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Development of tumour therapies: from target validation of TTLL12 to tests of a small molecule XRP44X in pre-clinical models of cancer

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Dedicated to the French Republic

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Contents

Abstract

Cancer of the prostate gland in France is the most prevalent cancer in men by incidence and the 3rd most deadly cancer type, partly due to limited therapeutic options. More specific therapeutic agents need to be developed.

TTLL12 protein and the Elk3 transcription factor are overexpressed in prostate cancer and have been validated as therapeutic targets. TTLL12 is involved in tubulin de-tyrosination and affects mitosis.

The aims of the project were to extend knowledge on the mechanisms of TTLL12's effects.

The results obtained demonstrate that TTLL12's involvement in tubulin tyrosination and H4K20 trimethylation are independent of its effect on mitosis.

Abbreviations

AR	Androgen receptor
ADT	Androgen deprivation therapy
ATP	Adenosine triphosphate
Arid1A	AT Rich interactive domain 1A (SWI-Like)
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
CRPC	Castration-resistant prostate cancer
CHD1	Chromodomain-helicase-DNA-binding protein 1
CCP	Cytosolic carboxypeptidase
CDK1	Cyclin-dependent kinase 1
DTT	Dithiothreitol
ETS	E-twenty-six transcription factor
EP300	E1A binding protein p300
ERG	ETS-related gene
EZH2	Enhancer of zeste homolog 2
ELISA	Enzyme-linked immunosorbent assay
FOXA1	Forkhead box protein A1
FOXP1	Forkhead box protein P1
FOXO3	Forkhead box O3
FACS	Fluorescence-activated cell sorting
GSTP1	Glutathione S-transferase P
GTPase	Guanosine 5'-triphosphatase
GEF	Guanine nucleotide exchange factor
GEM	Genetically engineered mouse models
GTP	Guanosine 5'-triphosphate
Glu-tub	Detyronised tubulin
HDAC6	Histone deacetylase 6
HIF-1α	Hypoxia-inducible factor 1-alpha
IGBMC	L'Institut de génétique et de biologie moléculaire et cellulaire
H4K20me3	Histone H4 trimethylated on lysine 20
K-Ras	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
KDM6A	Lysine (K)-specific demethylase 6A
KIF2A	Kinesin heavy chain member 2A
MYC	V-Myc avian myelocytomatosis viral oncogene homolog
MLL2/3	Histone-lysine N-methyltransferase MLL2/3
miR	MicroRNA
MCAK	Mitotic centromere-associated kinesin
MEM	Modified Eagle’s medium
NCOA2	Nuclear receptor coactivator 2
NCOR2	Nuclear receptor corepressor 2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PTEN	Phosphatase and tensin homolog
PHLPP1	PH domain and leucine rich repeat protein phosphatase 1
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PSA	Prostate-specific antigen
PCR	Polymerase chain reaction
p38	p38 mitogen-activated protein kinase
PMSF	Phenylmethanesulfonyl fluoride
PBS	Phosphate buffered saline
RAF1	V-Raf-1 murine leukemia viral oncogene homolog
Rb1	Retinoblastoma 1
RNAi	RNA interference
SPOP	Speckle-type POZ protein

SIRT2	NAD-dependent deacetylase sirtuin-2
SDS	Sodium dodecyl sulphate
SWI–SNF	SWItch/Sucrose non-fermentable complex
TMPRSS2	Transmembrane protease, serine 2
TTL	Tubulin tyrosine ligase
TG	Transglutaminase
TTLL12	Tubulin tyrosine ligase like 12
TBP	TATA binding protein
TRIS	Tris(hydroxymethyl)aminomethane
TBS	Tris-buffered saline
αTAT	Alpha-tubulin N-acetyltransferase
+TIP	plus-end tracking protein

1. General introduction

1.1 Cancer as a pathology

Cancer is a common pathology [1] characterized by abnormal cell growth and ability of cells to invade surrounding tissues. Malignant cells result in abnormal tissue growth referred to as a tumour. The growing number of genetic alterations associated with cancer suggest that tumour formation has a genetic basis [2]. However, only in less than 10% of cases cancer occurs due to hereditary factors, with lifestyle choices (e.g. diet and exercise) playing an increasingly central role disease formation [3]. Different types of cancer are categorized according to the tissue of origin: carcinoma, for example, is a cancer that derives from epithelial tissue. The most common treatment options for cancer therapy are surgery, chemotherapy and radiotherapy and prognosis often depends on the time of diagnosis and the location and type of cancer.

1.2 Prostate cancer

Prostate cancer is the most common solid tumour in Europe. World incidence and death from this disease are 127.7 and 10.8 cases per 100,000 men respectively [4]. Epidemiological studies associate prostate tumour incidence with risk factors such as age, ethnicity and heredity [5, 6]. The majority (95% of cases) of the disease is diagnosed as adenocarcinoma, which is defined as abnormal growth of glandular epithelial cells of prostate acini [7]. Prostate cancer is unique in its propensity to metastasize to bones, resulting typically in osteoblastic formations [8]. Common symptoms depend on the disease stage and applied treatment, and include difficulty in urinating, pain, erectile dysfunction, while advanced stage disease can be lethal.

Prostate imaging, digital rectal examination and biopsy are used to diagnose the disease [9, 10]. Prostate specific antigen (PSA) is a glycoprotein produced and secreted by prostate that is often

elevated in the serum of prostate cancer patients. It has been used for many years in prostate cancer diagnostics and screening. However, while increasingly popular, recently discovered limitations have made its use controversial [11].

Prognosis of prostate cancer is determined from a range of variables relating to abnormalities in normal prostate tissue differentiation, invasion of surrounding healthy tissues, and spreading and establishment of additional tumours at distant sites in the body. The degree of dedifferentiation of prostate tissue is determined on the Gleason grading system, and represented by the Gleason score [12]. The Gleason score ranges from 2 to 10, where a higher number describes more advanced disease, and is determined by architecture of acini when prostate tissue, taken by biopsy, is stained and examined microscopically. In addition, the TNM system (tumour, lymph node, metastasis) is used to distinguish between patients with localized and advance stage tumours [13]. TNM system evaluates tumour size, involvement of local lymph nodes and presence of distant metastases. Efforts are underway to establish a model of prostate cancer progression based on genetic alterations [14, 15].

Treatment options for prostate cancer are grade-specific and include radical prostatectomy, radiation therapy and hormonal therapy. At present, metastatic disease is considered as not curable [16].

Prostate cancer mechanisms are multifaceted. They may be genetic and epigenetic. Many classical and novel pathways are involved (reviewed in [17, 18]).

The phosphoinositide-3-kinase (PI3K) pathway is involved in cell proliferation, survival and invasion and is altered in 25-70% of cases of prostate cancer. The most common alterations being loss or mutations of PTEN, loss of PHLPP1 or amplification and gain of function of the PI3K catalytic subunit PIK3CA gene (reviewed in [19]).

Ras signalling pathway is commonly involved in metastatic stage of prostate cancer, however mutations in the pathway's components are not common. K-Ras, Raf1 and BRAF fusions can also be involved [17].

p53 tumour suppressor is lost in 25-40% or is dysfunctional (mutated) in 5-40% of cases of prostate cancer. p53 is responsible for arrest of the cell cycle, triggering of apoptosis and DNA repair in normal conditions. Retinoblastoma 1 (Rb1), another gene involved in the cell cycle progression check point, is inactivated in up to 45% of cases of castration-resistant prostate cancer (CRPC) [17]. The MYC oncogene, that promotes cycle progression and cell survival, is amplified in 5-40% of cases of neuroendocrine adenocarcinoma [18].

PI3K, Ras, p53, Rb and MYC pathways and proteins are not uniquely altered in prostate cancer, but in many other tumours too. There are, however, genes and pathways that are more specific to prostate tumours. They can be grouped to those affecting androgen receptor (AR) signalling and ETS genes rearrangements.

AR signalling is an important component of prostate physiology. AR is a ligand-dependent nuclear transcription factor that is activated by androgens (testosterone, dehydroepiandrosterone and dihydrotestosterone). In normal conditions, prostate development and maintenance depend on AR signalling, like for example prostate epithelium growth [20]. Consequently, it is not surprising that AR increased activity, rendered by gene amplification, point mutations and alteration in splicing, is frequent in prostate cancer (up to 58%). It is hypothesized that abnormal AR signalling becomes the driver of carcinogenesis as a result of therapies targeting AR receptor that put a selective pressure on cells [17, 21]. Additionally, genes involved in AR signalling can be altered in prostate cancer. They include NCOA2, NCOR2, EP300, FOXA1, FOXP1, FOXO3 [17].

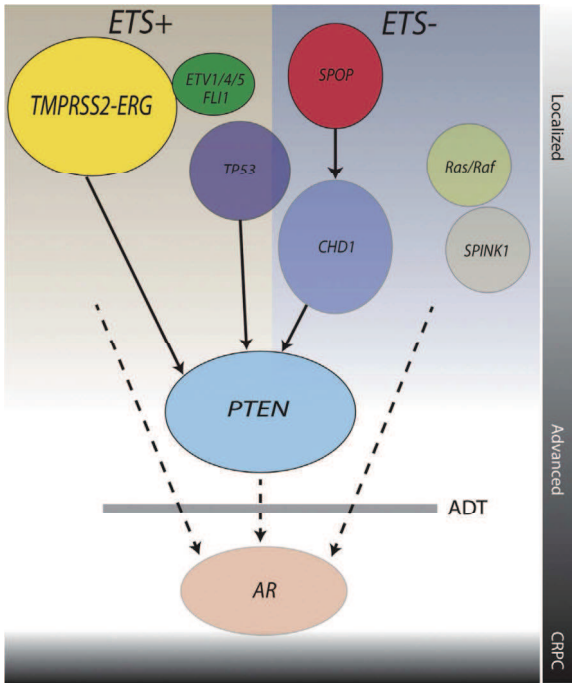

Figure 1. Genomic lesions in the timeline of prostate cancer. Genes with common genomic lesions (including mutation, rearrangement, or copy number alteration) are shown. Solid arrows designate a temporal relationship between events; presumptive "early" lesions are at the top, with "later" lesions below. Tumours with ETS fusions (ETS^+) are shown on the left; ETS^- tumours are shown on the right. ADT - androgen deprivation therapy, CRPC - castration-resistant prostate cancer [19].

The most common type of genetic alteration in prostate cancer is ETS gene fusions. They can be found in up to 79% of cases of prostate biopsies, and the most common fusions occur between the TMPRSS2 protease and ERG transcription factors of the ETS family. Many ETS genes are androgen-regulated [17].

At present, a number of systematic sequencing studies offer a new glimpse of the molecular subclasses of prostate cancer and their potential timeline (**Fig. 1**). However, this new scheme provides only a limited model for the development of disease. For example, CDH1 inactivation has been proposed to promote AR-dependent rearrangements, including TMPRSS2:ERG fusion [22].

A history of familial prostate cancer increases risk of developing the disease by 2-7 fold and is linked to approximately 10% to 15% of cases. Although 15-30 different loci from 10 distinct chromosomes are believed to predispose to prostate cancer development, no single highly-penetrant prostate cancer susceptibility gene has been identified. It is currently accepted that hereditary prostate cancer relies on the cumulative effect of a large number of genes which individually exert little influence on its development [23-26].

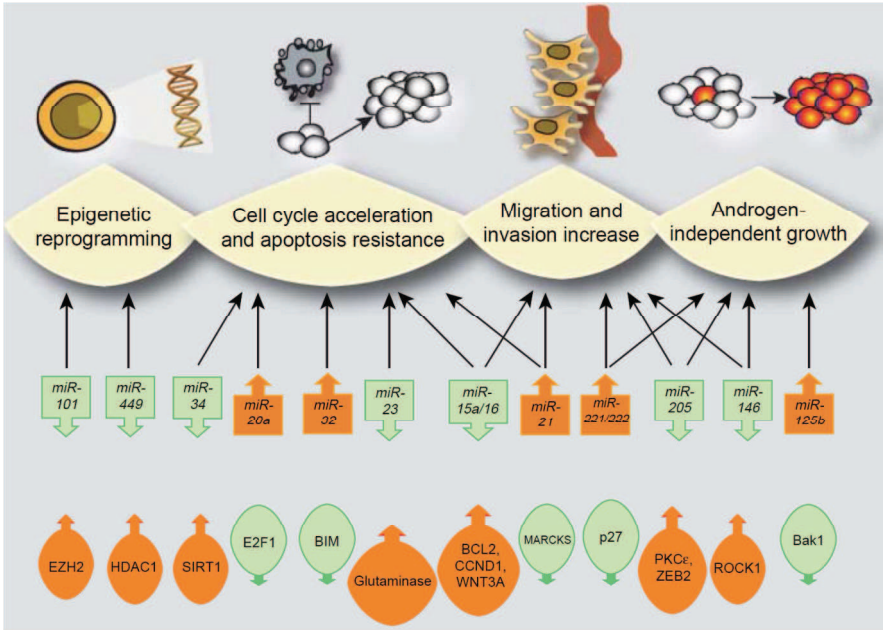

Figure 2. miRs whose physiological role in prostate cancer has been thoroughly investigated. miR targets and functions, such as those described in prostate cancer studies, are shown [27].

The epigenetic nature of prostate cancer pathogenesis is also an area of an increasing attention. SPOP, CHD1, KDM6A, EZH2, MLL2/3 are the genes involved in chromatin modifications that have been found altered in prostate cancer [17, 19]. Increased promoter methylation of the GSTP1

gene is seen in 90% of cases of prostate cancer and many others methylation patterns are associated with various stages of the disease (reviewed in [28]). MiR-21, miR-221/222, miR-15a/16-1 have been reported to alter apoptosis; miR-125b, miR-146a, miR-141, miRs-221/222, miR-331-3p were found to be AR responsive or to modulate AR signalling [28]. Some microRNAs are also involved in invasion and epigenetic modulation in prostate cancer (**Fig. 2**) (reviewed in [27]).

1.3 Microtubules: importance and as a therapeutic target

Microtubules are a ubiquitous component of the cellular cytoskeleton. The roles of microtubules are numerous and include cell division, maintaining the structure of the cell, cell polarity maintenance, intracellular transport and others. Microtubules are hollow prolonged structures made of polymerised α- and β-tubulin dimers and are dynamic, e. g. they polymerise and depolymerise depending on the needs of the cell [29]. Tubulin assembly and disassembly (polymerisation and depolymerisation) are GTP-dependent. When polymerising, α- and β-tubulin dimers carry two GTPs. GTPs can become hydrolysed and this triggers microtubule disassembly [30].

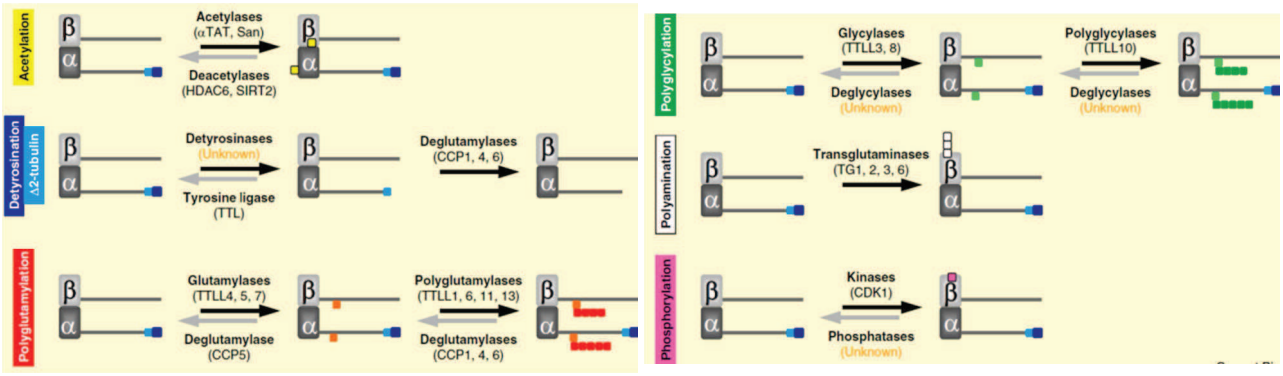

Figure 3. Enzymes involved in tubulin posttranslational modifications. αTAT - α-tubulin N-acetyltransferase, HDAC6 - histone deacetylase 6, SIRT2 - sirtuin 2, TTL - tubulin tyrosine ligase, TTLL - TTL-like, CCP - cytosolic carboxypeptidase, TG - transglutaminase, CDK1 - cyclin-dependent kinase 1 [31].

As many other proteins in the cell, tubulin is subject to a number of posttranslational modifications that occur mostly on polymerised tubulin (microtubules). These include tyrosination, acetylation, Δ2-tubulin formation, phosphorylation, glutamylation, glycylation and ubiquitination (reviewed in [32, 33]). Given that microtubules have a fairly homogenous structure, how is it possible

that microtubules perform so many different functions? It is proposed that the posttranslational modifications are the key [32]. These modifications regulate microtubules organelle-specific properties by allowing the recruitment of relevant protein complexes. This complex system has even been referred to as a "the tubulin code" [34, 35]. Among the numerous enzymes that modify tubulin, the TLL family is one of the most heavily implicated (**Fig. 3**). The modifications take place on microtubule polymers (microtubules), while their removal mostly occurs on soluble tubulin (reviewed in [31, 36]).

Compound class	Approved agents	Effect(s) on microtubules
Taxanes	Paclitaxel Docetaxel	Polymerization and/or stabilisation
Vinca alkaloids	Vinblastine Vincristine Vinorelbine	Depolymerization and/or destabilisation
Epothilones	Ixabepilone	
Halichondrin b		

Table 1. Classes of microtubule-biding agents (adapted from [37]).

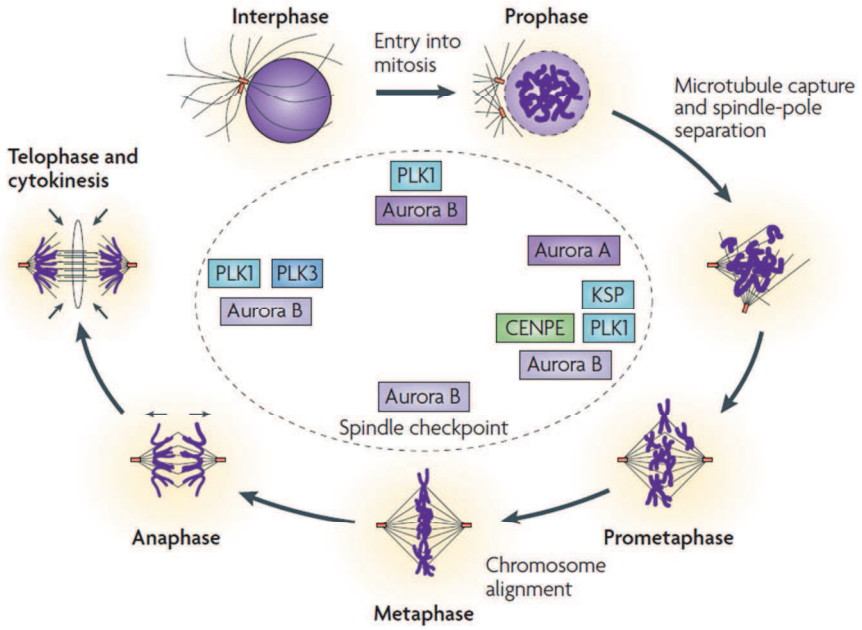

Figure 4. The phases of mitosis and proteins against which mitosis targeting compounds are being developed [38].

Given the important role microtubules play, it not surprising they have been pharmacologically targeted for a few decades in cancer therapy. The mitotic spindle is involved in chromosomal segregation during cell division. Microtubules are a key and dynamic component of mitotic spindles. Most of the compounds that target tubulin, and have seen use in cancer therapy, act by directly binding to tubulin in microtubules. They either stabilise microtubules so that they cannot disassemble when necessary, or destabilise so that microtubules are unable to elongate [39] (**Table 1**). Thus, frequently dividing cells, that often comprise a large part of a tumour, are particularly sensitive to microtubule-targeting therapy. However, microtubule-targeting cancer therapy has a few drawbacks, the major ones being high toxicity and eventual cancer resistance to treatment (reviewed in [37, 40]). Currently, new compounds that target tubulin directly are being developed [37] as well as new ways to target mitosis (reviewed in [38]) **(Fig. 4**).

Tyrosination/detyrosination, one of the tubulin posttranslational modifications, comprises addition/removal of tyrosine from/to α-tubulin on microtubules. The tyrosination/detyrosination cycle is involved in differential recruitment of molecular motors, plus-end tracking and microtubule protein binding. There is evidence that reduced tubulin tyrosination is associated with tumour invasiveness and poor prognosis, and proper tyrosination levels are necessary for correct neuronal organisation. Tyrosination is catalysed by Tubulin tyrosine ligase (TTL) and detyrosination by a not well characterised tubulinyl-tyr carboxypeptidase [32, 41]. Cytosolic carboxypeptidase 1 has been suggested for this role [33].

1.4 Histones and H4K20 methylation

Histones are the key component of chromatin. Histones hold chromosomal DNA in organised structures, called nucleosomes. Nucleosomes, in turn, are the structural units of chromatin. Histones are subject to numerous posttranslational modifications that affect gene expression, DNA repair, replication, mitosis, and oncogenesis (reviewed in [42-44]). Histone modifications either change chromatin structure or regulate the binding of chromatin factors, thus modifying access of transcriptional factors and complexes to DNA [42]. The fact that there are several families and subfamilies of histones, that they can be modified on different parts and that a number modifications exist, makes this area of biology particularly complex. Histone modifications are involved in epigenetics – where genetically relevant changes to genes do not implicate change in nucleotide sequence.

Methylation is one of the modifications of histones. It is “widespread type of chromatin modification that is known to influence biological processes in the context of development and cellular responses” [45]. Histone methylation is known to be involved in ageing, neurodegenerative diseases and DNA repair (reviewed in [45-47]). Methylation largely takes place on the side chains of arginines and lysines and does not change their electric charge. Methylation is a multi-layered modification: arginines may become mono-, symmetrically or asymmetrically di-methylated, and lysines - mono-, di- or tri-methylated [42]. Histone methyltransferases methylate lysines and generally have SET domains [42]. Methyl groups are removed from histones by histone demethylases.

Methylation of histone 4 on lysine 20 (H4K20) is important for DNA damage repair, DNA replication and chromatin compaction. In particular, mono- and dimethylated histone 4 on lysine 20 (H4K20me1,2) are involved in DNA replication and DNA damage repair, whereas trimethylated H4K20 (H4K20me3) is a mark of heterochromatic regions that are silenced. Pericentric chromatin of mitotic chromosomes is particularly enriched in H4K20me3 [43].

2. Tubulin tyrosine ligase-like 12 (TTLL12)

2.1 Introduction

Tubulin tyrosine ligase like 12 (TTLL12) is a Tubulin tyrosine ligase (TTL) family member. The TTL family of proteins has 14 members and TTLL12 is the least studied [48].

TTLL12 was identified in Dr. Wasylyks's laboratory as a differentially expressed RNA from macrodissected prostate tumours. They obtained pathologist-validated prostate cancer specimens, prepared RNA probes and hybridised them to comprehensive validated differential display [49] membranes spotted with differential display cloned cDNAs and reverse northern blotting. The signals were compared between control and anti-TTLL12 probes obtained from tumour and benign specimens. 58 clones gave differential signals, and subsequently 19 of them were selected for semiquantitative PCR analysis. Of these, one clone was selected for further analysis as it gave a differential signal on comprehensive validated differential display membranes; it was more expressed in tumour samples, as compared to benign samples, as shown by semiquantitative PCR, and was predicted to have enzymatic activity. Computational analysis revealed a highly conserved gene with TTL and SET-like domains [50], now called TTLL12. When compared to other TTL family members, a few features make it stand out: TTLL12 has a relatively long phylogenetic branch within the TTL family (**Fig. 5**), its TTL domain lacks some motifs that are present in other TTL family members and it has a SET-like domain [51].

TTL domains facilitate ligation of amino acids to tubulin and are around 350 amino acid residues long. Like many enzymes with an ATP-dependent carboxylate-amine/thiol ligase activity, the TTL domain has an ATP-grasp-like motif. TTL family proteins are known to ligate to the C-terminal ends of α/β tubulin tyrosine (TTL - tyrosine tubulin ligase, the founding member), glycine (TTLL3, 8, 10) or glutamate (TTLL1, 4, 5, 6, 7, 9, 11, 13) [51]

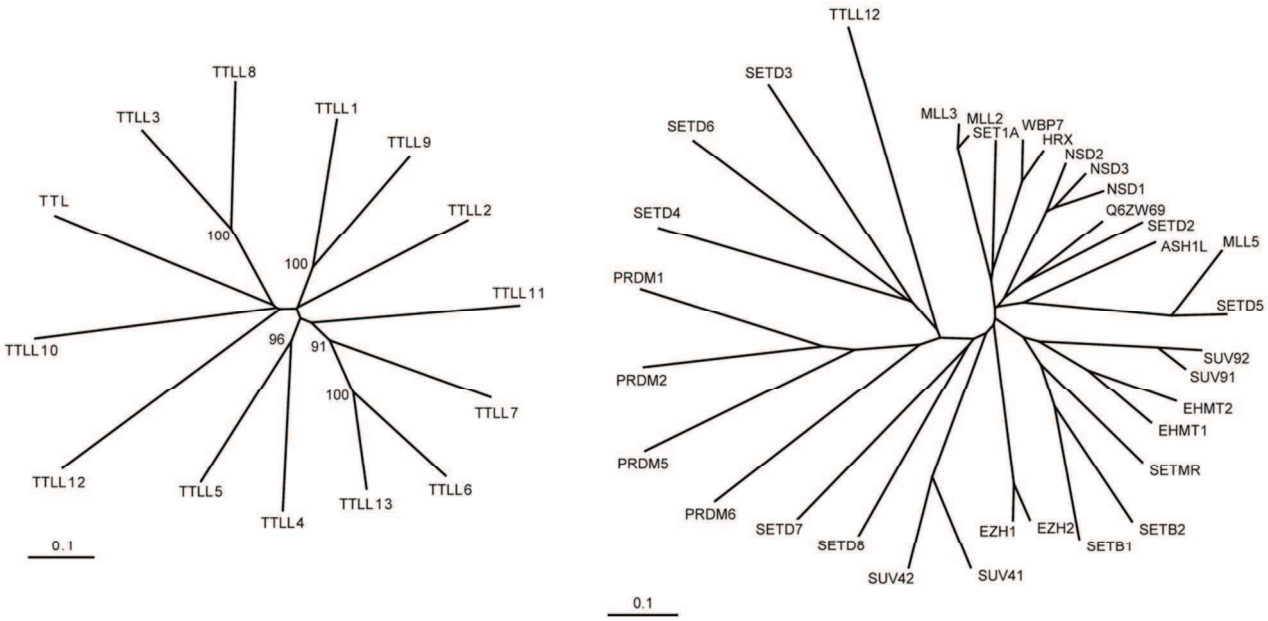

Figure 5. Phylogenetic analysis of the TTL domains and the SET-like domains of the human TTL family based on multiple sequence alignments. Bootstrap values are provided for significant nodes when they are > 80% [51].

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SET domains have been found in all known eukaryotes and are around 130 amino acids long. They facilitate methyl group transfer from S-adenosyl-L-methyonine to the N-amino group of lysine residues. In the case of histone lysine methyltransferases, transfer is to histones [52].

The Wasylyk laboratory developed antibodies against TTLL12, cloned the cDNA and explored TTLL12's expression levels in prostate cancer tissues by immunohistochemistry, tissue microarrays and *in situ* hybridisation. They found increased expression of TTLL12 in metastatic lesions, local recurrent tumours and prostatic intraepithelial neoplasia. Using immunocytochemistry and western blotting on prostate tissue and prostate tumour-derived cell lines, it was also shown that TTLL12's expression levels and subcellular localisation in cell lines reflect those in prostate tumours [50]. Additionally, the team used dual-label indirect immunofluorescence to explore TTLL12 localisation in DU145 prostate cancer cell line. They found TTLL12 to localise to intercellular bridges, midbodies, centrosomes, along mitotic spindles and vimentin filaments and that TTLL12, to a degree, colocalizes with α-tubulin and detyrosinated tubulin [50]. TTLL12 is similarly localised in HEp-2 cells [51].

More recently, TTLL12 was found to be the highest ranked discrimination autoantigen between benign and malignant prostate cancer tumours [53].

Since TTLL12 has a TTL domain, it might be involved in tubulin tyrosination. Dr. Wasylyk's laboratory thus explored the effect of TTLL12 levels on tubulin modifications. They found that

downregulation of TTLL12 expression by siRNAs in DU145 cells resulted in an increase of detyrosinated tubulin (glu-tub), Δ2-tubulin and polyglutamylated tubulin after 3 days. In HEp-2 clones that overexpress or underexpress TTLL12, detyrosinated tubulin was also found to be increased [50, 51].

As TTLL12 has a SET domain, indicating its possible involvement in histone methylation, and given that only H3 and H4 that can be methylated [54], levels of specific methylated forms of H3 and H4 were probed by western blotting in HEp-2 clones with stable overexpression or knockdown of TTLL12. These experiments revealed a positive correlation between H4K20 trimethylation and TTLL12 expression. Interestingly, heterochromatin protein HP1γ co-immunoprecipitates with TTLL12 from nuclear extracts of TTLL12-overexpressing HEp-2 cells [51].

The influence of TTLL12 on cell growth, the cell cycle and chromosome number was studied. Downregulation of TTLL12 in DU145, LNCaP, MDA-PCa and 22Rv1 prostate cancer cell lines resulted in reduced growth and prolonged mitosis duration. Prolonged mitotic duration was also found for HEp-2 cells with overexpressing or downregulated TTLL12. Overexpression of TTLL12 in HCT116 and HCT116 cells with depleted p53 results in increased chromosome copy number, and in HEp-2 cells affects spindle positioning [50, 51].

Microtubule-targeting drugs are known for their toxicity. This is largely due to the lack of specificity of their mode of action. Given the effect of TTLL12 on tubulin, TTLL12 activity has been targeted to indirectly modify tubulin [55]. Nitrotyrosine, which is structurally similar to tyrosine, is a molecule produced in some pathologic conditions, including cancer. It is used as a substitute for tyrosine in tubulin research, and its increased presence in tubulin correlates positively with inhibition of cell growth and survival. Nitrotyrosine was shown to be incorporated irreversibly in α-tubulin by TTL. TTL expression is relatively low is some tumours, while TTLL12 is overexpressed in prostate as well as head and neck cancers, suggesting an effect on the levels of nitrotyrosinated tubulin and an influence on cell survival. For this reason, a cell based ELISA screen was developed to discover molecules that could increase tubulin nitrotyrosination via inhibiting TTLL12. The screen used a small molecule library of 10,000 compounds and was designed to detect α-tubulin nitrotyrosination in the presence on nitrotyrosine in TTLL12-overexpressing cells. TTL-overexpressing cells were used as a positive control. The screen resulted in 74 hits. A number of these were then validated individually by treating a TTLL12-overexpressing clone and comparing levels of nitrotyrosinated α-tubulin.

The therapeutic potential of targeting TTLL12 is further substantiated by studies which found a strong inhibition of cell growth by paclitaxel or nocodazole in combination with nitrotyrosine, than by either compound alone. Additionally, in their respective mediums, highly metastatic prostate

cancer cells have less of nitrotyronised tubulin than non-metastatic prostate cancer cells PWR1E and RWPE1 [55].

2.2 Aims and outline

Being a key component of the cytoskeleton, microtubules have a vital role in many cellular processes. As such, they became a target of a major class of anti-cancer drugs and compounds that target tubulin. Although these drugs are efficient in controlling cell growth, their action is not precisely tumour cell-specific, resulting in a number of side effects in patients. Additionally, resistance to microtubule-targeting medicines is also an issue in cancer therapy. New ways to target tubulin are being developed. High TTLL12 levels lead to an increase in detyronised tubulin. Detyronised tubulin is increased in a number of cancers, similar to TTLL12 [50, 56-59]. High TTLL12 levels also lead to increased H4K20me3 (unpublished data). Additionally, TTLL12 prolongs the cell cycle (unpublished data) and it is not clear whether TTLL12's effect on detyronised tubulin and H4K20me3 levels is due to the cell cycle or not. Determining TTLL12 mechanism of action is an important task for further development of molecules against this protein.

The following strategy was set to achieve this aim: synchronizing TTLL12-overexpressing cells in cell cycle and measuring levels of detyrosinated tubulin and H4K20me3.

2.3 Results

2.3.1 TTLL12 increases tubulin detyrosination and H4K20 trimethylation independently of the cell cycle

To determine H4K20me3 and glu-tub levels in the context of TTL12 levels and the cell cycle, I used HEp-2 cell line clones [51] that overexpress TTLL12 (**Fig. 6A**) because they have a high amount of tyrosinated tubulin which makes it easy to measure its levels, and because HEp-2 clones overexpressing TTLL12 were already available in the laboratory. HEp-2 cells were synchronized to each cell cycle phase and western blotting was carried out to determine the levels of H4K20me3 and glu-tub in whole-cell protein extracts.

Although HEp-2 cells were originally derived from human larynx carcinoma, I tested the suitability of conditions used for the HeLa cell line [60, 61], since HEp-2 cells are considered now to be a HeLa contaminant [62].

Serum starvation was used to synchronize the cells in G1. In low serum, many types of cells stop proliferating, complete S and G2M phases and stop at the G1 checkpoint. The cells were plated and left for 24 hours to attach in 10% serum medium, the medium was changed to 0.1% serum for 24 hours, after which the cells were collected for cell cycle analysis by FACS. Under these conditions, there was no significant enrichment in the proportion of cells in G1 (**Fig. 6B**). Increasing the length of incubation in low serum to 68 hours, to allow slowly growing cells to reach the G1 checkpoint, did not result in a larger proportion of cells in G1 (data not shown).

Thymidine was used to synchronise cells in early S phase. Thymidine, due to its similarity with thymine, interferes with DNA synthesis and blocks cell cycle progression in S phase [63]. The cells were plated, incubated for 24 hours, medium with 2 mM thymidine was added and, after a further 24 hours, the cells were collected for cell cycle analysis by FACS. The majority of the cells (60% to 75%) were found to be in S phase (**Fig. 6B**).

For G2M synchronisation, a thymidine-nocodazole block was used. Nocodazole interferes with microtubules polymerisation thus blocking cell division. Cells were treated with thymidine, as described above, then kept in fresh medium for 3 hours (release from early S phase), then medium with nocodazole (100 ng/mL) was added and after 12 hours the cells were collected for cell cycle analysis by FACS. A large proportion of the cells were found to be in G2M phase (**Fig. 6B**). However, many cells were apoptotic (up to 35% according to FACS DNA content analysis and visual inspection, data not shown). Increasing the period of release from nocodazole to 4, 8, 16, 20 and 24 hours gave at best 67% in G1 at 8 hours.

The optimal approach was collection of cells at different time points following release from a double thymidine block (adapted from [60]). The cells (HEp-2 TTLL12-overexpressing and control clones) were plated and left for 24 hours to attach, thymidine (2 mM) was applied in fresh medium for 23 hours, the cells were released for 9 hours and thymidine was applied again for 23 hours. The cells were released by adding fresh medium and then collected every 2 hours for 24 hours and analysed for DNA content analysis by FACS (see **Fig. 6D**). Whole cell extracts were collected for western blotting for H4K20me3 and glu-tub. The cell cycle distributions for the released cells were around 85% in G1 after 14-16 hours, nearly 100% were in S phase after 2 hours and around 75% in G2M after 6-8 hours (**Fig. 6C, D**). Interestingly, TTLL12-overexpressing cells progressed through the cell cycle slower than controls, with an average delay of about 2 hours (**Fig. 6E, F**).

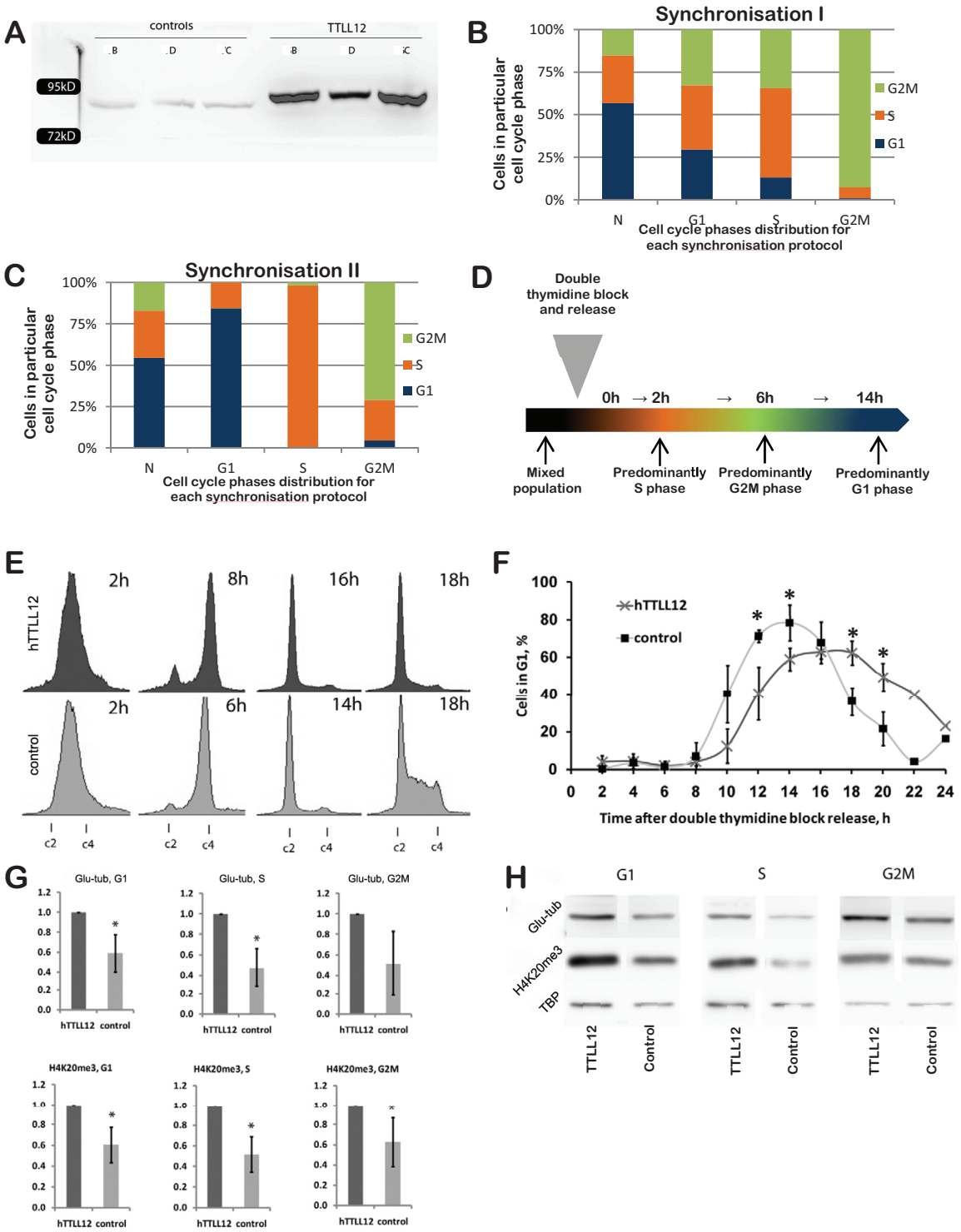

Figure 6. TTLL12 expression (**A**), cell cycle synchronisation (**B**, **C**, **D**), cell cycle progression (**E**, **F**), and detyronised tubulin (glu-tub) and H4K20 trimethylation levels (**G**, **H**) in HEp-2 cells and TTLL12-overexpressing clones. **A**. TTLL2 protein expression in TTLL12-overexpressing clones (hTTLL12 _B, _D, _C clones) and controls (Control_B, _D, _C clones). Ten times less concentrated whole cell protein extracts from TTLL12-overexpressing clones were blotted against controls with anti-TTLL12 antibody. The blotted nitrocellulose membrane was stained with ponceau to control for sample loading (data not shown). **B**. Proportion of cells in each cell cycle phase in normal growth conditions (N), synchronised with low serum for G1 phase (G1), with thymidine for S phase (S) and thymidine and nocodazole for G2M phase (G2M). Representative results from

one typical experiment are shown (Control_D clone). **C**. Proportion of cells in each cell cycle phase when synchronised for G1, S and G2M phases by double thymidine block followed by release for 16, 8 and 2 hours. Representative result from one typical experiment (hTTLL12 _D clone). **D**. Scheme of double thymidine block synchronisation. **E**. DNA content profile of TTLL12-overexpressing and control HEp-2 clones at time points at which there are maximum amounts of cells in a particular cell cycle phase after release from double thymidine block. C2 and C4 indicate standard HEp-2 chromosome set (G1 phase) and doubled chromosome set after S phase respectively. Representative image (hTTLL12 _C and Control _B clones). **F**. Amount of cells in G1 after double thymidine cell cycle block followed by release over the course of 24 hours. Average of 3 experiments (hTTLL12 _B, _C, _D vs. Control_B, _D clones). **G**, **H**. Glu-tub and H4K20me3 levels in HEp-2 cells and TTLL12-overexpressing clones at different stages of the cell cycle. Western blots (representative images) and their quantifications (3 repeats) of whole cell extracts for each cell cycle phase in TTLL12-overexpressing and control clones (hTTLL12 _C vs. Control_B clone for H4K20me3, hTTLL12 _C, _D vs. Control_B, _D clones for glu-tub). Cell cycle phases were determined by DNA content (FACS) as in E. Error bars represent standard deviation,*=P<0.05, Student's t-test.

Western blots were performed on whole-cell protein extracts obtained at those time points in which the cells were predominantly in the S1, G2/M and G1 phases (in most cases these were 2, 6 and 14 hours for control clones and 2, 8 and 16 hours for TTLL12-overexpressing clones). In TTLL12-overexpressing cells, the levels of H4K20me3 and glu-tub were higher in each cell cycle phase (**Fig. 6G, H**).

In summary, a double thymidine block proved to be the optimal method for synchronising cells. High TTLL12 levels result in increased H4K20 trimethylation and detyrosinated tubulin.

2.4 Discussion

2.4.1 TTLL12 increases tubulin detyrosination and H4K20 trimethylation independently of the cell cycle

It is known that TTLL12 overexpression results in increased detyrosinated tubulin, H4K20me3 levels and prolonged mitosis [50, 51]. TTLL12 has two domains of unknown function, SET and TTLL, which may confer the ability to modify tubulin tyrosination and H4K20 trimethylation. However, glu-tub and H4K20me3 levels are also likely to change during the cell cycle [43]. Thus it was not clear whether TTLL12 was affecting tubulin and H4K20 directly or as a secondary effect through the cell cycle. To investigate this, we used HEp-2 cells overexpressing TTLL12 which were synchronised, and levels of detyrosinated tubulin and H4K20me3 were measured.

Double-thymidine cell-cycle block with release allows efficient synchronisation of HEp-2 cells for S, G2M and G1 cell cycle phases. It also allows for accurate monitoring of cell cycle progression

after the release from the block. It might be expected that blocking cells in any cell cycle phase and releasing them from the block would be as useful as synchronising cells for each cell cycle phase separately. However in our hands it was not the case. The single thymidine block gave less synchronised cells than the double thymidine block. The reason for this might be that when thymidine is applied, some cells are already at their completion of S phase or at the beginning of G2M phase. These cells do not reach early S phase during the time when thymidine is present in the medium.

Moreover, cell synchronisation strategies, where synchronisation requires a block with a release, should be approached with caution. It is due to an alternative view of the cell cycle (where G1 phase used as a reference), called “continuum model”, which postulates that when cells are synchronised for a cell cycle phase (G1), they are not all resting at a particular cell cycle check point, but rather at different stages of a given cell cycle phase. This implies that when cells are released from the block, they would not enter the next cell cycle phase synchronously [64].

Serum starvation is used to synchronise cells for G1 phase. In our conditions the cell population was not enriched in G1 after serum starvation. Moreover, cell cycle distribution was different from normal distribution in HEp-2 cells. The reasons for this are unknown. Since serum starvation may influence cellular phenotype and experimental results [65], a decision was made not to proceed with this method. Additionally, synchronising cells for each cell cycle phase with different methods and then comparing biochemical features is not particularly a good strategy, as the compounds used for synchronisation are different. In our experiment, we used cells that were released from a compound (thymidine) into normal medium and only then analysed. This may have made our HEp-2 cell more comparable in their cell cycle phases.

Thymidine-nocodazole block resulted in many cells being in apoptosis. Nocodazole blocks mitotic spindle formation and cells cannot pass the spindle cell cycle checkpoint, resulting in cell death [66]. To succeed with this synchronisation strategy, a shorter time of exposure to nocodazole should be considered. Moreover, tubulin depolymerisation is associated with increased glu-tub levels [67] thus making it inappropriate to use nocodazole for the glu-tub level comparison because it depolymerises microtubules.

We found that in each cell cycle phase there is more glu-tub and H4K20me3. This indicates that it is not because TTLL12 affects mitosis that glu-tub and H4K20me3 levels are increased.

TTLL12 does not have enzymatic activity *in vitro* [51] and lacks amino acids essential for enzymatic activity in its TTLL and SET domains [50, 51]. Still, these domains are phylogenetically conserved, suggesting TTLL12 may have some basic function in the cell, like other conserved proteins,

and regulate tubulin detyrosination and H4K20 trimethylation indirectly. TTLL12 downregulation also increases glu-tub as well as polyglutamylated tubulin and Δ2-tubulin [50], indicating TTLL12 has a broader function as compared to other enzymes that normally catalyse a specific reaction.

Since the TTLL12-promoted increase in glu-tub appears to be a TLL12-dependent and not mitosis delay–dependent, could it be that higher glu-tub levels affect mitosis? Tubulin tyrosination determines microtubule interaction with kinesin 13 (motor protein) subfamily and microtubule plus-end tracking proteins (+TIPs). Two kinesin 13 proteins MCAK and KIF2A preferentially bind and actively depolymerise tyrosinated tubulin [68, 69]. Kinetochore microtubule depolymerisation is an event that occurs in mitosis and is facilitated by kinesins 13 [70]. This indicates that higher glu-tub levels could interfere with mitosis completion. +TIPs regulate microtubule dynamics and their interactions with other cellular components [71]. +TIPs with CAP-Gly domain associate with tyrosinated tubulin [33, 68], implying that increased glu-tub would alter the normal dynamics of microtubules. The mitotic spindle is a highly dynamic microtubule structure [71] and +TIPs regulate microtubules spatially and temporally during mitosis [72]. Therefore alterations to +TIPs binding to microtubules could affect mitosis. Further experiments are needed to test these hypotheses. Interestingly, TTLL12 colocalises with the mitotic spindle [50], suggesting that it is involved in mitosis. Additionally, RNAi profiling for genes involved in cell division identified TTLL12 as one of about 600 genes that are involved [73, 74], which is in agreement with our results .

With our present knowledge, it is difficult to predict how TTLL12 could indirectly increase H4K20 trimethylation.

TTLL12 is known to interact with SWI–SNF chromatin remodelling complex subunit Arid1A [75]. TTLL12 also interacts with SAP25 – a transcriptional corepressor mSin3 binding protein [76]. Expression of Rb and HP1α, genes that have indirect effect on H4K20 trimethylation [77-79], is altered by TTLL12 [51]. In addition, the presence of SET domain might not be related to histone methylations, since SET-domain proteins methylate other substrates as well [80]. Moreover, since histone lysine methylation is considered to be a relatively stable modification [43], it is perhaps not surprising that TTLL12's effect on H4K20me3 levels does not change during the cell cycle. Interestingly, whereas TTLL12 is now associated with cancer [50, 53, 81], high levels of H4K20me3 have only been shown to be a sign of poor prognosis in bladder cancer [82]. A number of other cancer types show low levels of H4K20me3 [83]. Taking everything into account, TTLL12 involvement in H4K20 trimethylation remains to be explored further.

3. Conclusion

Mitosis-targeting agents are only used in the last stages of prostate cancer. The reasons are high toxicity, resistance or simply ineffectiveness of compounds. More specific microtubule-targeting agents are needed. Posttranslational tubulin modifications provide such an opportunity, since tubulin modifications greatly affect microtubule functions. TTLL12 was discovered as a differentially expressed transcript in prostate cancer and *in vitro* analysis showed that it prolongs mitosis, inhibits tubulin tyrosination, promotes chromosome number increase and H4K20 trimethylation. Reduced tubulin tyrosination has been shown to be associated with cancer. This implies that there is an opportunity to target mitosis via TTLL12-modulated tubulin tyrosination. However, some mechanisms of TTLL12 involvement remained obscure: are TTLL12 effects cell cycle-dependent and could TTLL12, as a microtubule-modulator, be involved in cellular response to microtubule-targeting agents. The thesis results provide evidence to show that TTLL12 inhibits tubulin tyrosination and increases H4K20me3 in some more specific way and not via the cell cycle.

4. Materials and Methods

Cell culture. HEp-2 cells (ATCC CCL-23) were grown in modified Eagle's medium (MEM), 10% foetal calf serum, 0.1 mM nonessential amino acids, 1.0 mM Na-pyruvate, 40.0 mg/ml gentamicin at 37°C in a humidified 5% CO2 atmosphere.

The HEp-2 TTLL12-overexpressing and control clones are described in [50, 51]. Briefly, HEp-2 cells are stably transfected with the Flag-hTTLL12 expression plasmid or the empty pSG5-puro-Flag. Five positive and five control clones are available (hTTLL12 clones_A-E and Control_ A–E).

Cell cycle synchronisation by double thymidine block. HEp-2 control and TTLL12-overexpressing clones (Controls_B, _D, _C and hTTL12_B, _D, _C) $7.5x10^5$ cells were plated on 10 cm dishes to give 80% confluence in 4–5 days. 24 h after plating, the growth medium was replaced, and cells were grown for 23 h in the presence of 2 mM thymidine. Next, cells were washed three times with PBS and allowed to grow in fresh medium for 9 h prior to replacing it with thymidine supplemented medium for an additional 23 h. Cells were then washed three times in PBS, and fresh medium was applied. Thesse cells were collected at 2 h intervals over 18–24 h (**Fig. 1D**) and DNA content estimated using FACS analysis. In parallel, whole cell protein extracts were prepared to show protein expression levels. Time points at which cells were predominantly in the S1, G2/M and G1 phase were used in subsequent analysis through western blotting.

Cell cycle analysis. Cells were washed in PBS, fixed in ice-cold 70% ethanol for 1 h, washed in PBS to remove residual ethanol, treated with Rnase A (1 mg/mL) and triton X-1100 (0.1%) at room temperature for 2 h, and finally with 0.05 mg/mL propidium iodide prior to storage away from light at 4°C for subsequent DNA content analysis by FACS. A FACS Calibur cytometer (Becton Dickinson) and the CellQuest Pro software (ver. 5.2.1) were used for acquisition fluorometry and cell cycle analysis (ver. 6).

Antibodies for western blotting. See **Table 2**.

Antibody	Epitope	Reference	Use
hTTLL12, rabbit polyclonal	Amino acids 78–96	IGBMC, PAb2089	Western blotting 1:3000
H4K20me3 rabbit polyclonal	Residues 1 - 100 of Human Histone H4, tri methylated at K20	AbCam, ab9053	Western blotting 1:8000
GluT rabbit polyclonal	C-terminal amino acids of Glu tubulin GEEEGEE	AbCys, abC0101	Western blotting 1:1000
TBP mouse monoclonal	18 amino acids of human TBP	IGBMC, 3TF1/3G3	Western blotting 1:2000
Goat-anti-mouse IgG HRP	Mouse IgG heavy and light chain	Santa Cruz Biotechnology, SC-2005	Western blotting 1:7000
Goat-anti-rabbit IgG HRP	Rabbit IgG heavy and light chain	Santa Cruz Biotechnology, SC-2004	Western blotting 1:7000

Table 2. Antibodies used.

SDS-PAGE and western blotting. The cells were washed twice with cold PBS and lysed immediately with protein loading buffer (125 mM Tris base, 141 mM SDS, 185 mM HCl, 4.3 mM glycerol, 0.2 mM NaF, 0.1 mM NaVO4, 0.5 mM PMSF, 10 mM DTT, 0.625 mM β-mercaptoethanol). Even sample loading was ensured by immunobloting with antibodies against TBP protein. PageRulerTM Plus prestained protein ladder (Fermentas) was included as a marker of protein size. For TBP and hTTLL12 (3TF1/3G3) (1:2000) membranes were blocked

in PBS-T-milk (0.05% Tween, 10% fat-free milk); for H4K20me3 (1:8000) and detyrosinated tubulin (1:1000) in TBS-T-BSA (0.05% Tween, 3% bovine serum albumin (BSA, MP Biomedicals, 160069). Primary antibodies were diluted in 1% fat-free milk (Ragilait) (TLL12, TBP) or 1% BSA (H4K20me3, GluT), secondary (for TTLL12 and TBP detection) in 0.5% milk or (for H4K20me3 and detyrosinated tubulin detection) 0.1% BSA. Primary antibodies were applied overnight at 4°C, while secondary antibodies only for 1 h at room temperature; in between, and after antibody application, membranes were washed in PBS-T (TBP), or TBS-T (H4K20me3 and glu-tub). Chemiluminiscent signals were detected with ImageQuant LAS 4000 (General Electric); band intensity was quantified with the ImageQuant TL 7.0 software (GE).

Annexes

Publications

Semenchenko K., Wasylyk C., Zheng, H., Cheung H., Robertson D, Schlaken, J, van der Pluijm G., Wasylyk B. (2014). **Pre-clinical studies of a novel pyrazol XRP44X in prostate cancer mouse models.** *In preparation (Cancer Research)*

Robertson E. D., **Semenchenko K.** and Wasylyk B. (2013). **Crosstalk between Mdm2, p53 and HIF1-α: distinct responses to oxygen stress and implications for tumour hypoxia.** In S. Deb, and S. Deb, (Eds.), p53 mutations and MDM2 expression in cancer. Springer. *Accepted for publication*

Brants J., **Semenchenko K.,** Wasylyk C., Robert A., Carles A. et al. (2012) **Tubulin Tyrosine Ligase Like 12, a TTLL Family Member with SET- and TTL-Like Domains and Roles in Histone and Tubulin Modifications and Mitosis.** PLoS ONE 7(12): e51258. doi:10.1371/journal.pone.0051258

List of figures and tables

Thesis timeline

Year	2010								2011												2012													
Month	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2
TTLL12 in cell cycle																																		
Thesis + manuscript																																		
Conferences and meetings						→ 1.5 weeks per year, not 2014																														

1. Introduction générale

1.1 Le cancer comme pathologie

Le cancer est une pathologie courante [1] caractérisée par une croissance cellulaire anormale de cellules et la capacité à envahir les tissus environnants. Les cellules malignes donnent lieu à une croissance tissulaire anormale appelée tumeur. Le nombre croissant d'altérations génétiques associées au cancer suggèrent que la formation de tumeurs a une base génétique [2]. Cependant, seulement moins de 10% des cas de cancer sont due à des facteurs héréditaires, avec des choix de style de vie (par exemple de régime et d'exercice) jouant un rôle de plus en plus central dans la formation de la maladie [3]. Différents types de cancer sont classés selon le tissu d'origine: carcinome, par exemple, est un cancer qui dérive du tissu épithélial. Les options de traitement les plus courants pour le traitement du cancer sont la chirurgie, la chimiothérapie et la radiothérapie et le pronostic dépend souvent du moment du diagnostic, de la localisation et du type de cancer.

1.2 Le cancer de la prostate

Le cancer de la prostate est la tumeur solide la plus fréquente en Europe. L'incidence mondiale et de la mort de cette maladie sont 127,7 et 10,8 cas par 100 000 hommes respectivement [4]. Des études épidémiologiques associent l'incidence de la tumeur prostatique à des facteurs de risque tels que l'âge, l'origine ethnique et l'hérédité [5, 6]. La majorité (95% des cas) de la maladie est diagnostiquée comme adénocarcinome, qui est définie comme la croissance anormale des cellules épithéliales glandulaires des acini de la prostate [7]. Le cancer de la prostate est unique dans sa propension à former des métastases aux os, résultant généralement dans des formations ostéoblastiques [8]. Les symptômes courants dépendent du stade de la maladie et le traitement appliqués, et comprennent la difficulté à uriner, douleur, dysfonction érectile, alors que la maladie à un stade avancé peut être mortelle.

L’imagerie de la prostate, le toucher rectal et la biopsie sont utilisés pour diagnostiquer la maladie [9, 10]. L'antigène spécifique de la prostate (PSA) est une glycoprotéine produite et sécrétée par la prostate qui est souvent élevée dans le sérum de patients atteints de cancer de la prostate. Il a été utilisé pendant de nombreuses années dans le diagnostic du cancer de la prostate et le dépistage. Cependant, alors que de plus en plus populaire, des limitations récemment découvertes ont rendu son utilisation controversée [11].

Le pronostic du cancer de la prostate est déterminé à partir d'une gamme de variables relatives à des anomalies dans la différenciation des tissus de la prostate normale, l'invasion des tissus sains environnants, la diffusion et l'établissement de tumeurs supplémentaires à des sites distants dans le corps. Le degré de dédifférenciation du tissu de la prostate est déterminé par le système de classification de Gleason, et représenté par le score de Gleason [12]. Le score de Gleason va de 2 à 10, où un nombre plus élevé décrit une maladie plus avancée, et est déterminée par l'architecture des acini lorsque le tissu de la prostate, pris par biopsie, est coloré et examiné au microscope. De plus, le système TNM (tumeur, ganglion lymphatique, métastase) est utilisé pour faire la distinction entre les patients avec des tumeurs de stade localisé et avancé [13]. Le système TNM évalue la taille de la tumeur, l'implication des ganglions lymphatiques locaux et la présence de métastases à distance. Des efforts sont en cours pour établir un modèle de progression du cancer de la prostate fondé sur des altérations génétiques [14, 15].

Les options de traitement pour le cancer de la prostate sont spécifiques au grade et comprennent la prostatectomie radicale, la radiothérapie et l'hormonothérapie. À l'heure actuelle, la maladie métastatique est considérée comme incurable [16].

Les mécanismes du cancer de la prostate sont multiples. Ils peuvent être d'origine génétique et épigénétique. De nombreuses voies classiques et des nouvelles sont impliquées (revue dans [17, 18]).

La phosphoinositide-3-kinase (PI3K) est impliquée dans la prolifération cellulaire, la survie et l'invasion et est modifiée en 25 à 70% des cas de cancer de la prostate. Les altérations les plus courantes étant la perte de PTEN ou des mutations, perte de PHLPP1 ou amplification et gain de fonction du gène PIK3CA la sous-unité catalytique de la PI3K (revue dans [19]).

La voie de signalisation Ras est couramment impliquée dans le stade métastatique du cancer de la prostate, mais des mutations dans les composants de la voie ne sont pas communes. Des fusions K-Ras, Raf1 et BRAF peuvent également être impliqués [17].

Le suppresseur de tumeur p53 est perdu dans 25 à 40% ou bien il est dysfonctionnel (muté) dans 5 à 40% des cas de cancer de la prostate. p53 est responsable de l'arrêt du cycle cellulaire, le

déclenchement de l'apoptose et la réparation d'ADN dans des conditions normales. Le rétinoblastome 1 (Rb1), un autre gène impliqué dans le point de contrôle de la progression du cycle cellulaire, est inactivé dans jusqu'à 45% des cas de cancer de la prostate résistant à la castration (CRPC) [17]. L'oncogène MYC, qui favorise la progression du cycle cellulaire et la survie, est amplifié dans 5 à 40% des cas d'adénocarcinome neuroendocrine [18].

Les voies PI3K, Ras, p53, Rb et MYC et les protéines ne sont pas modifiées de manière unique dans le cancer de la prostate, mais dans de nombreuses autres tumeurs aussi. Il y a, cependant, des gènes et des voies qui sont plus spécifiques aux tumeurs de la prostate. Ils peuvent être regroupés comme ceux touchant la signalisation du récepteur aux androgènes (AR) et les réarrangements des gènes ETS.

La signalisation par l'AR est une composante importante de la physiologie de la prostate. L'AR est un facteur de transcription nucléaire ligand-dépendant qui est activé par les androgènes (la testostérone, la dihydrotestostérone et la déhydroépiandrostérone). Dans des conditions normales, le développement de la prostate et son maintien dépendent de la signalisation d'AR, comme par exemple la croissance de l'épithélium de la prostate [20]. Par conséquent, il n'est pas surprenant qu'une activité augmentée d'AR, rendue par amplification génique, des mutations ponctuelles et/ou l'altération dans l'épissage, sont fréquentes dans le cancer de la prostate (jusqu'à 58%). Il est supposé que la signalisation anormale d'AR devient le conducteur de la carcinogenèse à la suite de traitements ciblant le récepteur AR qui exercent une pression sélective sur les cellules [17, 21]. En plus, les gènes impliqués dans la signalisation d'AR peuvent aussi être modifiés dans le cancer de la prostate. Ils comprennent NCOA2, NCOR2, EP300, FOXA1, FOXP1, FOXO3 [17].

Le type le plus commun de modification génétique dans le cancer de la prostate est la fusion de gènes ETS. Ces fusions peuvent être trouvées dans jusqu'à 79% des cas de biopsies de la prostate, et les fusions les plus courantes se produisent entre les TMPRSS2 et ERG protéase facteurs de transcription de la famille ETS. De nombreux gènes ETS sont-androgènes réglementés [17].

À l'heure actuelle, un certain nombre d'études de séquençage systématique offrent un nouvel aperçu des sous-classes moléculaires du cancer de la prostate et de leur échelle de temps potentiel (**Fig. 1**). Toutefois, ce nouveau schéma ne fournit qu'un modèle limité du développement de la maladie. Par exemple, l'inactivation de CDH1 a été proposée de promouvoir des réarrangements AR-dépendants, y compris la fusion TMPRSS2: ERG [22].

Des antécédents de cancer de la prostate familial augmentent le risque de développer la maladie par 2-7 fois et sont liés de 10% à environ 15% des cas. Bien que 15 à 30 loci différents de 10 chromosomes différents sont soupçonnés de prédisposer au développement du cancer de la

prostate, aucun gène de prédisposition au cancer de la prostate hautement pénétrant unique a été identifié. Il est couramment admis que le cancer de la prostate héréditaire repose sur l'effet cumulatif d'un grand nombre de gènes qui exercent individuellement peu d'influence sur son développement [23-26].

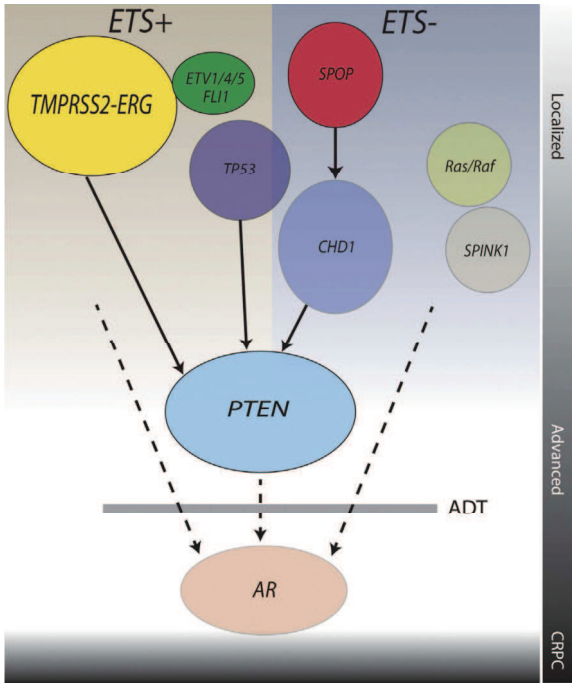

Figure 1. Des lésions génomiques dans la chronologie du cancer de la prostate. Les gènes présentant des lésions génomiques communes (y compris des mutations, réarrangements, ou du nombre de copies) sont présentés. Les flèches pleines désignent une relation temporelle entre les événements : les lésions présumées "précoces" sont en haut, avec les lésions «plus tardives» dessous. Les tumeurs avec des fusions de l'ETS (ETS +) sont indiquées sur la gauche; les tumeurs ETS- sont affichés sur la droite. ADT - hormonothérapie anti-androgénique, CRPC - cancer de la prostate résistant à la castration [19].

La nature épigénétique de la pathogenèse du cancer de la prostate attire actuellement une attention croissante. SPOP, CHD1, KDM6A, EZH2, MLL2/3 sont des gènes impliqués dans les modifications de la chromatine qui ont été trouvés altérés dans le cancer de la prostate [17, 19]. L'augmentation de la méthylation du promoteur du gène de la GSTP1 est vue dans 90% des cas de cancer de la prostate et de nombreux autres profils de méthylation sont associés à divers stades de la maladie (revue dans [28]). MiR-21, miR-221/222, miR-15a/16-1 ont été impliqués dans la régulation de l'apoptose; miR-125b, miR-146a, miR-141, mirs-221/222, miR-331-3p ont été retrouvés comme étant AR sensibles ou capables de moduler la signalisation par AR [28]. Certains microARNs participent également dans l'invasion et la modulation épigénétique dans le cancer de la prostate (**Fig. 2**) (revue dans [27]).

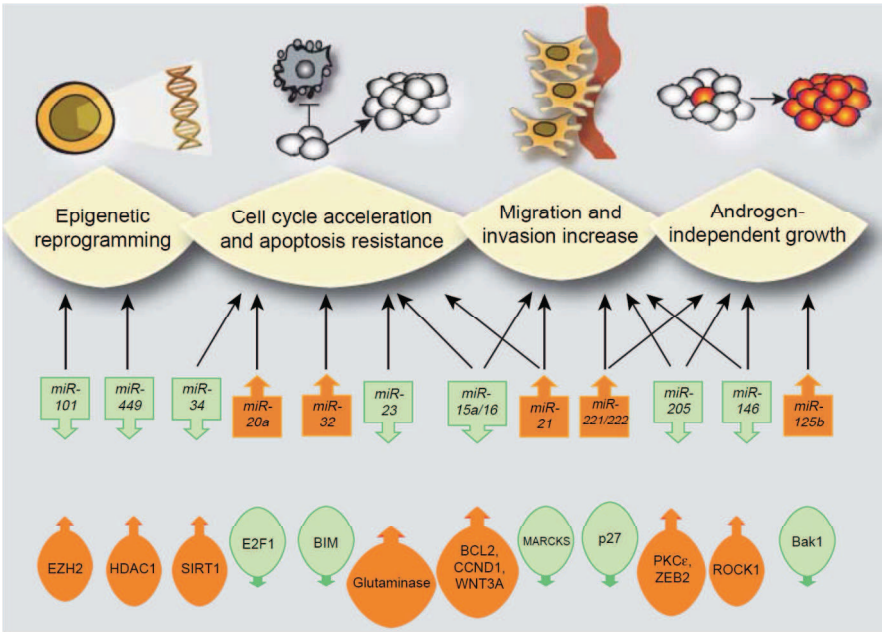

Figure 2. miARN dont le rôle physiologique dans le cancer de la prostate a été minutieusement étudié. Les cibles et les fonctions des mirs, tels que ceux décrits dans les études sur le cancer de la prostate, sont présentés [27].

1.6 Les microtubules: importance et comme cible thérapeutique

Les microtubules sont une composante omniprésente du cytosquelette cellulaire. Les rôles des microtubules sont nombreux et comprennent la division cellulaire, le maintien de la structure de la cellule, le maintien de la polarité cellulaire, le transport intracellulaire et d'autres. Les microtubules sont des structures creuses faites de longues dimères d'α- et β-tubuline polymérisée et sont dynamiques, e. g. ils se polymérisent et se dépolymérisent en fonction des besoins de la cellule [29]. Le montage et le démontage (polymérisation et dépolymérisation) de la tubuline sont GTP-dépendants. Lors de la polymérisation, les dimères α- et β-tubuline portent deux GTP. Le GTP peut être hydrolysé et cela déclenche le désassemblage des microtubules [30].

Comme de nombreuses autres protéines dans la cellule, la tubuline est soumise à un certain nombre de modifications post-traductionnelles qui se produisent principalement sur la tubuline polymérisée (les microtubules). Il s'agit notamment de la tyrosination, l'acétylation, la formation de Δ2-tubuline, la phosphorylation, la glutamylation, la glycylation et l'ubiquitination (revue dans [32,

33]). Étant donné que les microtubules ont une structure assez homogène, comment est-il possible que les microtubules effectuent autant de fonctions différentes? Il est proposé que les modifications post-traductionnelles sont la clé [32]. Ces modifications régulent les propriétés spécifiques des microtubules dans les organelles, en permettant le recrutement de complexes protéiques pertinentes. Ce système complexe a même été appelé "le code de la tubuline" [34, 35]. Parmi les nombreuses enzymes qui modifient la tubuline, la famille TLL est l'une des plus fortement impliquée (**Fig. 5**). Les modifications ont lieu sur des polymères de microtubules (les microtubules), tandis que leur retrait se produit principalement sur la tubuline soluble (revue dans [31, 36]).

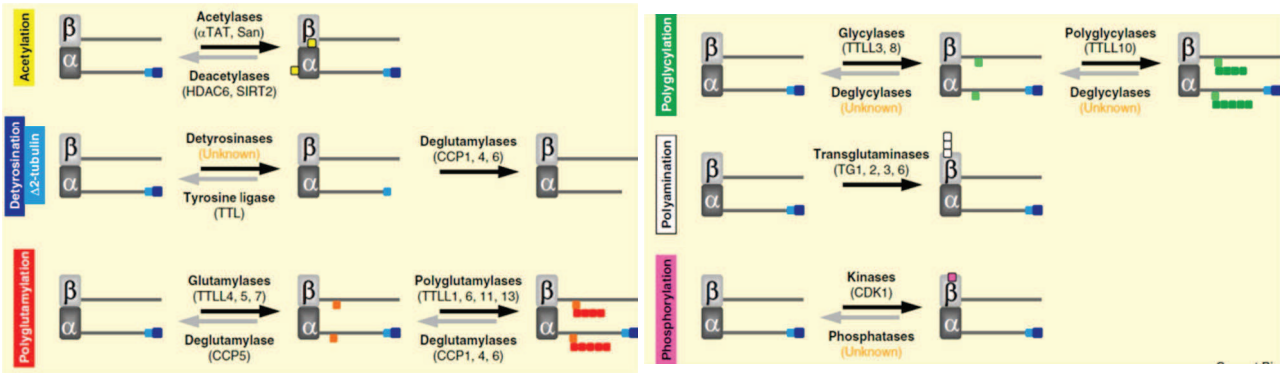

Figure 5. Enzymes impliquées dans les modifications post-traductionnelles de la tubuline. αTAT - α-tubuline N-acétyl, HDAC6 - déacétylase histone 6, SIRT2 - sirtuines 2, TTL - tubuline tyrosine ligase, TTLL - TTL-like, CCP - carboxypeptidase cytosolique, TG - transglutaminase, CDK1 - kinases cycline-dépendantes 1 [31].

Compte tenu de l'importance que les microtubules jouent, il n'est pas surprenant qu'ils ont été la cible pharmacologique dans le traitement du cancer pendant des décennies. Le fuseau mitotique est impliqué dans la ségrégation des chromosomes pendant la division cellulaire. Les microtubules sont un élément clé et dynamique du fuseau mitotique. La plupart des composés qui ciblent la tubuline, et ont vu son utilisation dans la thérapie du cancer, agissent en se liant directement à la tubuline dans les microtubules. Ils stabilisent les microtubules de sorte qu'ils ne peuvent pas se démonter si nécessaire, ou de sorte que les microtubules déstabilisés sont incapables de s'allonger [39] (**Tableau 1**). Ainsi, les cellules se divisant fréquemment, qui comprennent souvent une grande partie de la tumeur, sont particulièrement sensibles à une thérapie ciblant les microtubules. Cependant, la thérapie du cancer ciblant les microtubules présente quelques inconvénients, dont les principaux sont une toxicité élevée et la résistance du cancer à un éventuel traitement (revue dans [37, 40]). Actuellement, les nouveaux composés ciblant directement la

tubuline sont en cours d'élaboration [37] ainsi que de nouvelles façons de cibler la mitose (revue dans [38]) **(Fig. 6**).

Compound class	Approved agents	Effect(s) on microtubules
Taxanes	Paclitaxel Docetaxel	Polymerization and/or stabilisation
Vinca alkaloids	Vinblastine Vincristine Vinorelbine	Depolymerization and/or destabilisation
Epothilones	Ixabepilone	
Halichondrin b		

Tableau 1. Classes d'agents ciblant les microtubules (adapté de [37]).

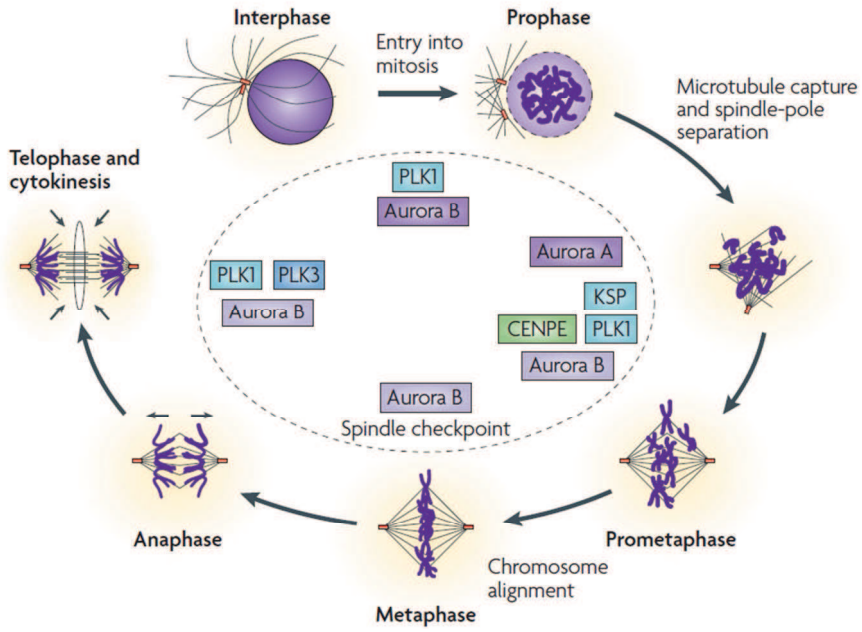

Figure 6. Les phases de la mitose et des protéines contre lesquelles des composés ciblant la mitose sont en cours de développement [38].

La tyrosination/détyrosination, une des modifications post-traductionnelles de la tubuline, comprend l'addition/suppression de tyrosine à partir de/vers l'α-tubuline dans les microtubules. Le cycle tyrosination/détyrosination est impliqué dans le recrutement différentiel des moteurs moléculaires, du « plus-end tracking » et de la liaison des protéines aux microtubules. Il est prouvé que la réduction de la tyrosination de la tubuline est associée aux propriétés invasives de la tumeur

et du mauvais pronostic, et des niveaux de tyrosination adéquats sont nécessaires pour l'organisation neuronale correcte. La tyrosination est catalysée par la tubuline tyrosine ligase (TTL) et la détyrosination par une tubulinyl-Tyr carboxypeptidase pas bien caractérisé [32, 41]. Une carboxypeptidase cytosolique a été suggérée pour ce rôle [33].

1.7 Les histones et la méthylation d'H4K20

Les histones sont un élément clé de la chromatine. Les histones détiennent l'ADN chromosomique dans des structures organisées appelées nucléosomes. Les nucléosomes, à leur tour, sont les unités de structure de la chromatine. Les histones sont soumises à de nombreuses modifications post-traductionnelles qui affectent l'expression des gènes, la réparation de l'ADN, la replication, la mitose, et l'oncogenèse (revue dans [42-44]). Les modifications des histones soit changent la structure de la chromatine ou régulent la liaison des facteurs de la chromatine, modifiant ainsi l'accès des facteurs de transcription et des complexes à l'ADN [42]. Le fait qu'il existe plusieurs familles et sous-familles d'histones, qu'elles peuvent être modifiées dans différentes parties et que plusieurs modifications existent, rende ce domaine de la biologie particulièrement complexe. Les modifications des histones sont impliquées dans l'épigénétique - où des changements pertinents aux gènes ne impliquent pas de changements dans la séquence nucléotidique.

La méthylation est l'une des modifications d'histones. Il est «une modification de la chromatine de type répandu qui est connu pour influencer des processus biologiques dans le contexte du développement et les réponses cellulaires» [45]. La méthylation des histones est connue pour être impliquée dans le vieillissement, les maladies neurodégénératives et la réparation de l'ADN (revue dans [45-47]). La méthylation se'exerce essentiellement sur les chaînes latérales des lysines et arginines et ne change pas leur charge électrique. La méthylation est une modification à plusieurs niveaux: les arginines peuvent devenir mono- ou di-méthylées, symétriques ou asymétriques, et des lysines - mono-, di- ou tri-méthylées [42]. Les méthyltransférases méthylent les lysines des histones et ont généralement des domaines SET [42]. Les groupes méthyle sont retirés des histones par les déméthylases.

La méthylation de l'histone 4 sur la lysine 20 (H4K20) est importante pour la réparation des dommages de l'ADN, la réplication de l'ADN et la compaction de la chromatine. En particulier, la mono- et diméthylation de l'histone 4 sur la lysine 20 (H4K20me1,2) sont impliquées dans la réplication de l'ADN et la réparation des dommages de l'ADN, alors que la triméthylation d'H4K20

(H4K20me3) est une marque de régions hétérochromatiques qui sont réduits au silence. La chromatine péricentrique de chromosomes mitotiques est particulièrement enrichie en H4K20me3 [43].

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Kostyantyn SEMENCHENKO

Development of tumour therapies: from target validation of TTLL12 to tests of a small molecule XRP44X in pre-clinical models of cancer

Résumé

Le cancer de la prostate, TTLL12, la tyrosination de la tubuline

Les modifications post-traductionnelles de la tubuline sont des cibles attrayantes pour la thérapie du cancer. TTLL12 est impliqué dans la détyrosination de la tubuline, la triméthylation de l'histone H4K20 et le cancer de la prostate. La thèse porte sur les effets de la surexpression de TTLL12 sur ces modifications à différents stades du cycle cellulaire. Les résultats montrent que TTLL12 affecte ces modifications indépendant du cycle cellulaire.

Résumé en anglais

Prostate cancer, TTLL12, tubulin tyrosination

Tubulin posttranslational modifications are an attractive target for cancer therapy. TTLL12 is involved in tubulin detyrosination, histone H4K20 trimethylation and prostate cancer. The thesis addresses the effects of TTLL12 overexpression on these tubulin and histone modifications at different stages of the cell cycle. The results show that TTLL12 overexpression affects tubulin detyrosination and H4K20 trimethylation independently of cell cycle phase.