too challenging to develop inhibitors targeting one phosphatase, without effecting their closely related counterparts with distinct functions. It is now becoming apparent that both these arguments do not hold true anymore. More and more phosphatases have been shown possess oncogenic potential, increasing their potential interest as therapeutic targets [59,60]. Moreover, due to increasing knowledge of the crystal structure of phosphatases, drug development is now able to targeting these enzymes more specifically. Interestingly, this is not only true for the phosphatases acting as oncogenes - tumor suppressor phosphatases can now be targeted as cancer treatment by reactivation of the dysregulated phosphatase [61].

Scope of this thesis

As stated above, the role of phosphatases in cancer is an underlit research field, mostly based on the dogma that phosphatases function as tumor suppressor genes. However, in our opinion dephosphorylation events by phosphatases can also enhance signaling in cancer. The current research was therefore focused on elucidating the role of several phosphatases in cancer, concentrating on phosphatases which have the potential to act as oncogenes rather than tumor suppressor genes, and which can have clinical implications as biomarker of future treatment target. More specifically, we investigated the role of the Low Molecular Weight protein tyrosine phosphatase (LMWPTP) in colorectal and prostate cancer, and studied the role of the protein tyrosine phosphatase 1B (PTP1B) and lipid phosphatase SH2 domain containing inositol 5-phosphatase 2 (SHIP2) in colorectal cancer.

Outline of this thesis

In order to highlight the hiatus in the current literature regarding phosphatases in cancer, this thesis starts with a review of the existing literature concerning the role of protein tyrosine phosphatases in colorectal cancer (**Chapter 2**), where it becomes clear that the current knowledge on phosphatases in cancer is mainly based on preliminary data, with relatively few studies concentrating on the possible oncogenic role of these enzymes. However, it also becomes apparent that phosphatases can indeed act as activators of cellular signaling, providing further rationale for oncogenic properties of these enzymes.

One of the phosphatases for which an oncogenic role has been proposed is the low molecular weight protein tyrosine phosphatase (LMWPTP). We investigated the role of this protein in the setting of prostate cancer (**Chapter 3**) and colorectal cancer (**Chapter 4**). We show that this enzyme acts as an oncogene in both these cancer types, by increasing the metastatic potential of cancer cells. Therefore LMWPTP has possible implications as biomarker and future treatment target in these diseases. Another phosphatase implicated in tumorigenesis is PTP1B. This enzyme is the first phosphatase ever to be identified, and mostly described as the “prototypical” PTP. Interestingly, it has been shown that PTP1B can act as tumor suppressor in certain cell types, while it acts as an oncogene in others. We therefore studied its role in colorectal cancer (**Chapter 5**), showing that PTP1B indeed acts as an oncogene in colorectal cancer as well. PTP1B expression, and more importantly, enzymatic activity are increased in CRC, thereby enhancing the oncogenic potential of this tumor. Interestingly, PTP1B inhibitors are slowly finding their way into the clinic for breast cancer. Our study now provides a rationale the use of these inhibitors in CRC.

In **chapter 6** we step away from the tyrosine phosphatases, and study the lipid phos-

phatase SHIP2. This enzyme acts as part of the PI3K-PKB-mTOR pathway by dephosphorylating phospholipids at the cell membrane. Since its function resembles the well-known tumor suppressor gene PTEN, it was long suggested SHIP2 must also function in a tumor suppressive way. However, our data shows that this enzyme acts as an oncogene in colorectal cancer, thereby suggesting that the use of SHIP2 inhibitors in a clinical setting might be warranted.

Since we identified several phosphatases as therapeutic targets, we reviewed the current status of drug targeting in the phosphatase field based on the recent Europhosphatase conference in **chapter 7**. This 5-day conference deals with all aspects of phosphatase research. We highlighted the latest findings on this class of enzymes in the oncological field, with special attention to phosphatases as treatment targets in cancer. It is becoming ever more clear that phosphatases can no longer be ignored in this sense. Targeting of oncogenic as well as tumor suppressor phosphatases by small molecules is now becoming available.

In **chapter 8** we dig deeper into one of the most important signaling pathway in colorectal cancer; the β-catenin pathway. Mutations in the tumor suppressor *APC* are the first step of the cancer process in the majority of the CRCs, resulting in increased β-catenin signaling. However, different mutations in *APC* result in different levels of β -catenin signaling dosage. We believe these different *APC* mutations do not occur entirely in a random manner, but rather in such a way that the cells reach an optimal level of enhanced β-catenin signaling, described as the 'just-right' signaling model. In this chapter we provide direct evidence for this theory, by showing that reducing the β-catenin levels leads to a shift from an intestinal to a mammary tumor phenotype in an *APC*-driven cancer model in mice.

The novel insights obtained in this thesis will be summarized and discussed in **chapter 9**, moving the role of phosphatases in cancer out of the shadow of the kinases, and into the big league of the cancer field.

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Chapter 2

The role of protein tyrosine phosphatases in colorectal cancer

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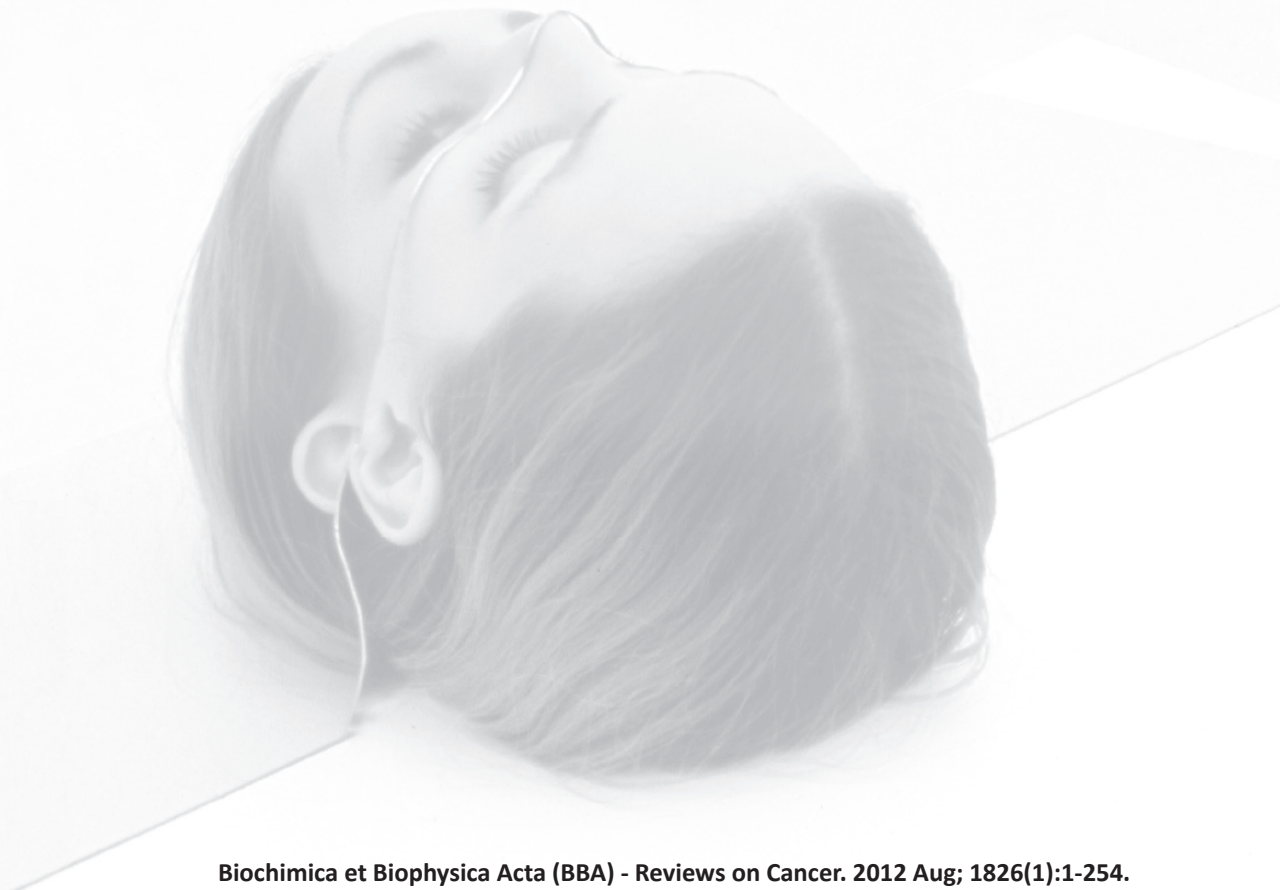

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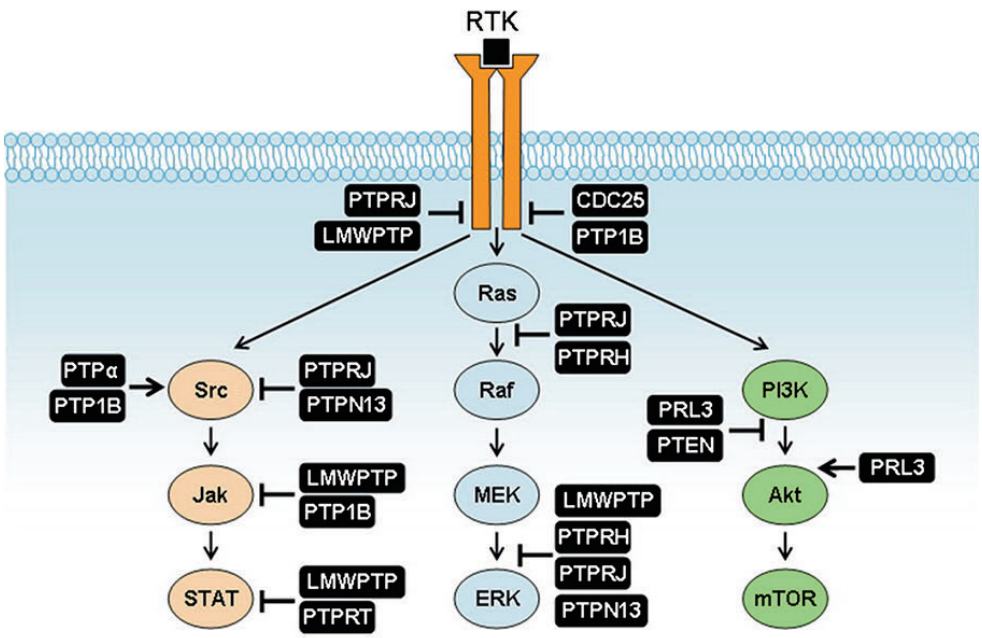
Abstract

Colorectal cancer is one of the most common oncogenic diseases in the Western world. Several cancer associated cellular pathways have been identified, in which protein phosphorylation and dephosphorylation, especially on tyrosine residues, is one of most abundant regulatory mechanisms. The balance between these processes is under tight control by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Aberrant activity of oncogenic PTKs is present in a large portion of human cancers. Because of the counteracting role of PTPs on phosphorylation-based activation of signal pathways, it has long been thought that PTPs must act as tumor suppressors. This dogma is now being challenged, with recent evidence showing that dephosphorylation events induced by some PTPs may actually stimulate tumor formation. As such, PTPs might form a novel attractive target for anticancer therapy. In this review, we summarize the action of different PTPs, the consequences of their altered expression in colorectal cancer, and their potential as target for treatment of this deadly disease.



Graphical abstract. Three major CRC related signaling pathways, and their modulation by the PTPs are reviewed.

Contents

- Characteristics of PTPα
- Role of PTPα in colorectal cancer

Cell cycle proteins

- CDC25
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Conclusions

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Introduction

Colorectal cancer (CRC) is a common form of cancer and a lethal disease. Approximately 945,000 new cases of colorectal cancer were diagnosed worldwide in the year 2000. In almost half of the cases this proves to be fatal [1]. Although CRC mortality has been progressively declining since 1990, it still remains the second most common cause of cancer death in the US and Europe [2-3]. There are environmental and genetic factors that contribute to the development of CRC. Although inherited susceptibility results in the most striking increases in risk, the majority of CRCs are sporadic rather than familial. Familial adenomatous polyposis (FAP) is a relatively common form of familial colon cancer syndrome, but accounts for less than 1 percent of the total CRC cases [4].

Many different genes and signaling pathways are involved in the development of colorectal cancer. These signaling pathways are involved in the control of cell proliferation, adhesion and migration and are under control of a delicate balance between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) [5]. Disturbance of this balance has been shown to play a role in the pathogenesis of numerous inherited or acquired human diseases such as cancer, immune deficiencies and inflammation [6]. Regulation of protein tyrosine phosphorylation contributes to many important physiological processes including cell growth, differentiation, and migration as well as glucose metabolism, synaptic transmission, and the immune response [5]. As such, the importance of protein kinases in health and disease has been extensively investigated and reviewed. These studies are boosted by the wide ranging efforts made to develop the large panel of kinase inhibitors currently available, of which some show clinical promise. In contrast, the equally important phosphatases have received much less attention. However, because of their role in human disease, it is also vitally important to gain more understanding in the function of PTPs in these processes.

The PTP gene superfamily is a large group of highly specific enzymes and comprises a total of 107 PTP genes in the human genome [7] (**Table 1**). They are composed of four main families based on their amino acid sequence in the phosphatase catalytic domains (**Table 1**). By far the largest family is the Class I cysteine based PTPs. These can be further divided into the classical PTPs, including the receptor PTPs (RPTPs) and nonreceptor PTPs (NRPTPs), and the dual specificity phosphatases (DUSPs), which can dephosphorylate serine and threonine in addition to tyrosine residues. Class II cysteine based PTPs is the smallest class of PTPs; presently only the low molecular weight phosphatases (LMWPTPs) are known to belong to this group. Class III cysteine based PTPs are tyrosine/threonine specific phosphatases. In humans this class contains three CDC25 phosphatase genes. Lastly, the four enzymes included in the Class IV aspartate based PTPs are shown to have Tyr/Ser phosphatase activity [7]. Several genomic modifications in genes encoding PTPs have been observed,

and emerging evidence suggests that these alterations play a role in various human cancers. Wang and colleagues performed a systematic screen for mutations in genes encoding PTPs in different human cancers. They identified 83 somatic mutations in six PTPs (*PTPRF, PTPRG, PTPRT, PTPN3, PTPN13, PTPN14*) affecting 26% of the colorectal cancers. With a frequency of 17%, PTPRT was most frequently mutated [8]. Interestingly, in their mutational screen on cancers with high level of microsatellite instability, Korff et al. described frameshift mutations in five different PTP genes (*PTPRA, PTPRS, PTPN5, PTPN23, PTPN21*, as well as the *PTPN13* found in the screen by Wang et al.) affecting 32% of the colorectal cancers [9]. In this review we will summarize the structure and role of different PTPs in the development of colorectal cancer.

Table 1. Classification and substrate specificity of the 107 PTPs

PTP Family	Members (N)	Substrate specificity
Class I cys-based PTPs	Receptor PTPs (21)	PTyr
	Non-receptor PTPs (17)	PTyr
	MAPKs (11)	PTyr, PThr
	Atypical DUSPs (19)	PTyr, PThr, mRNA
	Slingshots (3)	PSer
	PRLs (3)	PTyr
	CDC14 (4)	PSer, PThr
	PTENs (5)	D3-phosphoinositides
	Myotubularins (16)	PI(3)P
Class II cys-based PTPs	LMWPTP (1)	PTyr
Class III cys-based PTPs	CDC25 (3)	PTyr, PThr
Class IV asp-based PTPs	EyA (4)	PTyr, PSer

The table shows an overview of the PTP family, which is subdivided in 4 classes. PTP: protein tyrosine phosphatase; MKP: mitogen-activated protein kinases phosphatase; DUSP: dual-specificity phosphatase; PRL: phosphatase of regenerating the liver; CDC: cell division cycle; PTEN: phosphatase and tensin homolog; LMWPTP: low molecular weight protein tyrosine phosphatase; Eya: Eyes absent homolog. Based on Alonso et al. [7].

PI3K-PKB-mTOR pathway

The phosphatidyl inositol 3-OH kinase (PI3K) - protein kinase B (PKB/Akt) - mammalian target of rapamycin (mTOR) pathway is an important signal transduction axis and regulates survival, growth and proliferation in different human cells. The PI3K family are lipid kinases capable of phosphorylating the hydroxyl group at the 3' position of the inositol ring. After activation by various cytokines and growth factor receptors, this enzyme catalyzes the formation of phosphatidylinositol (3,4,5) trisphosphate (PtdIns(3,4,5)P3) by phosphorylation of PtdIns(4,5)P2. PtdIns(3,4,5)P3 is a critical second messenger for the membrane recruitment of several proteins, and binds to PKB through its pleckstrin homology (PH) domain. PKB plays a major role in cell survival by phosphorylating mTOR, GSK3 and forkhead transcription factors (reviewed in [10]). The PI3K pathway is regulated by the lipid phosphatases PTEN, SHIP1 and SHIP2, which will be discussed further in this review.

PTEN

Characteristics of PTEN

The tumor suppressor gene, phosphatase and tensin homolog (*PTEN*), originally described as MMAC (mutated in multiple advanced cancers), is located on chromosome 10q23.3 [11-12]. Mutations in PTEN are the second most common genetic alterations in human cancer, behind p53 mutations. Germline mutations in this gene lead to the development of rare autosomal dominant inherited disorders called Cowden disease, Lhermitte-Duclos disease, and Bannayan-Zonana syndrome [13-15]. These syndromes are characterized by numerous tumor-like growths, called hamartomas, and give an increased risk of developing certain forms of cancer, including CRC. The PTEN protein is a DUSP, which acts on both proteins and lipids, with a greater affinity for the latter. As a lipid phosphatase, it acts as a negative regulator of the PI3K/PKB/mTOR pathway by converting PtdIns(3,4,5)P3 to PtdIns(4,5)P2 at the plasma membrane. It has been shown that inactivation of PTEN results in constitutive activation of the PI3K/PKB pathway and increases cell proliferation, cell survival, migration and metastasis [16], all well-known characteristics of tumor cells.

Role of PTEN in colorectal cancer

As part of the PI3K/PKB/mTOR pathway, PTEN alterations are found in many different human neoplasms, including colorectal cancer. Langlois and colleagues observed that PTEN protein expression as determined by Western blot analysis is reduced by 40% in approximately 60% of the CRC samples [17]. This is in accordance with immunohistochemical reports from Li et al. and Jiang et al. who found PTEN expression in colorectal carcinoma to be statistically lower than the adjacent non-cancerous mucosa [18-19]. In vitro studies have shown that knocking down PTEN in colorectal cancer cell lines by using an siRNA approach, increases PKB phos-

phorylation, confirming that the PI3K-pathway is activated in PTEN-deficient cells. In these cells, migration and invasion capacity was also increased, thereby resulting in a higher oncogenic potential. In addition, PTEN knockdown was associated with a change in E-cadherin expression, showing that loss of PTEN induces cellular changes consistent with epithelial to mesenchymal transition (EMT), a common phenomenon in tumorigenesis [20]. These data lead to the conclusion that loss of PTEN expression is involved in pathogenesis, invasion and metastasis of colorectal carcinomas and that PTEN expression may be a good marker for prognosis of colorectal carcinoma.

SHIP2

Characteristics of SHIP2

Like PTEN, the SH2-domain-containing 5 inositol phosphatases, SHIP1 and SHIP2 are lipid phosphatases, although not characterized as PTP. Nevertheless, because of their potential role in CRC, we will discus this class of phosphatases here. SHIP1 and SHIP2 share 38% amino acid homology but whereas SHIP2 is ubiquitously expressed with high expression in adult heart, skeletal muscle and placenta [21], SHIP1 is predominantly found in cells of the haematopoietic lineage [22]. SHIP2 (*INPPL1*) is a 155-kDa protein containing a SH2-domain in the amino (N) terminus, a catalytic 5-phosphatase domain in the central region, potential phosphotyrosine binding (PTB) consensus sequences (NPXY), a proline-rich domain (PRD) and sterile alpha-motif (SAM) domain in the carboxy (C)-terminus [21, 23]. Just like PTEN, the lipid phosphatases SHIP1 and SHIP2 have long been thought to act as tumor suppressors by counteracting the PI3K survival signaling pathway. SHIP dephosphorylates the PI3K product PtdIns(3,4,5)P3 at the 5D position of the inositol ring, thereby generating PtdIns(3,4)P2. However, recent evidence suggests PtdIns(3,4)P2 has a higher affinity for PKB than PtdIns(3,4,5)P3. Although PtdIns(3,4,5)P3 is required for PKB phosphorylation, PtdIns(3,4)P2 may actually be necessary for full activation of PKB [24]. These findings indicate that both PtdIns may be required to achieve a malignant state, which has been referred to as the “two PIP hypothesis” [25].

Role of SHIP2 in colorectal cancer

Work performed by the group of N.K. Prasad showed upregulation of SHIP2 in breast cancer. 45% percent of the tumor specimens showed high SHIP2 levels, while only 15% of adjacent normal cells expressed high SHIP2 levels. Patients with higher SHIP2 levels in invasive carcinomas had significantly reduced disease-free and overall survival periods [26-27]. The observation that SHIP2 could play a role in breast cancer was further confirmed by the observation that novel pan-SHIP1/2 inhibitors inhibited cell growth of different breast cancer cell lines. In this study, SHIP inhibition resulted in decreased phosphorylation of PKB on both Threonine 308 and Serine 473 residues, and reduced cell viability of SHIP2 expressing breast cancer cells. Intriguingly, this process could be rescued by adding exogenous PtdIns(3,4)P2,

confirming the importance of PtdnIns(3,4)P2 in cell survival [28]. In the same light, Zhou et al. recently found elevated SHIP2 expression in laryngeal squamous cell carcinoma [29]. Reducing SHIP2 expression by siRNA resulted in a decreased epidermal growth factor (EGF) receptor-induced PKB phosphorylation in breast cancer cells. Considering the fact that increased phosphorylation of PKB and mTOR has been observed in a large number of colorectal cancer biopsies, and is attributed to some extent to increased EGF receptor signaling, it seems that SHIP2 could play a role in colorectal carcinomas. Indeed, silencing of SHIP2 was shown to increase EGFR degradation in some cells [30]. A report by Lincová et al. demonstrated that reduction of SHIP2 expression sensitizes colon cancer cells to the antiproliferative effect of the cytostatic indomethacin, although they attributed this effect to an increase in PKB activation [31]. Together, these data suggest that SHIP2 may be a target for decreasing CRC cell viability.

PRL-3

Characteristics of PRL-3

Phosphathase of regenerating the liver-3 (PRL-3, also known as PTP type IVA and encoded by *PTP4A3*) is part of a subfamily of PTPs which also contain PRL-1 and PRL-2. PRLs have a core PTP domain with the C(X)5R active site motif and are the only PTPs known to carry the membrane-targeting CAAX motif at their COOH-terminus [7, 32-33]. Among normal adult human tissues, PRL-3 mRNA is expressed primarily in skeletal muscle and heart, with lower expression levels in lung, pancreas, spleen, and testis. The exact function of PRL-3 in these tissues is currently unknown [34-36].

Role of PRL-3 in colorectal cancer

Using global gene expression profiling of colorectal cancer (CRC) samples, it was recently found that in metastatic colorectal carcinomas, PRL-3 expression levels were increased in comparison to normal epithelium and non-metastatic tumors, suggesting a role for PRL-3 in tumor survival and metastasis. PRL-3 expression has also been associated with poor prognosis in CRC [37]. Several proteins have been identified as possible PRL-3 target proteins. Forte et al. found evidence that indicates Ezrin pThr567 as a direct substrate of PRL-3 in the HCT116 colon cancer cell line. The identification of Ezrin as a target of PRL-3 action is of interest because of the role of the ERM family members (Ezrin-Radixin-Moesin) in several cellular processes involved in tumorigenesis, such as cell survival, proliferation, invasiveness and migration. Ezrin inactivation by PRL3 may enhance downstream activation of targets such as the Src kinase and Rho family of cytoskeletal proteins [38]. Other investigations led to the conclusion that PRL-3 is a direct target of TGF-beta signaling in colon cancer cells. The loss of TGF-beta signaling leads to the upregulation of PRL-3 and activation of the PI3K/PKB pathway [39]. Wang et al. found that PI3K/PKB activation as a result of PRL-3 activity can promote EMT. On further examination, they demonstrated that PTEN protein expression is downregulated by PRL-3. Con-

sequently, PI3K and PKB are activated, whereas GSK-3b is inhibited, which leads to the upregulation of mesenchymal markers and downregulation of epithelial markers [40]. Fiordalisi et al. have provided evidence that RhoA and RhoC family GTPases can also act downstream of PRL-1 and PRL-3 PTPs to promote motility, invasion, and possibly metastasis, although in this study the direct substrate of PRL activity was not identified [41].

PRL-3 is structurally similar to the lipid phosphatase PTEN, and carries a C-terminal CAAX box, which is not only featured in known lipid phosphatases but also localizes PRL-3 to the cellular compartments rich in phosphoinositides (PIPs). For this reason McParland et al. have speculated on a potential novel role for PRL-3 and provided the first evidence that PRL-3 may possess phosphatidylinositol 5-phosphatase activity [39]. This would suggest that, like PTEN, PRL-3 may act as tumorsuppressor. Nevertheless, as described above, other studies imply that PRL-3 and PTEN counteract each other both on protein level and in terms of PKB activation. Hence, whereas PTEN activity in cancer is most often decreased, PRL-3 expression is increased in CRC and can therefore possibly act as target for treatment.

JAK-STAT-signaling

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is an important signaling mechanism triggered by a large number of cytokines and growth factors, such as interleukin-6 (IL-6), and is involved in cell proliferation, differentiation, cell migration and apoptosis, as well as drug resistance. Cytokine receptor engagement activates receptor-associated JAKs, which in turn mediate phosphorylation of specific receptor tyrosine residues. This allows the recruitment of STATs, present in the in the cytoplasm. Activation of STATs occurs through their tyrosine phosphorylation, which permits STATs to dimerize, translocate to the nucleus and bind to specific regulatory DNA sequences to activate or repress transcription of target genes (reviewed in [42]). Enhanced STAT3 pathway activation is a characteristic of CRC and is associated with poor prognosis [43].

LMWPTP

Characteristics of LMWPTP

The family of low molecular weight protein tyrosine phosphatases (LMWPTP), represented by a single gene called *ACP1*, are a group of 18-kDa cytosolic enzymes that are widely expressed in different tissues [44]. LMWPTP is involved in cell growth regulation, in most instances as a negative regulator of growth factor-induced cell proliferation, but in some cases it may have an opposite role. Modulation of the activity of LMWPTP is based on its phosphorylation/dephosphorylation as well as reversible oxidation of its cysteine residues. LMWPTP contains two tyrosines, Tyr131 and Tyr132, which are preferential sites for phosphorylation by protein tyrosine

kinases such as Src [45-46]. Whereas Tyr131 phosphorylation causes a dramatic increase in enzymatic activity, phosphorylation of Tyr132 serves as a scaffold for the SH2 domain of growth factor receptor-bound protein 2 (Grb2), which may activate the Ras-ERK pathway (see below), but has no affect on its phosphatase activity [45]. In the presence of reactive oxygen species (ROS), oxidation of the cysteine residue in the catalytic site leads to oxidative inactivation of the enzyme. The ensuing prevention of LMWPTP auto-dephosphorylation causes an increase of Tyr132 phosphorylation, and may induce a prosurvival response.[47-48].

LMWPTP interacts with several, cancer relevant proteins, including platelet derived growth factor receptor (PDGFR) [49], Ephrin A2 receptor (Eph A2) [50], β-Catenin [51] and the JAK-STAT pathway. Oxidized LMWPTP is unable to dephosphorylate and inactivate JAK2 and STAT5, which contributes to its tumorigenicity [52]. On the other hand, LMWPTP oncogenic potential may also be associated with the receptor tyrosine kinase EphA2, which negatively regulates tumor cell growth, survival, migration, and invasion. Kikawa et al. showed that LMWPTP is a critical negative regulator of EphA2 tyrosine phosphorylation [50].

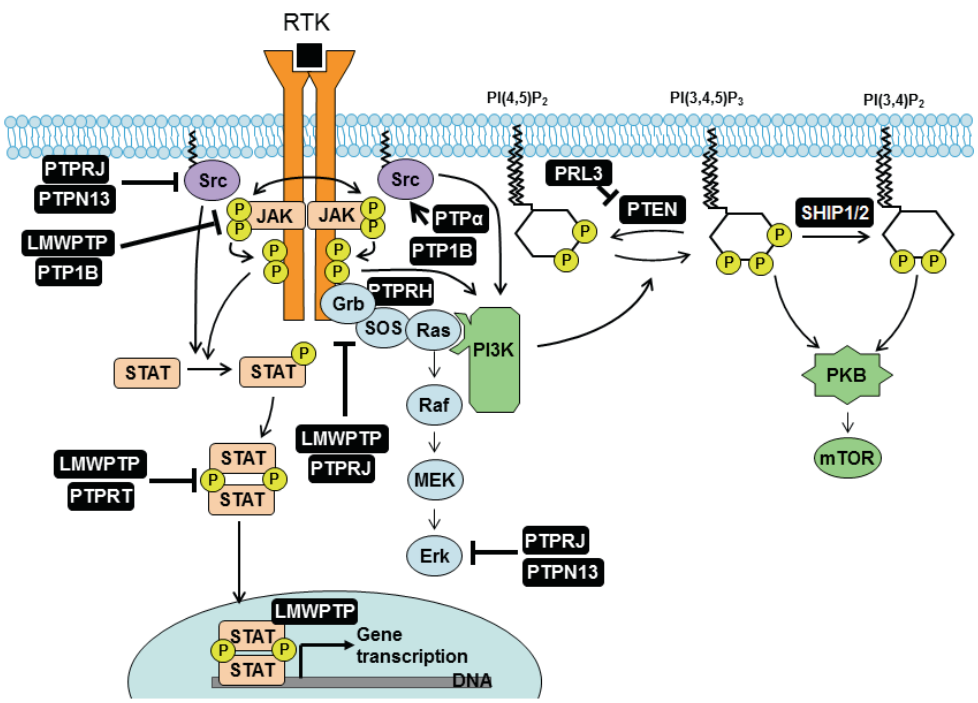

Figure 1. Schematic overview of the role of PTPs in colorectal cancer. Three main signaling pathways commonly over-activated in cancer are the PI3K–PKB–mTOR, the JAK–STAT and the Ras–Raf–MEK–ERK pathways (described in detail in the main body of the text). These pathways are generally activated through engagement of growth factors to receptor tyrosine kinases (RTK). Indicated in black are the phosphatases reviewed, and their interaction with these three important pathways.

Role of LMWPTP in colorectal cancer

When comparing the expression levels of LMWPTP mRNA in different human carcinomas (breast, colon, and lung) to their paired adjacent non-affected tissues, Malentacchi et al. found increased expression of LMWPTP mRNA in breast and colon cancers, but not in lung cancers [53]. This difference was most predominant in patient groups expressing negative predictive markers, such as G2–G4 patients, patients with lymph node involvement or advanced Dukes' stage, and in patients with a lower differentiation grade. Patients whose cancer LMWPTP mRNA expression is higher than that in paired non-affected tissue, have a significantly reduced survival probability in comparison to subjects who do not show an increase in LMWPTP mRNA in their tumours. The elevated mRNA also corresponded with an increase in LMWPTP protein, which was increased 2- to 20-fold in tumour samples compared to normal tissue. In the same light, Marzocchini et al. found a significant increase in LMWPTP expression in rat adenocarcinomas induced by DMH, especially proximal tumors, although the observed 2-fold increase did not reach statistical significance [54]. These results suggest that overexpression of LMWPTP is a phenomenon associated with the onset of malignancy.

There are three common *ACP1* alleles; A, B, and C, which give rise to six phenotypes. Subsequent alternative splicing of the ACP1 mRNA results in two isoforms of the LMWPTP protein, a slow migrating form (S) and a fast migrating form (F) [55]. The different genotypes of LMWPTP show different enzymatic activities and other molecular properties. Whereas the S-forms seems to be associated with enhanced LMWPTP catalytic activity, the F-variants appear to play a role in cell adhesion, migration and extracellular matrix degradation by metalloproteinases. In colorectal cancer, the LMWPTP genotype is shifted from *ACP1* S-forms to ACP1 F-forms, which may contribute to tumor invasiveness and metastasis potential [56].

Giving the possible oncogenic and metastatic roles in colorectal cancer, and the recent development of LMWPTP-inhibitors [57], it is tempting to speculate that there might be a future role for these inhibitors in a therapeutic setting.

PP2A

Characteristics of PP2A

Serine/threonine-specific protein phosphatase 2A (PP2A) is a major serine/tyrosine phosphatase protein involved in several cellular processes. Based on the finding that okadaic acid, a strong inhibitor of PP2A, is a potent tumor promoter, it was originally thought that PP2A acts as tumor supressor [58]. This dogma is now challenged by the finding that PP2A is essential for cell survival, and inhibition of PP2A with novel inhibitors may actually attenuate cancer cell growth [59]. PP2A is a protein composed of three regulatory subunits, A, B and C. The core enzyme consists of the 65-kDa scaffolding subunit (A), a 36-kDa catalytic subunit (C), and one of several

regulatory B subunits that can bind to the core enzyme. Each subunit has multiple isoforms which are all encoded by different genes, creating many forms of the PP2A holoenzyme, differing in expression patterns and specificity [60-61]. These different holoenzymes play a role in many fundamental cellular processes, such as signal transduction, DNA repair, transcription, translation, and growth control.

Role of PP2A in colorectal cancer

Because of a high frequency of loss of heterozygosity (LOH) in the region of chromosome 11q23 in colorectal cancers, Wang et al. systematically surveyed this specific region for candidate tumor suppressor genes. They found alterations in the *PPP2R1B* gene in colon and lung cancer [62]. This gene encodes the B isoform of the structural/regulatory A subunit PP2A-Ab which is necessary for interaction with the catalytic C-unit or regulatory B-unit. Similarly, others confirmed the presence of mutations in the Ab subunit, resulting in defective binding to the B and/or C unit [63-64]. These point mutations, deletions, frameshifts, and splicing abnormalities found in the *PPP2R1B* gene occur in 15% of the primary colon tumors suggesting a role for PP2A inactivation in colorectal cancer. One of the targets of PP2A is STAT6, whose dephosphorylation at certain serine residues results in its inactivation. Enhanced activation of this growth promoting signal transducer in colorectal cancer cells was attributed to the decreased expression of its positive regulators *PPP2CA* and *PPP2CB* [65].

PTPRT

Charcteristics of PTPRT

Protein tyrosine phosphatase receptor T (also designed as PTPρ) is a frequently mutated PTP in several human neoplasms, such as lung, gastric, skin and colorectal cancers [8]. Together with PTPRM, PTPRK and PTPRU, it belongs to the receptor type IIB PTP family. They share the same domain architecture, with an extracellular domain, a juxtamembrane region and two tandem intracellular catalytic domains (D1 and D2). The large extracellular domain contains a MAM (meprin-A5 antigen-PTP) domain, Ig-like domain and fibronectin type III-repeats [66]. The precise role of this PTP in signaling pathways has not been identified clearly yet.

Role of PTPRT in colorectal cancer

In the mutational screen performed by Wang et al. described earlier, *PTPRT* was found to be the most frequently mutated PTP in colorectal carcinomas. A large fraction of these mutations include nonsense and frameshift mutations, suggesting inactivating mutations. Many of the missense mutations in the catalytic domains (both D1 and D2) of PTPRT lead to diminished phosphatase activity. In addition, overexpression of PTPRT inhibits CRC cell growth, suggesting a tumor suppressive role for this PTP [8]. Recently, both paxillin and STAT3 were identified as substrates of PTPRT. Paxillin is a signal transduction adaptor protein which plays a role in

cell-cell adhesion. PTPRT dephosphorylates paxillin specifically at Y88 in CRC cells, which plays an important role in CRC tumorigenesis and CRC cell migration through established signaling pathways such as AKT, p130CAS and SHP2 [67]. STAT3 dephosphorylation by PTPRT negatively regulates its translocation to the nucleus and transcriptional activation of target genes, such as SOCS3 and Bcl-XL [68]. Hence, inactivation of PTPRT in colorectal cancer may contribute to the survival signals induced by STAT3 signaling in these cells.

PTP1B

Characteristics of PTP1B

PTP1B (or *PTPN1*) is the first mammalian PTP identified and is widely expressed [69]. The gene is located on human chromosome 20q13.1-q13.2 and has a molecular weight of approximately 50 kDa [70]. PTP1B is prototypic for the PTP superfamily, with an N-terminal catalytic (PTP) domain, followed by two proline-rich motifs, and localizes to the ER through a hydrophobic domain in its C-terminus [71]. PTP1B is involved in the regulation of a variety of cellular events through different molecular pathways, such as the epidermal growth factor receptor (EGFR) [72], platelet-derived growth factor receptor (PDGFR) [73], insulin receptor (IR) [74], insulin growth factor I receptor (IGF-IR) [75], p210 Bcr-Abl [76], Janus kinase 2 (JAK2), and TYK2 [77]. Given the central role PTP1B plays in the negative regulation of oncogenic signaling, by the dephosphorylating PTKs such as p210 Bcr-Abl, PTP1B inhibition is generally thought to act as a carcinogenic [76]. In contrast, PTP1B has also been identified as one of the major phosphatases that activate Src in breast cancer cells [78], thus suggesting a dual role for PTP1B in carcinogenesis [79].

Role of PTP1B in colorectal cancer

By evaluating distinct genomic DNA alterations for a large number of candidate oncogenes and tumor suppressor genes in 22 colorectal cancer samples, Lassmann et al. identified DNA amplifications of PTP1B in 22% of the cases, with the highest percentage in CIN-positive tumors [80]. Like earlier reports in breast cancer, Zhu et al. found PTP1B to be the major tyrosine phosphatase capable of specifically dephosphorylating Src at its negative regulatory site Y530 in six of seven human epithelial colon cancer cell lines. In addition, phosphatase activities were greatly elevated in these colon cancer cell lines compared to a normal colon epithelial cell line, thus resulting in enhanced Src activity in these tumor cells [81]. Enhanced Src activity is associated with the majority of CRC, and directs downstream activation of the PI3K-PKB, JAK-STAT, but also Ras-ERK pathways [82-83]. Interestingly, increased Src activity is based on increased expression/activity modulation rather then on Src mutations, since these are very rare, suggesting a role for PTPs in this process. Modulation of this important kinase through PTPs may therefore find clinical relevance. Giving the oncogenic role for PTP1B in this respect, PTP1B-inhibitors might be a useful therapeutic target for these malignancies.

RAS-RAF-ERK pathway

The Ras/Raf/MEK/ERK cascade is an important signaling system that controls fundamental cellular processes such as proliferation, differentiation, survival and apoptosis. Ras acts as a molecular switch by cycling between an active, GTP bound state, and an inactive, GDP bound state. A wide array of stimuli can recruit nucleotide exchange factors, such as the well characterized sons of sevenless (SOS), to the cell membrane where it binds to the adaptor protein growth-factor-receptor-bound protein 2 (Grb2). Once at the cell membrane, SOS promotes the exchange of GDP for GTP, thereby converting Ras into its active conformation. Activated Ras will result in a cascade of mitogen-activated protein kinase (MAPK) activation. The first to become activated is the MAPK kinase kinase (Raf), which subsequently activates MAPK kinase (MEK1/2), which in turn is followed by phosphorylation and activation of MAPK (ERK1/2). ERK1/2 phosphorylates numerous substrates involved in the regulation of proliferation, differentiation and survival. (reviewed in [84]).

PTPRJ

Characteristics of PTPRJ

Protein tyrosine phosphatase receptor type J, also designated as DEP-1 (high cell density-enhanced phosphatase-1) or CD148, is encoded by the *PTPRJ* gene located on human chromosome band 11p11. PTPRJ is a class III transmembrane protein tyrosine phosphatase that contains multiple fibronectin type III repeat domains in the extracellular portion, one transmembrane domain, and a single phosphatase domain in the intracellular portion [85]. PTPRJ can be activated by binding of various growth factors to its extracellular domain, and activated PTPRJ plays a role in several pathways such as VEGF [86], PDGFR [87-88] and HGFR [89] which are implicated in carcinogenesis. For instance, PDGFR mediated activation of Ras and Src, and to a lesser extent PI3K-PKB, can be inhibited by PTPRJ [88]. In addition, oncogenic Ras transformation of cells is inhibited by PTPRJ, and PTPRJ may directly dephosphorylate ERK1/2 [90-91].

Role of PTPRJ in colorectal cancer

Different *PTPRJ* haplotype associated single nucleotide polymorphisms (SNPs) have been identified, one of which reduces breast cancer risk [92]. However, there is no significant evidence for *PTPRJ* haplotype SNPs being either risk conferring or protective in colorectal cancer [93]. Ruivenkamp and collaborators demonstrated that one copy of the *PTPRJ* gene is deleted (LOH) in a large percentage of different sporadic human carcinomas, such as colorectal, breast and lung cancer [94]. Earlier research by the same group showed PTPRJ to be the only candidate gene for the mouse susceptibility locus to colon cancer, Scc1 [95]. In humans they screened sporadic colorectal adenomas for LOH along chromosome 11p11-11p12 and discovered the

PTPRJ containing region to be deleted in 49% of the carcinomas, suggesting a tumor suppressive role for PTPRJ. As no PTPRJ mutations were observed after sequence analysis of the individuals with and without LOH, it was suggested that loss of one copy of PTPRJ is sufficient to provide a growth advantage [94]. In line with this suggestion, Balavenkatraman et al. showed that reduction of PTPRJ protein expression by shRNA confers growth advantage in colorectal cancer cell lines [96]. Decreased PTPRJ expression was indeed observed in immunohistochemical stainings of high grade tumors, including some adenocarcinomas [97]. Based on the above, PTPRJ appears to have an antiproliferative effect in epithelial cells from the colon, where loss of a gene copy may contribute to cancer development.

PTPRH

Characteristics of PTPRH

Receptor-type tyrosine protein phosphatase H (*PTPRH*), also known as stomach cancer-associated protein tyrosine phosphatase-1 (SAP-1) was originally identified as a PTP expressed in a human stomach cancer cell line. This PTP with a molecular weight of 107kDa is located on chromosome 19q13, and is structurally similar to PTPRJ [98]. It binds to Grb2, and as such may play a role in Ras signaling [99]. PTPRH is specifically overexpressed in human colon and pancreatic cancers [100] suggesting a role in carcinogenesis. In contrast, overexpression of this PTP in cultured fibroblasts inhibits cell proliferation, through either inhibition of growth factor-induced activation of MAP kinase or by caspase-dependent apoptosis [101-102]. Based on this, and the earlier finding that there is a reduced expression of PTPRH in advanced cancers [100, 103], it has also been suggested that PTPRH may act as a tumor suppressor.

Role of PTPRH in colorectal cancer

As mentioned above, PTPRH overexpression has been found in colorectal cancers. Seo and co-workers examined the immunohistochemical PTPRH expression in specimens from 65 patients, and demonstrated PTPRH expression in colonic adenomas showing moderate or severe dysplasia, but not in normal colonic epithelial cells or adenomas with mild dysplasia [100]. Others suggested PTPRH can promote intestinal tumorigenesis in mice, as loss of PTPRH inhibits tumorigenesis in mice that are heterozygous for a mutation of *APC* that induces tumors of the colon [104]. The mutation of APC leads to stabilization and the accumulation of β-catenin, which initiates transformation and promotes tumorigenesis through activation of transcription factor 4 (reviewed in [105]). It thus appears that PTPRH cooperates with the canonical Wnt-signaling pathway in the formation of tumors, although the exact mechanism remains elusive.

PTPN13

Characteristics of PTPN13

Non-receptor type protein tyrosine phosphatase 13 (*PTPN13*), also known as FAP-1 and PTPL1, is a cytoplasmic PTP located on chromosome 4q21 and the largest among all PTPs with a molecular weight of 270 kDa [106-109]. It contains a phosphatase domain in the C-terminal portion and many non-catalytic domains, including five PSD-95/Drosophila disc large zonula occludens (PDZ) domains, an N-terminal kinase non-catalytic C-lobe (KIND) domain [110] and a four-point-one/ezrin/radixin/moesin (FERM) domain [111]. These non-catalytic domains can interact with several different proteins, causing PTPN13 to have diverse functions. For instance, the third PDZ domain (PDZ2) intracellularly inhibits FasR-mediated apoptosis by interacting with the cytoplasmic death domain of Fas receptors [109]. In addition, PTPN13 is a negative modulator of Src-ERK signaling in cancer cells [112].

Role of PTPN13 in colorectal cancer

Several studies in colorectal cancer have shown conflicting results for the role of PTPN13, as either a tumor suppressive or an oncogenic protein (reviewed in [113]). Elevated levels of PTPN13 expression have been seen in Ewing's sarcoma family tumors (ESFT), as it is a direct transcriptional target of the ESFT oncogene, EWS-FL11 [114]. In the same light, Yao et al. reported a high incidence of PTPN13 expression in colon carcinomas, and demonstrated that this is related to resistance to FasR-mediated apoptosis, thus acting as a survival mechanism and enhancing tumor growth [115]. In complete contrast, others found that ectopic expression of PTPN13 enhances, rather then decreases, apoptotic cell death in colon adenocarcinomas, suggesting a more tumor suppressive role for PTPN13 [116]. These observations are further supported by the previously mentioned reports by Wang et al. and Korff et al., who identified *PTPN13* among the PTPs most frequently mutated in colorectal cancer, suggesting they are likely to act as tumor suppressor genes [8-9].

PTPα

Characteristics of PTPα

PTPα is a transmembrane tyrosine phosphatase with a short, unique extracellular domain and two tandem catalytic domains. In comparison to other receptor-like PTPs which are often restricted to a lineage-specific expression, PTPα is widely expressed [117-118]. Two isoforms of PTPα exist, only differing in their extracellular domain, which arise by alternative splicing. The major substrates of PTPα are the Src family kinases (SFKs) [119-120]. By dephosphorylating the inhibitory COOH-terminal tyrosine residue of SFKs, PTPα is capable of activating Src family members such as Src and Fyn.

Role of PTPα in colorectal cancer

Tabiti et al. first explored the possible involvement of PTPα in colorectal cancer by looking at the mRNA levels of PTPα in tumors compared to adjacent healthy colon mucosa [121]. In the Dukes' D stage carcinoma they found a 2- to 10-fold increase in mRNA levels. As mentioned above, a large fraction of human colon cancers present with increased Src protein tyrosine kinase activity. RNAi against PTPα reduces Src specific activity in different breast cancer and colorectal cancer cell lines, which suppresses anchorage-independent growth and induces apoptosis [122]. More recently, Krndija et al. reported that in normal tissue PTPα expression was restricted to smooth muscle cells, whereas over 70% of the colon cancer samples showed expression of PTPα, supporting a role for PTPα in colorectal cancer, and identifying this PTP as a potential target for treatment [123].

Cell cycle proteins

Cdc25

Characteristics of Cdc25

Cdc25 (cell division cycle) phosphatase is a family of human cyclin-dependent kinase activating phosphatases composed of three different members, Cdc25A, Cdc25B, and Cdc25C. They serve as Cdk/cyclin-activating phosphatases, by removing inhibitory phosphates from the threonine and/or tyrosine residues of these cell cycle proteins. This action is imperative for normal cell cycle progression [124]. Cdc25A is expressed in late G1 phase, where it is essential for G1/S transition through the activation of the cyclin E/CDK2 complex, while Cdc25B and C mainly regulate G2 and G2/M transition [125].

Role of Cdc25 in colorectal cancer

Different studies have indicated that unlike Cdc25C, Cdc25A and B may have oncogenic potential. Cdc25A and B overexpression has been observed in different cancer cell lines and human tumors, suggesting that these phosphatases are implicated in human neoplasms [126]. Hernández et al. examined the structure and expression of Cdc25A, B, C, and several splicing variants in a series of 34 paired tumor and normal colorectal tissues. They observed Cdc25B mRNA overexpression in 56% of the tumors, whereas Cdc25A and C were overexpressed in 12% and 26% of cases, respectively [127]. Takesama et al. confirmed a 60% overexpression of Cdc25B mRNA level by RT-PCR in colorectal carcinoma [128]. In their unpublished data of a preliminary study they found that Cdc25B expression was relatively low in hepatocellular carcinoma and in esophageal squamous cell carcinoma, although these carcinomas frequently expressed Cdc25A at high levels. They imply that high levels of Cdc25B may be a characteristic to colorectal carcinoma. and went on to demonstrate high Cdc25B in CRC as an independent predictive factor of death with a relative risk of 3.7. As this risk is higher than the relative risk for lymph node metastasis (2.4),

which is considered one of the strongest predictors of poor prognosis in colorectal carcinoma, they proposed Cdc25B as a novel prognostic marker in patients with colorectal carcinoma. On the other hand, Talvinen et al. did not find statistical evidence for Cdc25B as a good marker for colorectal carcinoma [129]. Clearly, further studies are required to specify the role of Cdc25 in CRC.

Conclusions

Protein phosphorylation and dephosphorylation, and in particular phosphorylation on tyrosine, plays an important role in several cellular processes, such as the control of cell proliferation, adhesion and migration. These processes are controlled by a tight balance between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). So far, most of the research has focussed on the PTKs. In part, this is probably due to an historical cause, as the first PTP was only purified [130] ten years after the first PTK [131]. A variety of PTKs have since been linked to tumorigenesis, and overexpression of PTK activity results in malignant transformation [132]. Because of their antagonizing role on PTKs, PTPs have long been thought to act solely as tumor suppressors. However, emerging evidence, some of which has been discussed in this review, suggests that PTPs act in different roles, both as tumor suppressor genes and oncogenes. Thanks to comprehensive screens, such as those from Wang et al. and Korff et al. [8-9], the role of PTPs in colorectal cancer is starting to receive more attention. Here, we discuss the role of different PTPs in colorectal cancer (summerized in **Table 2**). Based on the studies included, we conclude that PTPs not only play an imperative role in oncogenesis, but that some of these phosphatases may form an attractive target in the battle against colorectal cancer.

Table 2. PTPs discussed in this review with their potential role in colorectal cancer.

Phosphatase	Role in colorectal cancer	Ref.
PTEN	Tumor suppressor gene, acts a negative regulator of the PI3K/PKB/mTOR pathway by converting PtdIns(3,4,5)P3 (PIP3) to PtdIns(4,5)P2 (PIP2).	[17-20]
SHIP2	Possible oncogene, as PtdIns(3,4)P2 may actually be necessary for full activation of PKB.	[27-28]
PRL-3	Oncogene, increased in metastatic colorectal tumors.	[37]
LMWPTP	Oncogene, increased levels in colorectal tumors with negative predictors.	[52-53]
PP2A	Tumor suppressor gene, mutations in subunit A results in defective binding and impaired function.	[60-62]
PTPRT	Tumor suppressor gene, trough interaction with paxillin and STAT3	[65-66]
PTP1B	Oncogenic role, by activating Src-family members.	[78-79]
PTPRJ	Tumor suppressive role, LOH in the PTPRJ containing region is deleted in 49% of the CRCs.	[92]
PTPRH	Oncogenic role, possibly through the canonical Wnt-pathway.	[98, 100, 102]
PTPN13	Dual role, through Fas mediated apoptosis	[8-9, 113-114, 131]
PTPα	Oncogene, upregulation in colorectal carcinomas by increasing Src activity.	[119, 121]
CDC25B	Oncogene, prognostic marker for survival.	[124-127]

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Chapter 3

Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) predicts prostate cancer outcome by increasing the metastatic potential

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Abstract

Background: Low-risk patients suffering from prostate cancer are currently placed under active surveillance rather than undergoing radical prostatectomy. However, clear parameters for selecting the right patient for each strategy are currently not available, and new biomarkers as well as treatment modalities are needed. Low molecular weight protein tyrosine phosphatase could present such a target.

Objective: To correlate expression levels of LMWPTP in primary PCa to clinical outcome, and determine the role of LMWPTP in prostate tumor cell biology.

Design, Setting, and Participants: *ACP1* expression was analyzed on microarray datasets, which were subsequently used in an Ingenuity Pathway Analysis. Immunohistochemistry was performed on a tissue micro array containing material of 481 PCa patients, of which the clinicopathological data were recorded. PCa cell line models were used to investigate the role of LMWPTP on cell proliferation, migration, adhesion and anoikis resistance.

Outcome Measurements and Statistical Analysis: The association between LMWPTP expression and clinical and pathologic outcomes was calculated using chi-square correlations and (multivariable) cox regression analysis. Functional consequences of LMWPTP overexpression or downregulation were determined using migration and adhesion assays, confocal microscopy, western blotting and proliferation assays.

Results and Limitations: LMWPTP expression is significantly increased in human prostate cancer (PCa), and correlates with earlier recurrence of disease and reduced patient survival (HR:1.99; P<0.001, and HR:1.53; P=0.04). Unbiased Ingenuity analysis comparing cancer and normal prostate suggests migratory propensities in PCa. Indeed, overexpression of LMWPTP increases PCa cell migration, anoikis resistance and reduces activation of Fak/Paxillin, corresponding to decreased adherence.

Conclusions: Overexpression of LMWPTP in PCa confers a malignant phenotype, with worse clinical outcome. Prospective follow-up should determine the clinical potential of LMWPTP overexpression.

Patient summary: These findings implicate LMWPTP as novel oncogene in prostate cancer, and could offer the possibility of using this protein as biomarker or target for treatment in this disease.

Introduction

Kinases and phosphatases that control intracellular phosphorylation are critical regulators of cell proliferation, adhesion, migration and death. Deregulation of oncogenic kinases has been shown to contribute to cancer development. In general, it is assumed that phosphatases, by counteracting kinase activities, act as tumor suppressors. However, a dual role for the Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) in tumor cell biology is emerging. Enhanced expression of LMWPTP has been correlated to poor prognosis for some human tumors [1]. This may be related to the fact that this 18kDa protein tyrosine phosphatase interacts with cancer related molecules such as PDGFR, β-catenin, EphA2, JAK2 and STAT5, resulting in a positive effect on cell growth and proliferation signaling [2–7].
Prostate cancer (PCa) is one of the most frequently diagnosed cancers in males [8]. There are several treatment strategies available for non-metastasized primary prostate tumors, i.e. radical prostatectomy, radiation therapy (either external beam or brachytherapy), and active surveillance. However, while several treatment guidelines are available, prognostic biomarkers to aid clinical decision making are warranted. Furthermore, since prostate cancer is highly metastatic to the bone and lymph nodes, new treatments should be directed at avoiding this progression towards metastatic clinical state. The aim of this study was to examine expression levels of LMWPTP in primary PCa, correlate these to clinical phenotype, and to study the role of LMWPTP in prostate tumor cell proliferation, migration and stromal cell interaction.

Materials and Methods

Gene expression profiling and pathway analysis.
Use of clinical samples was approved by the Erasmus Medical Center Medical Ethics Committee according to the Medical Research Involving Human Subjects Act in protocol MEC-2004-261. Samples and Affymetrix analysis are described in [9]. Differentially regulated genes, their log2 fold change and associated p-value for LN-PCa, TURP and PCa as compared against NAP were filtered for a p-value ≤ 0.05, then the corresponding expression values and p-value were imported into Ingenuity Pathway Analysis software for an unbiased analysis of the functional role of *ACP1* in canonical pathways (supplementary material).

Immunohistochemistry
FFPE tissue sections from seven non-prostate cancerous patients and fifteen radically resected prostate cancer patients with a Gleason score of at least 4+3=7 were immunohistochemically stained for LMWPTP (ACP1 antibody, sc-100343, Santa Cruz Biotechnologies, Dallax, Tx) ([10] and supplement). In addition, a tissue mi-

croarray (TMA) of 481 PCa patients [11] was stained. All patients had undergone radical prostatectomy for their disease without previous adjuvant therapy and were evaluated for Gleason score, pT-stage and surgical margins. Biochemical recurrence was defined as an increase in serum PSA after two different measurements, at least 3 months apart. In a subpopulation of these patients, local recurrence as suspected by clinical presentation was confirmed by needle-biopsy. Death and death due to disease were registered by the physician who last treated the patient. We tested the hypothesis that increased LMWPTP status is related to a more invasive tumor (Gleason score and pT-stage) using Rank-sum and $\chi 2$ tests, and whether increased LMWPTP expression is predictive for worse clinical outcome using Cox regression analysis with the following covariates; age and PSA at diagnosis, Gleason score, pT-stage, Surgical margins, and LMWPTP status. Patient selection and statistics were performed as described in supplement.

Cell culture and transfections
Cells (PC3, DU145, PNT2C2, MG63) were cultured as described in supplementary material. Overexpression plasmid pCS2+MT-LMWPTP was a kind gift from Prof. J. den Hertog, Hubrecht Institute, Utrecht, The Netherlands. Transfection of the LMWPTP construct was performed using Lipofectamine™ 2000 from Invitrogen (Bleiswijk, The Netherlands) according to the manufacturer's directions (see supplement). Analyses were performed 48h after transfection.

LMWPTP immunoprecipitation and phosphatase assay
LMWPTP activity assays were performed as described in [12] and supplement.

Migration Assays
Transwell migration assay was performed as described in [13] and supplement. In scratch-wound assays, cell monolayers were scratched with a pipette tip, washed twice and photographs were taken (Axiovert200M microscope,Carl Zeiss, BV, the Netherlands) to analyze the percentage of open wound area at 24h (Image J software).

Adhesion assay
Cells in serum-free medium were allowed to adhere to rat-tail type I collagen (Sigma, St-Louis, MO) coated, bovine serum albumin (BSA) blocked plates for 10, 30 and 60 min. The attached cells were stained with Cristal Violet, and absorbance after extraction with 10% acetic acid was measured.

Fluorescence immunohistochemistry
PC3 cells were grown on coverslips, transfected and fixed with 3.7% formaldehyde. Fixed cells were permeabilized and blocked in PBS/0.1% Triton X-100/10%FBS, stained with 200ng/mL DAPI (4', 6'-diamidino-2-phenylindole, Sigma, St Louis, MO) and actin filaments visualized with 10µg/mL phalloidin-TRITC (tetramethyl

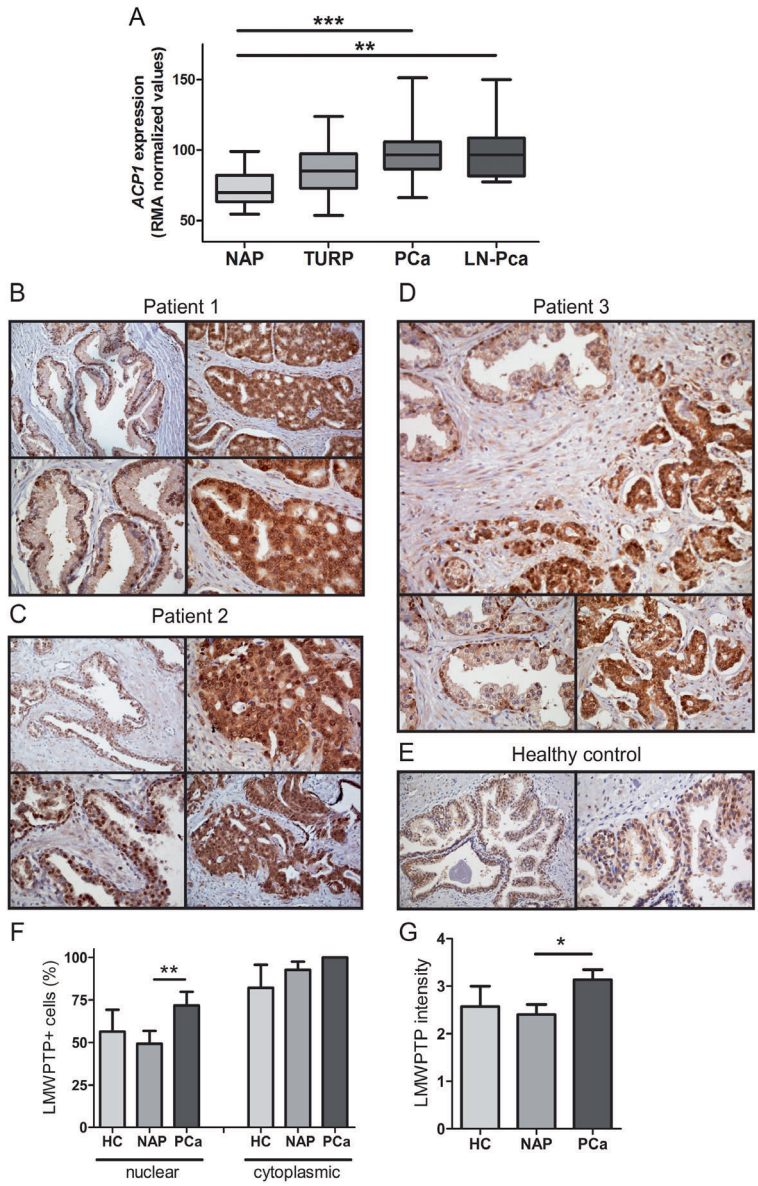

Figure 1. Gene expression profiling shows increased *ACP1* mRNA expression in PCa, which corresponds to increased LMWPTP protein levels. A) Using gene expression array data of a previously described cohort of patient samples collected at the EMC, expression of *ACP1* (transcript 2466141, NM_007099) was compared between tumor tissue (PCa, n=56), lymph node metastasis of PCs (LN-PCa, n=12), TURP (n=10) and adjacent normal prostate tissue (NAP, n=12, p = 0.0008 by ANOVA). Significantly higher expression of *ACP1* mRNA expression was observed in prostate tumor and LN-PCa compared to NAP (***=0.0002; **=0.003, RMA normalized expression values). **(B-E)** 15 PCa patients with Gleason score >7 and 7 healthy controls were selected for staining. Representative examples of immunohistochemistry of three individual patients **(B-D)**, and one healthy control (HC) **(E)** are shown. Magnification of 10X and inset of 40x are presented. **(F-G)** The number of cells exhibiting nuclear and cytoplasmic staining as well as intensity of staining were scored (**= 0.0055; *=0.014).

Rhodamine Isothiocyanate; Sigma St-Louis, MO). Upon mounting in Mowiol DABCO aqueous mounting medium (Vector Laboratories, Burlingame, CA), images were acquired using an epifluorescence microscope Axiovert200M (Carl Zeiss, BV, The Netherlands).

Western blotting
Protein extraction and blotting were performed as described in [14] and supplement.

Co-culture with stromal cells
Transfected PC3 cells were labeled with 1μM CFSE (carboxyfluorescein diacetate succinimidyl esther; Molecular Probes, Eugene, OR). Cells were resuspended in F12K medium and added on top of a confluent MG63 cell monolayer. After 24h the suspension-cell fraction and adherent-cell fraction were analyzed by flow cytometry (FACS Canto, BD Biosciences, Bedford, MA) and 10000 events were acquired. The total number of CFSE-labeled PC3 cells was calculated by reference of a known number of beads (BD Bioscience, San Jose, CA) in the tubes.

Cell cycle analysis
Cell cycle analysis was performed as described [15]. Cells were stained for 1h in sodium citrate-dihydrate (1g/L) solution, containing 20μg/mL propidiumiodide, 0.1% triton X-100 and 100μg/mL ribonuclease A. Cell cycle distributions were analyzed using Modfit LT software (verity software House, Topsham, ME, USA)

Zymography Analysis
Proteolytic activity of MMP-2 and MMP-9 was assayed by gelatin zymography as described by Souza et al [16] and supplement.

Results

ACP1 mRNA and protein are overexpressed in primary human prostate cancer

Using gene expression array data from a cohort of patient samples [9] we observed a significantly higher mRNA expression of the LMWPTP gene *ACP1* in primary prostate tumors, compared to normal adjacent prostate (NAP) (transcript 2466141, NM_007099, p=0.0001, **Figure 1A**). In addition, increased levels of *ACP1* mRNA were found in prostate tumor cells metastasized to the lymph nodes (p=0.0024) as compared to NAP tissue, showing that enhanced *ACP1* expression in prostate tumor cells is maintained after metastasis.

To validate whether increased *ACP1* mRNA levels in PCa correspond to increased protein levels, we performed immunohistochemistry on PCa biopsy tissue sections

Table 1. Clinicopathological characteristics of Tissue Micro Array (TMA), divided according to LMWPTP intensity (High/Low). (* p<0.05; ** p<0.01)

Parameter	Overall Median (quart) or N (%)	LMWPTP low expression Median (quart) or N (%)	LMWPTP high expression Median (quart) or N (%)	p-value
Number of Pts	481	314	147	
Age at diagnosis	64 (61-67)	64 (61-67)	65 (61-67)	0.33
PSA at diagnosis				
Total	5.2 (3.6-7.8)	5.1 (3.5-7.7)	5.4 (3.9-8.9)	0.17
<10 ng/ml	418 (87%)	277 (88%)	125 (85%)	
>10 ng/ml	62 (13%)	37 (12%)	21 (15%)	
FU time (months)	113 (89-139)	119 (98-137)	112 (74-137)	0.52
Gleason score				
<7	265 (56%)	187 (60%)	69 (47%)	**0.013 ***
3+4 = 7	152 (28%)	95 (30%)	49 (33%)	
4+3 = 7	35 (6%)	16 (5%)	16 (11%)	
>7	28 (5%)	15 (5%)	13 (9%)	
Surgical margin				
Negative	362 (75%)	239 (76%)	108 (73%)	0.54
Positive	119 (25%)	75 (24%)	39 (27%)	
pT-stage				
T2	344 (72%)	239 (76%)	92 (63%)	**0.005 ****
T3a	92 (19%)	46 (15%)	41 (28%)	
T3b	17 (4%)	10 (3%)	7 (5%)	
T4	28 (6%)	19 (6%)	7 (5%)	
Lymph node positive	1 (0.2%)[a]	0	1	n.a.
KI-67				
<1%	353 (73%)	246 (78%)	107 (73%)	0.23
1-5%	40 (8%)	27 (9%)	19 (13%)	
>5%	9 (2%)	5 (2%)	4 (3%)	
unknown	73 (17%)	36 (11%)	17 (12%)	

a. Since lymph node metastasis was only observed in 1 patient, lymph node metastasis was not taken into account in the further analysis.

(Gleason score ≥7) and non-cancerous prostate sections (examples shown in **Figure 1B-E**). Staining of LMWPTP is evident in both the nucleus and the cytoplasm, and significantly increased in PCa as compared to either NAP or healthy controls (**Figure 1F-G**).

Increased LMWPTP expression is correlated to worse clinical outcome

Next, we performed LMWPTP immunohistochemical staining on a tissue micro array (TMA) containing representative cores of 481 prostate cancer patients who underwent radical prostatectomy (**Table 1**), with at least 2 cores per patient. Median follow-up of the TMA cohort is 113 months. Patients were divided in two groups based on intensity of LMWPTP staining (examples in **Figure S1**). High intensity of LMWPTP staining was significantly associated with well-known parameters for prostate cancer aggressiveness, such as Gleason score (P=0.01) and pT-stage (P=0.005) (**Table 1**). Furthermore, high LMWPTP expression in prostate cancer correlates to earlier biochemical and local recurrence (Logrank; P<0.001 and P=0.05), an incrementally increased prostate cancer related death (Logrank; P=0.005) (Figure 2), and significantly reduced overall patient survival (Logrank; P=0.017) (Figure S2). In multivariate analysis, high LMWPTP expression resulted in a significant hazard ratio for time to biochemical (N=119; HR:1.99; P<0.001), and time to death (overall) (N=109; HR: 1.53; P=0.04) (**supplementary tables S1-S3**), suggesting a role for LMWPTP in increasing tumor aggressiveness. Due to the limited number of events for local recurrence (N=21) and prostate cancer related death (N=12), we could not perform multivariate analysis, however in univariate analysis high LMWPTP status results in HRs of 2.35 and 4.77, respectively (0.98-5.66; P=0.056, and 1.44-15.84; P=0.01), **Tables S2 and S4**. Staining of the proliferation marker KI-67 (examples in **Figure S3**)

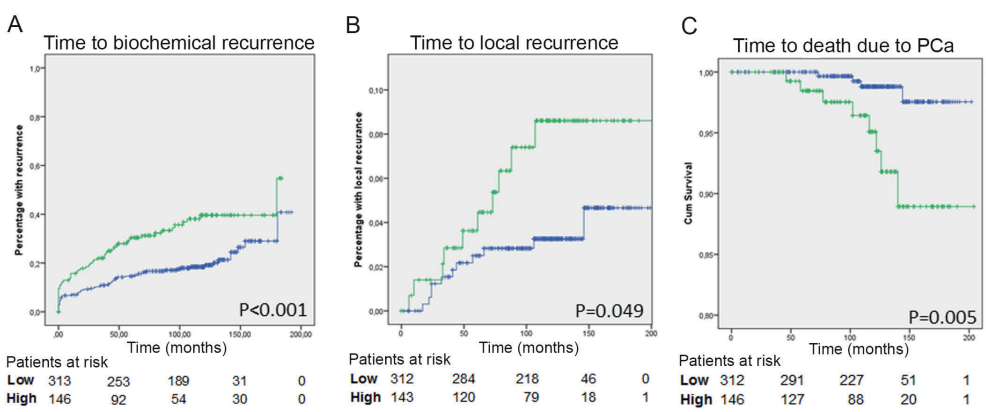

Figure 2. Increased LMWPTP expression is correlated to worse clinical outcome.
LMWPTP staining intensity within the TMA was divided in two groups (High expression [>1.5] in green /and Low expression [<1.5] in blue) and correlated to clinical characteristics. Kaplan Meier curves for Time to biochemical recurrence **(A)**, Time to local recurrence **(B)**, and time to death due to prostate cancer **(C)**, reveal a significant correlation between high LMWPTP expression and these clinical parameters. (P = logrank test)

did not correlate to LMPWTP intensity, nor did it correlate to poor outcome or serve as independent prognostic marker for any clinical parameter. These data suggest that in our cohort, signaling induced by LMWPTP is more conductive to aggressive tumor behaviour of PCa cells than their proliferative capacity.

Identification of canonical pathways regulated by *ACP1*

Using the Ingenuity network platform, we performed an unbiased analysis of *ACP1*-related signaling differences in PCa in comparison to NAP. The top canonical pathways which are statistically significant for each result set (PCa-NAP, TURP-NAP and LN-PCa-NAP) were determined in IPA (**Figure S4A-B**), demonstrating a significantly differential expression of Ephrin Receptor Signaling (ERS) (-log[p-value] > 4.0), with only two other pathways achieving greater significance. Closer inspection of the ERS pathway (**Figure 3**) revealed the protein-protein interaction between Ephrin-B and LMWPTP (or *ACP1*) in IPA, but the regulatory effect of *ACP1* determined by Stein et al. [17] lacked in the canonical pathway. We amended this pathway to include *ACP1*, which regulates Ephrin-A (EphA) [18–20]. MAP analysis of the

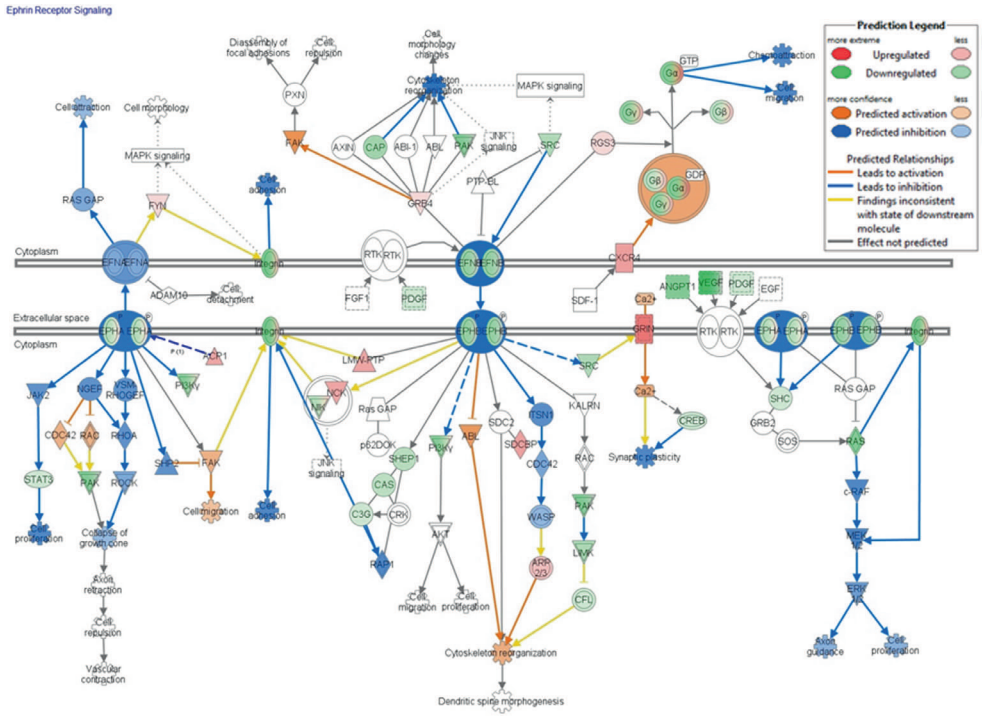

Figure 3. MAP analysis using the ERS pathway with differentially expressed genes from PCa versus NAP results. Schematic representation the Ephrin-Receptor Pathway (ERS), comparing the PCa and NAP datasets. Pink and green are up- and down-regulated genes respectively, with blue and orange representing the negative and positive outcomes predicted by IPA based on the differentially expressed genes in prostate cancer. Note the increased prediction of migration and reduced prediction of proliferation based on the presence of related gene interactions in PCa.

amended ERS pathway was consistent between all PCa datasets (**Figure 3, Supplementary figures S5-6**), with the overriding outcome that cell migration is predicted to be upregulated in PCa, TURP and LN-PCa as compared to NAP, through regulation of cytoskeletal rearrangement mediators. Furthermore, STAT3 expression is downregulated, and the STAT3-pathway as known in IPA is predicted to be inhibited in PCa. While Jak-STAT signaling is upregulated in many cancers, Spiotto and Chung showed that in prostate cancer loss of STAT3 transcriptional activity is correlated to oncogenic potential and disease progression [21]. Furthermore, proliferative potential is predicted to be downregulated.

These unbiased predictions were validated by definition of a set of known LMWPTP-interacting genes (**supplementary table S5**), again predicting STAT3 pathway inhibition, and demonstrating increased IGF1 and drug resistance pumps expression in conjunction with *ACP1* expression (**Figure S7**).

Overexpression of LMWPTP in PC3 prostate cancer cells increases migration

To study the effect of LMWPTP on PCa cell growth, we overexpressed this phosphatase in the prostate cancer cell line PC3. Changes in cell morphology (i.e. loss of spindle shape) suggested that overexpression of LMWPTP may enhance cell proliferative capacity (**Figure S8A**). However, no changes in G1, S and G2/M cell cycle distribution of LMWPTP-overexpressing cells were observed (**Figure S8B**). When filamentous actin (F-actin) was visualized using phalloidin-TRITC staining, LMWPTP-overexpressing cells were not only rounded, but displayed an F-actin distribution along the periphery of the cell, resembling lamellipodia and indicative of migratory cells (**Figure S8C**).

We therefore analyzed migration in three different prostate cell lines and showed that overexpression of LMWPTP augmented chemokinesis capacity of PC3, DU145 and PNT2C2 prostate cells >twofold (**Figure 4A-D**). The PNT2C2 prostate cell line, derived from non-tumorigenic prostate epithelial cells, is regarded as less malignant than PC3 or DU145, which were derived from prostate tumors. Spontaneous random migration of PNT2C2 cells is minimal compared to PC3 and DU145 (Figure 4E), consistent with a lower LMWPTP expression and activity in these cells compared to PC3 cells (**Figure 4F**). Indeed, when LMWPTP expression was reduced in PC3 cells by siRNA, a 31% reduction in migration of these cells was observed (**Figure S9A, B**). Accordingly, in LMWPTP silenced PCa cells, matrix metalloproteinase 2 (MMP-2) was less active (**Figure S9C, D**), indicative of decreased invasive potential. When the low LMWPTP expression observed in PNT2C2 cells was further reduced by siRNA, their migratory capacity, as measured in wound closing assays, was also diminished (**Figure 4G**). Together, these results demonstrate that overexpression of LMWPTP in prostate tumor cells results in their enhanced migration.

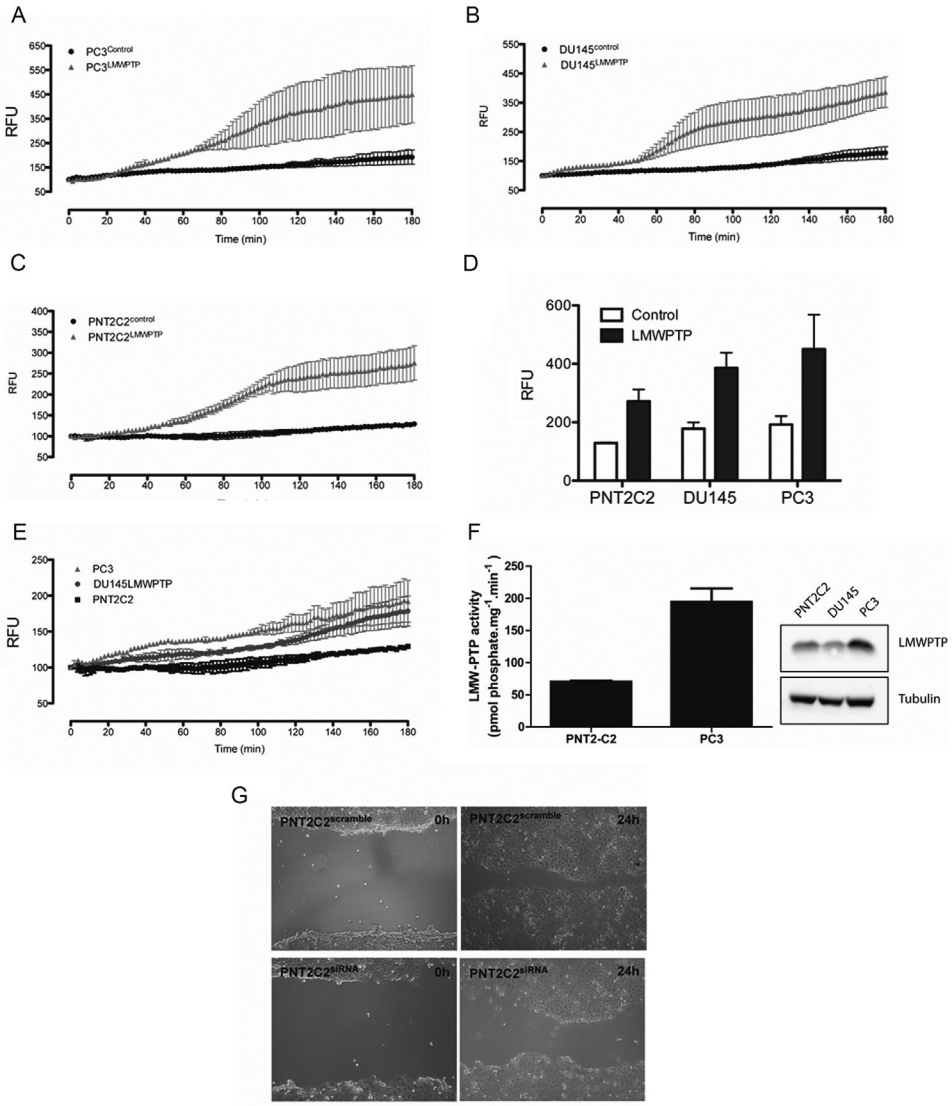

Figure 4. LMWPTP promotes PCa cell migration. A) PCa cell lines were transfected with either empty vector (control) or LMWPTP and Transwell migration of CellTracker Green labeled cells was measured over time. Values represent the number of cells on the bottom side of the insert. Representative examples of migrating PC3 cells (A) DU145 (B) and PNT2C2 (C) are shown. (D) Quantification of migratory PNT2C, DU145 and PC3 cells at t=180 min. (E) Migration of untransfected PC3, DU145 and PNT2C2 cells compared side-by-side. Spontaneous random migration of PNT2C2 cells is minimal when compared to the tumor lines PC3 and DU145 (129.3% RFU at t=180 min vs 192.2% and 178.5 %, respectively). (F) LMWPTP activity was compared between PCa cell lines with the highest and lowest migratiory response (PC3 and PNT2C2). LMWPTP activity was immunoprecipitated from equal amounts of protein, and specific phosphatase activity was measured. In addition, equal amounts of protein (as determined by Tubulin staining) were assessed for LMWPTP expression by western blot analysis. (G) PNT2C2 cells were transfected with either Scrambled siRNA or siRNA against LMWPTP. As these cells migrate too slow to detect a further reduction with transwell migration, we performed a scratch assay. Reducing LMWPTP in PNT2C2 cells decreased the speed with which the induced scratch is healed.

Modulation of LMWPTP expression affects actin-cytoskeletal-related signaling and adhesive properties

Next, we investigated the signaling moieties involved in cytoskeletal rearrangement. The vasodilator-stimulated phosphoprotein (VASP) is involved in the regulation of actin nucleation, with phosphorylation on Ser239 inhibiting actin accumulation and affecting filopodia formation during migration [22]. Overexpression of LMWPTP in PC3 cells reduced phosphorylation of VASP on Ser239, while downmodulation of LMWPTP increased it (**Figure 5B**).

Furthermore, phosphorylation of Focal adhesion kinase (Fak) and its target Paxillin, two proteins involved in cytoskeletal rearrangement [23] were reduced upon overexpression of LMWPTP, and increased when LMWPTP was silenced (**Figure 5A-C**). Cytoskeletal signaling in LMWPTP-overexpressing cells being consistent with reduced inhibition of actin nucleation and affected cell adhesive properties, we subsequently measured adhesion of PC3 cells on collagen I-coated plates. Overexpression of LMWPTP significantly reduced cell adherence to collagen (**Figure 5D**). However, in vivo, adhesion of cells is modulated through integrin binding and matrix proteins. When non-adherent LMWPTP-overexpressing PC3 cells were replated on MG63 osteoblastic cells, these floating PC3 cells adhered efficiently to this stromal cell line (**Figure 5E, F**). Furthermore, the total number of viable PC3 cells in MG63 co-cultures was enhanced upon overexpression of LMWPTP (**Figure 5G**), showing that LMWPTP mediates anoikis-resistance in PC3 cells, one of the tumor hall-marks required for metastatic potential.

Discussion

An oncogenic rather than tumor suppressive role for phosphatases has been postulated before [1,24]. Overexpression of LMWPTP alone is sufficient to drive transformation of epithelial cells, and increased levels of LMWPTP have been reported in several human tumors, including neuroblastoma and breast cancer [19,25]. In this study, we show for the first time that LMWPTP is overexpressed in prostate tumor biopsies, which corresponds to a significantly worse clinical outcome - i.e. earlier time to recurrence and to disease-related death, suggesting that this staining could potentially function as a prognostic marker, and may help with the identification of patients eligible for the active surveillance strategy. However, the number of events for PCa related death in our study was low, and prospective follow-up studies will need to show the clinical implications of this phosphatase in prostate cancer. Interestingly, our data (KI-67 staining and IPA analysis) suggest that invasive potential rather than proliferation confers tumor aggressiveness. Our in vitro studies support a role for LMWPTP in cellular migration and anoikis-independence, through modulation of adhesion-associated cytoskeletal signaling. These data fit well with earlier

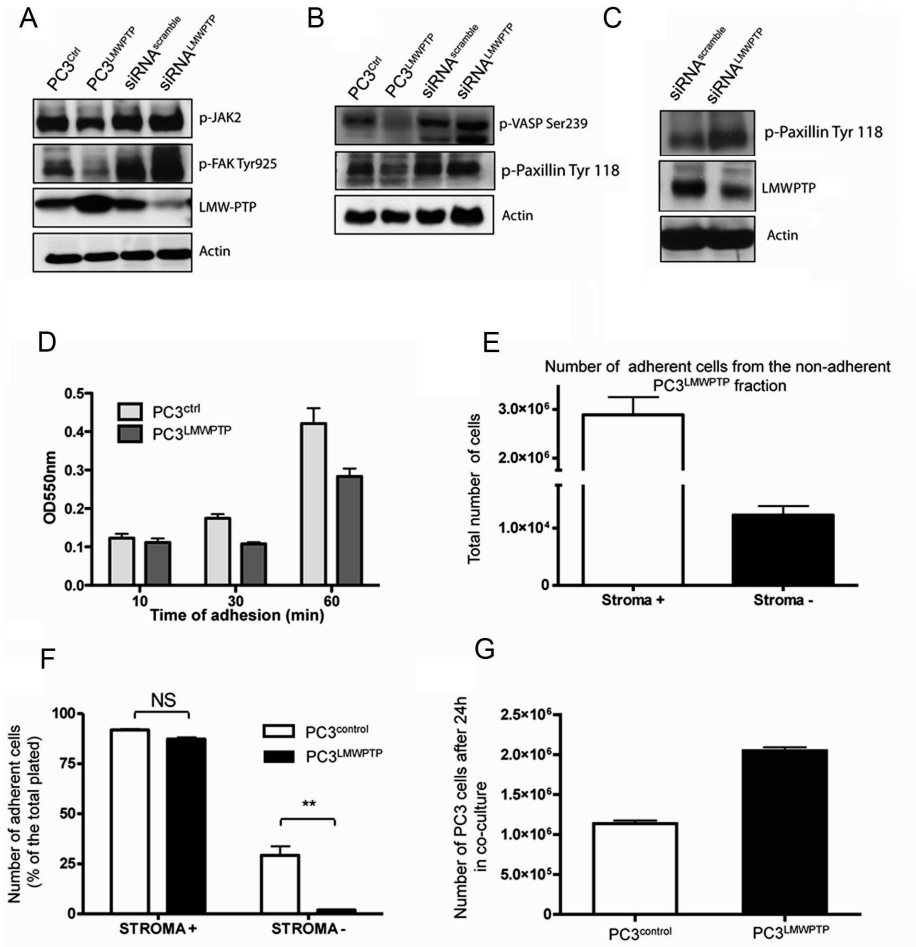

Figure 5. Modulation of LMPWTP expression affects cytoskeletal signaling and influences adherence-dependent cell survival. LMWPTP expression in PC3 cells was increased with overexpression vectors or decreased with siRNA. Functional activity of LMWPTP protein was evident from decreased JAK phosphorylation in LMWPTP overexpressing cells and decreased p-JAK2 in LMWPTP knockdown cells **(A)**. Next, phosphorylated Fak **(A)**, phosphorylated VASP **(B)**, and phosphorylated Paxillin **(B,C)** were determined by Western blot analysis. Equal loading was confirmed by anti-Actin antibodies. Representative examples of experiments are shown. **D)** Control vector or LMWPTP transfected PC3 cells were plated on collagen-I coated wells. Non-adherent cells were removed at different time intervals, and presence of adherent cells was determined by MTT assay. LMWPTP over-expression reduces the number of adherent cells. **E)** Non-adherent LMWPTP over-expressing cells were labelled with CFSE and re-plated on either stromal cells or empty wells. After 24h, non-adherent cells were gently removed, the culture was trypsinized, and the number of fluorescent cells was determined by flow cytometry. Floating LMWPTP over-expressing cells are viable and adhere well to stromal cells. **F)** Control vector and LMWPTP transfected PC3 cells were labelled with CFSE and plated on either stromal cells (Stroma +) or empty wells (Stroma -). Adherence of LMWPTP over-expressing cells to empty wells is significantly decreased compared to control cells. Adherence to stroma is more efficient than adherence to empty wells, for both control and LMPTP expressing cells. **G)** The total number of viable PC3 cells in co-cultures of PC3LMWPTP cells with stromal cells is higher than in co-cultures of PC3control cells with stromal cells. Fluorescent PC3 cells were determined by flow cytometry.

studies showing increased migration and reduced Fak activity upon overexpression of LMWPTP in fibroblasts [19].

Thus far, relatively few other phosphatases have been found to perform an oncogenic function in PCa. The prostatic acid phosphatase (PAP) was shown to be highly expressed in metastatic PCa, and enhanced expression of the dual specificity phosphatase (DUSP)-1 was found to be inversely correlated with apoptosis in PCa, and is likely involved in early phases of neoplastic transformation [26,27]. LMWPTP may now be added to this list.

Conclusions

We report that expression of the phosphatase LMWPTP is increased in primary prostate tumor, which leads to a worse clinical outcome. Overexpression of LMWPTP in PCa affects cytoskeletal signaling, increases anoikis resistance and enhances migratory potential. Our data indicate that LMWPTP could potentially be used as biomarker, and a future treatment target for prostate cancer. Future prospective studies will need to confirm the clinical implications of our findings.

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Competing interests: Authors declare no conflicts of interest.

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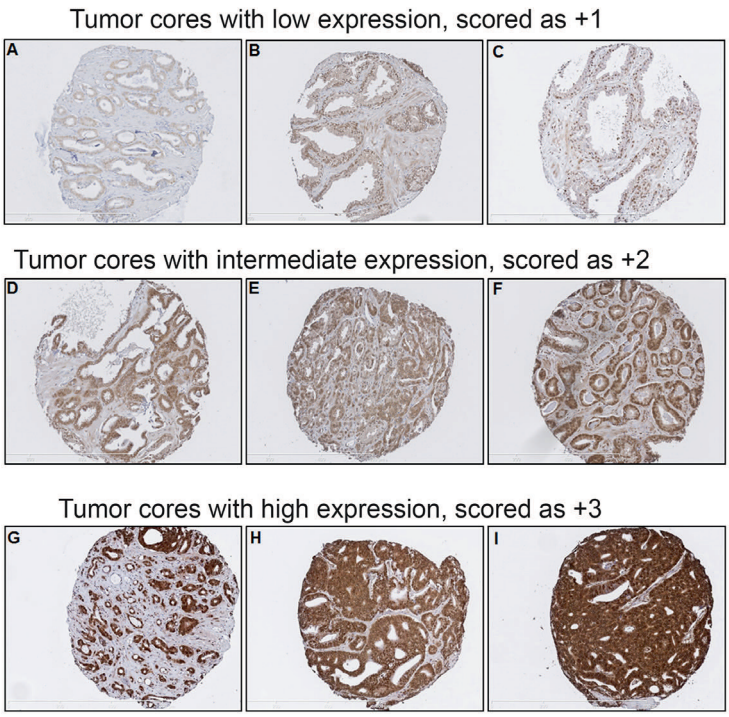
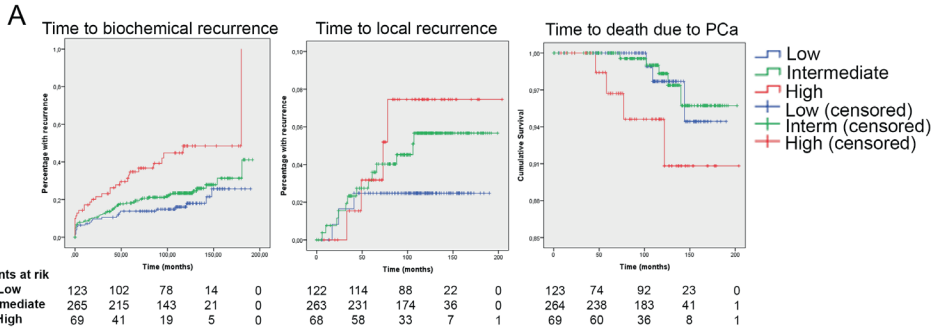
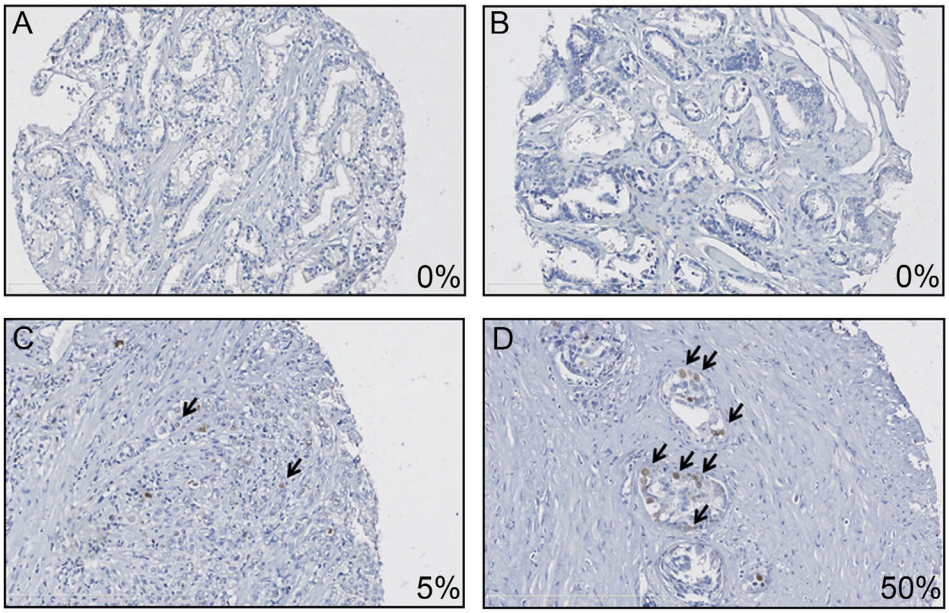
Supplementary Figures



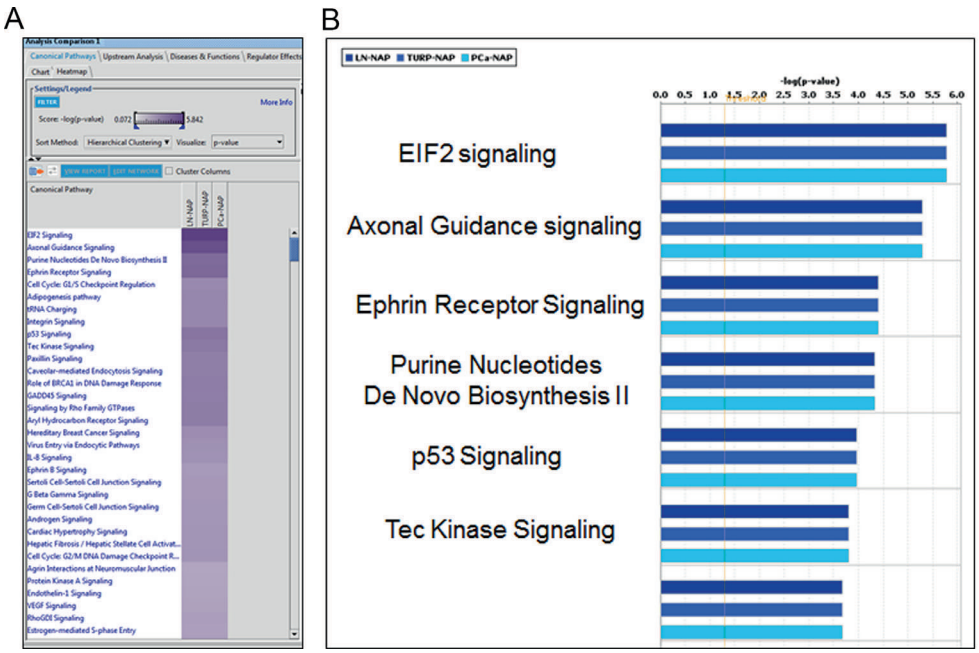
Supplementary Figure 1. Immunohistochemical analysis of LMWPTP expression on tissue micro array (TMA). Representative cores of prostate specimens, stained for LMWPTP expression. Since most epithelial cells were positive for the staining, we focused on LMWPTP intensity. Low intensity **(A,B,C)**, was scored as scored as +1, intermediate intensity **(C,D,E)** were scored as +2, and high intensity **(F,G,H)** scored as +3**.**



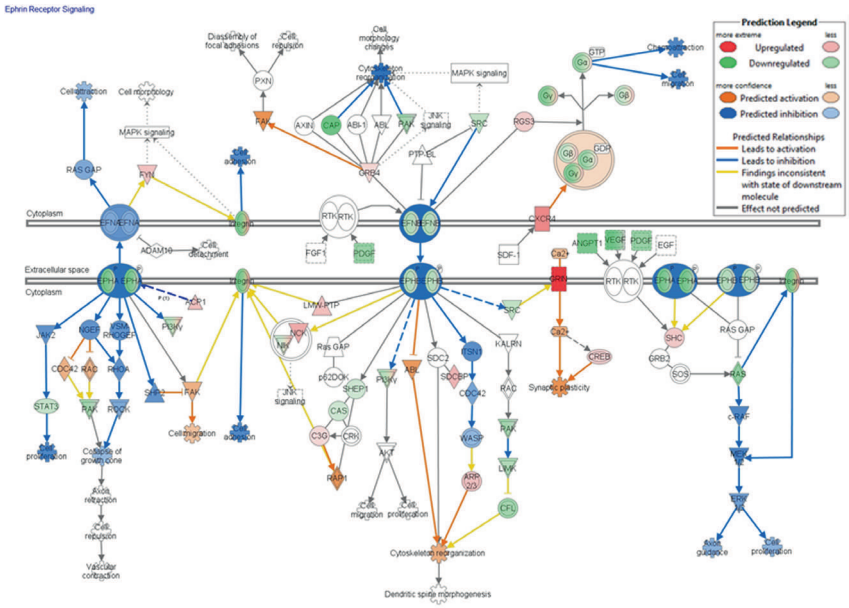
Supplementary Figure 2. Increased LMWPTP expression is correlated to worse clinical outcome. Patients were divided into three groups according to the intensity of their LMWPTP staining (Low expression in blue / intermediate in green / high in red) and LMWPTP expression was correlated to clinical characteristics. Kaplan Meier curves for Time to biochemical recurrence, Time to local recurrence, and Time to death due to prostate cancer confirmed an increase in time to recurrence and death upon increasing LMWPTP expression.



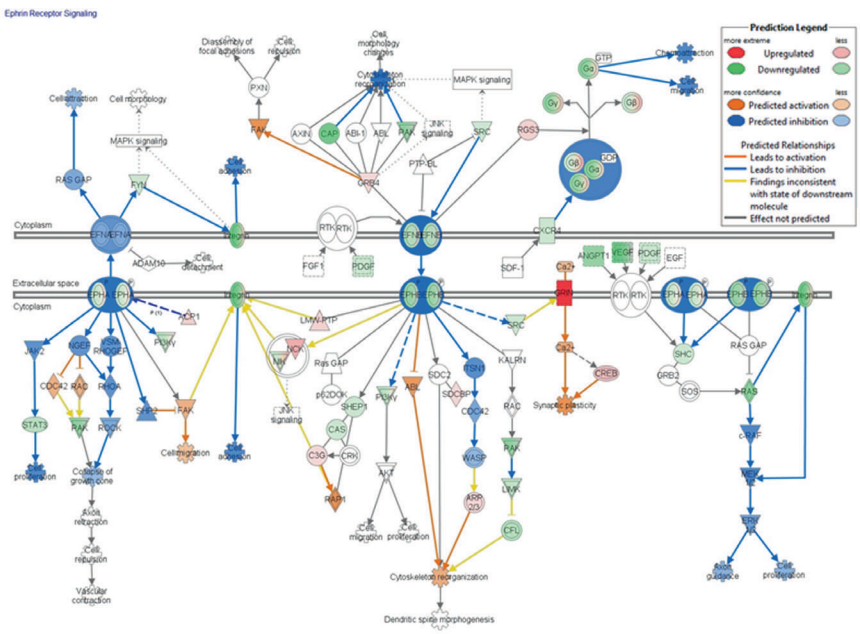
Supplementary Figure 3. Representative cores of prostate cancer specimens, stained for the proliferation marker KI-67. Representative cores of prostate specimens stained for KI-67. Expression was absent in **A** and **B**, in **C** less than 5% of the nuclei were positive (indicated by arrows), while in **D** around 50% of the nuclei are positive for the marker.



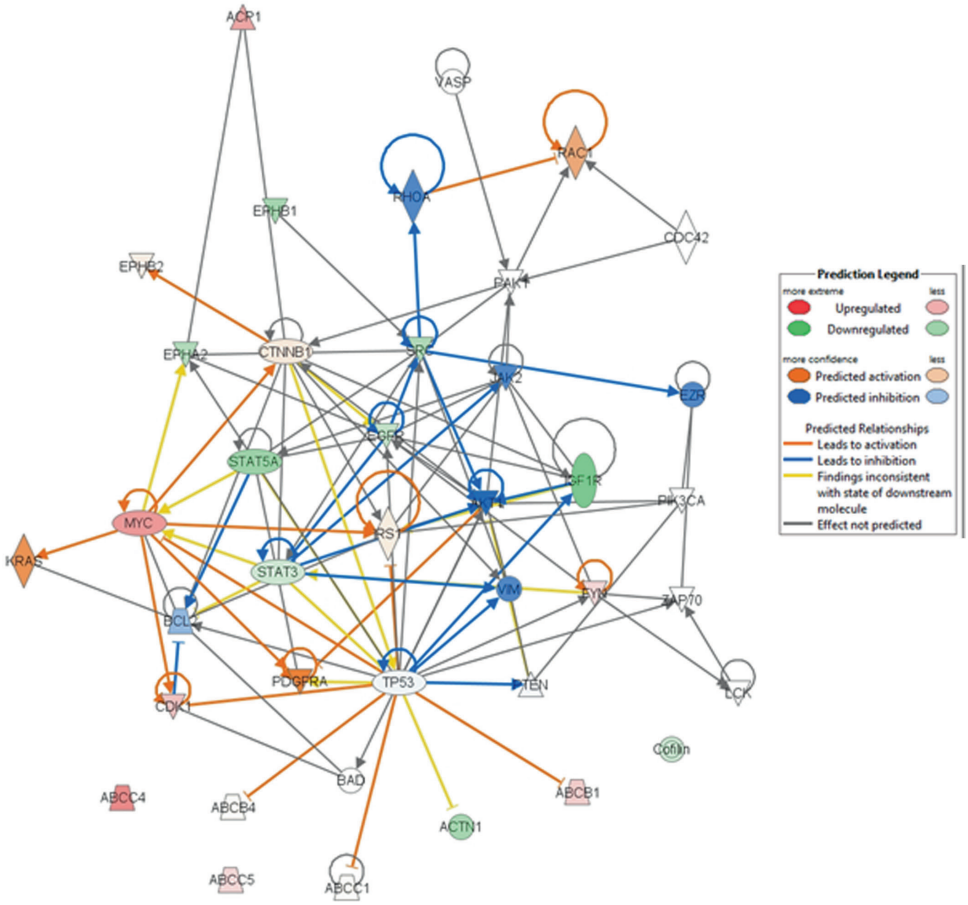
Supplementary figure 4. Differentially regulated canonical pathways identified using IPA.
A) Hierarchical clustering of canonical pathways in the 3 datasets (LN-PCa-NAP, TURP-NAP and PCa-NAP). **B)** Close-up of the top canonical pathway identified with log-values.



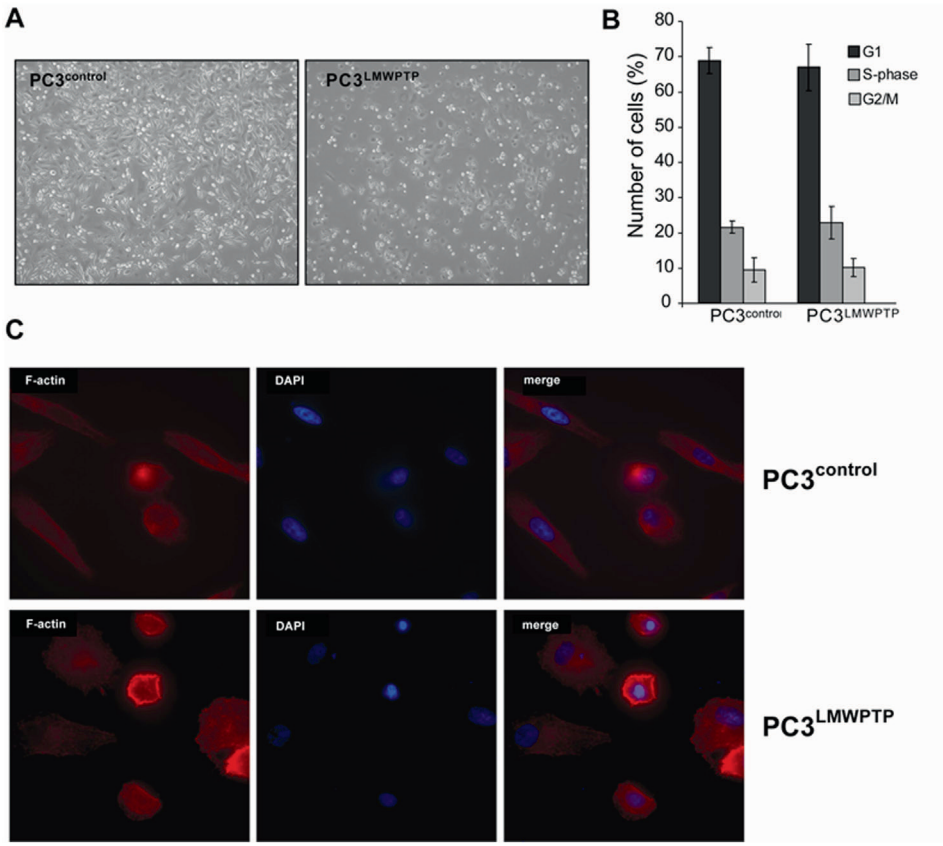
Supplementary figure 5. MAP analysis using the ERS pathway with differentially expressed genes from LN-PCa-NAP results. Pink and green are up- and down-regulated genes respectively with blue and orange representing the negative and positive predicted outcome the differentially expressed genes in LN-PCa



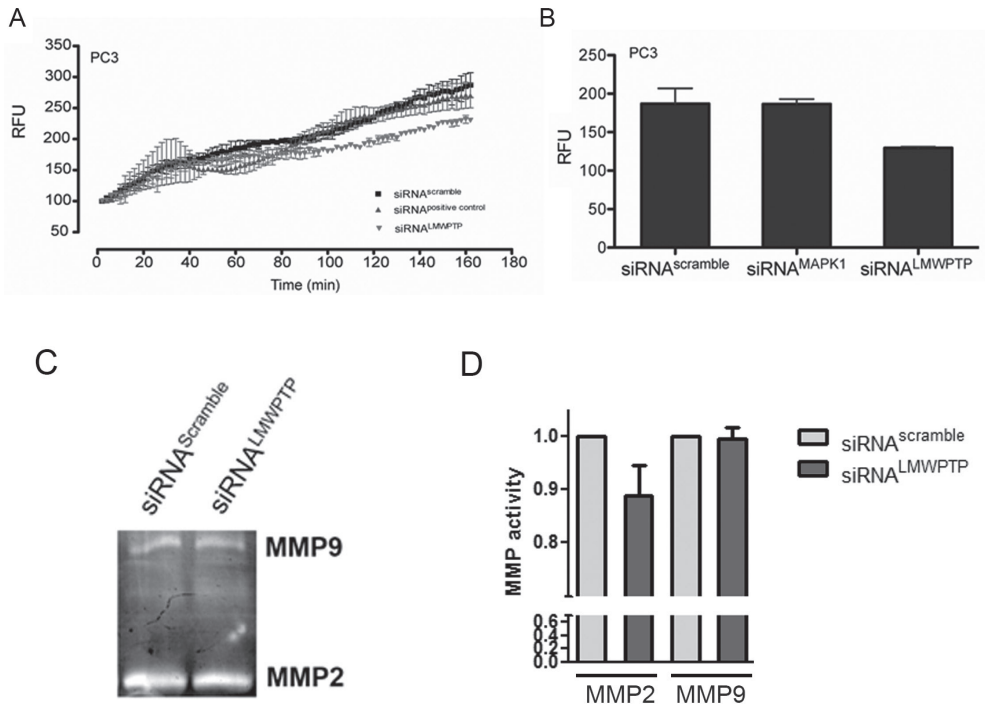
Supplementary figure 6. MAP analysis using the ERS pathway with differentially expressed genes from LN-PCa-NAP results. Pink and green are up- and down-regulated genes respectively with blue and orange representing the negative and positive predicted outcome the differentially expressed genes in TURP



Supplementary figure 7. MAP analysis of target network based on the known interactions and experimentally validated connections of *ACP1* (or LMWPTP) stored within the IPA platform.
Gene expression patters of PCa vs NAP. Associations known in IPA are indicated with arrows. Not all literature-described associations are annotated in IPA, these are represented as separately from the IPA-drawn network, but correspond with the expected increase in multidrug resistance pumps in PCa, which is in agreement with literature indicating a role for LMWPTP in drug resistance.



Supplementary figure 8. LMWPTP overexpression alters PCa phenotype.
A) PC3 prostate cancer cells over-expressing LMWPTP show a rounded phenotype compared to control vector transduced cells. Fewer cells are seen per field of view. **B)** PC3 cell cycle progression is not affected by overexpression of LMPWTP. Cell cycle analysis of control vector and LMWPTP overexpressing cells was determined by staining with propidium iodine, followed by flow cytometry. **C)** Cells transfected with either control vector or LMWPTP were stained with phalloiding-TRITC to visualize F-actin, and DAPI nuclear stain. LMWPTP overexpressing cells are rounded, lose their characteristic spindle-shape and gain lammelipodia-like actin structures at the periphery of the cell.



Supplementary figure 9. Modulation of LMWPTP expression influences PCa cell migration and MMP2 activity. (A) Representative example of PC3 cells transfected with scrambled siRNA, siRNA against MAPK1 or siRNA against LMWPTP, and used in the migration assay. **(B)** Whereas siRNA against MAPK1 did not affect PC3 migratory response, PC3LMWPTPsiRNA cells presented 129% RFU after 180 min, compared to 187%RFU in control cells. **C)** Equal amounts of protein from each sample were applied to gel zymography. The activity of MMP-9 and MMP-2 was measured by densitometry of the gelatin zymography in PC3 cells, showing reduced MMP-2, but not MMP-9 activity. **D)** Quantification of gelatin zymography of MMP activity in PCa cells (n=2).

Supplementary Tables

Supplementary table 1. Multivariable cox-regression analysis for time to biochemical recurrence. (number of events = 119)

Parameter	Multivariate analysis	
	HR (95% CI)	p-value
Age	1.023 (0.98-1.07)	0.339
PSA	1.02 (1.01-1.03)	0.009
Gleason score		
*<7	1	0.001
3+4 = 7	1.50 (0.91-2.38)	
4+3 = 7	3.41 (1.85-6.30)	
>7	1.94 (0.95-4.00)	
pT-stage		
*pT2	1	<0.001
pT3a	1.12 (0.68-1.84)	
pT3b	3.16 (1.57-6.37)	
pT4	2.89 (1.60-5.21)	
Surgical margin	2.32 (1.55-3.48)	<0.001
LMWPTP status	1.99 (1.36-2.91)	<0.001

Cox- regression analysis with continuous variables age and PSA level, and categorical variables Gleason score,and pT-stage, where * represents the category of reference.

Supplementary table 2. Univariate cox-regression analysis for time to local recurrence. (number of events = 21)

Parameter	Univariate analysis	
	HR (95% CI)	p-value
Age	0.96 (0.87-1.05)	0.37
PSA	1.03 (1.01-1.05)	0.002
Gleason score		
*<7	1	0.001
3+4 = 7	1.79 (0.58-5.55)	
4+3 = 7	8.78 (2.83-27.28)	
>7	6.06 (1.51-24.30)	
pT-stage		
*pT2	1	0.02
pT3a	0.55 (0.12-2.44)	
pT3b	1.59 (0.21-12.19)	
pT4	4.31 (1.53-12.19)	
Surgical margin	4.10 (1.73-9.75)	0.001
LMWPTP status	2.35 (0.98-5.66)	0.056

Cox-regression analysis with continuous variables age and PSA level, and categorical variables Gleason score and pT-stage, where * represents the category of reference.

Supplementary table 3. Multivariable cox-regression analysis for time to overall death. (number of events = 109)

Parameter	Multivariate analysis	
	HR (95% CI)	**p-value**
Age	1.10 (1.05-1.16)	<0.001
PSA	1.02 (1.00-1.03)	0.02
Gleason score		
***<7**	1	0.11
3+4 = 7	1.29 (0.83-2.02)	
4+3 = 7	2.08 (1.13-3.81)	
>7	1.02 (0.37-2.78)	
pT-stage		
***pT2**	1	0.06
pT3a	0.51 (0.30-0.87)	
pT3b	0.44 (0.15-1.29)	
pT4	0.63 (0.29-1.37)	
Surgical margin	1.10 (0.71-1.72)	0.68
LMWPTP status	1.53 (1.03-2.27)	0.04

Cox-regression analysis with continuous variables age and PSA level, and categorical variables Gleason score and pT-stage, where * represents the category of reference.

Supplementary table 4. Univariate cox-regression analysis for time to prostate cancer related death. (number of events = 12)

Parameter	Univariate analysis	
	HR (95% CI)	**p-value**
Age	1.05 (0.92-1.20)	0.46
PSA	1.03 (1.01-1.05)	0.001
Gleason score		
***<7**	1	0.001
[a]3+4 = 7	-	
4+3 = 7	14.80 (3.70-59.28)	
>7	13.23 (2.66-65.78)	
pT-stage		
***pT2**	1	0.005
pT3a	3.06 (0.61-15.31)	
pT3b	5.86 (0.61-56.60)	
pT4	13.18 (3.09-56.15)	
Surgical margin	1.29 (0.387-4.31)	0.68
LMWPTP status	4.77 (1.44-15.84)	0.01

Cox-regression analysis with continuous variables age and PSA level, and categorical variables Gleason score and pT-stage, where * represents the category of reference. (a) not analyzable due to lack of events.

Supplementary table 5. Gene list used to construct an *ACP1*-related Target Network in IPA.

Genes involved in migration	Genes involved in proliferation/apoptosis	Possible *ACP1* upstream/ downstream targets
RhoA	TP53	ZAP70
PAK	BAD	FYN
Vimentin	BCL2	LCK
Rac1	CDK1	CTNNB1
CDC42	MYC	EphA2
EGFR	KRAS	EphB1
Actn1	PIK3CA	EphB2
Vasp	PTEN	IRS-1
Ezr	AKT1	STAT3
Cofilin		STAT5
		FAK
		SRC
		PDGFRA
		JAK2
		IGF1R
		ABCB1
		ABCB4
		ABCC1
		ABCC4
		ABCC5

Supplementary material and methods

Gene expression profiling

Use of clinical samples was approved by the Erasmus Medical Center Medical Ethics Committee according to the Medical Research Involving Human Subjects Act in protocol MEC-2004-261. Initial treatment of primary tumors was radical prostatectomy. Hematoxilin/eosin-stained, frozen tissue sections were evaluated by two pathologists (T.van der Kwast and G.J.L.H. van Leenders). Samples were collected between 1984 and 2001, and specimens containing >70% tumor tissue or specimens without the presence of cancer (normal adjacent prostate (NAP)) were selected [1]. The samples with and without PCa are from different specimens. RNA from 30 consecutive, 30-µm tissue sections obtained from primary resected prostate cancers (PCa, n=56), lymph node metastasis (LN-PCa; n=12), Transurethral resected prostates (TURP; n=10) and normal adjacent prostate (NAP; n=12) samples were isolated using RNA-Bee (Campro Scientific, Berlin, Germany). The lymph node tissue itself was not analyzed, so comparison of LN-PCa is compared to NAP. The tumor percentage of two flanking sections and one internal section was determined. RNA for microarray analysis were processed according to the protocol of the supplier (Affymetrix, Santa Clara, CA, USA). Expression profiles were determined using GeneChip Human Exon 1.0 ST (Affymetrix) at the Erasmus Center for Biomics, Erasmus Medical Center, and ServiceXS (Leiden, the Netherlands) (GSE41410 and GSE59745) [2]. Microarray data were RMA normalized using Partek Genomics Suite (Partek Inc, St Louis, MO, USA). Next, we also performed an analysis of the *ACP1* expression levels in prostate cancer compared to normal prostate using the Oncomine tool, using publicly available databases (website).

Ingenuity Pathway analysis

Gene array data generated as described above were analysed as follows: differentially regulated gene list, their log2 fold change and associated p-value for LN-PCa, TURP and PCa as compared against NAP were filtered for a p-value ≤ 0.05, then the corresponding expression values and p-value were imported into the Ingenuity Pathway Analysis software (IPA; Ingenuity Systems, Redwood City, CA) [3]. A rank list of canonical pathways from IPA's library of canonical pathways, based on statistical significance was determined independently for each PCa result set (PCa-NAP, LN-PCa-NAP and TURP-NAP) with a Fisher's exact test to determine the probability that each biological function assigned to that data set was due to chance alone. The threshold for a significant pathway is determined as the corrected p-values based on the Benjamini-Hochberg method of accounting for multiple testing [4] and set maximum False Discovery Rate of 5%.

Subsequently IPA’s Comparison Analysis was used to rank the most common significant pathways which were determined from the differentially expressed genes for all three data set comparisons (PCa-NAP, LN-PCa-NAP and TURP-NAP). To assess the

functional role of *ACP1* in prostate cancer, the most significant canonical pathway(s) containing *ACP1* common to all three results sets was used for Molecule Activity Predictor (MAP). MAP is used to interrogate canonical pathways (and networks) and to generate hypotheses by selecting a molecule of interest, indicating up or downregulation, and simulating directional consequences of downstream molecules and the inferred activity upstream in the pathway (or network). The three PCa results sets (PCa-NAP, LN-PCa-NAP and TURP-NAP) were used as quantitative measures for MAP analysis.

In parallel and as a validation to the predictions generated from the canonical pathway analysis, a set of genes was defined by the authors based on their knowledge of the literature with respect to the functional aspects of *ACP1* (STAT-signaling [5], growth factor signalling [6], drug resistance [7]), or their involvement in cell proliferation or migration. These genes were used to generate a network based on the known interactions and experimentally validated connections stored within the IPA platform. MAP analysis was repeated as with the canonical pathway analysis to determine if *ACP1* has a functional role in this network.

Patient Information

We selected all men from the European Randomized Study of Screening for Prostate Cancer (ERSPC), Rotterdam section, who had undergone radical prostatectomy as part of the normal clinical care for non-metastasized prostate adenocarcinoma in Erasmus Medical Center between 1987 and 2010 [8]. In this study, men aged between 55 and 74 years were invited for a screening visit every 4 years. Recruitment and randomization started in December 1993 and ended December 1999. Up until May 1997, patients with a serum prostate-specific antigen (PSA) level of ≥4.0 ng/ml, an abnormal digital rectal examination and/or abnormal transrectal ultrasound underwent lateralized sextant prostate needle-biopsies. As from May 1997, a biopsy was indicated by a PSA level of ≥3.0 ng/ml or abnormal digital rectal examination and/or transrectal ultrasound.

Directly after surgery, radical prostatectomy specimens were transported on ice to the pathology department. After fixation in neutral-buffered formaldehyde, the radical prostatectomy specimens were routinely cut in 4-mm transverse slices with additional perpendicular slicing of the apex and basis to allow optimal evaluation of surgical margins, and totally embedded in paraffin. Hematoxylin/eosin (HE) slides were microscopically evaluated by two board-certified pathologists with expertise in urological pathology. Histological slides of all patients (n=509) were retrieved from the pathology archives together with corresponding paraffin blocks containing the largest tumor volume (with tumor of at least 0.5 cm in diameter per paraffin block). In 28 cases, the tumor diameter was less than 0.5 cm, or no paraffin tissue was available, resulting in 481 patients to be included. Median age at diagnosis was 64 (55-75) years, median PSA at time of diagnosis was 5.2 ng/ml (IQR 3.6-7.8 ng/

ml). Gleason score was <7 in 265 (56%), 3+4=7 in 152 (28%), 4+3=7 in 35 (6%), and >7 in 28 (5%) cases, respectively. Extra-prostatic progression (pT3/4) was observed in 137 (28%) cases and surgical margins were positive in 119 (25%) cases. Only one of the patients (0.2%) had positive lymph nodes, and all the patients were distant metastasis free (since this was a contraindication for radical prostatectomy), and therefore these variables were excluded from further analysis (clinicopathological characteristics are listed in table 1).

Clinical follow-up was recorded after each control at our outpatient clinic, and data were transmitted to the central study database. Use of samples for research purposes was approved by the Erasmus Medical Center Medical Ethics Committee according to the Medical Research Involving Human Subjects Act (MEC-2004-261).

Immunohistochemistry

The FFPE tissue sections were immunohistologically stained for LMWPTP (ACP1 antibody, sc-100343, Santa Cruz Biotechnologies, Dallax, Tx) as described [9]. Briefly, 5µm sections were deparaffinized in xylene and rehydrated through graded alcohols. Antigen-retrieval was performed by boiling the slides in citrate buffer pH 6.0 for 15 minutes. Endogenous peroxidases were blocked by immersing the slides for 10 minutes in 3% H2O2 in phosphate buffered saline (PBS). Next, slides were blocked by incubation in PBS/10% goat serum for one hour at room temperature. Primary antibody was added 1:800 in blocking buffer and incubated overnight at 4°C. Envision goat anti-mouse-horseradish peroxidase (Dako) was used as secondary antibody. The slides were scored for percentage and intensity of positive epithelial cells in the cancerous tissue as well as the directly adjacent normal prostate tissue.

TMA analysis and statistics

Scoring ability of the staining was assessed by a trained urological pathologist (GL), after which the tissue micro array sections were scanned by a Hamamatsu Nanozoomers virtual microscope. Next, TMA was scored independently by two researchers (EH and JL; kappa =0.65), and number of positive epithelial cells as well as intensity of the staining was recorded. The intensity of LMWPTP expression was scored as negative (0; very low or no staining), weak (1+; intensity just above background level), moderate (2+; clearly visible at low magnification) and strong (3+; striking at low magnification). For each patient on the TMA, at least two tissues cores were available. Based on the average intensity of the staining, two patient groups were formed using arbitrarily chosen cut-off points (low <1.5 and high >1.5). When dividing our patients group into three groups, cut-off points of <1, 1<x<2, and >2 are used.

We tested the hypothesis that increased LMWPTP status is related to a more invasive tumor and therefore correlated to prostate cancer parameters such as Gleason

score and pT-stage, and that increased expression is predictive of worse clinical outcome. Statistical associations between expression of LMWPTP (as categorical variable) and continuous clinico-pathological parameters (age and PSA at the time of diagnosis) were tested using independent sample rank sum test, and with categorical parameters (Gleason score, pT-stage, surgical margins, KI-67) using Pearson's $\chi 2$ test.

To determine whether LMWPTP expression was predictive for biochemical recurrence, local recurrence, overall death or disease-specific death, we used univariate and multivariate Cox regression with following covariates; age at diagnosis and PSA at diagnosis (as continuous variables), Surgical margins and LMWPTP status (as categorical variables), Gleason score and pT-stage as multiple categories (Gleason <7, Gleason 3+4=7, Gleason 4+3=7, Gleason >7), (pT-stage pT2a, pT3a, pT3b, pT4). We have checked the linearity of age and PSA at diagnosis assumption by adding quadratic terms (AGE*AGE; PSA*PSA) to the model, and performed a restricted cubic splines analysis with 4 knots based on the method by F.E. Harrel [10], which were all not significant. Due to the low number of events in the local recurrence (N=21) and prostate cancer related death group (N=12), we could not perform a multivariate analysis, and therefore only reported the univariate analysis. The proportionality assumption for LMWPTP positive versus negative cases was visually assessed in Kaplan–Meier curves. P-value <0.05 was considered significant. All statistics were performed using SPSS 21 (SPSS, Chicago, IL, USA).

Cell lines

PC3 (purchased from ATCC) and DU145 cells (obtained from Prof Dr Jack Schalken, Radboud University Medical Center, Nijmegen, the Netherlands) were cultured in F12K medium, supplemented with 10% fetal bovine serum (FBS, PAA, Etobicoke, Ontario, Canada). PNT2C2 cells were kindly provided by Professor N. J. Maitland, University of York, York, UK, and cultured in RPMI1640 (PAA, Etobicoke, Ontario, Canada), containing 10% FBS. MG63 osteoblastic cells, a kind gift from Dr. R. van Bezooyen, LUMC, Leiden, the Netherlands, were cultured in Dulbecco's Modified Eagles Medium (DMEM, PAA, Etobicoke, Ontario, Canada) containing 10% FBS. All cell cultures were supplemented with 100U/ml penicillin, 100mg/ml streptomycin, and propagated at 37ºC in a 5% CO2 humidified atmosphere.

Cell culture and transfections

Overexpression plasmid pCS2+MT-LMWPTP was a kind gift from Prof. J. den Hertog, Hubrecht Institute, Utrecht, The Netherlands. Transfection of the LMWPTP construct was performed using Lipofectamine™ 2000 from Invitrogen (Bleiswijk, The Netherlands) according to the manufacturer's directions. Briefly, 3x105 cells were seeded in a 6-well plate and after 24h, transfections were performed in serum free media using LMWPTP (4µg DNA/well) or negative control (4µg DNA/well) expression vectors. Four hours later, the medium was replaced for media containing 10%

FBS for 24h. For LMWPTP knockdown, cells were transfected with 5nM of siRNA (Qiagen, Germantown, MD) in a 6-well plate using Hiperfect Transfection Reagent (Qiagen, Germantown, MD) according to the manufacture's specification. All analysis were performed 48h after transfection.

LMWPTP immunoprecipitation and phosphatase assay

LMWPTP activity assays were performed as described [11]. To quantify the phosphatase activity, cells were lysed with 200µL of Lysis Buffer (20 mM HEPES, pH7.7 with 2.5mM MgCl2, 0.1mM EDTA, 1mM PMSF, 1mM DTT, 10µg/mL aprotinin and 10µg/mL leupeptin) on ice for 2h. After clarifying by centrifugation, the cell extracts were incubated overnight at 4ºC under rotation with antibodies against LMWPTP (Abcam, Zwijndrecht, The Netherlands). A-Sepharose beads were added to cell homogenates and incubated for 2h at 4ºC. Cell extracts were washed 3 times with lysis buffer and 2 times with acetate buffer 100 mM pH5.5. The precipitate was resuspended in acetate buffer (100 mM pH 5.5) and immediately used for enzymatic assay.

The PTP activity was measured using the Protein Tyrosine Phosphatase Assay kit Non-Radioactive from Sigma (St. Louis, MO). Reaction medium contained 80mM Acetate Buffer pH5.5, and 0.2mM of PTP phosphorylated substrate. The reaction was carried out at 37°C for 20 minutes and stopped with an equal volume of Malachite Green/Ammonium Molybdate reagent (1:100). The amount of phosphate produced in the reaction was measured at 650nm and compared to a standard curve.

Migration Assays

The migration assay was performed as described [12]. Briefly, transfected and non-transfected cells in 6-well plates were labeled for 1h with 10µM CellTracker Green (Invitrogen, Bleiswijk, the Netherlands) in serum-free medium. Subsequently, cells were incubated for 1h in medium with 10% FCS to retain the dye, washed, detached with trypsin and resuspended in serum-free medium. The cell suspension was transferred to 8µM pore size HTS FluoroBlok Cell Culture Inserts from BD Falcon which were inserted in a 24-well in which contained 600µL of serum free media. Fluorescence was measured every 2 minutes on a microplate reader (Synergy HT, Biotek) and the values represent the number of cells on the bottom side of the insert. Migration start points were set to zero.

Another migration assay, the scratch-wound assay was used to confirm the migratory response of PNT2C2 cells. 28h after transfection with siRNA, PNT2C2 cells were grown to confluence in a 6-well plate, serum-starved overnight and scratched with a pipette tip to create a wound. After wounding, cells were washed twice with PBS to remove cell debris. Photographs were taken by Axiovert200M microscope (Carl Zeiss, BV, the Netherlands) with coupled camera (Axiocam HR, Carl Zeiss BV, the Netherlands), and the percentage of the open wound area at 24h was analyzed

using Image J software (http://rsbweb.nih.gov/ij/).

Adhesion assay

Rat-tail type I collagen (Sigma, St-Louis, MO) 0.01% in 20mM acetic acid was used to coat 96-well plates for 1h at room temperature. Plates were washed with PBS and blocked with 3% (w/v) heat-denatured bovine serum albumin (BSA) for 1h at 37°C followed by washings with PBS. Cells were harvested with 0.5mM EDTA, washed in serum-free media, resuspended in serum-free media/0.1% (w/v) BSA and plated at a concentration of 2.5x104 cells per 100µl. The assay was terminated at 10, 30 and 60 min by washing the wells twice with serum-free F12K medium. The attached cells were fixed and stained with Cristal Violet stain, which was extracted with 10% acetic acid. The absorbance was measured at OD 550nm.

Zymography Analysis

Proteolytic activity of MMP-2 and MMP-9 was assayed by gelatin zymography as described by Souza et al [12]. In brief, culture medium was collected and stored at −20°C until further use. Samples were diluted in non-reducing buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 1% SDS and 0.001% bromophenol blue), and the volume of the samples loaded was proportional to the protein concentration. The samples were resolved by SDS–polyacrylamide gel (10%) and 4% gelatin (Sigma-Aldrich). Protein renaturation was done using 2% Triton X-100 for 1 h followed by incubation with 50 mM Tris-HCl and 10 mM CaCl2 (pH 7.4) at 37°C for 18 h. Gels were stained with 0.5% Coomassie blue G 250 for 30 min and then washed in a 30% methanol and 10% glacial acetic acid solution.

Western blotting

Cells (2.5x107) were lysed on ice for 2h, in 200µL cell lysis buffer (50mM Tris-HCl, pH7.4, containing 1% Tween 20, 150mM NaCl, 1mM EGTA, 1mM o-vanadate, 1mM sodium fluoride, 1mg/ml aprotinin, 10mg/ml leupeptin, and 1mM PMSF). The protein extract was clarified by centrifugation and the protein concentration was quantified using RC DC protein assay (BioRad, Hercules, CA). Equal volume of 2x concentrated SDS gel loading buffer (100mM Tris–HCl (pH6.8), 200mM dithiothreitol, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added and samples were boiled for 10 min. Cell extracts were resolved by SDS–PAGE (12%) and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% Bovine Serum Albumin in TBS/0.05% Tween-20 and incubated overnight at 4ºC with appropriate primary antibody at 1:1000 dilutions. After washing in TBS/0.05% Tween-20, membranes were incubated with antirabbit, antigoat or antimouse horseradish peroxidase-conjugated secondary antibodies at 1:2000 dilutions in blocking buffer for 1h. Detection was performed using ECL (Amersham, Buckinghamshire, United Kingdom) according to the manufacturer's instructions.

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Chapter 4

Low molecular weight protein tyrosine phosphatase (LMWPTP) upregulation mediates malignant potential in colorectal cancer

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Abstract

Phosphatases have long been regarded as tumor suppressors, however there is emerging evidence for a tumor initiating role for some phosphatases in several forms of cancer. Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP; acid phosphatase 1 [*ACP1*]) is an 18 kDa enzyme that influences the phosphorylation of signaling pathway mediators involved in cancer and is thus postulated to be a tumor-promoting enzyme, but neither unequivocal clinical evidence nor convincing mechanistic actions for a role of LMWPTP have been identified. In the present study, we show that LMWPTP expression is not only significantly increased in colorectal cancer (CRC), but also follows a step-wise increase in different levels of dysplasia. Chemical inhibition of LMWPTP significantly reduces CRC growth. Furthermore, downregulation of LMWPTP in CRC leads to a reduced migration ability in both 2D- and 3D-migration assays, and sensitizes tumor cells to the chemotherapeutic agent 5-FU. In conclusion, this study shows that LMWPTP is not only overexpressed in colorectal cancer, but it is correlated with the malignant potential of this cancer, suggesting that this phosphatase may act as a predictive biomaker of CRC stage and represents a rational novel target in the treatment of this disease.

Introduction

Colorectal cancer (CRC) is one of the most common forms of cancer in the Western world. Although CRC mortality has been progressively declining since 1990, it still remains the second most common cause of cancer death in the US and Europe [1]. When in a non-metastatic state, surgery of the primary tumor is considered a curative treatment. Unfortunately, around 20% of the CRC patients already present with metastatic disease, dropping the 5-year survival rate from 90% to a dramatic 12% [2]. For this reason, treatments focusing on the prevention of this progression into the metastatic state are urgently called for. All cellular functions are under tight control of the balance between phosphorylation and dephosphorylation of proteins. Like many neoplasms, disturbed protein phosphorylation patterns, indicating imbalanced kinase and/or phosphatase activities, are often observed in colorectal cancer [3]. So far, kinases have received most of the attention in cancer studies, as it is well established that deregulation of these enzymes can contribute to the development of multiple neoplasms [4]. Inhibitors of kinase activities, such as EGFR- and BRAF-inhibitors, are amongst the novel potential treatments currently explored for CRC. Despite the fact that these drugs have shown some promising results [5, 6], there is still a need for new, additional, classes of molecules as potential targets. Phosphatases could present such a class.

Generally assumed to be tumor suppressive by counteracting kinase activities, phosphatases have largely been ignored as viable targets for treatment. However, a tumor promoting role has also been suggested for certain phosphatases [7]. One of these is the ubiquitously expressed Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP), encoded by the gene *ACP1*. Enhanced mRNA expression of this phosphatase has been reported for some human tumors [8, 9]. This 18kDa protein tyrosine phosphatase can have a positive effect on cell growth and proliferation signaling by interacting with several molecules involved in these processes, such as Ephrin A2 receptor (EphA2) [10], β-catenin [11], platelet-derived growth factor receptor (PDGFR) [12], Janus kinase (JAK)-2 [13], and signal transducer and activator of transcription (STAT)-5 [14].

The aim of this study was to examine the expression levels and potential role of LMWPTP in colorectal cancer. Our study reveals a novel unexpected action of hypomethylation-mediated upregulation of LMWPTP mRNA and protein levels in primary colorectal cancer and shows that this upregulation mediates chemoresistance and increased migration that characterizes this infaust disease.

Materials and Methods

Gene expression profiles
Expression profiles from publicly available NCBI GEO datasets were browsed to find comparisons of CRC or colorectal adenoma samples to their adjacent normal tissue. Information on *ACP1* expression was available in 2 arrays. Dataset Record GDS4382 (transcript 215227_x_at), based on the Affymetrix Human Genome U133 Plus 2.0 Array, was used to compare 17 paired CRC and adjacent normal tissue samples [15]. The same platform was used in dataset record GDS2947 (transcript 215227_x_at), used to compare 32 paired colorectal adenoma and adjacent normal tissue samples [16]. P-values were calculated per probe using Student's t-tests.

Patient selection
At the Erasmus MC Formalin fixed paraffin embedded (FFPE) colorectal tissue specimens were collected from the department of pathology for 9 low grade dysplasia (LGD) patients, 5 high grade dysplasia (HGD) patients, 7 adenocarcinoma (CRC) patients and 5 patients with CRC-related liver metastasis. Patients with active and inactive ulcerative colitis (n=8) served as controls. In addition, a tissue micro array was constructed at the Leiden University Medical Centre (LUMC), containing material from 72 patients with colorectal cancer. Representative cores of healthy adjacent tissue were available for 65 patients, 25 patients had available adenoma cores, and 62 patients had representative carcinoma cores.

Immunohistochemistry
The FFPE tissue sections and TMA were immunohistochemically stained for LMWPTP (Acp1 antibody, sc-100343, Santa Cruz Biotechnologies, Dallas, Tx) as described [17]. Briefly, 5µm sections were deparaffinized in xylene and rehydrated through graded alcohols. Antigen-retrieval was performed by boiling the slides in citrate buffer pH 6.0 for 15 minutes. Endogenous peroxidases were blocked by immersing the slides for 10 minutes in 3% H2O2 in phosphate buffered saline (PBS). Next, slides were blocked by incubation in PBS containing 10% goat serum in for 1h at RT. Primary antibody was added 1:100 in blocking buffer and incubated overnight at 4°C. Envision goat anti-mouse-horseradish peroxidase (Dako, Heverlee, Belgium) was used as secondary antibody. The slides were scored for the percentage of positive epithelial cells as well as intensity of the staining on a 4 scale scoring system. P-values were calculated using Student's t-tests and Wilcoxon signed-rank test for the paired samples.

Cell lines
HCT116 and CACO-2 colorectal cancer cells were purchased from ATCC (Manassas, USA) and cultured in Dulbecco's Modified Eagles Medium (DMEM, Lonza, Basel,

Switzerland), supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, USA). EPC2-hTERT cells are cultured in Keratinocyte-SFM medium (Life technologies, Bleiswijk, NL), supplemented with Epidermal Growth Factor (EGF) and Bovine Pituitary Extract (BPE). All cell cultures were supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (Life technologies, Bleiswijk, NL), and propagated at 37°C in a 5% CO2 humidified atmosphere. Cell lines were routinely tested for Mycoplasm infection using MycoAlert (Lonza, Basel, Switserland).

Cell culture and transfections

Using a lentiviral system, stably transfected LMWPTP knockdown cells were generated. In brief, HEK293T cells were transfected with LMWPTP or non-target control shRNA (Sigma-Aldrich, St. Louis, USA) and viral plasmid, generating virus containing medium. CRC cells were incubated with the conditioned medium for 48 hours after which transfected cells were selected using puromycin (2µg/ml, Sigma-Aldrich, St. Louis, USA).

Western blotting

Subconfluent cells were lysed on ice in 2x concentrated Laemmli buffer (100mM Tris–HCl (pH 6.8), 200mM dithiothreitol, 4% SDS, 0.1% bromophenol blue and 20% glycerol) and samples were boiled for 10min. Cell extracts were resolved by SDS–PAGE and transferred to polyvinylidene difluoride membranes (Merck chemicals BV, Amsterdam, the Netherlands). Membranes were blocked in 50% odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) in PBS/0.05% Tween-20 and incubated overnight at 4°C with primary antibody. After washing in PBS-T, membranes were incubated with IRDye® antibodies (LI-COR Biosciences, Lincoln, NE) for 1h. Detection was performed using Odyssey reader and analyzed using manufacturers software. For antibodies used see supplementary table 1.

Immunoprecipitation and phosphatase assay

To quantify the phosphatase activity, cells were lysed with 200µL of Lysis Buffer (20 mM HEPES, pH7.7 with 2.5mM MgCl2, 0.1mM EDTA, 1mM PMSF, 1mM DTT, 10µg/mL aprotinin and 10µg/mL leupeptin) on ice for 2h. After clarifying by centrifugation, the cell extracts were incubated overnight at 4°C under rotation with antibodies against LMWPTP (Acp1 antibody, sc-100343, Santa Cruz Biotechnologies, Dallas, Tx) PTP1B (PTP1B antibody, sc-14021, Santa Cruz Biotechnologies, Dallas, Tx) or SHP-1 (SH-PTP1 antibody, sc-7289). A/G-Sepharose beads were added to cell homogenates and incubated for 2h at 4°C. Cell extracts were washed 3 times with lysis buffer and 2 times with acetate buffer (100 mM pH5.5). The precipitate was resuspended in acetate buffer containing PNPP as substrate. 45 min after incubation at 37°C, equal volume of 1N HCl was added, and phosphatase activity was measured using a spectrophotometer at 405nm.

Cell viability assay
Using a colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, proliferation was measured. In brief, 10.000 cells were seeded in 96 wells plate, after 24, 48, 72 or 96 hours 5mM MTT was added, and incubated for 2 hours. Next, cells were resuspended in 100µl of Dimethyl sulfoxide and wavelength was measured using a spectrophotometer at 490 and 595nm. Each assay was performed at least three times in duplicate.

Cell cycle analysis
Cell cycle analysis was performed by staining the cells for 1h in sodiumcitrate-dihydrate (1g/L) solution, containing 20 µg/mL propidiumiodide, 0.1% triton-X100 and 100 µg/mL ribonuclease A. The cell cycle distributions were analyzed using Modfit LT software. Each assay was performed twice.

P-Glycoprotein expression levels
P-gp expression levels were measured using standard flow cytometric analysis. Cells were incubated with primary anti-mouse P-gp antibody (Imunnotech, Marseille, France). After washing, cells were incubated with anti-mouse FITC labelled antibody, and analyzed by flow cytometry. Data is shown as mean fluorescence intensity (MFI).

Adhesion assay
50.000 cells were loaded into 96 wells plates and allowed to adhere for different time points to the plate surface either coated with fibronectin, or without coating. After 30 min, 1h and 1.5 hours non-adherent cells were washed away. After 1.5 hours, MTT was added to the plate in order to quantify the amount of adhered cells. Cells adhering to fibronectin coating for 1.5 hours served as control. Assays were performed twice, with 8 duplicates averaged in each assay.

Scratch migration assay
Cells in 6-well plates were grown to semi confluence. Using a yellow Gilson pipette tip simple scratch wounds were made. After washing the cells, the persisting areas of clear plastic were measured at 0, 24 and 48 hours using Axiovision 3.0 software (Carl Zeiss Vision GmbH) and the reduction in scratch wound area from time 0 was calculated. Each assay was performed twice, in duplicate.

Cell migration assay "ring barrier system"
Cell migration assays were performed using the ring-barrier migration assay previously described [18]. Briefly, sterile coverslips placed in an Attofluor incubation chamber were coated with gelatin (1mg/ml) and incubated for 1h at 37°C, prior to cell seeding. A removable circular sterile migration barrier was inserted into the chamber, which prevents cell growth in the center of the coverslip. 4x105 HCT116 and 2,5x105 CACO-2 knockdown and control cells were seeded around this barri-

er and the rings were incubated at 37 °C for 24h, thereby generating a confluent monolayer in the periphery and a cell-free area in the center of the coverslip. After removing the migration barrier, time-lapse imaging was conducted at 37°C under humidified 5% CO2 air flow for 24h on an Axiovert 100M inverted microscopes, equipped with an AxioCam MRC digital cameras, using a 10X/0.30 Plan-Neofluar objective (Carl Zeiss B.V., Sliedrecht, Netherlands). Time-lapse movies (images taken every 12 min) were used to quantify cell migration using AxioVision 4.5 software. For each movie, 10 cells at the migration front were randomly selected and tracked for the analysis. The net track movement of cells in 24 h was termed 'total migration', while the directional movement of cells to the cell-free center of the coverslip was termed 'effective migration'. Migration efficiency was determined as the percentage of directional movement over the total track distance. For each cell line, at least three independent migration assays were performed.

3D-migration using cell dispersion assay

Cytodex-3 microcarrier beads (Sigma–Aldrich) were mixed with 5x105 CACO-2 and HCT116 knockdown and control cell suspensions, considering a density of 40 cells per bead, and incubated at 37°C for 6h with gentle mixing. The bead suspension was transferred to a 25 cm2 tissue culture flask and incubated for 48h to ensure complete coating of beads and to remove unattached cells. Coated beads were embedded in 1.6mg/ml collagen gel (collagen: modified Eagle's medium:7.5% w/v NaHCO3 in the ratio 8:1:1) in a 24-well plate such that each well had approximately 150 beads. Plates were incubated at 37°C for 2h for the beads to settle in the gel and the polymerized gels were covered with 500µl DMEM, 10% FBS, 1% p/s. Cell dispersion was measured as the maximum migrated distance from the surface of the bead into the collagen gel. All measurements were performed using AxioVision 4.5 software and assays were performed three times in duplicate. Two-way analysis of variance was performed to calculate P-values.

Immunofluorescence

Subconfluent cells, cultured on glass coverslips, were fixed 15 min in 4% paraformaldehyde in PBS and permeabilized in 0.1% Triton-X100 in PBS (PBS-T). Actin filaments were stained with 10 µg/mL phalloidin-TRITC in PBS-T. Cell nuclei were stained with 200 ng/mL DAPI (4', 6'-diamidino-2-phenylindole) in PBS-T for 30min and coverslips were mounted using Vectashield mounting medium. Immunofluorescent images were taken using Zeiss LSM510Meta confocal microscope with x40 OilFLUAR lens. Morphometric analysis was performed by measuring the length/width ratio of 26 randomly selected cells.

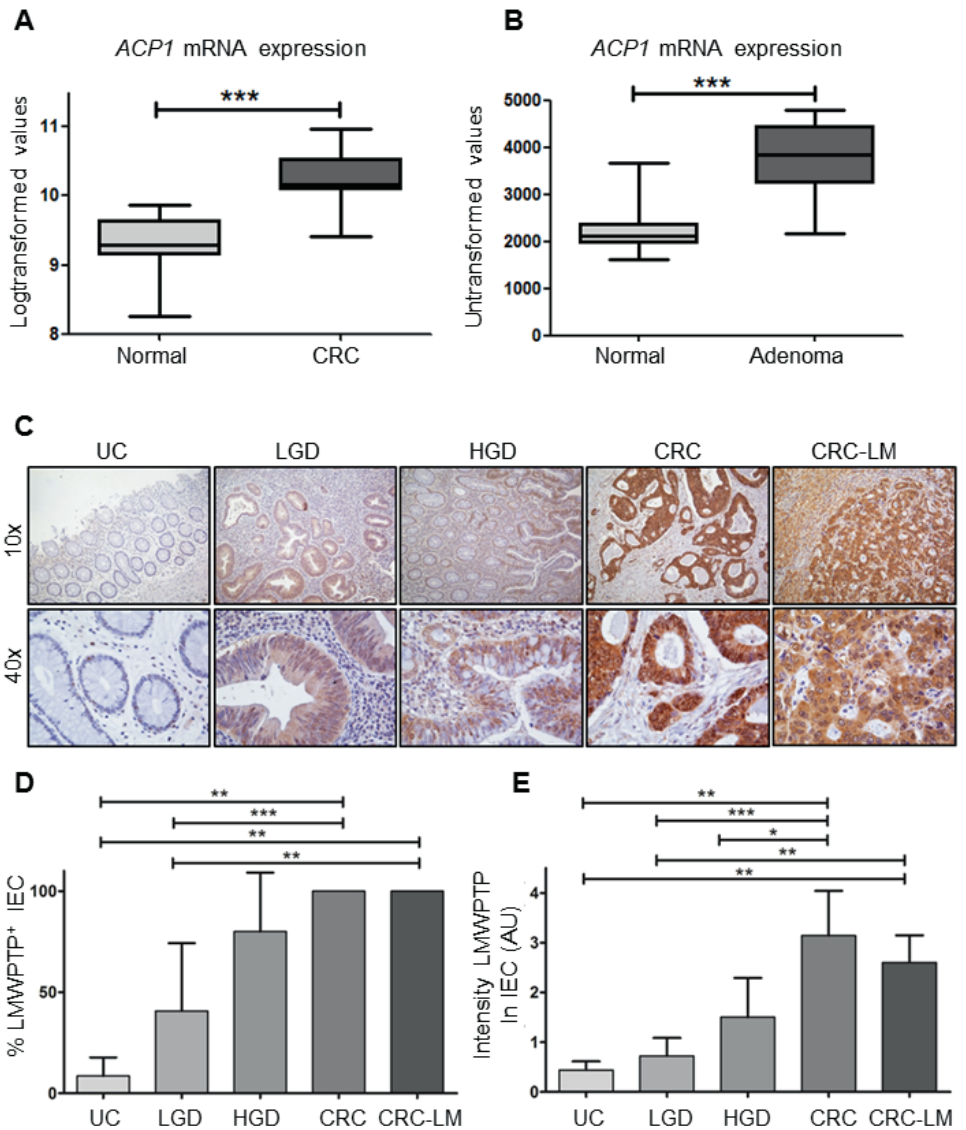

Figure 1. *ACP1* mRNA and LMWPTP protein expression are increased in colorectal dysplasia and carcinoma as compared to non-dysplastic tissue. (A,B) Using publicly available gene expression data from Affymetrix platforms, *ACP1* expression (transcript 215227) was analyzed in carcinoma (CRC) and adjacent normal colon. Significantly higher expression of *ACP1* mRNA expression was observed in carcinoma tissues (n=17, P=0.0005 by student's T-test). Gene expression array comparing *ACP1* expression in colorectal adenoma to normal adjacent tissue shows increased *ACP1* mRNA expression in cancer tissues (n=32, P<0.0001) **(C)**. Tissues of patients with inactive ulcerative colitis (UC, n=8), low grade dysplasia (n=8), high grade dysplasia (HGD, n=6), colorectal cancer (CRC, n=12) and CRC liver metastasis (n=5) were stained for LMWPTP by immunohistochemistry. Representative examples (10x and 40x magnifications) of UC, LGD, HGD, CRC and liver metastasis are shown. **(D,E)** LMWPTP staining was scored for percentage of positive intestinal epithelial cells as well as intensity of staining and statistical analysis was performed using Mann-Whitney t-test. (*P >0.05; ** P >0.01, *** P >0.001).

Results

***ACP1* mRNA expression is increased in colorectal adenomas and carcinomas.**

To understand the role of LMWPTP in colorectal cancer, we first investigated the gene expression levels of *ACP1* using publicly available microarray datasets from Affymetrix Platforms. Expression of the LMWPTP encoding gene *ACP1* (transcript 215227) was compared between CRC and normal adjacent colonic tissue (n=17), and found to be significantly increased in the carcinoma group (P=0.0005, **Figure 1A**). Colon cancer follows the adenoma to carcinoma sequence, and most cancers arise from dysplastic adenomas. Therefore, we also examined *ACP1* expression levels in adenoma samples and again observed an increased mRNA expression in these samples (n=32) compared to their normal adjacent colon tissue (P<0.0001, F**igure 1B**).

LMWPTP protein is overexpressed in primary colorectal cancer samples.

Next, we examined whether the increased *ACP1* expression corresponds to increased protein levels of LMWPTP in CRC samples. Immunohistochemistry was performed on tissue sections of biopsies of low grade dysplasia (LGD; n=9), high grade dysplasia (HGD; n=7) adenocarcinoma (n=12) and controls (n=8) (**Figure 1C**). LMWPTP expression in intestinal epithelial cells (IEC) was limited to 9±9% of cells in non-cancerous tissues. In contrast, expression of LMWPTP was significantly increased with subsequent levels of dysplasia (41±33% and 80±29% positive IEC in LGD and HGD, respectively), with up to 100% of LMWPTP-positive IECs in adenocarcinoma (**Figure 1D**). In addition to increasing numbers of positive cells, the intensity of the staining also increased in the untransformed-to-colorectal cancer sequence (0.44±0.18, 0,72±0.36, 1.50±0.79 and 3.14±0.90 in control, LGD, HGD, and CRC respectively, **Figure 1E**). Furthermore, LMWPTP overexpression is preserved in liver-metastasized CRC tumor cells, with 100% of IECs highly positive for this phosphatase (note that the normal liver tissue stains negative for LMWPTP) (n=5).

To validate these results using a different technique, we examined LMWPTP expression in 6 paired freshly frozen tumor and normal adjacent tissues by Western blotting, again demonstrating a significant increase in the total levels of this phosphatase in the tumor tissue (**Figure 2A,B**).

To confirm the increased LMWPTP protein expression in a larger sample group, the staining was subsequently performed on a tissue micro array (TMA) containing samples of 72 colorectal adenoma and/or carcinoma patients (**Table 1**; representative samples shown in **Figure 2C**). After excluding poor quality cores, 62 cores of CRC tissue, 25 cores of adenoma tissue and 65 cores of healthy adjacent tissue were available for analysis. Again, the cores were scored for percentage positive

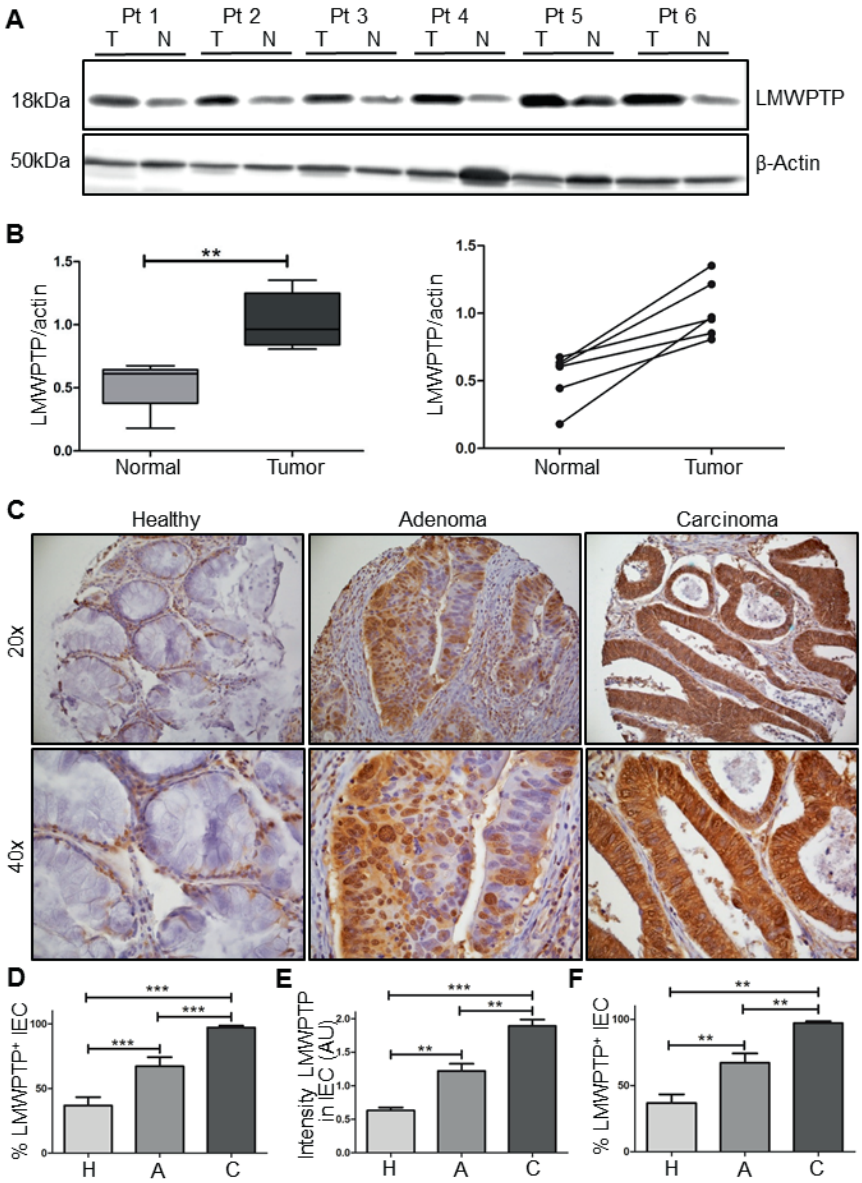

Figure 2. LMWPTP protein expression is increased in CRC as compared to paired normal adjacent tissue, and expression increases during the canonical progression sequence from normal tissue via adenoma to carcinoma. (A) Freshly frozen paired tumor (T) and normal adjacent (N) colonic tissue samples of 6 patients were lysed, and LMWPTP expression was determined using western blot analysis. β-Actin served as loading control. **(B)** Quantification of western blot using Li-Cor Odyssey software (*P<0.05; **P<0.01). **(C)** IHC analysis of LMWPTP on a tissue micro array (TMA) of patients with colorectal cancer (n=65), colorectal adenoma (n=25), and healthy adjacent tissue (n=62). Representative stainings (20x and 40X inset) of CRC , adenoma and healthy tissue. **(D-E)** Staining was scored for percentage of LMWPTP positive IEC as well as intensity. Statistical analysis using Mann-Whitney t-test. **(F)** Percentage positive IEC were compared in patients from whom normal, adenoma and CRC tissue were all available (n=15, Wilkinson paired t-test) (*P< 0.05; **P<0.01; ***P < 0.001).

IECs and intensity of the staining. The mean percentage positive IEC was 27±3% in normal adjacent tissues compared to 64±4% in adenoma and 90±3% in carcinoma (P<0.001, **Figure 2D**). In addition, the intensity of the staining similarly increased from healthy tissue to adenoma and CRC (0.63±0.05, 1.22±0.10 and 1.90±0.09, respectively, P<0.001, **Figure 2E**). For 15 patients there was material available for all three stages. In these patients, a significant increase in LMWPTP expression from normal to adenoma, and adenoma to carcinoma tissue was observed (37±6%, 67±7% and 97±1%, respectively, P<0.001, **Figure 2F**), suggesting a role for LMWPTP in the oncogenic transformation of colonic epithelial cells. Due to the high expression of LMWPTP in all our carcinoma samples, we were unable to correlate clinical parameters such as Dukes' stage or patient survival to LMWPTP expression in cancer. However, when correlating patient survival to LMWPTP expression in their normal tissue, a higher LMWPTP expression in healthy tissue was significantly cor-

Table 1. Patients characteristics of tissue micro array.

Parameter	Mean (SD) or N (%)
Number of Patients	72 (100%)
Healthy cores available	65 (90%)
Adenoma cores available	25 (35%)
Carcinoma available	62 (86%)
Age at presentation	
Mean	69.85 (11.8)
Median	70
Range	30-92
Sex, N (%)	
Male	37 (51.4%)
Female	35 (48.6%)
Dukes' stage	
A	1 (1%)
B	38 (47%)
C	23 (28%)
D	10 (12%)
Status	
Living	44 (61%)
Non-CRC-related death	13 (18%)
CRC-related death	15 (21%)

related to increased disease related mortality (Spearmans rho correlation P=0.026, **Figure S1A**). These data suggest either that there are infiltrating tumor cells present in what we denominate as normal adjacent tissue, or that an increased LMWPTP expression in normal cells is predictive or conductive to cellular transformation. Together, these results show that LMWPTP is overexpressed in a stepwise manner from normal tissue to carcinoma.

One of the mechanisms which may contribute to upregulation of LMWPTP expression levels, could be based on an altered methylation pattern of *ACP1*. Using an in silico analysis with the online database MENT (http://mgrc.kribb.re.kr:8080/MENT/) [19], we observed that *ACP1* is hypomethylated in colon cancer as compared to normal colonic tissue (N=680; P<0.0001), providing a possible explanation for the observed upregulation of gene expression (**Figure S1B**).

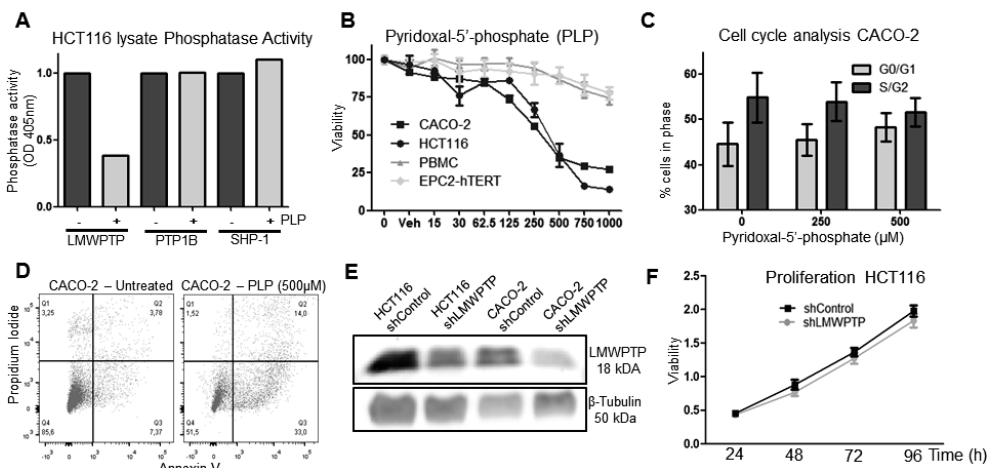

Figure 3. Effects of chemical inhibition and knockdown of LMWPTP on the oncogenic potential of colorectal cancer cells. (A) Immunoprecipitated phosphatases (LMWPTP, PTP1B and SHP-1) from HCT116 lysates were incubated with the only known inhibitor of LMWPTP, PLP, resulting in reduction of LMWPTP phosphatase activity in LMWPTP precipitates, while enzymatic activity of the two other PNPP phosphatases remain unaffected upon PLP treatment. **(B)** Treatment of CRC cell lines (HCT116 and CACO-2) with PLP dose-dependently reduced viable cell numbers as determined by MTT assay, while non-transformed cell lines (EPC2-hTERT and PBMCs) are hardly effected. **(C)** Propidium-iodine staining of CACO-2 cells followed by FACS analysis shows that PLP treatment induces a G0/G1 cell cycle arrest. **(D)** PLP treatment of CRC cells results in apoptosis, as shown by FACS analysis with Annexin V/PI staining on CACO-2 cells treated either with 500uM PLP or vehicle. **(E)** Stably transfected cell lines were created, by lentiviral transfection of HCT116 and CACO-2 cells with shRNA against LMWPTP, resulting in approximatly 50% reduction of LMWPTP expression compared to non-target controls. **(F)** LMWPTP knockdown does not affect overall cell proliferation as shown by MTT assay.

Effect of inhibition of LMWPTP on cell survival

As our data in primary CRC indicates that an increased LMWPTP expression may contribute to tumor progression, we wondered whether inhibition of LMWPTP might reverse any of the oncogenic processes involved. The only LMWPTP inhibitor available to date is PLP, an active derivative of Vitamin B6, which has been shown to inhibit LMWPTP activity by interacting with the Asp129 site [20]. To confirm the effectiveness and selectivity of this compound, we precipitated LMWPTP and two other phosphatases with activity towards the substrate PNPP (SHP-1 and PTP1B) from CRC cells and demonstrated that the phosphatase activity of LMWPTP was indeed decreased in the presence of PLP, while the activity of SHP-1 and PTP1B were unaffected (**Figure 3A** and **S2A**). Next, we treated CRC cells (CACO-2 and HCT116), as well as the non-transformed human gastrointestinal epithelial cell line EPC2-hTERT and freshly isolated PBMCs, with this compound and assessed cell viability. As shown in **Figure 3B**, PLP dose-dependently reduced viable cell numbers of the CRC lines, while non-transformed cells are hardly affected by this treatment. PLP induced a G0/G1 cell cycle arrest in the cancer cells (**Figure 3C** and **S2C**). Furthermore, PLP treatment concomitantly caused apoptosis in CRC cells, as shown by an increased Annexin V staining (**Figure 3D** and **S2B**). Thus, these data suggest that chemical inhibition of LMWPTP may reduce CRC growth.
As PLP may have some off-target effects (LMWPTP is not the only molecular target to be inhibited by this compound), we decided to further investigate the effect of inhibition of LMWPTP by specifically reducing its expression. We employed shRNA against *ACP1* to stably knock down LMWPTP in HCT116 and CACO-2 cells, which reduced the expression of this phosphatase by 50% (**Figure 3E**). However, while chemical inhibition of LMWPTP affected cell viability and cell cycling, knockdown of LMWPTP did not (**Figure 3F** and **S2D-F**). This is perhaps not surprising, as knock down of LMWPTP was not complete, and the creation of stable cell lines would necessarily select for cells escaping cell death.

LMWPTP induces drug resistance

Whilst not inducing cell death, knock down of LMWPTP in CRC lines allowed us to further investigate the role of LMWPTP in other oncogenic processes. We started by determining some of the molecular targets of LMWPTP. **Figure 4A** shows that knock down of LMWPTP resulted in the downregulation of several cancer-associated signaling pathways. Most noticeably, we observed a reduced phosphorylation of the epidermal growth factor receptor (EGFR) and diminished phosphorylation of protein kinase B (PKB) both on the threonine 308 and serine 473 sites in LMWPTP knockdown cells (**Figure 4A**). In addition to proliferation, these molecules are implicated in cell survival, and we therefor speculated that LMWPTP knockdown cells might be more susceptible to cytostatic agents. Indeed, treatment of CRC cells with 5-fluorouracil (5-FU), a commonly used chemotherapeutic, caused a dose depend-

ent decrease in viable cell numbers, which was significantly more pronounced in LMWPTP knock down cells (**Figure 4B** and **S3A**). Thus, silencing of LMWPTP confers drug sensitivity of CRC cells, possibly through loss of EGFR and PKB activity. Other mechanisms used by tumor cells to escape drug effects include the expression and regulation of multidrug resistance efflux pumps. These include p-glycoprotein, also known as multidrug resistance protein 1 (MDR1), which transports several substrates across the extracellular membrane. Interestingly, P-gp was expressed on CACO-2 cells, and its expression was reduced upon silencing of LMWPTP (**Figure 4C**). In contrast, HCT116 cells did not express this particular efflux pump (and hence no decrease was observed in LMWPTP knock down cells), suggesting that different mechanisms may contribute to drug sensitivity in different CRC lines (**Figure S3B**).

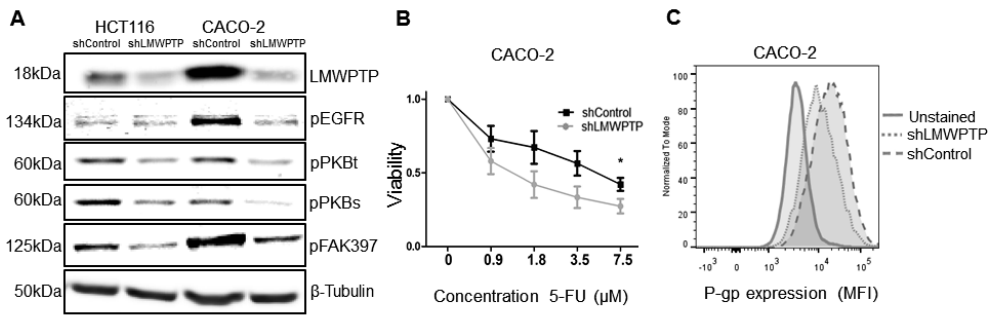

Figure 4. LMWPTP influences cell survival pathways and desensitizes colorectal cancer cells to chemotherapy. (A) Western blot analysis of LMWPTP knockdown and control cells reveals reduced phosphorylation of Epidermal Growth Factor Receptor (EGFR), Protein Kinase B (PKBt and PKBs) and FAK in the knockdown cells. **(B)** CACO-2 LMWPTP knockdown and control cells were treated with increasing concentrations of 5-fluorouracil and cell viability was assessed by MTT assay after 96h. CACO-2 knockdown cells were more susceptible to 5-fluorouracil as compared to control (*P<0.05). **(C)**. P-glycoprotein expression on CACO-2 cells was determined by FACS analysis, using anti-P-gp and anti-mouse-FITC antibodies. P-gp expression is reduced upon LMWPTP knockdown in CACO-2 cells, as shown by FACS mean fluorescence intensity (MFI).

LMWPTP targets migration signaling

Colorectal cancer is a frequently fatal disease because of its high propensity to migrate and invade other tissues, preventing curative surgical treatment. Cellular migration is dependent on the tight regulation of assembly and disassembly of focal adhesion sites. This process is mediated the by the formation of a FAK–Src complex, and phosphorylation of FAK-associated substrates such as paxillin and p130cas, all known to be required for cell motility [21]. Our biochemical analysis of LMWPTP deficient cells revealed reduced FAK Tyr-397 phosphorylation in these cells, suggesting that LMWPTP may function in this pathway to promote FAK Tyr-397 phosphorylation and the formation of membrane extensions characteristic of migrating cells (**Figure 4A**). We therefore investigated the effects of LMWPTP downregula-

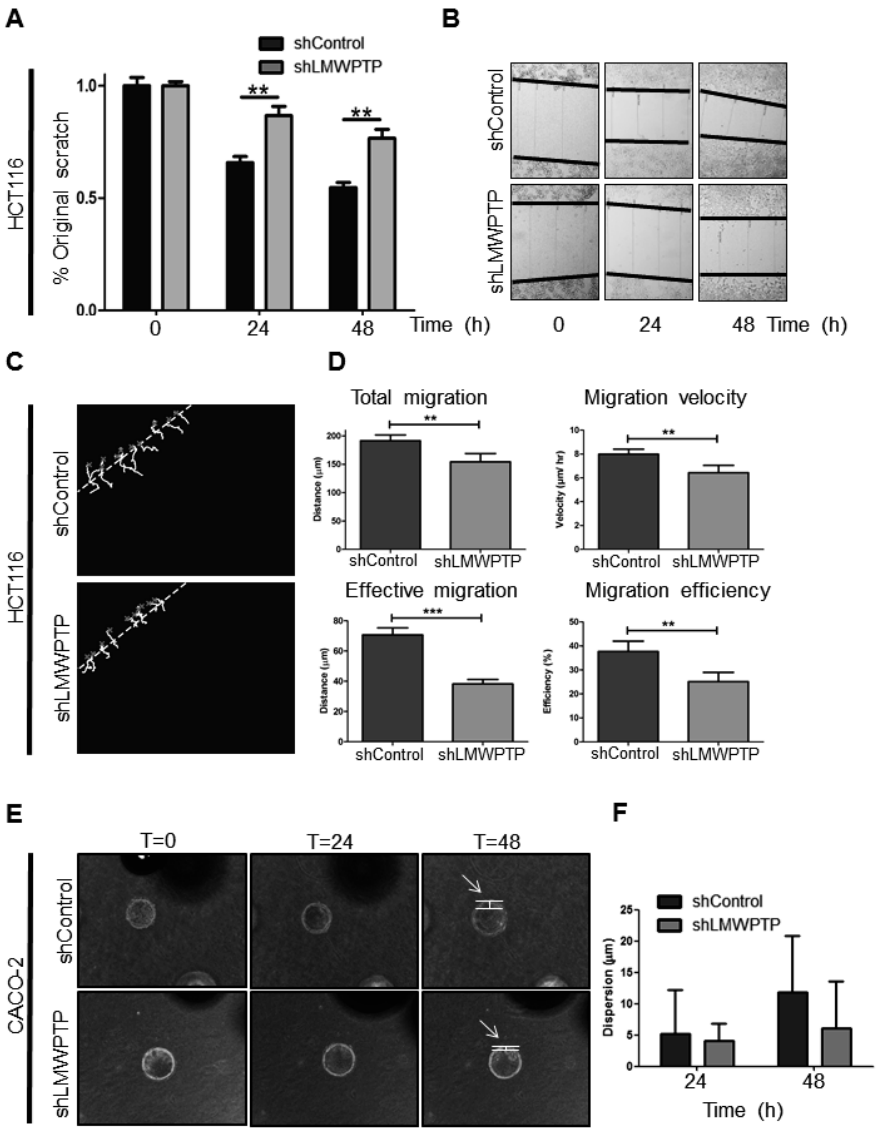

Figure 5. Modulation of LMWPTP results in impaired migration and invasion in colorectal cancer cells. (A,B) HCT116 cell migration was measured by scratch assays, simple scratch wounds were made, and pictures are taken at 0h, 24h, and 48h. Persistent area of clear plastic was measured and statistical analysis was performed using student's T-test. (C,D) Two-dimensional migration was analyzed using a ring-barrier system. HCT116 cell migration on gelatin was tracked during 24h, with locations being captured using time-lapse microscopy every 12min (x=start, line=cell track)(C). Quantification of migrated path indicates that the total migration and velocity were significantly reduced in LMWPTP knockdown cells. Effective migration and thereby efficiency are even further reduced. (D; *P<0.05; **P<0.01; ***P<0.001). (E,F) Beads were coated with either CACO-2 LMWPTP knockdown or control cells for 24 hours, and embedded in a collagen gel matrix. Cells were allowed to invade the collagen matrix, and pictures were taking at 0h, 24h, and 48h (examples in E). The cell dispersion from the bead (arrow) into the collagen matrix was measured, and a trend towards reduced invasion was observed in LMWPTP knockdown cells. Data represents at least four beads (F).

tion in colorectal cancer cell lines on their ability to migrate. Confluent plates of CACO-2 and HCT116 cells were scratched using a pipet tip, and cell migration into the wound was assessed after 24h and 48h. LMWPTP knock down cells showed a significant delay in the ability to migrate into the empty space (**Figure 5A-B** and **Fig S4A-B**, N.B. that HCT116 is a slower migrating cell line). To verify the positive role of LMWPTP in cell migration, we used a second, different approach to investigate cellular movement, which does not rely on wounding the CRC monolayer. Using time-lapse microscopy of cell migration we again observed that CACO-2 and HCT116 LMWPTP knockdown cells are significantly impaired in their total migration, and thereby also the cell velocity. Strikingly, the effective migration, which is defined as the directional movement of the cells to the cell-free center, was even more reduced (**Figure 5C-D** and **S4C-D**). We subsequently went on to assess the role of LMWPTP on migration in a 3D-setting, representing the invasive capacity of these cells. Beads were coated with CACO-2 and HCT116 knockdown or control cells, and were settled in a collagen matrix. Cell dispersion from the bead into the surrounding collagen matrix was measured. Although not reaching statistical significance, we observed a trend towards reduced invasive capacity upon LMWPTP knock down for both cell lines (**Figure 5E-F** and **S4E-F**). Together these data demonstrate that knocking down LMWPTP in colorectal cancer cells reduces their migratory capacity, and is especially important for directional cell migration.

Different mechanisms for reduced migratory responses in CRC cells

As both CACO-2 and HCT116 cell lines demonstrated reduced FAK activity and subsequent migration upon LMWPTP knock down, we next set out to further examine the underlying mechanism of this reduced migration. First we investigated the adhesive capacity of these cell lines, adhesion being indispensable for proper migration, and FAK being a major regulator of this process. As shown in **Figure 6A**, full adherence of CACO-2 non-target cells to the glass surface was reached after 30 minutes and 1h, while only 50% of the knockdown cells adhered to the bottom of the wells within these time points ($P>0.05$). In contrast, HCT116 cells were much slower to adhere, and no differences could be observed between LMWPTP knock down and control cells (**Figure S5A**).

Next, we employed confocal microscopy to investigate the actin distribution as well as cellular morphology of the cells. We found alterations in the cytoskeletal F-actin composition in HCT116 cells downregulated for LMWPTP. Loss of LMWPTP resulted in a more stretched appearance of cells, suggestive of a less migratory phenotype (**Figure 6B**). This is also reflected by our morphometric analysis (**Figure 6C**), which reveals a significantly higher length/width ratio in the LMWPTP knockdown cells (1.1 ± 0.02 in control cells and 1.7 ± 0.18 knockdown cells; $P<0.0001$). In contrast, CACO-2 cells, which have a cuboidal appearance, did not show the stretching in the knockdown cells observed in the HCT116 cell line (**Figure S5B**). These results

indicate that while the net effect of LMWPTP on migration is similar in CACO-2 and HCT116 cell lines, the underlying mechanisms differ, emphasizing the heterogeneity of CRC.

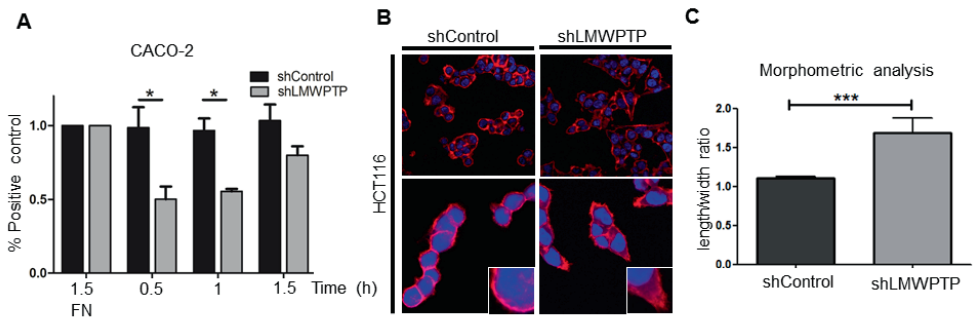

Figure 6. LMWPTP influences cell adhesion and cell morphology. (A) CRC cell adhesion was determined by MTT assay of adherent cells after indicated time points, with fibronectin (FN) coating serving as control. CACO-2 LMWPTP knockdown cells adhere less than control cells (*P<0.05; **P<0.01). **(B,C)** Confocal microscopy of Phalloidin-rhodamine stained cells was employed to examine cell morphology. Immunofluorescence reveals a more elongated morphology in HCT116 LMWPTP knockdown cells as compared to control **(B)**. To quantify the changes in morphology, the ratio between the length and width of the cells was calculated, with a higher ratio indicating a more elongated shape. Non-target control cells have a significantly lower ratio compared to LMWPTP knockdown cells. (1.1±0.02 vs 1.7±0.18, n=26; P<0.0001) **(C)**

Discussion

In this study we identify LMWPTP overexpression as a mediator for increased chemoresistance and migration in colorectal cancer. We show for the first time, that LMWPTP protein expression is drastically increased in primary colorectal cancer samples as compared to normal adjacent tissue. LMWPTP expression appears to follow a step-wise increase from healthy tissue, to dysplastic adenoma, and to carcinoma. Deregulated transcription and translation is a common phenomenon of cancer cells, and many signaling pathways can concomitantly be affected in malignant cells. However, analysis of other phosphatases in CRC sections does not indicate a general upregulation of PTPs in this tumor type (unpublished results), indicating a specific oncogenic role for LMWPTP in the transformation of colon epithelial cells. While the cause of the specific upregulation of *ACP1* mRNA has yet to be identified, interestingly, an initial database analysis (MENT methylation and expression database) suggests that this gene is hypomethylated in adenoma and cancer samples.

Thus far, while several phosphatases have been shown to act as tumor suppressors, few phosphatases have been implicated as oncogenes in colorectal cancer. Phos-

phatase of regenerating liver-3 (PRL-3), also known as *PTP4A3*, was shown to be upregulated in up to 100% of CRC liver metastasis cases, and is overexpressed in primary tumors [22]. Furthermore, PRL-3 can promote migration and invasion by enhancing MMP2 activity [23]. Similarly, the phosphatase SAP-1 has been shown to be overexpressed in colorectal tumors, and especially promotes the tumorigenic potential of intestinal epithelial cells [24, 25]. Likewise, PTPα is capable of activating Src protein kinase activity in colorectal cancer, further supporting the idea that phosphatases not only act as inhibitors of oncogenic kinases, but can function as tumor promoters as well [26]. To this relatively short list, we may now add LMWPTP. Although these other phosphatases may also be implicated in the colorectal cancer process, convincing demonstration of their dysregulation in a large cohort of well-characterized colon cancer patients as well as detailed mechanistic insight into their exact role in the cancer process is largely lacking. In contrast, LMWPTP appears a bona-fide mediator of the CRC invasiveness and chemoresistance.

After our initial finding that LMWPTP is significantly overexpressed in colorectal cancer, we evaluated the effect of LMWPTP in vitro. Up to now, the most potent compound known to inhibit LMWPTP activity is the active form of vitamin B6, PLP. Interestingly, earlier reports have shown that both vitamin B6 and PLP serum levels are inversely correlated with colorectal cancer risk [27]. Our data suggest that inhibition of LMWPTP through Vitamin B6 may provide one possible explanation for these findings. Indeed, in pituitary cells PLP has been shown to induce cell cycle arrest [28], which corresponds well with the cell cycle block and apoptosis observed in our study, and suggests that LMWPTP inhibition may be a valuable avenue for treatment of CRC. Earlier reports have suggested that LMWPTP could modulate chemoresistance of cancer cells [29]. The current treatment protocol for advanced CRC contains chemotherapy, such as 5-fluorouracil. Typically, the first course of chemotherapy is highly beneficial, but tumor cells tend to make use of several mechanisms to escape the therapy and become resistant. We now show that LMWPTP could be involved in these mechanisms in CRC. In addition to an increased sensitivity to chemotherapy, LMWPTP knock down cells showed a great reduction in phosphorylated EGFR and PKB, making it tempting to speculate that decreased activity of these survival signals contributes to drug-sensitivity of these cells. In addition, multidrug resistance pumps, such as P-gp are often upregulated on cancer cells and can contribute to decreased drug-sensitivity of tumors [30, 31]. Our study suggests that LMWPTP can act on these transporters as well, thereby contributing to the chemoresistance of CRC cells.

In this study, we observed clear differences between HCT116 and CACO-2 cells. While LMWPTP knock down conferred drug sensitivity in both cell lines, no modulation of P-gp was observed in HCT116 cells, suggesting different underlying mechanisms. In addition, while migratory and invasive behavior was impaired in both LMWPTP knock down cell lines, the molecular pathways contributing to migratory

defects appeared to differ. Both cell lines demonstrated reduced phosphorylation of the focal adhesion kinase FAK. This kinase, by forming a complex with p120RasGAP and p190RhoGAP (p190A), leads to phosphorylation of p190A, resulting in polarity cues and increased directional movement [32]. Impaired formation of the Ras-Rho complex as a result of LMWPTP downmodulation may thus contribute to the inefficient cell movement and polarity observed in our migration assays. FAK is a major regulator in the adhesion to matrix, which is also essential for proper migration. However, while we observed adhesion defects in LMWPTP knock down CACO-2 cells, the same was not observed in HCT116 cells. This second LMWPTP knock down cell line selectively demonstrated a changed morphology and F-actin rearrangement, characteristic with sessile behavior of cells. These latter findings are reminiscent of (colorectal) cancer cells in which a more rounded morphology as a result of overexpression of RhoA correlated to increased ability to migrate in vitro and metastasize in vivo [33]. Thus, while inhibition of LMWPTP in both of these colorectal cancer lines appears beneficial in terms of reducing cell growth, drug resistance and metastatic potential, the mechanisms through which this is achieved may rely on genetic identity of the tumor cells. These data highlight the need for personalized medicine in cancer treatment, as different genetic backgrounds may affect the usefulness of treatment regimens as well as the molecular mechanisms behind them. This was recently very clearly demonstrated by the report of a selective benefit of mTOR inhibitors only in patients carrying PTEN-deficient tumors [34].

In summary, we show that low molecular weight protein tyrosine phosphatase is overexpressed in primary human colorectal cancers at both mRNA and protein level and that this phosphatase can function as an oncogene, by enhancing the migration, adhesion and chemoresistance in colorectal cancer cells. Together, this indicates that LMWPTP expression is a determining factor in the malignant potential of colorectal cancer, and suggests that this phosphatase provides a target in the fight against this devastating disease.

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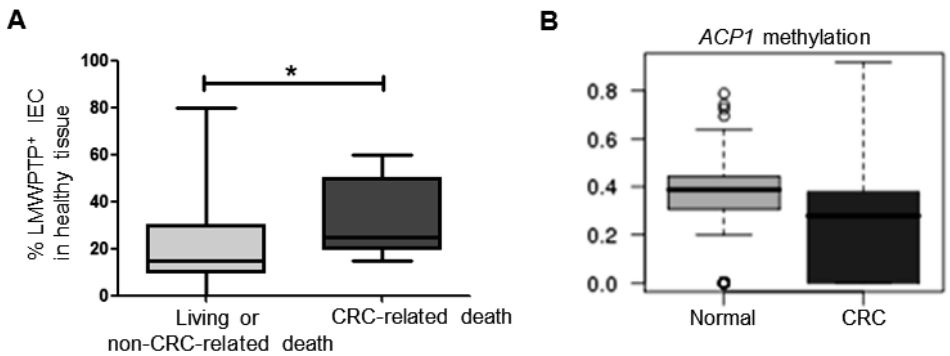
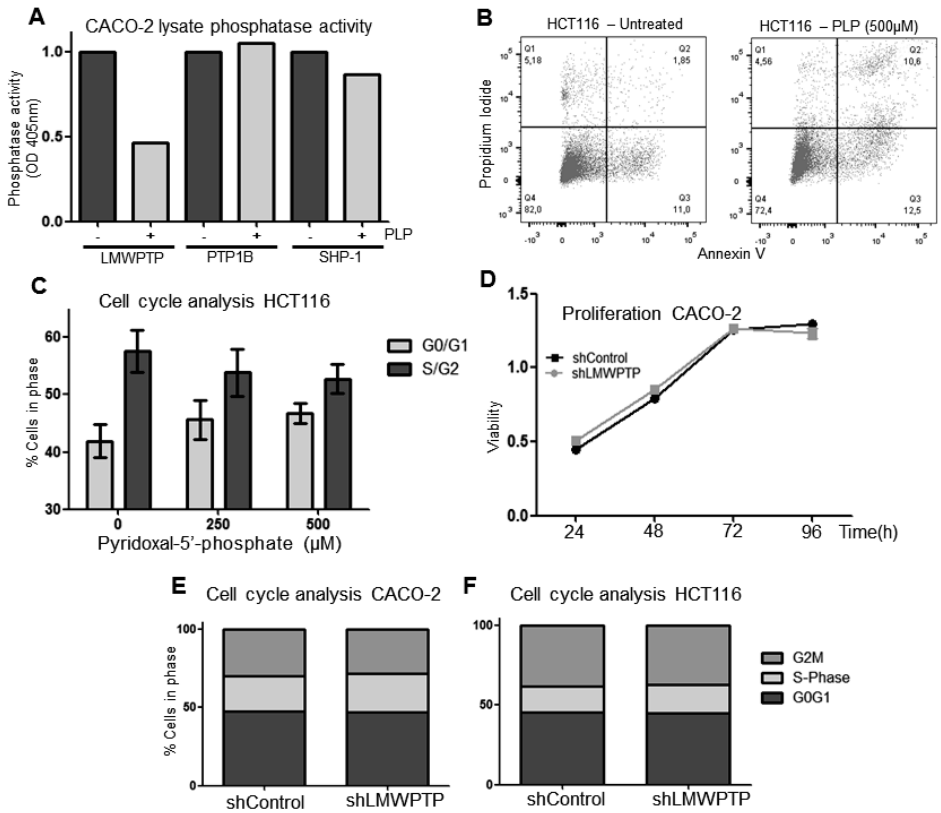
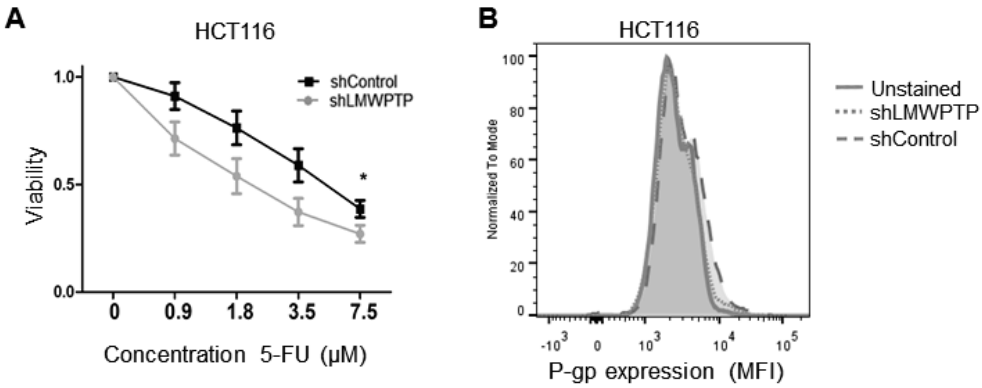
Supplementary Figures



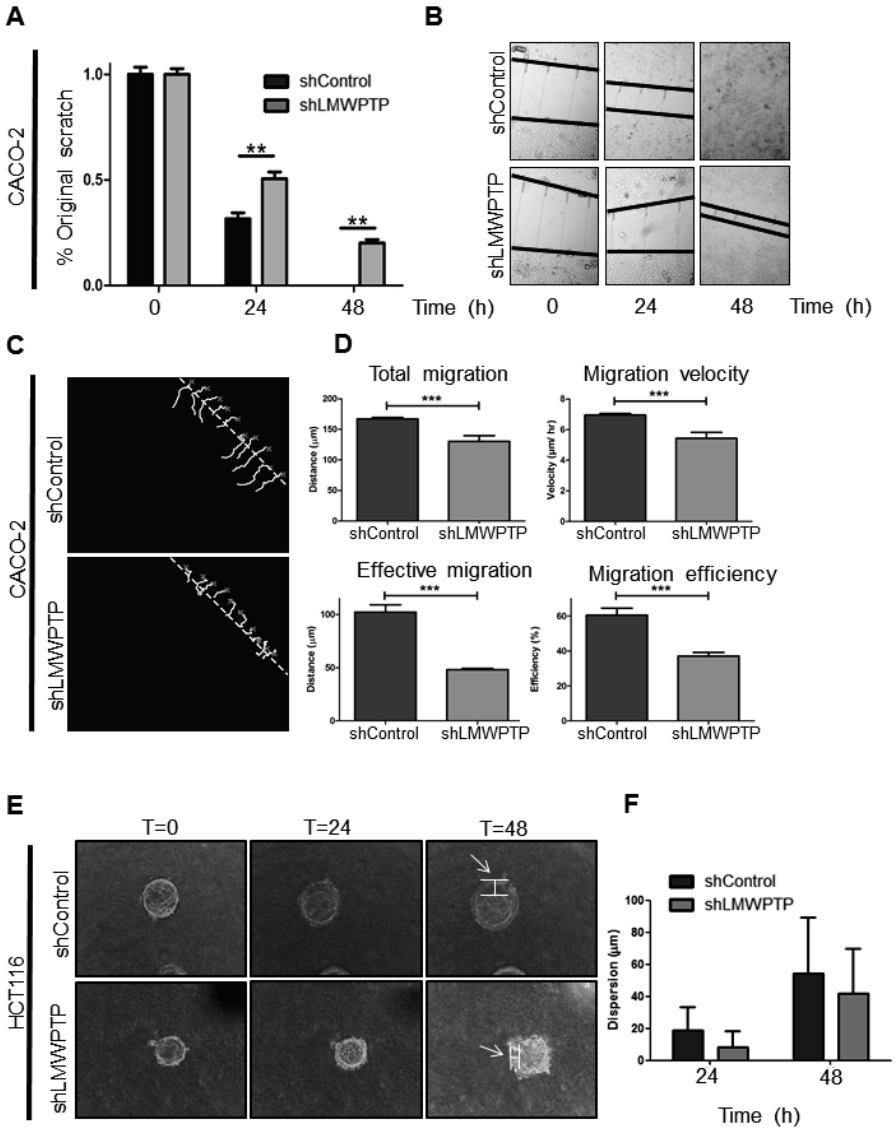
Supplementary figure 1. LMWPTP upregulation is related to *ACP1* hypo-methylation and corresponds to patient survival in normal adjacent colonic tissue. (A) Analysis of percentage LMWPTP positive IECs in normal adjacent tissue cores, related to patient outcome. Patient group is divided in patients still alive or suffering a non-CRC-related death, and patients deceased due to colorectal cancer. **(B)** In silico analysis of *ACP1* methylation in colorectal cancer and normal colonic tissue, using MENT database. *ACP1* is significantly hypo-methylated in colorectal cancer as compared to normal tissue (-0.24032; P>0.00001).



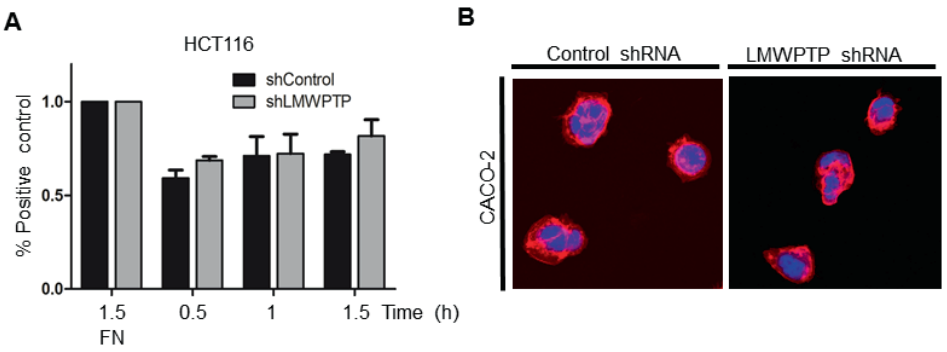
Supplementary figure 2. Effects of chemical inhibition and knockdown of LMWPTP on colorectal cancer cells. (A) Immunprecipitated phosphatases (LMWPTP, PTP1B and SHP-1) from CACO-2 lysates were incubated with the only known inhibitor of LMWPTP, PLP, resulting in reduction of LMWPTP phosphatase activity, while the two other PNPP phosphatases remain unaffected upon PLP treatment. **(B)** Treatment of HCT116 cells with 500uM of PLP induces apoptosis as determined by Annexin V/Pi FACS analysis. **(C)** Treatment of HCT116 cells with PLP induces cell cycle arrest as determined by ploidity through FACS analysis of propidium iodide staining. **(D)** MTT analysis shows that stable knockdown of LMWPTP in CACO-2 cells does not affect viable cell numbers in culture. **(E,F)** Analysis of cell cycle by propidium iodide staining shows no differences between LMWPTP knockdown and control cells for CACO-2 **(E)** and HCT116 **(F)** CRC cell lines.



Supplementary figure 3. LMWPTP knockdown sensitizes colorectal cancer cells to chemotherapy. (A) HCT116 knockdown and control cells were treated with increasing concentrations of 5-fluorouracil (5-FU) for 96 hours. Knockdown cells are more susceptible to 5-fluorouracil treatment as compared to non-target control cells. **(B)** In contrast to CACO-2 cells, HCT116 cells show little P-glycoprotein expression, as determined by FACS analysis.



Supplementary figure 4. Downmodulation of LMWPTP results in reduced migration and invasion in colorectal cancer cells. (A,B) CACO-2 cell migration was measured by scratch assays, where simple scratch wounds were made, and pictures are taken at 0h, 24h, and 48h. Persistent area of clear plastic was measured and statistical analysis was performed using student's T-test. (C,D). Two-dimensional migration was analyzed using a ring-barrier system. CACO-2 cell migration on gelatin was tracked during 24h, with locations being captured using time-lapse microscopy every 12min (x=start, line=cell track)**(C)**. Quantification of migrated path indicates that the total migration and velocity were significantly reduced upon LMWPTP knockdown. Effective migration and thereby efficiency are even further reduced. (D; *P< 0.05; **P<0.01; ***P<0.001). **(E,F)** Beads were coated with either HCT116 knockdown or control cells for 24h, and embedded in collagen gel matrix. Cells were allowed to invade the matrix, and pictures were taking at 0h, 24h, and 48h (examples in **E**). The cell dispersion from the bead (arrow) was measured and quantified in **F**. Data represents at least four beads (F).



Supplementary figure 5. LMWPTP influences cell adhesion and cell morphology. (A) CRC cell adhesion was determined by MTT assay of adherent cells at indicated timepoints, with fibronectin (FN) coating serving as control. HCT116, a slow migrating cell line, does not show reduced adhesion upon LMWPTP knockdown. **(B)** Confocal microscopy of Phalloidin-rhodamine stained CACO-2 cells was employed to examine cell morphology. The more stretched appearance upon knockdown of LMWPTP in HCT116 cells was not observed in CACO-2 cells, which have a more cuboid appearance, form clumps when cell numbers are low and grow out as complete sheets as cell numbers increase.

Supplementary Tables

Supplementary table 1. Summary of primary antibodies

Antibody	Dilution	Company	Catalog number
Mouse-anti-ACP1α/β	1:500	Santa Cruz	sc-100343
Rabbit-anti-Tubulin	1:10000	Abcam	Ab-6046
Mouse-anti-Actin	1:2500	Santa Cruz	Sc-47778
Rabbit-anti-phospho-AKT (T308)	1:1000	Cell signaling	#2965
Rabbit-anti-phospho-AKT (S473)	1:1000	Cell signaling	#9271
Rabbit-anti-phospho-EGFR (Y1068)	1:1000	Cell signaling	#3777
Rabbit-anti-phospho-FAK (Y397)	1:1000	Invitrogen	44-625G
P-Glycoprotein	1:20	Immunotech	1864
Phalloidin-Rhodamin	1:200	Invitrogen	R415
DAPI (4', 6'-diamidino-2-phenylindole)	1:1000	Sigma	D9542

Supplementary table 2. Summary of secondary antibodies

Antibody	Dilution	Company	Catalog number
HRP-conjugated goat-anti-mouse IgG (EnVision™)	n/a	DAKO	K4007
HRP-conjugated goat-anti-Rabbit IgG (EnVision™)	n/a	DAKO	K4011
Goat-anti-rabbit IgG IRDye 800	1:10000	Westburg	926-32211
Goat-anti-rabbit IgG IRDye 680	1:10000	Westburg	926-68072
Goat-anti-mouse IgG DyLight 488	1:50	Biolegend	405310

Chapter 5

PTP1B expression and phosphatase activity are increased in colorectal cancer, leading to a more invasive phenotype and worse patient outcome

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Submitted

Chapter 6

The lipid phosphatase SHIP2 functions as oncogene in colorectal cancer and can be effectively targeted using novel small molecule inhibitors

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Submitted

Chapter 7

Meeting report: Europhosphatase 2015; Phosphatases as drug targets in cancer

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Conditionally accepted, Cancer Research

Abstract

Phosphatases are key regulators of cellular signaling and as such play an important role in nearly all cellular processes governing diseases, including cancer. However due to their highly conserved structure and highly charged and reactive catalytic site, they have been regarded as “undruggable”. Fortunately, during the recent Europhosphatase meeting (Turku, Finland), it became most clear that phosphatases can no longer be ignored as potential targets in cancer therapy. Since reactivation of tumor suppressor phosphatases or direct inhibition of phosphatases acting as oncogenes is becoming available, this class of enzymes can now be considered as feasible drug targets.

Introduction

A delicate balance between protein kinases and protein phosphatases regulates many different processes to maintain tissue homeostasis. Alterations in phosphorylation patterns are linked to numerous diseases, and are a common phenomenon in several types of cancer, making this an interesting target for therapeutic intervention. In the last decades, much of the attention was focused on targeting the kinases, resulting in over 20 kinase inhibitors currently used in the clinic [1]. However, the equally important phosphatases were neglected for a long time. In part, this is probably due to historical reasons, as the first PTP was only purified ten years after the first protein tyrosine kinase (PTK), but another very important reason is that phosphatases have been long been regarded as "undruggable". This is in part due to the close similarity of the catalytic domains of phosphatases, which argues against the use of catalytic domain inhibitors due to specificity issues. However, the greatest challenge in the development of phosphatase inhibitors is to identify inhibitory compounds with pharmacologically acceptable bioavailability. Due to their highly charged active sites, most of the inhibitory compounds contain negatively charged nonhydrolyzable phosphotyrosine (pTyr) mimetics, limiting the cell-permeability of the designed compounds [2]. The recently held Europhosphatase meeting has made it clear that phosphatases should no longer be ignored, and are in fact feasible targets for cancer treatment, with the potential to aid the resistance to other targeted treatment strategies. Europhosphatase 2015 was the fifth meeting in the EMBO Conference Series on Protein Phosphatases. This biannual meeting brings together over 180 participants with a wide range of backgrounds and research fields, all with a special interest in phosphatases. This years' conference had special emphasis on the importance of the equilibrium between phosphatases and kinases (phosphoswitches) in regulating tissue homeostasis in different physiological and pathological conditions, amongst which cancer. This report highlights the latest findings on this class of enzymes in the oncological field as presented at this meeting, with special attention to phosphatases as treatment targets in cancer.

Catalytic mechanisms phosphatase families

Based on their structure, phosphatases are subdivided into distinct functional and structural superfamilies, all with their own catalytic mechanism. Within the protein tyrosine phosphatases (PTPs), four distinct families can be distinguished: class I, II and III Cys-based PTPs, and the Asp-based tyrosine phosphatases, represented by the EYA-family. They all carry the highly conserved active "PTP-signature" motif C(X-)5R, and (apart from the Asp-bases PTPs) use the same basic catalytic mechanism, in which the cysteine residue in the signature motif executes a nucleophilic attack on the phosphate group in the substrate. Simultaneously, a conserved acidic residue plays two critical roles in catalysis. First, it acts as a general acid, adding a proton to the leaving group oxygen in the substrate. Second, it serves as a general base,

activating a water molecule to promote hydrolysis of the phosphoenzyme intermediate (extensively reviewed in [3,4]). The aspartate-based tyrosine phosphatases family, carry out catalysis differently from the other PTP families, since they use an aspartic acid residue rather than a cysteine in the active site, for a cation-dependent nucleophilic attack on the phosphoryl group of the substrate [5].

The protein serine/threonine phosphatases (PSPs) are subdivided into three main families, the phosphoprotein phosphatases (PPPs), the metal-dependent protein phosphatases (PPMs), and Mg2+-dependent FCP family. Unlike the PTPs, which are large multi domain proteins, the PPPs are comprised of multiple individual subunits to form holoenzymes. This way the PPP-family, which amongst others includes PP1 and PP2A, can potentially form hundreds of different holoenzymes by combining a small number of conserved catalytic domains with a very large number of regulatory subunits, each with different biological functions. Therefore, inhibition of the catalytic subunit of PPPs might not be feasible, but targeting the subunits which restrict access to the active site may be possible. The PPM family, with their prototypical member PP2C, do not have regulatory subunits, but instead contain additional domains and conserved sequence motifs for substrate specificity. They were identified on the basis of the requirement for an exogenous divalent ion (Mg2+, Mn2+) for activity, as well as for the insensitivity to okadaic acid, a known inhibitor of PPPs. Both the PPMs and the PPPs use metal-activated water molecules as nucleophiles to catalyze dephosphorylation (extensively reviewed by Yigong Shi [6]).

Tumor suppressor phosphatases as potential targets for treatment

Due to the emerging knowledge of the structure, protein complexes (holoenzymes) and catalytic function of protein phosphatases, the possibility of targeting these enzymes using small molecules is gaining interest. Intuitively, phosphatases that act as tumor suppressor are less suitable for targeting, since inhibition of their enzymatic activity would be expected to result in further activation of the oncogenic pathways involved. However, rather than inhibiting these enzymes, reactivation of tumor suppressive phosphatases is now emerging as a potential strategy in cancer treatment. As described above, most serine/threonine phosphatases exert their function by forming a holoenzyme complex with scaffolding proteins and regulatory units together with the catalytic unit, making the holoenzyme an interesting target for chemical manipulation. In addition, the activity of most phosphatases is under the regulation of other proteins, offering the possibility of activating a specific phosphatase via manipulation of one of its upstream regulators. However, it is important to realize that targeting or reactivating tumor suppressor phosphatases is only useful when the original function of the protein is not disrupted due to inactivating or truncating mutations, as is the case for the well-known tumor suppressor phosphatase PTEN - in such cases, there is obviously no protein to reactivate.

PP2A reactivation as cancer treatment

Much of the attention at the Europhosphatase 2015 meeting was directed at the known tumor suppressor group of protein phosphatase 2A (PP2A) enzymes [7]. Together with PP1, the PP2A family of phosphatases accounts for more than 90% of Ser/Thr phosphatase activity in human tissues and cells. PP2A phosphatases consist of a holoenzyme containing a scaffold A subunit, a catalytic C subunit, and one of many possible regulatory B subunits. They have a well-established role as tumor suppressor by counteracting diverse kinase-driven oncogenic pathways. Although mutations in PP2A are almost never found in human cancer, overexpression of its inhibitor CIP2A, or downregulation of its promotor PTPA is often observed. Therefore, the possibility of targeting PP2A reactivation as possible cancer treatment was extensively discussed at the conference (e.g. by Goutham Narla, Case Western Reserve University, Pepper Pike, US and Rosalie Sears of the Oregon Health and Science University, Portland, US). It is known that PP2A negatively regulates oncogenic MEK/ERK and PI3K/PKB signaling. The work presented by Sears et al now demonstrates that via its B56 regulatory subunit, PP2A also dephosphorylates the Serine 62 site of c-Myc, thereby enhancing its oncogenic activity and providing further rationale for the use of PP2A activators. Such activators have been described before, and indeed, the use of several of these small molecule PP2A activators resulted in a reduction of c-Myc Ser62 phosphorylation, and reduced the oncogenic potential of breast cancer cells both in vitro and in vivo. Goutham Narla and colleagues performed a screen on FDA-approved drugs in order to find novel PP2A activators. Whole transcriptome and pathway analyses were performed on the potential small molecule activators of PP2A, again showing that these agonists inhibit multiple oncogenic pathways including ERK, PKB, and c-Myc. Together these studies show that targeting PP2A, either by stimulating its activity, or by blocking inhibitors such as CIP2A, is a promising therapeutic opportunity in human cancer. Furthermore, it also encourages investigation into the potential for the reactivation of other known tumor suppressor phosphatases as treatment target.

Other phosphatases with tumor suppressive potential

Other phosphatases for which such an application could be feasible in the future are the dual specificity phosphatases (DUSPs), also known as MAP kinase phosphatases (MKPs), so named because they can dephosphorylate both tyrosine and serine/threonine residues. They are, amongst others, key negative regulators of the RAS-RAF-ERK pathway. Stephen Keyse et al (University of Dundee, Dundee, United Kingdom) demonstrated a novel tumor suppressive function for DUSP5, by regulation of ERK signaling activity. DUSP5 expression in the nucleus causes translocation of ERK2 to the nucleus and serves as anchor protein for the inactive kinase. They now demonstrated that DUSP5 knockout mice show twice as many tumors as their WT counterparts in a DMBA/TPA skin carcinogenesis model, providing clear evidence

that this phosphatase is a potential target for therapeutic agonists. Although specific DUSP inhibitors are available, the search for DUSP agonists is as yet ongoing.
One of the phosphatases previously identified by Newton and colleagues as tumor suppressor is PHLPP, which targets the well-known oncogenic PKB pathway. In the current meeting, Anne Castro (CRBM-CNRS, Montpellier, France) described a novel mechanism of PHLPP modulation through the oncogenic kinase Greatwall. Although the direct role of this kinase in human cancer is not yet investigated, Greatwall is known to promote cell proliferation by controlling mitotic division via inhibition of the phosphatase PP2AB55. Castro et al now report that this kinase also mediates degradation of the PHLPP phosphatase, again leading to phosphorylation of the cell survival kinase PKB on Ser374. These findings indicate Greatwall as novel regulator of PHLPP, and suggest that inhibition of Greatwall or stimulation of PHLPP may inhibit oncogenesis.

Two other non-receptor type PTPs are also found to act as tumor suppressor genes, mostly by affecting migration and epithelial to mesenchymal transformation, a migrating tumor cell hallmark. André Veilette (IRCM, Montreal, Canada) discussed PTPN12, also known as PTP-PEST, previously described to be a positive regulator of migration in endothelial and immune cells. However, Veilette et al now show that in an ErbB2-driven breast cancer animal model, PTPN12 deficiency leads to enhanced tumor development and metastasis. In vitro, PTPN12-deficient cells show enhanced cell migration and invasion. Likewise, Yeesim Knew-Goodall and colleagues (SA Pathology and University of South Australia, Adelaide, Australia) showed that while knockdown of PTPN14 (or PTPpez) hardly affects the proliferation of primary breast tumor cells, it can significantly promote metastasis by altering protein trafficking. Injection of PTPN14 knockdown cells or even conditioned medium from PTPN14-deficient cell cultures promoted the growth and metastasis of breast cancer xenografts. Indeed, loss of catalytically functional PTPN14 increased the secretion of growth factors and cytokines, such as IL-8, and increased the abundance of EGFR at the cell surface of cancer cells. Therefore PTPN14 can prevent metastasis by restricting trafficking of both soluble and membrane-bound proteins. In line with this theory, PTPN14 expression was found to be decreased in invasive breast cancer, and patient survival was worse in low expressing tumors. While there are already a number of phosphatases known to act as tumor suppressors, the above phosphatases can now be added to this list of potential treatment targets.

Oncogenic phosphatases

Nowadays, it is becoming more clear that dephosphorylation of proteins and lipids does not necessarily terminate cellular signaling, but can also activate signaling. For example, oncogenic Src kinase is kept in an inactive state by its phosphorylation at Tyr527 and dephosphorylation of this residue by SHP1 or PTP1B relieves this inhibition. Therefore, as for oncogenic kinases, efforts have now been directed towards

the development of small molecule inhibitors of phosphatases. This has been quite an endeavor, with rather limited success so far, mostly due to low inhibitor selectivity. However, by combining large drug screens of existing drugs, with a more detailed knowledge of the crystal structures of these proteins, development of more directed small molecules is now being improved. For instance, Zhong-Yin Zhang (Indiana University School of Medicine, Indianapolis, United States) is developing selective inhibitors by combining ligands that can target both the active PTP site together with the unique peripheral binding sites, resulting in more specific inhibitors. On the other hand, Nicholas Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, United States) used a different approach, aiming to develop allosteric inhibitors that bind to unique sites on the enzyme distinct from the active site. Targeting these unique, non-catalytic segments that are unrelated to any other member of the PTP family will create inhibitors highly selective for the specific phosphatase.

PTP1B inhibition as breast cancer therapy

Due to the efforts of three independent groups in the phosphatase field, Benjamin Neel (University Health Network/Princess Margaret Cancer Center, Toronto, Canada), Michel Tremblay (McGill University, Montreal, Canada) and Nicholas Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, United States), it has become clear that PTP1B can be a feasible target for cancer (and diabetes) treatment. During this conference these groups again provided valuable novel insights in the oncogenic role of this phosphatase. Neel described a novel role for PTP1B in breast cancer, showing that PTP1B depletion results in increased hypoxia and necrosis in breast cancer cells, which were subsequently more sensitive to hypoxia-induced cell death. Interestingly however, none of the known hypoxia pathways were affected in PTP1B knockdown cells. Instead, PTP1B seems to regulate the RNF213 protein, which in turn can control the activity of α-ketoglutarate dioxygenases, thereby controlling the tumor cell response to hypoxia. This novel PTP1B/RNF213 hypoxia pathway appears to be imperative for the survival of breast cancer cells. Nicholas Tonks also discussed the role of PTP1B in breast cancer, focusing on the recent development of an enzymatic inhibitor of this phosphatase called 'Trodusquemine' or 'MSI-1436'. This small molecule inhibitor induces a conformational change of PTP1B by binding to the disordered C-terminus of the protein, thereby reducing its enzymatic activity. This inhibitor was employed in a mouse model of HER2-dependent breast cancer. Strikingly, Trodusquemine treatment significantly reduced tumor formation and completely abrogated cancer metastasis. Due to these very promising results, the first clinical trials with this compound will be started soon. This treatment strategy might not only be worthwhile in the setting of breast cancer, since Tremblay and colleagues have previously shown that PTP1B is an androgen receptor (AR) regulated phosphatase, capable of promoting the progression of prostate cancer. Moreover, in his work presented at this conference, he showed a frequent amplification of the PTP1B-encoding chromosomal region 20q13 in metastatic pros-

tate cancer, often in co-occurrence with amplification of the AR. Interestingly, they presented that AR can bind and upregulate the genes within this commonly amplified 20q13 region, such as PTP1B, resulting in a more aggressive tumor. While these data argue for a tumor-promoting role for PTP1B, the reverse was true in a PTEN null background. When PTP1B was removed from PTEN-deficient mice, which were subsequently fed a high fat diet, the initial tumor and its growth became much more aggressive. Thus, caution is required when implementing PTP1B inhibitors for cancer treatment– they may not be useful in PTEN-deficient tumors.

Development of PTP inhibitors against SHP2

Another phosphatase known to act as oncogene is the Src-homology 2 domain-containing phosphatase 2 (SHP2). Activating SHP2 mutations are found in leukemia and solid tumors which can promote invasion, proliferation and loss of cell polarity. Zhong-Yin Zhang (Indiana University School of Medicine, Indianapolis, United States) presented a novel strategy for the discovery of PTP inhibitors by exploring existing drug databases, using SHP2 as an example. By screening the Johns Hopkins Drug Library for compounds capable of inhibiting SHP2, he found that Cefsulodin, a third generation β-lactam antibiotic, acts as a reversible and competitive SHP2 inhibitor. X-ray crystallography of SHP2 in complex with Cefsulodin was used as a guide for the subsequent design of cefsulodin-based SHP2 inhibitors, resulting in compounds with high specificity and low IC50-values. Since SHP2 phosphatase activity is required for the full activation of the Ras-pathway, EGF-induced ERK1/2 activation was assessed in the presence of these compounds. As expected, these inhibitors effectively reduced the phosphorylation of ERK in a dose-dependent manner. Likewise, cell survival of a number of human cancer cell lines was also reduced upon the use of these SHP2 inhibitors. Furthermore, as eluded to by Zhang, using the same strategy, other PTP inhibitors with promising specificity and IC50-values have now been developed, amongst which an inhibitor against Low Molecular Weight PTP (LMWPTP). Interestingly, our own work, presented at this conference, demonstrates that LMWPTP can play an oncogenic role in prostate cancer. This enzyme enhances the metastatic potential of prostate cancer cells, resulting in a worse patient survival. We showed that LMWPTP can be used as a biomarker and potential treatment target for this disease. Prostate cancer may not be the only tumor to potentially benefit from LMWPTP inhibitors, as we have previously demonstrated a role from LMWPTP in leukemic cells, and have shown that LMWPTP is also overexpressed and active in primary colorectal cancer specimens, where it also contributes to migratory behavior of cells.

Other phosphatases with oncogenic potential

In addition to LMWPTP, phosphatase of regenerating liver 3 (PRL-3) is overexpressed in colorectal cancer metastasis and acts as oncogene in cell proliferation and migra-

tion. However, the underlying mechanism remains to be elucidated. Maja Köhn (European Molecular Biology Laboratory, Heidelberg, Germany) reported on the role of PRL-3 in cell polarity, a fundamental process in tumor progression. Overexpression of this phosphatase increases cell migration, and results in fully specified lumens in 3D-cultures, a phenotype which could be reversed with their newly developed PRL-3 phosphatase activity inhibitor, providing additional evidence that with this novel inhibitor, PRL3 becomes a druggable target.

Members of the SIX family of homeodomain proteins promote cell proliferation through the regulation transcription of multiple cell cycle genes involved in G1/S and G2/M transitions, as well as S-phase progression. As presented during this Europhosphatase conference (Heidi Ford, University of Colorado, Aurora, United States), some of these proteins do not have their own transcriptional activation domain, but require a family of co-activators to mediate transcriptional activation; the Eya1-4 phosphatases. Eyas are pro-tumorigenic and overexpressed in many forms of cancers including glioblastoma, ovarian cancer, and breast cancer. This overexpression is commonly correlated with earlier recurrence and decreased overall survival. This oncogenic potential is linked to the transcriptional activity of the Eya/SIX1 complex, but also requires EYA tyrosine phosphatase activity. By making use of high-throughput screening, Ford et al identified several small molecule inhibitors that specifically target the Six/Eya complex, as well as inhibitors of Eya2 phosphatase activity per se. Interestingly, these compounds decreased cell viability and migration potential in several cancer models overexpressing the Six/Eya2 complex.

Serine/threonine/tyrosine-interacting protein (STYX) is a catalytically inactive protein, homologous to the dual-specificity phosphatases (DUSPs) family. Since it does not possess phosphatase activity it is called a “pseudophosphatase”, which means they are able to signal in the absence of enzymatic activity. Hesso Farhan (University of Konstanz, Kreuzlingen, Switserland) previously showed that STYX can regulate ERK signaling, however in the current meeting he presented that STYX can bind with, and negatively regulate the F-box protein FBXW7. This protein is a tumor suppressor gene which is altered in 10-15% of all human cancers. Farhan showed that indeed FBXW7 was significantly downregulated, in breast cancer, concomitant with an overexpression over the pseudophosphatase STYX. However, it is as yet unclear how inhibition of STYX may be brought about.

Concluding remarks

In an era where kinase inhibitors are emerging as part of daily clinical practice, resistance to these targeted therapies is becoming an ever larger problem. New strategies for combating cancer from multiple angles will therefore be imperative. Phosphatases can no longer be ignored as a novel therapeutic option. Fortunately, it is now becoming clear that targeting phosphatases is indeed a feasible option in

cancer treatment. Inhibition of oncogenic phosphatases or the reactivation of tumor suppressor phosphatases seems within reach. For most of the potential target phosphatases presented here, substantial basic research still needs to be performed before the leap from bench to clinic can be made. However, with the development of PTP1B inhibitors and PP2A activators, the first phosphatase-based clinical cancer therapies will be available soon. With only a few of the more than 100 phosphatases in the human genome investigated to date as potential targets for cancer treatment, there is hope that an increased attention of the scientific community directed towards this interesting class of enzymes may uncover additional therapeutic targets, as was in previous decades the case for the kinases.

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Chapter 8

β-catenin signaling dosage dicatates tissue-specific tumor predisposition in *Apc*-driven cancer

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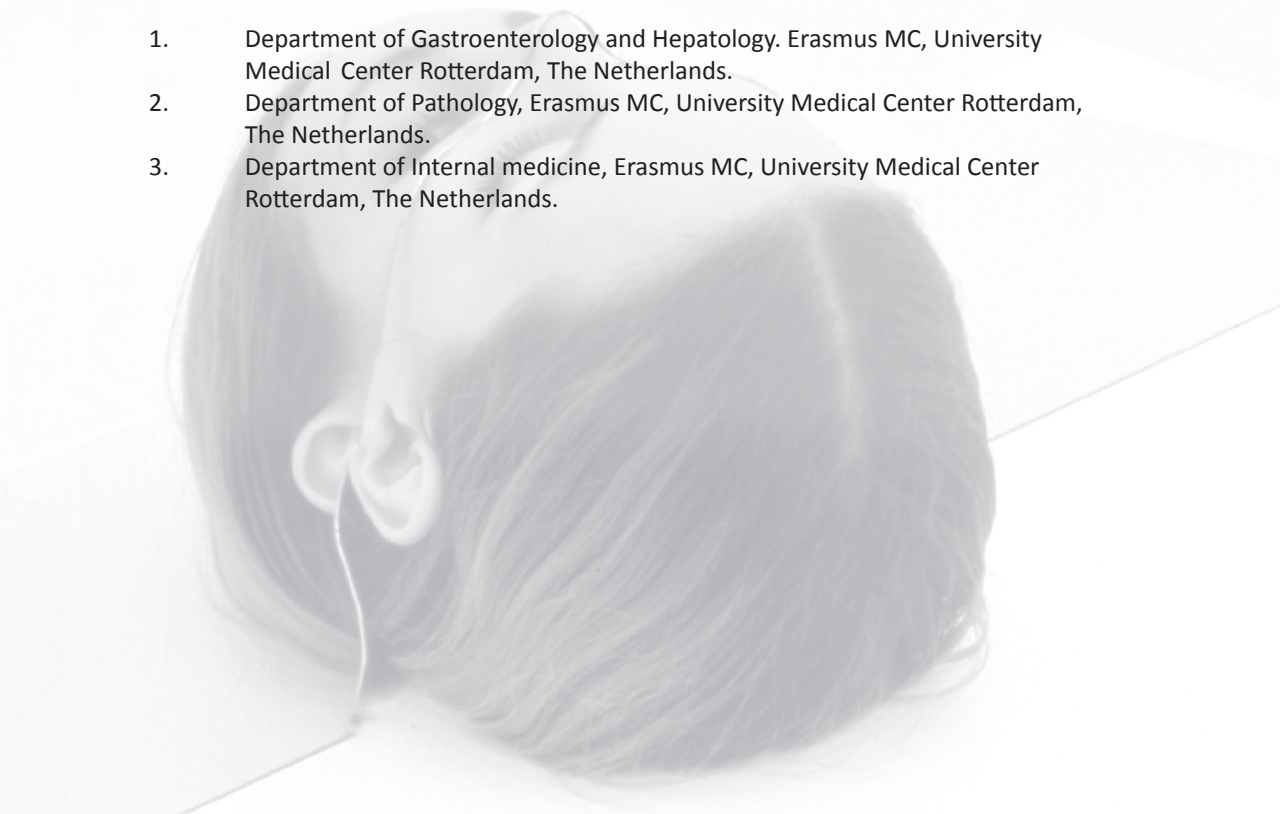

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Abstract

Apc-driven tumor formation in patients and *Apc*-mutant mouse models is generally attributed to increased levels of β-catenin signaling. We and others have proposed that a specific level of β-catenin signaling is required to successfully initiate tumor formation, and that each tissue prefers different dosages of signaling. This is illustrated by *APC* genotype-tumor phenotype correlations in cancer patients and by the different tumor phenotypes displayed by different *Apc*-mutant mouse models. *Apc*1638N mice, associated with intermediate β-catenin signaling, characteristically develop intestinal tumors (<10) and extra-intestinal tumors including cysts and desmoids. *Apc*1572T mice associated with lower levels of β-catenin signaling, are free of intestinal tumors but instead develop mammary tumors. Although the concept of β-catenin signaling dosage and its impact on tumor growth among tissues is gaining acceptance, it has not been formally proven. Additionally, alternative explanations for *Apc*-driven tumor formation have been proposed. To obtain direct evidence for the dominant role of β-catenin dosage in tumor formation and tissue-specific tumor predisposition, we crossed *Apc*1638N mice with heterozygous β-catenin knockout mice, thereby reducing β-catenin levels. Whereas all *Apc*1638N;*Ctnnb1*$^{+/+}$ mice developed gastrointestinal tumors, none were present in the *Apc*1638N;*Ctnnb1*$^{-/+}$ mice. Incidence of other *Apc*1638N-associated lesions including desmoids and cysts was strongly reduced as well. Interestingly, *Apc*1638N;*Ctnnb1*$^{-/+}$ females showed an increased incidence of mammary tumors, normally rarely observed in *Apc*1638N mice, and histological composition of the tumors resembled that of *Apc*1572T-related tumors. Hereby, we provide in vivo genetic evidence confirming the dominant role of β-catenin dosage in tumor formation and in dictating tumor predisposition among tissues in *Apc*-driven cancer.

Introduction

The Wnt/β-catenin signaling pathway represents one of the main regulatory mechanisms to retain tissue homeostasis in the adult organism by balancing self-renewal, differentiation and apoptosis in several adult stem cell niches [1]. Underscoring the relevance of this pathway, many tumor types exhibit enhanced Wnt/β-catenin signaling that strongly contributes to tumor growth. In the Wnt/β-catenin signaling pathway, the adenomatous polyposis coli (APC) protein is a central component regulating the degradation and concomitantly the transcriptional activity of β-catenin in the nucleus. As depicted in **Figure 1A**, several motifs in the central domain of APC are responsible for regulating intracellular β-catenin levels. Four 15 amino acid repeats (AAR) bind β-catenin, whereas seven 20-AARs are involved in both binding and downregulation. Interspersed within those 20-AARs are three binding sites for Axin required for an optimal recruitment of APC into the destruction complex. Inactivation of APC perturbs the formation of the β-catenin degradation complex, leading to increased nuclear translocation and target gene expression, thereby affecting important cellular decisions and favoring a genetic program that initiates tumor formation. In case of colorectal cancer, a small subset (~1-2%) of tumors acquires activating mutations in β-catenin itself, whereas most others result from inactivating biallelic *APC* mutations [2]. The vast majority of these *APC* mutations result in truncated proteins that lack all Axin binding motifs while retaining between one and three 20-AARs. As a result, these truncated proteins still have residual activity in downregulating β-catenin signaling. Accordingly, an inverse correlation is observed between the number of retaining 20-AARs and the resulting level of β-catenin signaling, i.e. more repeats means a lower β-catenin signaling level to the nucleus. Based on these observations, we and others have proposed that *APC* mutations do not occur entirely randomly but rather occur in respect to one another to reach an optimal level of enhanced β-catenin signaling, described as the 'just-right' signaling model [2–6]. According to this model, levels beneath the optimal β-catenin signaling window will not provide cells with sufficient activation of target genes to gain growth advantage and trigger tumor formation, whereas levels exceeding the optimal window will trigger apoptosis instead. As reviewed in Albuquerque et al, optimal β-catenin signaling dosages favoring tumorigenesis differ throughout the body, indicated by different *APC* genotypes that are observed in tumors on different locations [2]. Both sporadic as well as familial forms of desmoid and duodenal tumors contain *APC* mutations retaining 2-3 20AARs associated with moderate β-catenin signaling activation. On the other hand, most colorectal tumors are associated with shorter truncating proteins resulting in higher levels of β-catenin signaling. Interestingly, correlations are observed even within the colorectal tract, where right-sided colon tumors generally retain more 20-AARs than left-sided ones [2,7,8]. Although in human breast cancer patients mutations in β-catenin or *APC* are rarely found, aberrant activation of β-catenin signaling is observed frequently [9].

Phenotypes of *Apc*-mutant mouse models strongly support *Apc* genotype-tumor phenotype correlations (**Figure 1B**). *Apc*$^{Min/+}$ mice have high levels of β-catenin signaling and develop intestinal tumors at high multiplicity (>100). Animals carrying the hypomorphic *Apc*1638N mutation, associated with intermediate β-catenin signaling, characteristically develop intestinal tumors at lower multiplicity (<10) and in parallel show a high susceptibility for extra-intestinal tumor types such as cutaneous cysts and desmoid tumors [10]. The *Apc*1572T mouse model, associated with lower levels of β-catenin signaling, is free of intestinal tumors but instead develops mammary tumors with high penetrance, in addition to cysts and desmoids albeit with reduced numbers compared to *Apc*$^{1638N/+}$ mice [11]. Taken together, this indicates that tissue-specific dosages of β-catenin signaling are required to efficiently

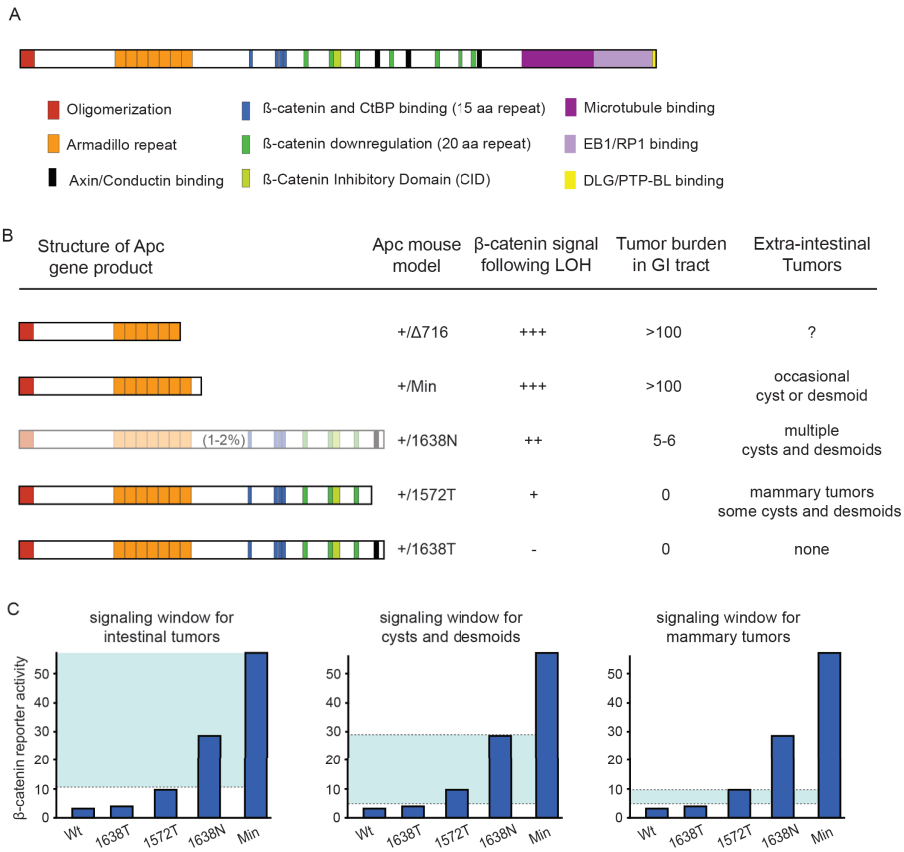

Figure 1. APC structure and associated β-catenin signaling dosage. (A) Structure of the Apc protein containing multiple regulatory domains. **(B)** Truncated Apc in different *Apc*-mutant mouse models. The number of remaining 20-AARs is inversely correlated with the β-catenin signaling dosage and associated with tumor development in different tissues. **(C)** Windows of β-catenin signaling dosages associated with specific tumor types in *Apc*-mutant mouse models. Whereas intestinal tumorigenesis requires high to moderate β-catenin signaling, cysts and desmoid development is associated with moderate β-catenin signaling and mammary tumors occur with the low level of β-catenin signaling as observed in *Apc*1572T mice. Figure modified from [2] with permission of BBA Reviews on Cancer.

trigger tumorigenesis, where intestinal tumors are associated with higher levels of β-catenin signaling than cysts and desmoids, which in turn are associated with higher β-catenin signaling than mammary tumors (**Figure 1C**).
Although the concept of β-catenin signaling dosage and its impact on tumor growth among tissues is gaining acceptance, tissue-specific tumor predisposition has not been formally proven to be a direct consequence of β-catenin signaling dosage. Furthermore, alternative explanations for *APC*-driven tumor formation have been proposed. APC is a large, multifunctional protein and in addition to downregulating β-catenin signaling it is implicated in various other cellular processes, as APC can affect chromosomal segregation, cytoskeletal organization and bind C-terminal binding protein (CtBP) [12–16]. Here, we provide direct genetic evidence for the dominant role of β-catenin in tumor formation and establish the impact of β-catenin signaling dosage in dictating tissue-specific tumor predisposition. To this aim, we reduced the pool of available β-catenin in *Apc*$^{1638N/+}$ (*Apc*1638N) mice by heterozygous β-catenin (*Ctnnb1*) knockout. Consequently, gastrointestinal tumor formation was completely prevented while mammary tumor predisposition was enhanced, shifting the phenotype towards the *Apc*1572T-related tumor phenotype.

Materials and methods

Cells and β-catenin reporter assay
Mouse embryonic fibroblasts (MEFs) were isolated from embryos of embryonic day (E)13.5-15.5, cultured and transfected as described previously [17]. The β-catenin reporter assay was performed as described previously [17]. Assays were performed in duplicate three times.

Mouse strains
Mouse strains (C57BL/6J) used in this study were: *Apc*$^{+/1638N}$, *CAG-Cre* and *Ctnnb1*$^{-/+}$ [10,18]. *Ctnnb1*$^{fl/+}$ males (gift from Dr. J. Huelsken) had been crossed previously with *CAG-Cre* females, obtaining *Ctnnb1*$^{-/+}$ knockout mice. Subsequently, these *Ctnnb1*$^{-/+}$ mice were crossed with *Apc*$^{+/1638N}$, resulting in compound heterozygous *Apc*$^{+/1638N}$/*Ctnnb1*$^{-/+}$ animals and corresponding single transgenic controls *Apc*$^{+/1638N}$/*Ctnnb1*$^{+/+}$. All mice were maintained under specific pathogen-free conditions at the animal facility of the Erasmus Medical Centre. Mice were examined for tumor formation at the age of 8 months. All experiments were approved by the Animal Ethics Committee and carried out in accordance with Dutch and international legislation.

Histology and immunohistochemistry
Tissues were washed in PBS and fixed overnight in 4% PBS-buffered paraformaldehyde at 4°C. Paraffin embedding and Haematoxylin Eosin (HE) staining were performed according to routine protocols. For immunohistochemistry, antigen retrieval citrate pH6 preceded staining for SMA (1:200, DAKO), Cytokeratin-14 (1:10000,

Covance) and CD44 (1:1000, BD Biosciences), Tris-EDTA pH9 for β-catenin (1:2000, Epitomics), Cyclin D1 (1:200, Vector Laboratories) and Ki67 (1:200, DAKO) and 0.1% pronase for Cytokeratin-8 (1:800, DSHB).

RNA isolation, cDNA synthesis and quantitative PCR

RNA was isolated from cultured MEFs followed by cDNA generation using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed with TaqMan Gene Expression Assays (Applied Biosystems) and run in the IQ5 Real time PCR detection system (Bio-Rad). Expression levels were corrected for expression of Actb. Assay was performed in duplicate three times.

Results and discussion

The *Apc*1638N mouse model is a representative model to investigate intestinal cancer, where mice characteristically develop about 1-7 gastrointestinal tumors [19]. In addition, these mice are highly susceptible for extra-intestinal tumor types including desmoids and cutaneous cysts [10]. To reduce their dosage of β-catenin, we crossed *Apc*$^{1638N/+}$ mice with *Ctnnb1*$^{-/+}$ knockout mice [18]. First, non-tumorigenic intestinal tissues were characterized of both *Apc*$^{1638N/+}$/*Ctnnb1*$^{+/+}$ and *Apc*$^{1638N/+}$/*Ctnnb1*$^{-/+}$ mice. Haematoxylin Eosin (HE) staining showed normal intestinal histology in both groups (**Figure 2A**). Also, no discernible alterations in β-catenin protein expression were detected using β-catenin immunohistochemistry (**Figure 2A**). These results were in line with expectations, since normal tissues only harbor the heterozygous germline *Apc*-mutation and intestinal tissue homeostasis of mice harboring the *Apc*1638N mutation is also unaltered compared to that of wildtype mice [20]. Furthermore, β-catenin immunohistochemistry is not a very sensitive technique to detect differences in β-catenin protein expression and to determine associated alterations in β-catenin signaling. To verify reduced β-catenin signaling as a consequence of heterozygous *Ctnnb1* knockout, we used a more sensitive approach. For this, β-catenin reporter assays were performed to measure the intrinsic β-catenin signaling of mouse embryonic fibroblasts (MEFs) that we generated of embryos of the different genotypes. In the absence of *Apc* mutation, levels of β-catenin signaling were low and not detectably reduced by heterozygous β-catenin knockout (**Figure 2B**). *Apc*$^{1638N/+}$ MEFs showed slightly enhanced β-catenin signaling, and here, heterozygous β-catenin knockout clearly resulted in reduced β-catenin signaling. Moreover, *Apc*$^{1638N/+}$ MEFs with heterozygous β-catenin knockout showed intrinsic β-catenin signaling levels that approached the β-catenin signaling dosage as observed in *Apc*$^{+/1572T}$ MEFs. Comparable results were obtained when we assessed β-catenin signaling by determining RNA levels of the β-catenin target gene *Axin2* in these MEFs (**Figure 2C**). In the absence of *Apc* mutation, *Axin2* levels were slightly reduced by heterozygous β-catenin knockout. *Apc*$^{1638N/+}$ MEFs showed enhanced *Axin2* levels, which again were reduced by heterozygous β-catenin knockout, reaching levels

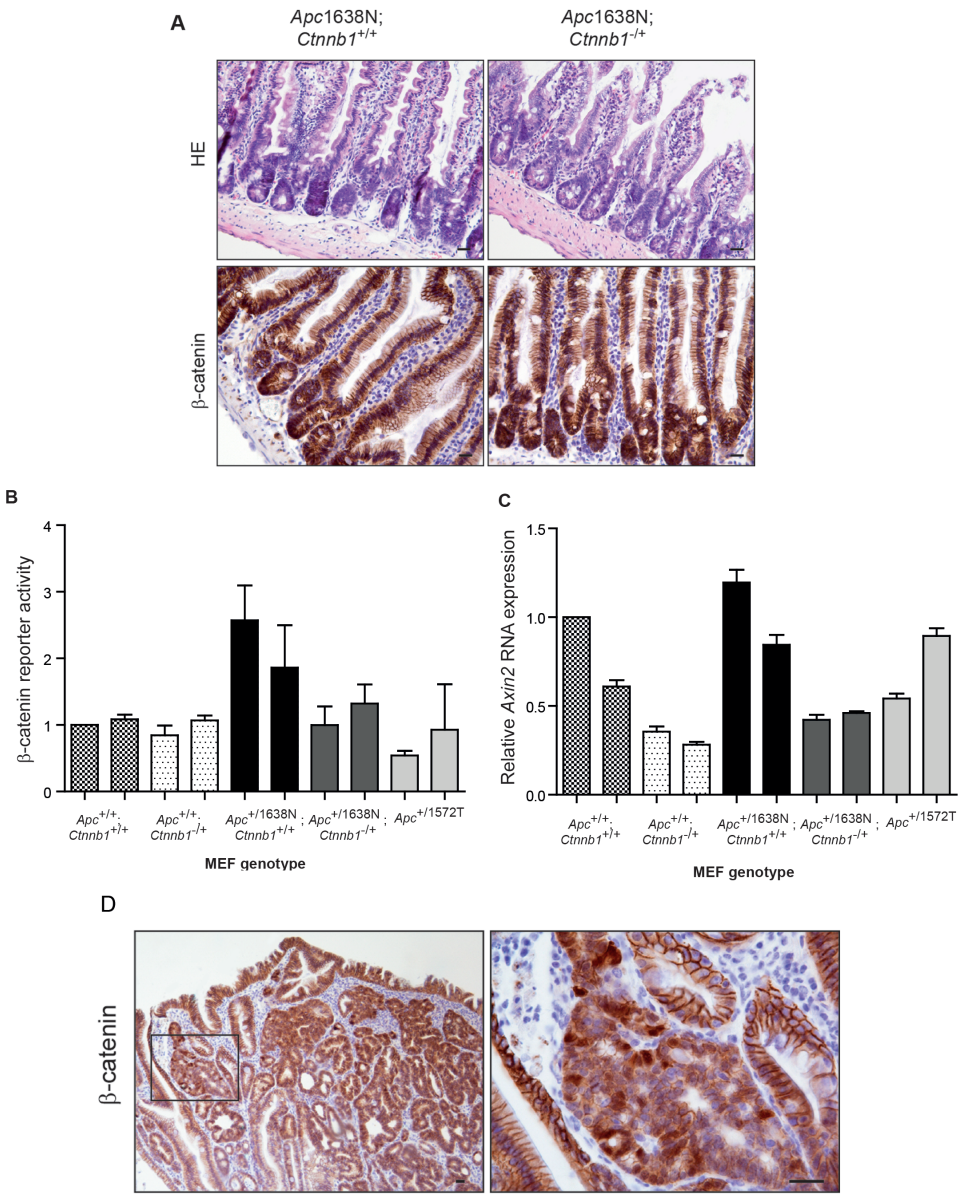

Figure 2. Heterozygous β-catenin knockout reduces β-catenin signaling. (A) HE and β-catenin staining of non-tumorigenic intestine, showing no gross differences between groups. **(B)** β-catenin reporter assay of MEFs of *Apc*$^{+/+}$ or *Apc*$^{+/1638N}$ genotype, each in combination with *Ctnnb1*$^{+/+}$ or *Ctnnb1*$^{-/+}$, in addition to *Apc*$^{+/1572T}$, and **(C)** *Axin2* mRNA expression in these MEFs determined by quantitative PCR. Two bars per genotype indicate two individual MEF lines that were used. **(D)** β-catenin staining of an intestinal tumor of an Apc$^{+/1638N}$ mouse. Nuclear β-catenin staining is clearly detected.

comparable to those observed in *Apc*$^{+/1572T}$ MEFs. Together these data verify that heterozygous β-catenin knockout indeed substantially reduces β-catenin signaling in *Apc*1638N mice, moreover, in such a way that β-catenin signaling levels approach the β-catenin dosage of *Apc*1572T mice. These observations are in line with those of Buchert et al, who showed that halving β-catenin levels consistently reduced β-catenin signaling in various *Apc*-mutant MEFs [21].

We then determined β-catenin expression in the intestinal tumors that developed in the *Apc*1638N mice, since Phelps et al have proposed that following *Apc* loss, activation of oncogenic KRAS is required to impose nuclear accumulation of β-catenin [14]. In the Apc1638N intestinal tumors, we clearly detected nuclear β-catenin staining in the epithelial tumor cells in addition to membranous β-catenin expression (**Figure 2D**), in accordance with previous results [22]. Importantly, *Apc*1638N intestinal tumors and those of other *Apc*-mutant models do not spontaneously acquire Ras mutations [23–25]. This shows that oncogenic Ras is not required for nuclear accumulation in mouse intestinal tumors, which recently has also been confirmed for human colorectal tumors [26,27].

Subsequently, we examined tumor phenotypes of 8-months aged *Apc*1638N/*Ctnnb1*$^{+/+}$ and *Apc*1638N/*Ctnnb1*$^{-/+}$ mice. Strikingly, whereas all 19 *Apc*1638N/*Ctnnb1*$^{+/+}$ mice developed gastrointestinal tumors as characteristic for *Apc*1638N mice, none of the 21 *Apc*1638N/*Ctnnb1*$^{-/+}$ mice developed any gastrointestinal tumor nor was any microlesion detected (**Figure 3A**). This provides direct evidence for the absolute requirement of a sufficiently enhanced β-catenin level for intestinal tumorigenesis. The complete absence of intestinal tumors in mice with heterozygous β-catenin knockout precluded us to compare β-catenin and associated signaling characteristics in intestinal tumors between groups following second hit *Apc* mutation. *Apc*1638N-associated extra-intestinal lesions were still observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice with characteristic gender-specific distribution, although with a clearly reduced incidence (**Figure 3B,C**). Desmoid numbers were reduced from 8.6 ± 3.0 to 0.2 ± 0.4 in females and from 61.4 ± 14.4 to 19.1 ± 8.1 in males (**Figure 3B**). Histological appearance of the desmoids was similar in both groups, showing abundant collagen fibers interspersed by fibroblast-like cells, as has been described for *Apc*1638N mice previously [10]. Cyst numbers were lowered from 5.6 ± 3.8 to 0.4 ± 0.6 in females and from 29.8 ± 19.5 to 2.4 ± 1.5 in males (**Figure 3C**). Thus, reducing β-catenin levels in *Apc*1638N mice prevented gastrointestinal tumor formation and significantly reduced the incidence of other lesions associated with the *Apc*1638N mouse model. Most strikingly, we observed that half of the *Apc*1638N/*Ctnnb1*$^{-/+}$ females developed mammary lesions, reflecting a strongly enhanced incidence of mammary lesions following heterozygous β-catenin knockout (**Figure 3D**). Normally, mammary lesions are rarely observed in *Apc*1638N mice but are characteristic for *Apc*1572T mice. Our findings nicely illustrate that by reducing β-catenin in *Apc*1638N mice, the tumor phenotype shifts towards an *Apc*1572T-related phenotype,

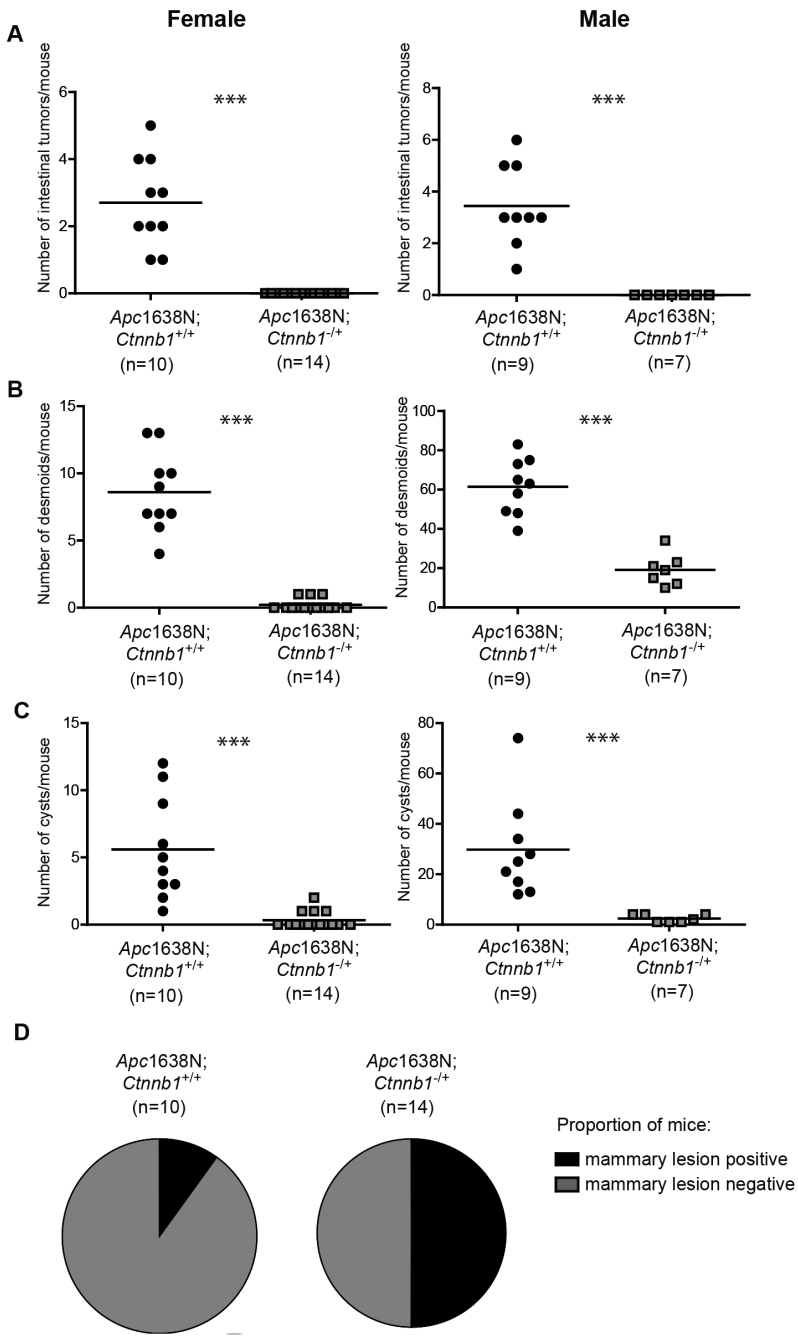

Figure 3. Heterozygous β-catenin knockout prevents gastrointestinal tumor formation but predisposes for mammary tumors in *Apc*1638N mice. Compound heterozygous *Apc*$^{+/1638N}$; *Ctnnb1*$^{-/+}$ animals and corresponding single transgenic *Apc*$^{+/1638N}$ control mice were examined for tumor formation at the age of 8 months. Number of **(A)** gastrointestinal tumors **(B)** desmoids and **(C)** cysts per mouse, distinguishing distribution in females (left) and males (right). ***p<0.001 (Student t-test) **(D)** Proportion of females with/without mammary tumor development. *p<0.05 (Chi-square test).

which is associated with a relatively lower activation level of β-catenin signaling. This confirms that β-catenin signaling dosage by itself dictates tissue-specific tumor predisposition in *Apc*-mutant mice.

The mammary lesions we observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice were relatively small, showing an average diameter of 2.6 ± 1.4 mm, compared to those generally observed in *Apc*1572T mice, reaching 2 cm^3 by 4-5 months of age [11]. In accordance with this relatively mild mammary tumor phenotype in our *Apc*1638N/*Ctnnb1*$^{-/+}$ mice, metastases were not observed. Microscopic characterization of the identified mammary lesions revealed a heterogeneous histology, displaying glandular and squamous regions, keratinizing components and inflammatory cells (**Figure 4A**). Immunohistochemical analyses further established the heterogeneity of the mammary tumors found in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice. Hence, staining for cytokeratin-8 confirmed luminal epithelial differentiation, cytokeratin-14 indicated areas of squamous differentiation and smooth muscle actin showed myoepithelial cell types (**Figure 4A**). This histological composition is virtually identical to that of the mammary lesions observed in *Apc*1572T mice [11]. In the mammary lesions of the *Apc*1638N/*Ctnnb1*$^{-/+}$ mice, staining for Ki67 revealed moderate proliferation (**Figure 4B**). Expression of β-catenin was observed in epithelial cells displaying membrane-bound and nuclear β-catenin (**Figure 4B**). Accordingly, expression of the Wnt/β-catenin targets CD44 and Cyclin D1 was observed in the epithelial compartment of *Apc*1638N/*Ctnnb1*$^{-/+}$ mammary lesions (**Figure 4B**). Thus, *Apc*1638N/*Ctnnb1*$^{-/+}$ mice develop heterogeneous mammary tumors resembling those observed in *Apc*1572T mice histologically and showing active Wnt/β-catenin signaling.

Our data show that by reducing β-catenin levels, the characteristic *Apc*1638N-related intestinal tumor phenotype shifts towards mammary tissues, where tumors typically develop in *Apc*1572T mice [11]. Also, the reduced incidence of cysts and desmoids is in accordance with that observed in *Apc*1572T mice. The mammary tumors observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice resembled those of *Apc*1572T mice histologically, although remaining smaller. We propose that in *Apc*1638N mice following loss of the wild type *Apc* allele required for tumor initiation, we reduced the β-catenin dosage by heterozygous β-catenin knockout to levels approaching those associated with *Apc*1572T mice, thereby enabling successful mammary tumorigenesis [10,11,28]. However, the exact β-catenin signaling level preferred to sustain fully penetrant mammary tumor growth and metastasis may not have been reached most optimally, explaining the smaller tumors observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice compared to *Apc*1572T mice.

Uncovering this shift in tumor phenotype from the gastrointestinal tract towards mammary tissues following β-catenin dosage reduction provides direct in vivo evidence that β-catenin dosage by itself dictates tissue-specific tumor predisposition in the setting of *Apc*-driven cancer. This is in accordance with previously described

APC genotype–tumor phenotype correlations and associated β-catenin signaling dosages among *Apc*-mutant mouse models and sporadic and familial cancer patients [2]. Comparably, tissue-specific biological output being determined by specific dosage has been reported for the proto-oncogene *c-Myc* as well, which is one of the main target genes of β-catenin signaling [29,30]. In line with our findings, Buchert et al presented specific β-catenin signaling thresholds being important for hepatic and intestinal tumorigenesis [21]. Hence, they showed that hepatic tumor formation as observed in a hypomorphic *Apc*-mutant model was prevented by heterozygous *Ctnnb1* knockout [21]. In addition, following heterozygous *Ctnnb1* loss they observed a reduced intestinal tumor incidence and multiplicity in an *Apc*-mutant mouse model associated with a relatively high β-catenin signaling dosage [21]. We currently show that intestinal tumor formation is even prevented completely by reducing β-catenin levels below a hypothetical threshold, using the *Apc*1638N mouse model associated with intermediate β-catenin signaling dosage. Importantly, we hereby demonstrate that for *Apc*-driven tumor formation in the gut, enhanced β-catenin signaling is absolutely required. These results contradict suggested alternative explanations for *Apc*-driven cancer and strongly argue against the model presented by Phelps et al, who recently proposed that *APC*-driven tumor formation is independent of β-catenin, but instead requires the transcriptional corepressor CtBP [10]. CtBP has been shown to interact with APC at its 15-AARs thereby competing with β-catenin binding, and CtBP's levels appear to increase upon *Apc* loss in early adenomas [14,15]. In their paper, Phelps and coworkers suggest that in contrast to CtBP, nuclear β-catenin cannot be detected following Apc loss alone using immunofluorescence, and suggested the additional activation of oncogenic KRAS to impose nuclear accumulation of β-catenin [14]. As discussed by Fodde and Tom-

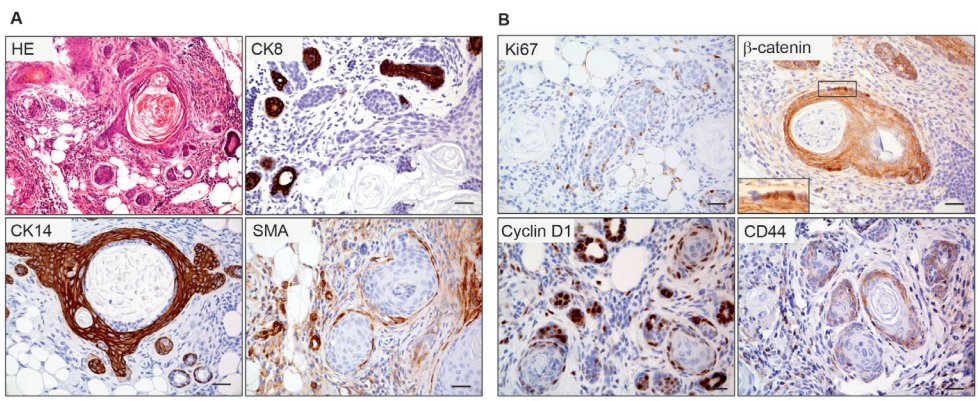

Figure 4. Characterization of *Apc*1638N/*Ctnnb1*$^{-/+}$ mammary tumors. (A) HE staining reveals heterogeneous histology. Immunohistochemical staining for Cytokeratin-8 showing luminal epithelial differentiation, cytokeratin-14 indicating squamous differentiation and smooth muscle actin showing myoepithelial cells. **(B)** Ki67 indicating moderate proliferation and β-catenin staining revealing membrane-bound and nuclear localization. Active Wnt/β-catenin signaling is further indicated by the expression of Cyclin D1 and CD44.

linson, nuclear staining of β-catenin is a reliable indicator of active Wnt signaling, but its absence does not exclude the robust activation of β-catenin target genes [27]. Using immunoperoxidase-based methods most investigators detect nuclear β-catenin accumulation in early adenomas, independent of KRAS mutation status [26,27]. As described above, nuclear β-catenin can be detected in *Apc*1638N intestinal tumors (**Figure 2D**), while *K-*, *N-*, or *H-Ras* mutations are not found in these tumors [23]. In addition, whereas oncogenic *CTNNB1* mutations have been detected in a large number of tumor types and expression of oncogenic β-catenin leads to the development of numerous tumors in the mouse intestine, equivalent data indicating tumor-initiating capacity of CtBP do not exist. Furthermore, as CtBP binds the more N-terminal located 15-AARs of APC, it can not explain the selection of specific truncated APC proteins retaining between 1-3 20-AARs that is observed in tumors, whereas this is the case for β-catenin [2,31]. The same argument holds true for the C-terminal microtubular functions of APC, which are completely lost in all APC-mutant proteins. Although loss of these C-terminal regions has been implicated in disturbed cell migration and chromosomal segregation [12,13], *Apc*-mutant mouse studies have shown that the C-terminal domains of Apc do not influence intestinal tumorigenesis. Hence, *Apc*1638T mice lacking the C-terminal regions of Apc but retaining an axin-binding repeat remain tumor-free [32], and the tumor phenotype of *Apc*1322T mice expressing a truncated Apc retaining only 1 20-AAR is not influenced by reintroduction of the C-terminal regions of Apc [33].

Although our findings provide genetic evidence for the dominant role of β-catenin signaling dosage in dictating tissue-specific predisposition for *Apc*-driven tumorigenesis, mechanisms underlying tissue preferences for specific levels of β-catenin signaling remain largely unknown. In the intestine, β-catenin signaling is one of the main regulatory pathways, however, it operates in concerted action with multiple other signaling routes. This complex interplay is poorly understood. Other tissues including the mammary gland have unique architectural organizations and other signaling pathways are likely to play a role into different degrees. Unravelling how the complexity of all those signaling pathways influences which β-catenin signaling dosage dictates tissue-specific tumor predisposition in *Apc*-driven tumorigenesis represents a challenge for future investigation.

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Chapter 9

Summary and General discussion

Summary

Cancer is a generic term for a group of diseases all characterized by abnormal, uncontrolled cell proliferation, due to a disturbance in the cellular signaling pathways which tightly control tissue homeostasis. These signaling pathways are under strict regulation of two opposing enzymes; kinases and phosphatases. Indeed, alterations in phosphorylation patterns is a common phenomenon in several types of cancer, making this an interesting target for therapeutic intervention. As such, the importance of protein kinases in health and disease has been extensively investigated. These studies are boosted by the wide ranging efforts made to develop a large panel of clinically promising kinase inhibitors. In contrast, extensive knowledge of the equally important group of phosphatase enzymes is still lacking. Therefore the aim of this thesis was to study the role and possible clinical applications of phosphatases in cancer. Our findings will be summarized and discussed in this chapter. We will also provide future perspectives and issues that need to addressed in additional studies.

In **chapter 2** we have prepared a comprehensive review on the action of protein tyrosine phosphatases in colorectal cancer, focusing on the consequences of altered expression of phosphatases, and their potential as targeted treatment. Phosphatases have long been regarded as tumor suppressor genes, since dephosphorylation events were thought to primarily result in termination of signaling. Indeed, phosphatases can act as important negative regulators of phosphorylation patterns. However, what becomes clear in **chapter 2**, is that phosphatases do not only act as signal transduction inhibitors, but can also play positive roles in cellular processes. As such, their role in pathogenesis may need to be redefined.

One of the phosphatases for which a possible oncogenic role is proposed in chapter 2 is low molecular weight protein tyrosine phosphatase (LMWPTP). In **chapters 3 and 4** we further studied the role of this phosphatase in prostate and colorectal cancer. We showed that while LMWPTP is expressed in normal prostate epithelial cells, its expression was significantly increased in prostate cancer. Interestingly, high LMWPTP expression in prostate cancer was correlated to a worse clinical outcome i.e. earlier time to recurrence and disease-related death, suggesting that LMWPTP acts as an oncogene in prostate cancer, and could potentially be used as a prognostic marker. Furthermore, LMWPTP expression did not correlate to the proliferation marker KI-67, suggesting that the signaling induced by LMWPTP is more conductive to other PCa tumor cell characteristics than their proliferative capacity. This was further supported by our *in vitro* studies, in which we show that overexpression or downregulation of LMWPTP in prostate cancer cell lines regulates the migration potential of these cells, without affecting the cell proliferation.

Similar results were obtained in **chapter 4**, where we studied the role of LMWPTP in colorectal cancer. While in healthy prostate tissue the LMWPTP expression was

quite abundant, the expression in normal colonic epithelial cells was rather limited. However, all the colorectal cancer samples showed a massive overexpression of this phosphatase in the intestinal epithelial cells. Furthermore, the expression followed a step-wise increase in different levels of dysplasia, again suggesting a role for LMWPTP in the oncogenic transformation of colorectal epithelial cells. Next, we investigated the effect of LMWPTP on colorectal cancer cells *in vitro*. As observed in prostate cancer cells, LMWPTP downregulation in colorectal cancer cells reduced cell migration and invasion. Furthermore, LMWPTP interfered with cell survival signaling pathways, while we observed no effect on the proliferation of these stably transfected cell lines. On the other hand, treatment with the only known inhibitor of LMWPTP resulted in CRC cell death. Furthermore, we found that LMWPTP downregulation made CRC cells more susceptible to the chemotherapeutic agent 5-fluorouracil, possibly a result of reduced activation of survival signals. In addition, we also observed that LMWPTP can directly interact with multidrug resistance pumps, such as P-glycoprotein. In summary, we found that LMWPTP acts as an oncogene in both prostate and colorectal cancer, mostly by increasing the metastatic potential of the epithelial cells.

Another phosphatase for which a role in cancer has been proposed is protein tyrosine phosphatase 1B (PTP1B). Interestingly, it has been shown that PTP1B acts as tumor suppressor gene in lymphomas and hepatocellular carcinoma, while it plays an oncogenic role in breast cancer and prostate cancer. A tumor promoting role for PTP1B in colorectal cancer has also been proposed, although its contribution to cellular cancer hallmarks and signaling remained unclear. Therefore, in **chapter 5**, we further elucidated the role of the PTP1B phosphatase in CRC. We show that protein tyrosine phosphatase 1B is overexpressed in colorectal cancer on both protein and mRNA level, which corresponds with significantly reduced patient survival. Furthermore, PTP1B is an independent prognostic marker for disease free survival, and a borderline significant independent predictor for overall survival. Of even more importance, we show that not only the expression, but also the intrinsic enzymatic activity of PTP1B is greatly increased in the colorectal tumors. *In vitro*, this results in a more malignant phenotype by enhancing the proliferation, migration, and anoikis resistance of intestinal epithelial cells. Interestingly, PTP1B downregulation also reduced the levels of β-catenin signaling dosage, a critical driver of colorectal cancer carcinogenesis which was further pursued in chapter 8. Together these data suggest that targeting the activity of this phosphatase could be a viable treatment option for CRC, and a step forward in the fight against colorectal cancer.

Phosphorylation and dephosphorylation does not only take place on protein residues, but also on lipids. PI3-kinase acts as an oncogene by phosphorylating membrane bound inositol lipids to produce phosphatidylinositol(3,4,5) triphosphate (PIP_3), which subsequently recruits and allows activation of PKB. Dephosphorylation of PIP_3 is therefore generally assumed to terminate PKB signaling, as is the case for

the well-known tumor suppressor gene and lipid phosphatase PTEN, which hydrolyses PIP_3 to produce $PI(4,5)P_2$. The action of the lipid phosphatase SHIP2 resembles that of PTEN, however, the hydrolysis product of SHIP2 is not $PI(4,5)P_2$ but $PI(3,4)P_2$. Interestingly, $PI(3,4)P_2$ has an even higher affinity for PKB than PIP_3, and is required for full activation of this kinase. Thus, both PIP_3 and $PI(3,4)P_2$ are suggested to play a role in cancer development, suggesting that SHIP2 could be a possible oncogene rather than tumor suppressor. We therefore investigated the role of this phosphatase in colorectal cancer in **chapter 6**. We found that the expression and intrinsic phosphatase activity of SHIP2 is increased in human colorectal cancer, and that increased expression within a large cohort of CRC patient is correlated to a worse patient survival. SHIP2 enhances cell migration and invasion, and reduces cell adhesion in colorectal cancer cells. Furthermore, treatment with a SHIP2 activity inhibitor resulted in dose-dependent cell death, and this inhibitor sensitizes CRC cells to chemotherapy treatment. These data led us to conclude that SHIP2 is another phosphatase which contributes to the malignant potential of CRC, and may present yet another therapeutic target for this disease.

After our discovery that phosphatases indeed pose as potential treatment targets, we looked at the realistic possibility of targeting such phosphatases in cancer in **chapter 7**. For a long time researchers and industry were not interested in phosphatases as potential therapeutic option, since they were regarded as “undruggable” due to their high similarity. However, based on novel data presented at the Europhosphatase conference 2015, it is becoming clear that due to increasing knowledge of crystal structures and mechanisms-of-action, the possibility of targeting these enzymes is no longer in the realm of fantasy. Intuitively, phosphatases that act as tumor suppressor are less suitable for targeting, since inhibition of their enzymatic activity would be expected to result in further activation of the oncogenic pathways involved. However, rather than inhibiting these enzymes, reactivation of tumor suppressive phosphatases is now emerging as a potential strategy in cancer treatment. Even so, in the previous chapters, we identified phosphatases acting as oncogenes rather than tumor suppressor genes. Therefore, as previously done for oncogenic kinases, efforts are now directed towards the development of small molecule inhibitors of such phosphatases. This has already lead to the development of PTP1B activity inhibitors with promising success *in vitro* and *in vivo*, which will soon be tested in clinical trials. Furthermore, small molecule inhibitors against oncogenic phosphatases SHP2 and LMWPTP are under development. Hopefully these novel compounds directed against phosphatases can broaden the spectrum of targeted therapies, providing additional options for personalized medicine in cancer therapy.

In **chapter 8**, we studied the canonical Wnt/β-catenin signaling pathway. The vast majority of CRC cases arise as a result of aberrant activation of this signaling pathway due to *APC* or β-catenin mutations resulting in enhanced β-catenin -induced proliferation. Most mutations in *APC* result in a truncated protein which loses the

ability to downregulate β-catenin signaling. However, depending on the location of the APC mutation, residual β-catenin downregulation may occur. Accordingly, there is an inverse correlation between the length of the truncated protein and amount of β-catenin signaling. The distribution of the several APC-mutations along different tissues or tumor locations do not seem to occur in an entirely random fashion, but appear selected to reach an optimal window of signaling. This is described as the "just-right" signaling model. This model proposes that signaling levels beneath the optimal window will not give a growth advantage, while exceeding levels will result in apoptosis of the affected cell. The concept of the just right signaling dosage for β-catenin in tumor development is gaining a lot of acceptance, however direct evidence was still lacking. Therefore in chapter 8, we aimed to formally prove this concept by lowering the β-catenin signaling dosage in an APC-mutant mouse model, shifting the signaling window towards a different tumor phenotype. By crossing APC1638N mice with heterozygous β-catenin knockout mice, we were able to reduce the β-catenin signaling in this model to a level comparable to that in APC1572T mice. While all the APC1638N mice developed gastrointestinal tumors, none were present in the gastrointestinal tract of the heterozygous β-catenin knockouts. Strikingly, the female 1638N/ heterozygous β-catenin knockout mice developed mammary tumors, which are observed in APC1572T mice, but rarely in APC1638N mice, thereby providing direct *in vivo* genetic evidence for the dominant role for β-catenin signaling dosage in tumor formation.

General discussion

This thesis offers novel insights into the role of phosphatases in cancer. While aberrant phosphorylation is often studied with respect to kinases, phosphatases are somewhat overlooked. We believe that the data described in this thesis demonstrate that a closer look to the phosphatases is warranted.

Phosphatase overexpression

Genetic alterations that regularly occur in cancer include mutations, copy number loss/deletion, and copy number amplification [1] studies of different stages of colorectal neoplasia may shed light on the genetic alterations involved in tumor progression. We looked for four genetic alterations (ras-gene mutations and allelic deletions of chromosomes 5, 17, and 18. Gain-of-function alterations may increase the expression or activity of an oncogene [2,3], while loss-of-function mutations can silence a tumor suppressor gene. In the studies described in this thesis, we have observed overexpression in cancer samples of three distinct phosphatases. In these cancer types, overexpression of other oncogenes, such as COX-2, c-Myc but also PI3K, PKB, STAT3 and its phosphorylation are described to be increased [4–8].

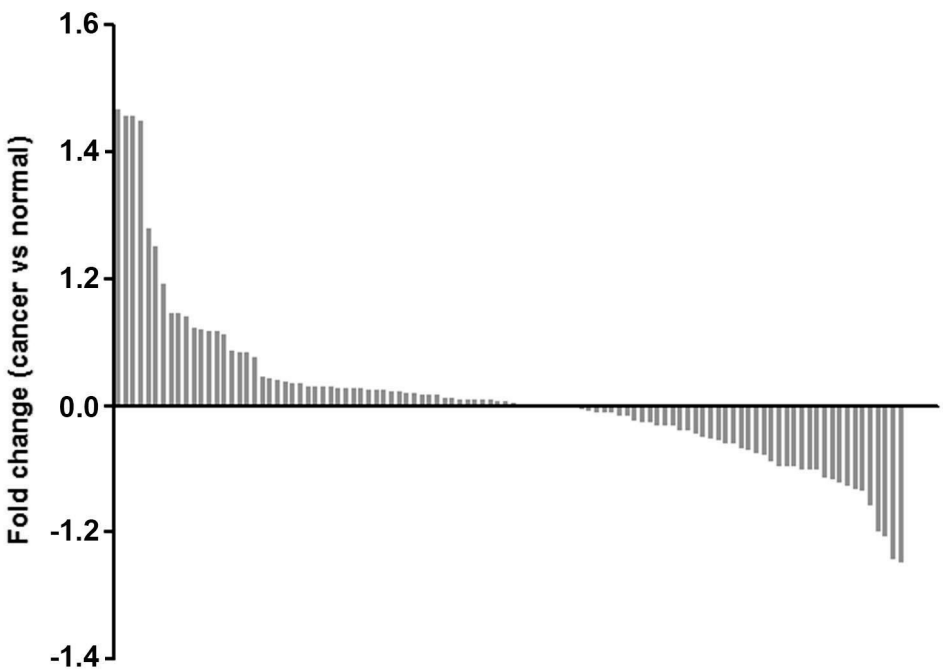

Figure 1. Expression patterns of all phosphatases in carcinoma tissue compared to normal colonic tissue in the TCGA colorectal cancer 2 dataset. Almost as many phosphatases are upregulated as are downregulated in colorectal cancer.

So how is it that both these oncogenes and the phosphatases that are traditionally regarded as negative modulators of these oncogenic pathways are increased in these tumors? It is tempting to speculate that their upregulation is a general phenomenon of cells turning malignant, where transcriptional machinery goes haywire, as it is known that deregulated transcription and translation is a common event in cancer cells, resulting in the concomitant dysregulation of many signaling pathways [9]. To address this notion, we analyzed the expression pattern of all known phosphatases, based on the TCGGA colorectal 2 dataset [10](**Figure 1A-B**). We observed that almost as many phosphatases are significantly upregulated as are downregulated in colorectal cancer. This shows that overexpression of a certain phosphatase is not based on a general increase in transcription, but a specific event in the development of cancer. Off course this dataset describes the overall upregulation in a group of tumors, and it not representative of the events happening in a single tumor. However, while our studies showed LMWPTP expression was increased in all the colorectal cancer samples we analyzed, PTP1B and SHIP2 expression differ drastically between different individual tumors, with some tumors being completely negative for one or the other. Since we analyzed the expression of SHIP2 and PTP1B on the same cohort, we could also compare individual tumors. Again, we observed discrepancies in expression patterns of both phosphatases, with some being negative for one, and positive for the other. Another interesting observation made in this cohort, is that the expression of SHIP2 is correlated to MSI-status, while this was not the case for PTP1B. Interestingly, BRAF mutations are commonly associated with mismatch repair tumors, and recently it has been shown that for effective treatment of these colorectal tumors a concomitant BRAF and PI3K/mTOR blockade is required [11]. Since we showed that SHIP2 is a regulator of the PI3K/mTOR pathway, we can speculate that SHIP2 inhibitors can be an important addition for these tumors. Altogether, this suggests that certain mutations or activated pathways benefit from the upregulation of a specific phosphatase.

Of course this raises the question, how are these phosphatases upregulated? Mutations in phosphatase genes are described for a number of phosphatases, however most of these are inactivating mutations in the tumor suppressor phosphatases [12,13], which result in disturbed negative feedback of cancer signaling. While mutations in PTP1B are found, the gene is most frequently overexpressed through amplification of the chromosome 20q13 region. Furthermore, epigenetic mechanisms such as methylation can also play a role in the regulation of expression. We observed a hypomethylation of LMWPTP in colorectal cancer as compared to normal colon tissue, providing a possible explanation for the increased LMWPTP expression in CRC. However, such alterations for PTP1B and SHIP2 are not observed, and other mechanisms are likely to contribute to the upregulation of these proteins. Another hypothesis is these phosphatases are not upregulated as a result of mutations or amplifications in the gene itself, but as cellular response to overactive oncogenic signaling. Cells will generally attempt to maintain homeostasis, and counteract

these hyperactive pathways, by increasing negative mediators of cellular signaling. Nevertheless, the upregulated phosphatases provide growth and metastasis advantage for the tumor cell and is therefore maintained. As yet, the actual mechanism for upregulation of LMWPTP, PTP1B and SHIP2 remains to be elucidated, and likely differs between phosphatases as well as individual tumors.

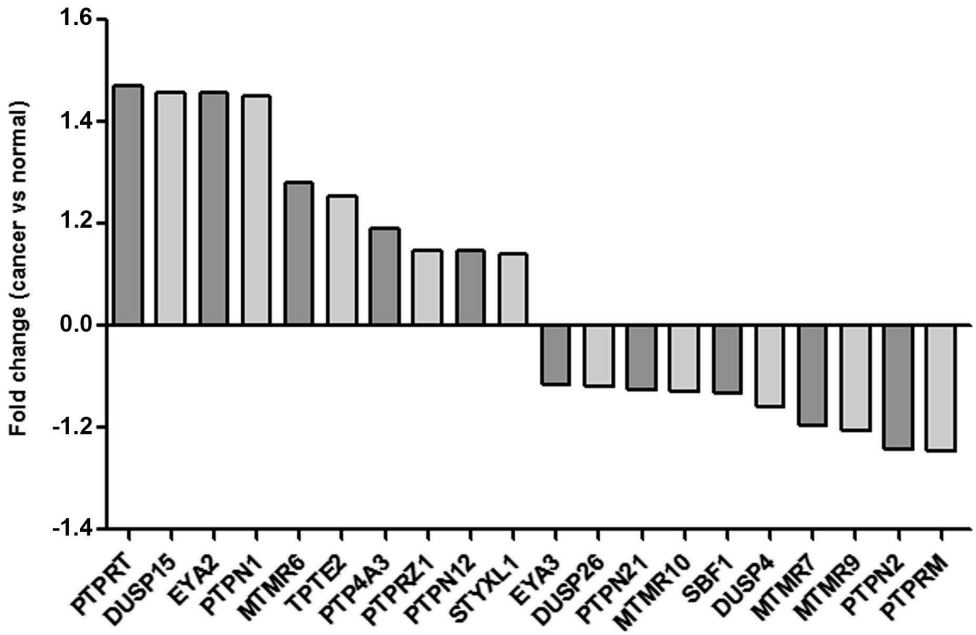

Figure 2. Expression patterns of the 10 most up- and downregulated phosphatases in carcinoma tissue compared to normal colonic tissue in the TCGA colorectal cancer 2.

As described in chapters 4 and 5, phosphatases are not only regulated through their expression, but also through their activity. There are many post-translational mechanisms for modulating phosphatase activity [14,15]. Phosphatases can be phosphorylated themselves, resulting in a change in their activity, as has been described for both LMWPTP and SHIP2 [16–18]. However, there are also other mechanisms for phosphatase modulation, such as inactivation by reactive oxygen species, as is the case for LMWPTP and PTP1B [19–21]. Based on the expression and activity patterns we observed in our studies, it seems that the intrinsic activities of the studied phosphatases are specifically upregulated, irrespective of their expression patterns, and hence the overall enhanced effect of these phosphatases is not the result of an overactive transcriptional machinery in general.

The effect of phosphatases on the process of metastasis

Interestingly, for all three phosphatases studied in this thesis, we found significant effects on cell adhesion and migration. Cell adhesion and migration are dynamic multi-step processes, that require quick and precise regulation [22]. Since phosphorylation is a rapid and reversible modification process, this is an ideal system for the control of these dynamic events [23]. Cell migration is composed of 4 integrated steps which together result in coordinated cellular movement, and all of these steps are tightly regulated by phosphorylation. To start migration, cells become asymmetric and polarized – they form a clear distinction between cell front and rear. This is done via the protrusion of lamellipodia and filopodia at the leading edge of the cell. Lamellipodia are broad, flat, sheet-like structures, whereas filopodia are thin, cylindrical, needle-like projections. Following these protrusions, cells form new sites of cell attachment to the extracellular matrix (ECM) via focal complexes or focal adhesion, which link the ECM to the cells actin skeleton. Next, through the force generated by actomyosin interactions, the cell body contracts and translocates in the direction of the leading edge. The final phase of cell migration is a release of cell contact at the rear end of the cell and recycling of membrane receptors from the rear to the front of the cell. Phosphatases play instrumental roles in regulating the timing, duration and localization of all these independent events. In our studies, we observed that knockdown of LMWPTP, PTP1B, and SHIP2 all reduce cancer cell migration. However, while knockdown of LMWPTP and PTP1B both also resulted in a reduced cell adhesion, this was not the case for SHIP2 downregulation. SHIP2 knockdown cells apparently adhered more efficiently to culture plates as compared to the control cells. While cell migration and adhesion are closely linked, they are not the same. As described above, in order to efficiently migrate, the leading edge of the cell needs to form new complexes and adhere to the extracellular matrix, while on the other hand cells also need to efficiently release the rear end. The adhesion assay as described in our work does not distinguish between these two events. Signaling in a migrating cell acquires a local intracellular gradient. For instance, PI3K and Rac GTPase activity are found at the leading edge of the cell, whereas Rho GTPase activity is required for the retraction of the uropod during migration [22]. It is conceivable that differences in cellular localization of the studied phosphatases influences their effect on cellular adhesion. For instance, as PI3K signaling needs to be terminated at the trailing edge of the cell during migration [24], SHIP2 might be found at the uropod, where reducing its activity may thus decrease the detaching capacity of the cell. It would be of interest to further study the subcellular localization of these phosphatases, and their interaction with the Rac and Rho GTPases. Nevertheless, both a too strong and a too weak attachment of cells may hamper cellular migration, which may explain why, despite differences in effect on adhesion, SHIP2, PTP1B and LMWPTP knockdown all result in a reduced migratory response.

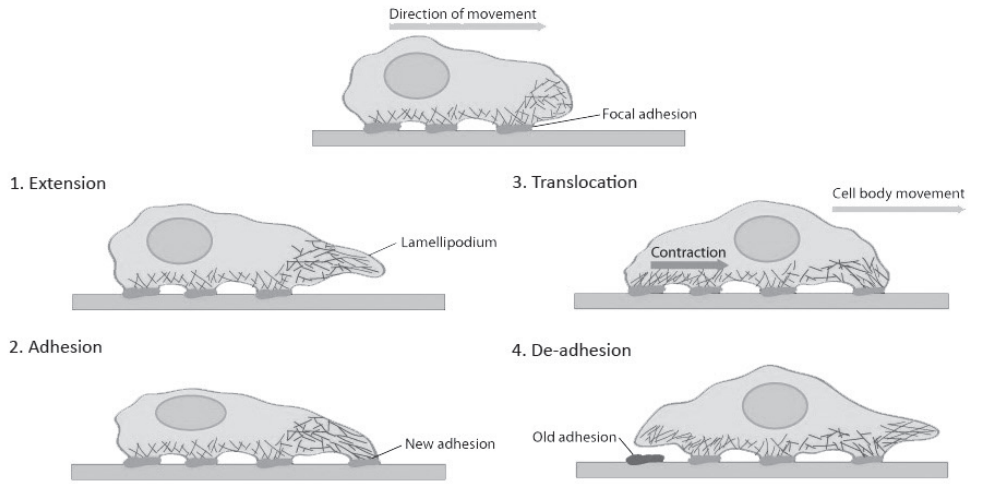

Figure 4. Schematic representation of the four steps of cell migration. 1. Polymerization of actin filaments at the leading edge is translated into protrusive force. 2. Membrane protrusion facilitates the binding of transmembrane cell surface receptors to the substratum components. New adhesions are rapidly linked to the network of actin filaments. 3. The combined activity of retrograde actin movement and contractile forces produced by stress fibers generate tension to pull the cell body forward. 4. The forces produced by the contractile network combined with actin filament and focal adhesion disassembly, helps to retract the trailing cell edge. Used with permission from MBInfo: www.mechanobio.info; Mechanobiology Institute, National University of Singapore

Phosphatases and their substrates

Besides the influence of the studied phosphatases in relation to cellular migration and adhesion, we also tried to elucidate which cellular signaling cascades are under the influence or regulation of our target proteins. We have found several important cancer related signaling pathways altered upon the knockdown of our phosphatases of interest. However, there are still some interesting discrepancies, or unexplained findings. For instance, in LMWPTP knockdown CRC cells, we found that phosphorylation - and thus activation - of PKB and EGFR was reduced. This is of course of interest, since both are known to be frequently mutated/upregulated in several types of cancer [25–27], and corresponds well with known pathways to be deregulated in cancer. However, when the levels of a certain phosphatase are downregulated, as is the case in our LMWPTP knockdown cells, one would expect to find an increased phosphorylation of its substrates. This suggests that PKB and EGFR are not direct substrates of LMWPTP, but rather indirect targets. It is likely that an intermediate phosphatase or kinase is the direct substrate of LMWPTP, and that the phosphorylation levels of PKB and EGFR are reduced through these intermediate LMWPTP substrates. Similarly, we observed a clear reduction in phosphorylation levels of ERK upon PTP1B knockdown. However, this phosphatase was

previously shown to dephosphorylate the Src kinase at its inhibitory Y527 position, thereby activating this oncogenic kinase. As such, Src could act as an intermediate activator of ERK phosphorylation in the PTP1B cascade. Future research should be aimed at identifying more direct substrates of these interesting oncogenic phosphatases. Furthermore, while LMWPTP was upregulated in both prostate and colorectal cancer, we observed discrepancies in the signaling routes that were activated upon LMWPTP modulation between these tumor types. This means that substrates can even differ between several tissue types, or at least that the specificity towards these substrates differ. This directly raises the question whether LMWPTP also acts as an oncogene in other forms of cancer – and if so – which substrates are affected in these tumor types.

Future perspectives

We have identified several phosphatases acting as oncogenes in colorectal and prostate cancer, based on human expression data and *in vitro* experiments. However, the ultimate goal of future research will be to understand the functions of each of the phosphatases *in vivo*. Generation of more conditional and/or tissue-specific knockout animal models will therefore be necessary, especially using the Cre/Lox-system to analyze intestine specific effects [28]. Once available, they can be crossed with existing tumor models like the *APC*-mutants for colorectal cancer, to gain more insights into their role in the pathogenesis of cancer. Since we found the most prominent effects for these phosphatases on cell migration rather than proliferation, animal models for metastasis might be of more interest than models for tumor initiation. The same hold true for the newly developed phosphatase targeting drugs. Huge discrepancies can occur in sensitivity to chemical manipulation between *in vitro* and *in vivo* experiments. In order to find the clinically relevant drugs, animal models are the first step in this translation.

Nevertheless, with the realization that expression of certain phosphatases is correlated to patient outcome, a clinical application as biomarker for phosphatases emerges. We already addressed the possibility for the use of the studied phosphatases as biomarker, however, this is based on relatively small cohorts. In order to find correct cut-off values, sensitivity and specificity, and true correlations to clinical parameters, our findings need to be repeated in larger and independent tissue cohorts, preferably collected in a prospective manner. Also, the immunohistochemical analysis in our studies was performed on tissue micro arrays constructed from resected cancer material. For biomarker studies it would be more valuable to perform the staining on biopsies, since the most important application of a biomarker will be to help decide whether a patient is – or is not – in need of invasive surgical treatment. Future biomarker studies should therefore focus on the prospective collection of biopsies in which the immunohistochemical marker can be analyzed. Besides the use as biomarker for surgical decision making, these stainings could also be of value to decide whether the use of additional chemotherapy might be beneficial, especially since we observed that both LMWPTP and SHIP2 interfere with chemoresistance to the widely used chemotherapeutic agent 5-fluorouracil.

The ultimate challenge for the coming years, will be to further extent the knowledge regarding phosphatase regulation and function *in vivo*, which will identify the most promising targets for therapeutic intervention. Next, by combining the knowledge on the crystal structures of these enzymes, the appropriate animal models, and large drug screens, phosphatase targeting drugs should become become available to prevent or cure diseases in the future.

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Chapter 10

Nederlandse samenvatting
Dutch summary

Nederlandse samenvatting

Kanker is een algemene term voor een groep aandoeningen die wordt gekenmerkt door cellen die zich abnormaal en ongecontroleerd gaan delen (proliferatie), waarna deze woekerende klomp cellen, de tumor, uitlopers kan vormen die het omringende weefsel binnen dringen (infiltratie). Soms kunnen cellen van de tumor zich afsplitsen en zich verplaatsen naar andere delen van het lichaam om hier nieuwe tumoren te vormen, we noemen dit metastasering of uitzaaiing. Normaliter bevatten cellen regulerende mechanismen die er voor zorgen dat een cel op het juiste moment gaat delen, maar bij kankercellen zijn deze mechanismen vaak verstoord. Veel van de processen die bijdragen tot tumorvorming staan onder nauwgezette controle van eiwitten, die aan- en uitgezet kunnen worden middels fosforylering. Fosforylering houdt in dat een fosfaat-groep aan een eiwit wordt gekoppeld, wat meestal leidt tot de activering van dit eiwit. Het toevoegen van deze fosfaat-groepen wordt geregeld door een specifieke groep eiwitten, de '**kinase enzymen**'. Het tegenovergestelde proces, het verwijderen van de fosfaat-groep, ook wel defosforylering genoemd, wordt gedaan door de '**fosfatase enzymen**'. Aangezien er in kankercellen vaak een overtollige fosforylering van eiwitten wordt gevonden, is er in de onderzoekswereld veel aandacht geweest voor de rol die kinasen spelen in kanker. Dit heeft geleid tot de ontwikkeling van nieuwe medicijnen die de activiteit van verschillende kinasen kunnen verminderen, en sommige hiervan hebben zeer veelbelovende resultaten laten zien in de kliniek. De rol van de even belangrijke fosfatasen in het kankerproces is daarentegen erg onderbelicht gebleven. Het doel van dit proefschrift was dan ook om de rol en de mogelijke klinische toepassingen van fosfatasen in kanker in kaart te brengen. Onze bevindingen zullen worden samengevat en besproken in dit hoofdstuk.

Fosfaat-groepen worden niet willekeurig aan een eiwit vast gemaakt, maar slechts op enkele anker-plaatsen die in deze eiwitten voorkomen, de aminozuren serine, threonine of tyrosine. Een cel bevat meer dan 100 eiwit fosfatasen, waarbij de belangrijkste onderverdeling te maken is in serine/threonine-specifieke fosfatasen en tyrosine-specifieke fosfatasen. In **hoofdstuk 2** hebben we een uitgebreid overzicht gemaakt van de werking van met name de tyrosine fosfatasen in darmkanker, met de nadruk op de gevolgen van een veranderde expressie van deze eiwitten om kankercellen, en de mogelijkheid om deze eiwitten te gebruiken als aangrijpingspunt voor behandeling. Fosfatasen werden lange tijd beschouwd als 'tumor suppressor eiwitten', eiwitten die de groei van kankercellen remmen. Dit idee kwam voort uit de gedachte dat defosforylering door fosfatasen vooral zou leiden tot het remmen van signalering die verantwoordelijk is voor de ontwikkeling van kanker. Het verlies van deze eiwitten zou derhalve leiden tot verhoogde celdeling. Er is inderdaad voor een aantal fosfatasen aangetoond dat ze op deze manier kunnen fungeren in kanker. Echter, wat duidelijk wordt in **hoofdstuk 2**, is dat sommige fosfatasen niet uitsluitend als remmers van signalering werken, maar ook een activerende rol kun-

nen spelen in processen die bijdragen aan kankerontwikkeling, en dat de rol van fosfatasen niet in alle type tumoren hetzelfde is. Hierdoor is het belangrijk dat hun rol in de ontwikkeling van kanker opnieuw wordt bekeken.

Eén van de fosfatasen waarvan in hoofdstuk 2 bleek dat zij een tumor-versterkende (oncogene) rol zou kunnen spelen is 'low molecular weight protein tyrosine phoshatase' (LMWPTP). In de **hoofdstukken 3 en 4** hebben we de rol van dit enzym nader onderzocht in prostaat- en darmkanker. We toonden aan dat LMWPTP aanwezig is in normale prostaat cellen, maar dat de hoeveelheid LMWPTP significant verhoogd is in prostaatkanker. Daarnaast zagen we dat een verhoogde hoeveelheid LMWPTP in prostaatkanker correleert met een slechtere uitkomst voor patiënten, zoals een eerdere terugkeer van de tumor. Dit suggereert dat LMWPTP inderdaad fungeert als een oncogen in prostaatkanker. Daarnaast laten we zien dat de expressie (de hoeveelheid van het eiwit) van LMWPTP gebruikt zou kunnen worden als voorspellende marker in deze ziekte. De expressie van LMWPTP correleerde niet met een marker voor de hoeveelheid celdeling, wat er op zou kunnen wijzen dat de signalering die wordt geactiveerd door dit enzym geen grote invloed heeft op de celdelingscapaciteit, maar op andere tumor kenmerken. Dit vermoeden werd bevestigd door onze *in vitro* studies, waaruit bleek dat wanneer wij de hoeveelheid LMWPTP eiwit in prostaat kanker cellen verlaagden, deze cellen minder goed konden migreren, terwijl de celdeling niet beïnvloed werd.

Vergelijkbare resultaten werden verkregen in **hoofdstuk 4**, waar we de rol van LMWPTP in darmkanker onderzochten. In tegenstelling tot normale prostaatcellen, komt LMWPTP in normale darm epitheelcellen nauwelijks voor. De expressie van LMWPTP in afwijkende cellen was echter verhoogd, en nam nog verder toe in echte kanker cellen, wat opnieuw suggereert dat LMWPTP een rol speelt in het proces van kankerontwikkeling in de darm. Vervolgens hebben we het effect van LMWPTP op darmkankercellen onderzocht in het laboratorium. Zoals we eerder al zagen in prostaatkankercellen, resulteert ook het verminderen van de expressie van LMWPTP in darmkankercellen in een verlaagde cel migratie en invasie, twee belangrijke kenmerken voor het uitzaaiingsproces. Er werd geen direct effect gevonden op de celdeling, hoewel we wel aanwijzingen verkregen dat LMWPTP van invloed is op signaleringsroutes die belangrijk zijn voor de overleving van cellen. Het verminderen van de hoeveelheid LMWPTP maakte de darmkanker cellen dan ook gevoeliger maakt voor chemotherapie. Bovendien gingen darmkanker cellen dood wanneer we ze behandelden met een chemische remmer van LMWPTP. Deze resultaten laten samen zien dat LMWPTP als oncogen fungeert in zowel prostaat- en darmkanker, waar het met name het vermogen van de cellen om te migreren bevordert, en dus metastasering kan bespoedigen.

Een ander fosfatase waarvoor een rol in kanker is beschreven is 'protein tyrosine phosphatase 1B' (PTP1B). Al eerder is aangetoond dat PTP1B als tumor suppressor kan fungeren in lymfklierkanker en leverkanker, terwijl het bij borstkanker en pros-

taatkanker juist een tumor bevorderende rol speelt. Ook in darmkanker zou PTP1B tumorgroei kunnen bespoedigen, maar de precieze toedracht was tot op heden onduidelijk. Daarom hebben we in **hoofdstuk 5** de rol van de PTP1B in darmkanker nader onderzocht. We toonden aan dat PTP1B verhoogd tot expressie komt in darmkanker, wat gepaard gaat met een significant slechtere overleving van darmkanker patiënten. PTP1B expressie zou zelfs als voorspellende marker voor ziekte uitkomst gebruikt kunnen worden. Van groter belang is echter dat we konden laten zien dat niet alleen de expressie, maar ook de enzymatische activiteit van PTP1B sterk is toegenomen in de darmtumoren. Uit *in vitro* proeven bleek dat PTP1B expressie resulteert in een meer kwaadaardige tumor, via bevordering van zowel celdeling als celmigratie van darmcellen. Verlaging van PTP1B eiwit niveaus reduceerde β-catenin signalering, een van de belangrijkste regulatoren in de ontwikkeling van darmkanker (verder besproken in hoofdstuk 8). Deze bevindingen samen suggereren dat een gerichte behandeling tegen de activiteit van PTP1B een veelbelovende behandelingsoptie voor dit type kanker zou kunnen zijn, en een belangrijke stap voorwaarts in de strijd tegen darmkanker.

Het proces van fosforylatie en defosforylatie vindt niet alleen plaats op eiwitten, maar ook fosfaat-groepen kunnen ook aan vetachtige stoffen worden gekoppeld. Een voorbeeld hiervan is het bekende oncogen 'PI3-kinase', dat een fosfaatgroep overbrengt op het zogenaamde $PI(4,5)P_2$ inositol lipide, een lipide met twee fosfaatgroepen (aangeduid met 'P') dat verankerd is aan de binnenkant van de celmembraan. Het ontstane product, $PI(3,4,5)P_3$ (het heeft een extra fosfaatgroep verkregen), zorgt vervolgens voor de activering van de 'PKB' signaleringscascade, een belangrijke route voor de overleving en deling van cellen. Defosforylering van $PI(3,4,5)P_3$ tot $PI(4,5)P_2$ wordt bewerkstelligd door een van de meest bekende tumor suppressor eiwitten; PTEN. De fosfatase 'SHIP2' heeft een functie die vergelijkbaar is met die van PTEN. Het herkent ook het gefosforyleerde lipide $PI(3,4,5)P_3$, maar haalt hier een andere fosfaatgroep vanaf, waarmee het product $PI(3,4)P_2$ ontstaat. Recentelijk is aangetoond dat in tegenstelling tot $PI(4,5)P_2$, $PI(3,4)P_2$ ook in staat is het PKB overlevings-signaal aan de cel door te geven. Hierdoor is het goed mogelijk dat in tegenstelling tot PTEN, SHIP2 misschien wel als oncogen werkt in kanker. In **hoofdstuk 6** hebben we daarom de rol van SHIP2 fosfatase in darmkanker onderzocht. We hebben gevonden dat zowel de expressie als de activiteit van SHIP2 verhoogd zijn in kanker. Daarnaast zagen we dat een verhoogde SHIP2 expressie gecorreleerd is met een slechtere uitkomst voor patiënten. SHIP2 verbeterde cel migratie en invasie *in vitro*, en verminderde de celadhesie van darmkanker cellen. Wanneer tumor cellen behandeld werden met een remmer van het SHIP2 enzym resulteerde dit in een dosis-afhankelijke sterfte van darmkanker cellen. Daarnaast zorgde deze remmer ervoor dat de cellen vatbaarder werden voor chemotherapie. Uit deze resultaten bleek derhalve dat SHIP2 inderdaad werkt als oncogen in darmkanker, waarmee we een nieuw therapeutisch doelwit voor deze ziekte hebben geïdentificeerd.

Nadat we hadden ontdekt dat fosfatasen potentieel gebruikt zouden kunnen worden als aangrijpingspunten voor behandeling, zijn we in **hoofdstuk 7** gaan kijken naar de realistische mogelijkheden om dit te doen. Jarenlang zijn onderzoekers, maar ook de farmaceutische industrie, niet geïnteresseerd geweest in de ontwikkeling van medicijnen tegen fosfatasen, omdat dit als 'onmogelijk' of 'te moeilijk' werd geacht. Fosfatasen hebben namelijk onderling grote overeenkomsten, waardoor het specifiek benaderen van 1 fosfatase erg lastig zou zijn. Echter, op basis van gegevens die gepresenteerd werden op het 'Europhosphatase 2015' congres, is duidelijk geworden dat de algemene mening hierover veranderd is. Er is steeds meer bekend over de structuur en het werkingsmechanisme van deze eiwitten, waardoor specifiek gerichte behandelingen niet langer onmogelijk zijn. Het lijkt aannemelijk dat de fosfatasen die werken als tumor suppressor minder geschikt zijn als behandeldoelwit, aangezien remmen van de activiteit deze eiwitten juist tot verdere activering van kanker signalen zou leiden. Er is echter gebleken dat het mogelijk is om de tumor onderdrukkende fosfatasen te reactiveren in tumor cellen, wat een mogelijke nieuwe strategie kan zijn voor de behandeling van kanker. Bovendien hebben wij in de vorige hoofstukken een aantal fosfatasen geïdentificeerd die als oncogenen werken in plaats van tumor suppressor genen. Net als de voorgaande jaren gedaan is voor de oncogene kinasen, is er nu veel aandacht voor het ontwikkelen van inhibitoren van dergelijke oncogene fosfatasen. Dit heeft inmiddels geleidt tot de ontwikkeling van activiteits-remmers van PTP1B, die veelbelovende resultaten hebben laten zien in het laboratorium en daarom binnenkort getest zullen gaan worden op patiënten. Vergelijkbare inhibitoren worden nu ook ontwikkeld tegen oncogene fosfatasen als SHP2 en LMWPTP. Hopelijk kunnen deze nieuwe ontwikkelingen in de nabije toekomst bijdragen aan de behandeling van kankerpatiënten.

Tot slot hebben wij in in **hoofdstuk 8** de zogenaamde Wnt/β-catenine signaalroute onderzocht. De overgrote meerderheid van darmkankers hebben een afwijking in deze signaalroute, als gevolg van een mutatie in het β-catenine gen zelf of het aanverwante gen APC. Dergelijke mutaties leiden tot een verhoogde β-catenine signalering, resulterend in een ongeremde celdeling. Het type APC mutatie kan verschillen tussen tumoren, en elke mutatie zorgt voor een eigen mate van β-catenine signalering. Ieder celtype lijkt een ander niveau van β-catenine signalering nodig te hebben om uit te groeien tot een tumor. Dit principe is omschreven als het "just-right" model, maar hoewel dit concept algemeen geaccepteerd is, ontbrak het directe bewijs hiervoornog. In **hoofdstuk 8** hebben wij geprobeerd om dit directe bewijs te leveren, door het β-catenine signaleringsniveau te verlagen in een muis model. Het gevolg hiervan was dat het type tumoren wat deze muizen ontwikkelen verschoof van darmtumoren naar borsttumoren, waarmee wij het directe bewijs hebben geleverd dat het niveau van β-catenine signalering een bepalende factor is in de ontwikkeling van kankers van verschillende origine.

In het kort zouden we kunnen zeggen dat wij, met het werk gepresenteerd in dit proefschrift, laten zien dat fosfatasen een stuk belangrijker zijn voor de maligniteit van tumoren dan men voorheen aan nam. Wij presenteren drie fosfatasen, LMWPTP, SHIP2 en PTP1B, die allen verhoogd zijn in kankercellen, en daar bijdragen aan cel overleving, resistentie en metastaserings-processen. Hoewel wij ons nu hebben beperkt tot prostaat en darm-tumoren is het heel wel mogelijk dat fosfatasen ook in andere tumor-typen een oncogene rol vervullen. Het remmen van deze fosfatasen is dan ook mogelijk een goede strategie in de bestrijding van verschillende tumoren. Verder onderzoek zal moeten aantonen in hoeverre andere fosfatasen tumorgroei bevorderen en in welke typen kanker. Wij hopen dan ook dat ons werk internationaal onderzoekers zal aansporen fosfatasen nader te bestuderen, en dat dit onderzoek een stap is in de richting van nieuwe medicamenteuze oplossingen voor deze, nog altijd dodelijke, ziekte.

Chapter 11

Appendix

Dankwoord
List of Publications
PhD portfolio
About the author

Dankwoord

Ondanks dat mijn naam op de voorkant van dit proefschrift staat, had dit boek niet tot stand kunnen komen zonder de enorme support die ik van allerlei kanten heb mogen ontvangen, op wat voor manier dan ook.

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Beste **Maikel**, je bent echt een professor zoals een professor moet zijn. Je zit vol met spectaculaire ideeën die je op de mooiste manieren kunt formuleren, en voor elk probleem of onverwachtse uitkomst weet jij weer een alternatieve pathway tevoorschijn te toveren waar de mogelijke oplossing in verscholen zit. Ik heb daar heel veel respect voor en heb daar ook enorm van genoten en veel van geleerd. Op het begin is het lastig om je echt te leren kennen, maar gaandeweg mijn promotie merkte ik dat ook bij jou de deur altijd voor iedereen open staat. Hier heb ik ook gretig gebruikt van gemaakt toen ik bezig was met mijn sollicitaties voor de MDL-opleiding. Je hebt me in die tijd ook altijd van waardevolle adviezen voorzien, ook hier ben ik je erg dankbaar voor!

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Mijn team aan paranimphen (je hebt er eentje extra nodig als de allochtoon wellicht het land uitgezet wordt..) **Wesley**, **Rik** en **Evelyn**! We zijn met z'n allen zo goed als tegelijk begonnen aan onze promoties, en daardoor kregen we gelijk een band. Dit is ook uitgegroeid van collega's naar goede vrienden. Doordat jullie er waren (en een aantal anderen, maar daar kom ik later op terug), ben ik nooit met een naar gevoel naar mijn werk gekomen. De eindeloze bakjes koffie als er leuks - of minder leuks - te melden was. Het put-putten op onze kamer, de feestjes, Cadzand, borrels, verjaardagen, het was allemaal leuker doordat we zo'n geweldig groepje hadden. Ik ben dan ook vereerd dat jullie mijn team aan paranimphen willen vormen!!

Wesley, m'n maatje vanaf dag 1, omdat we allebei als arts begonnen aan een labonderzoek. Hierdoor hebben we elkaar veel kunnen helpen en liepen we tegen de zelfde problemen aan. We hebben je gelukkig snel kunnen overhalen om naar onze "Man Cave" te komen in de L-vleugel, en daarna heb je deze plaats nooit meer af hoeven staan. Ik heb ook genoten van onze congressen samen. Eerst samen naar Orlando, waar ik zelfs nog even met jou en Cin mee kon naar Universal Studios, daarna nog naar Chicago, waar we denk ik een fantastische tijd hebben gehad! Ook buiten het werk het vaak dingen samen gedaan, waardoor je echt een waardevolle vriend bent geworden. Ik hoop dan ook echt met heel mijn hart dat je in de toekomst naast me staat als MDL-arts, want er is niemand die ik het meer gun dan jij!

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List of publications

1. **Elmer Hoekstra**, Maikel P. Peppelenbosch, Gwenny M. Fuhler. The role of protein tyrosine phosphatases in colorectal cancer, *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 2012*

2. Elvira R.M. Bakker, **Elmer Hoekstra**, Patrick F. Franken, Werner Helvensteijn, Carolien H.M. van Deurzen, Wendy van Veelen, Ernst J. Kuipers and Ron Smits. β-Catenin signaling dosage dictates tissue-specific tumor predisposition in *Apc*-driven cancer, *Oncogene (2012)*

3. Auke P. Verhaar, **Elmer Hoekstra**, Angela S. W. Tjon, Wesley K. Utomo, J. Jasper Deuring, Elvira R. M. Bakker, Vanesa Muncan & Maikel P. Peppelenbosch. Dichotomal effect of space flight-associated microgravity on stress-activated protein kinases in innate immunity. *Scientific reports (2014)*

4. **Elmer Hoekstra**, Liudmila L. Kodach, Asha M. Das, Roberta R. Ruela-de-Sousa, Carmen V. Ferreira, J.C. Hardwick, C. Janneke van der Woude, Maikel P. Peppelenbosch, Timo L.M. ten Hagen, Gwenny M. Fuhler. Low molecular weight protein tyrosine phosphatase (LMWPTP) upregulation mediates malignant potential in colorectal cancer. *Accepted for publication Oncotarget 2015*

5. **Elmer Hoekstra*,** Roberta R Ruela-de-Sousa*, A. Marije Hoogenland, Karla C. Queiroz, Maikel. P Peppelenbosch, Andrew P. Stubbs, Geert J.L.H. van Leenders, Guido Jenster, Carmen V. Ferreira, Hiroshi Aoyama, Gwenny M. Fuhler. *authors share first authorship. Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) predicts prostate cancer outcome by increasing the metastatic potential. *Accepted for publication European Urology 2015*

6. **Elmer Hoekstra**, Maikel P. Peppelenbosch, Gwenny M. Fuhler. Meeting report Europhosphatase 2015; Phosphatases as drug targets in cancer. *Conditionally accepted, Cancer Research*

7. **Elmer Hoekstra**, Asha M. Das, Marloes Swets, Wanlu Cao, C. Janneke van der Woude, Marco J. Bruno, Maikel P. Peppelenbosch, Peter J.K. Kuppen, Timo L.M. ten Hagen, Gwenny M. Fuhler. Increased PTP1B expression and phosphatase activity in colorectal cancer, results in a more invasive phenotype and worse patient outcome. *Submitted*

8. **Elmer Hoekstra,** Asha M. Das, Marcella Willemsen, Marloes Swets, Peter J.K. Kuppen, Christien J. van der Woude, Marco J. Bruno, Jigisha P. Shah, Timo L.M. ten Hagen, John D. Chisholm, William G. Kerr, Maikel P. Peppelenbosch and Gwenny M. Fuhler. *Submitted*

PhD portfolio

Name PhD student:	Elmer Hoekstra
Erasmus MC department:	Gastroenterology and Hepatology
PhD period:	November 2011-November 2015
Promotor:	Prof. Dr. Maikel P. Peppelenbosch
Co-promotor:	Dr. Gwenny M. Fuhler

General courses

2012 Basic and translational oncology, Molecular Medicine Postgraduate school, Erasmus MC

2013 Biomedical English Writing Course, Molecular Medicine Postgraduate school, Erasmus MC

2013 Selected for UEG basic science course, Use of human tissue in gastroenterology research, London, UK

2014 Selected for UEG basic science course, Hot topics in experimental GI cancer, Munich, Germany

Conferences - Oral presentations

2013 Spring Meeting of Dutch society for Gastroenterology (NVGE), Veldhoven, the Netherlands: *LMW-PTP is upregulated in primary colorectal cancer and affects cancer signaling pathways.*

2014 Spring Meeting of Dutch society for Gastroenterology (NVGE), Veldhoven, the Netherlands: *PTP1B expression and phosphatase activity are increased in primary colorectal cancer which leads to a more invasive phenotype.*

2014 Annual Day of the Molecular Medicine Postgraduate School, Rotterdam, the Netherlands: *PTP1B expression and phosphatase activity are increased in primary colorectal cancer which leads to a more invasive phenotype.*

2015 Spring Meeting of Dutch society for Gastroenterology (NVGE), Veldhoven, the Netherlands: *Lipid phosphatase SHIP2 functions as oncogene in colorectal cancer by regulating PKB activation.*

2015 Europhosphatase 2015; Phosphorylation switches and cellular homeostasis, Turku, Finland: *Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) predicts prostate cancer outcome by increasing the metastatic potential*

Conferences - Poster presentations

2013 Digestive Disease Week 2013, Orlando, United States of America: *LMW-PTP is upregulated in primary colorectal cancer and affects cancer signaling pathways.*

2013 Annual Day of the Molecular Medicine Postgraduate School, Rotterdam, the Netherlands: *LMW-PTP is upregulated in primary colorectal cancer and affects cancer signaling pathways.*

2013 United European Gastroenterology Week, Berlin, Germany: *LMW-PTP is upregulated in primary colorectal cancer and affects cancer signaling pathways.*

2014 Digestive Disease Week 2014, Chicago, United States of America: *PTP1B expression and phosphatase activity are increased in primary colorectal cancer which leads to a more invasive phenotype.*

2014 United European Gastroenterology Week 2014, Vienna, Austria: *PTP1B expression and phosphatase activity are increased in primary colorectal cancer which leads to a more invasive phenotype.*

2015 Annual Day of the Molecular Medicine Postgraduate School, Rotterdam, the Netherlands: *Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) predicts prostate cancer outcome by increasing the metastatic potential*

2015 Digestive Disease Week 2015, Washington, United States of America: *Lipid phosphatase SHIP2 functions as oncogene in colorectal cancer by regulating PKB activation.*

Scientific awards and grants

2012 9th Mark van Blankensteijn SLO student award – award for best internship at Gastroenterology Department Erasmus MC

2013 Erasmus Trustfonds Travel Grant

2014 Erasmus Trustfonds Travel Grant

2014 Dutch Society for Gastroenterology Travel Grant

2015 Erasmus Trustfonds Travel Grant

2015 Europhosphatase Travel Grant, Turku, Finland

Teaching activities

2014 Marcella Willemsen, Bachelor student Health and Life sciences

Other activity

2012 Rocket Launch Esrange Space Center, Kiruna, Sweden

About the author

Elmer Hoekstra was born in Leiderdorp, The Netherlands, on July 11th, 1986. He was raised by his beloved parents Teun en Sonja Hoekstra, and grew up together with his brother and sister, Menno and Femke. In 2004, he finished his VWO-atheneum secondary school and started studying biomedical sciences at the University of Amsterdam (UvA). However, after one year he switched to the Erasmus University Rotterdam to start his medical school. In 2011 he performed his graduation research at the laboratory of Gastroenterology and Hepatology, under supervision of Dr. Ron Smits. His research was entitled "The role of ß-catenin signaling dosage in colorectal cancer". After obtaining his medical degree in 2011, he started a PhD project on the role of phosphatases in cancer at the department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam, The Netherlands. This project under supervision of Dr. Gwenny Fuhler and Prof. Maikel Peppelenbosch. After finishing his PhD, Elmer is going to start his Gastroenterology training at the Leiden University Medical Center.