The role of ubiquitin specific proteases in cancer development through interaction with critical cellular processes

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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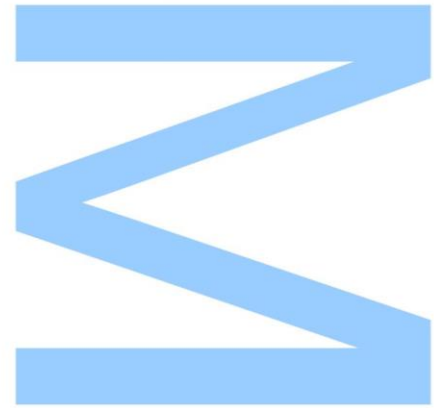

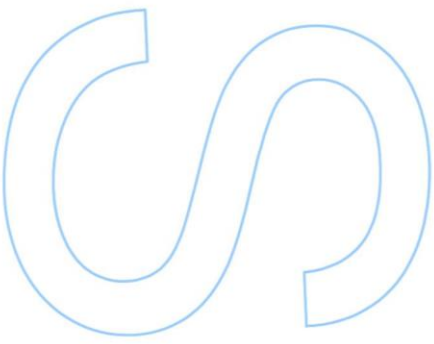

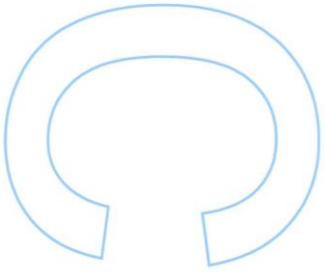

“I know you can do everything. You make plans, and nothing can change or stop them.”

Job 42:2

Agradecimentos

Como transformar algo abstrato em palavras concretas e inteligíveis? Tenho pouca habilidade, mas espero ser fiel ao sentimento que emana desde o primeiro contacto, num tempo em que as ruas se babavam de gente e as casas cativavam sem nos manterem cativos. Esse ("o tal contacto") ocorreu com a ilustre Doutora Paula Soares, uma mulher pioneira na investigação de excelência feita em Portugal. Os seus atributos profissionais e pessoais são mais que muitos e amplamente reconhecidos. No entanto, se a tivesse de definir numa palavra (exercício altamente redutor) seria Liderança. Essa idiossincrasia é refletida na forma inteligente como doseia afeto e exigência. Obrigado pelo cuidado, simpatia e por ter tornado possível, para além da realização da tese, conhecer este incrível grupo. Neste grupo conheci um trabalhador nato e um inigualável pedagogo, Marcelo Correia. A designação "MC", tantas vezes vista em cartazes e propagandas festivas, ganhou uma nova interpretação e imagem mental para mim :) Obrigado pela proatividade, discernimento em tempos dúbios, orientação entusiástica e paciência de santo. Desejo-te uma vida incrível, repleta de alegria e boas surpresas.

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Obrigado a todos por nunca desistirem de mim e nunca me deixarem desistir.

Abstract

The ubiquitination process represents a post-translational modification in which 3 families of enzymes (E1, E2 and E3) intervene and are responsible for allowing the connection between ubiquitin and the substrate. However, ubiquitination can be reversed through the action of deubiquitinating enzymes (DUBs). These enzymes have the ability to remove the mono or polyubiquitination signals and are involved in the maturation, recycling, editing and rearrangement of the ubiquitin(s).

Most DUBs belong to the family of ubiquitin-specific proteases (USPs). This family is responsible for various cellular functions through the interaction with different targets (p53, Klf5, ELK1, Cyclins, Claspin, XPC, BRCA2, H2A, APC, Smads, β-catenin, etc.). Over the past few years, several studies have focused on the role of USPs in carcinogenesis, which has led to an increasing development of therapies based on USP inhibitors.

In this bibliographic review, we intend to relate the different cellular functions, such as cell cycle, DNA damage repair, chromatin remodelling and several signalling pathways, in which USPs are involved in the development of cancer. In addition, we describe the existing therapy, targeting the inhibition of USPs.

Keywords: Cancer, ubiquitination, deubiquitination, deubiquitinating enzymes (DUBs), ubiquitin specific proteases (USPs), USP inhibitors.

Resumo

O processo de ubiquitinação representa uma modificação pós-tradução na qual intervêm 3 famílias de enzimas (E1, E2 e E3) responsáveis por permitir a ligação entre a ubiquitina e o substrato. No entanto, a ubiquitinação pode ser revertida através da ação de enzimas deubiquitinantes (DUBs). Estas enzimas têm a capacidade de remover os sinais de mono ou poliubiquitinação e estão envolvidas na maturação, reciclagem, edição e rearranjo da(s) ubiquitina(s).

A maioria dos DUBs pertence à família das ubiquitin-specific proteases (USPs). Esta família é responsável por regular diversas funções celulares através da interação com diversos targets (p53, Klf5, ELK1, Cyclins, Claspin, XPC, BRCA2, H2A, APC, Smads, β-catenin, etc.). Ao longo dos últimos anos, diversos estudos têm mostrado existir relação entre USPs e carcinogénese, o que tem provocado um crescente desenvolvimento de terapias baseadas em inibidores de USPs.

Neste trabalho de revisão bibliográfica, pretendemos relacionar as diversas funções celulares, como ciclo celular, reparação de danos no DNA, remodelação da cromatina e diversas vias de sinalização, nos quais os USPs estão envolvidos com o desenvolvimento de cancro. E para além disto, descrevemos a terapêutica existente, tendo como alvo a inibição dos USPs.

Palavra-chave: Cancro, ubiquitinação, deubiquitinação, deubiquitinating enzymes (DUBs), ubiquitin specific proteases (USPs), inibidores de USP.

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Abbreviations

ALK5 – Activin receptor-Like Kinase 5

APC – Adenomatous polyposis coli

ATM – Ataxia-Telangiectasia Mutated

ATR – ATM-and Rad3-related

BRCA1 – Breast Cancer type 1 susceptibility gene

BRCA2 - Breast Cancer type 2 susceptibility gene

CARP – Caspase 8/10 Associated RING Protein

CBL – Casitas B-lineage Lymphoma

CDC25 – Cell Division Cycle 25

CDK – Cyclin-Dependent Kinases

CDKI – Cyclin-Dependent Kinase Inhibitor

c-Fos – Fos proto-oncogene

ChK1 – Checkpoint Kinase 1

ChK2 – Checkpoint Kinase 2

c-Jun – Jun proto-oncogene

CK1 – Casein Kinase 1

c-Met – Tyrosin-protein kinase Met

c-Myc – Myc proto-oncogene

COPS5 - COP9 Signalosome Subunit 5

DDB – Double-strand DNA Break

DDR – DNA Damage Repair

DNA – Deoxyribonucleic Acid

DUB – Deubiquitinating enzyme

e.g. – for example

EGFR – Epidermal Growth Factor Receptor

EGR1 – Early Growth Response 1

EGR2 – Early Growth Response 2

ELK1 – ETS transcription factor ELK1

EMT – Epithelial-Mesenchymal Transition

ERBB3 – Erb-B2 Receptor Tyrosine kinase 3

ERK – Extracellular signal-Regulated Kinase

EZH2 – Enhancer of Zeste Homolog 2

FANCD2 – Faconi Anemia Complementation Group D2

FBW7 – F-Box/WD repeat-containing protein 7

GSK3 – Glycogen Synthase Kinase 3 beta

H2Aub1 – Histone H2A monoubiquitination

H2AX – H2A histone family member X

γH2AX - H2AX phosphorylated

H2Bub1 - Histone H2B monoubiquitination

HDAC – Histone Deacetylase

HIF1 – Hypoxia-Inducible Factor 1

Hsp90 – Heat shock protein 90

IER2 – Immediate Early Response 2

IL-3 – Interleukin-3

IL-6 – Interleukin-6

JAMM – JAB1/MPN/Mov34 metalloenzyme

Klf5 – Krueppel-Like Factor 5

MCF7 – Michigan Cancer Foundation-7

MCPIP – Monocyte Chemotactic Protein-Induced Protein

MDC1 – Mediator of DNA damage protein 1

Mdm2 – Mouse double minute 2 homolog

MEF – Mouse Embryonic Fibroblast

miR-7 – micro RNA-7

MJD – Machado-Joseph Disease protein domain protease

MMP – Matrix Metalloproteinase

Notch1 – Notch homolog 1 translocation-associated

OTU – Otubain protease

PCNA – Proliferating Cell Nuclear Antigen

PHF8 – Plant Homeodomain Finger protein 8

Pirh2 – p53-induced RING-H2 protein

PLK1 – Polo-like Kinase 1

PRC1 – Polycomb Repressive Complex 1

PRC2 - Polycomb Repressive Complex 2

PTM – Posttranslational Modifications

RBM4 – RNA-Binding protein 4

RhoA – Ras homolog family member A

R-Smad – Receptor-regulated Smad

RTK – Receptor Tyrosine Kinase

SDB – Single-strand DNA Break

SDS3 - Suppressor of Defective Silencing 3

SET8 – [Su(var) 3-9, Enhancer -of-zeste and Trithorax] domain containing protein 8

TCF7 – Transcription Factor 7

TGFβ - Transforming Growth Factor-beta

TGFβR – TGF Beta Receptor

TNM – Tumour Nodes Metastases

UAF1 – USP1 Associated Factor 1 WDR48

Ub – Ubiquitin

UBA1 – Ubiquitin-like modifier activating enzyme 1

UBE3A – Ubiquitin Protein Ligase E3A

UCH – Ubiquitin Carboxyl-terminal Hydrolases

USP – Ubiquitin-Specific Protease

USP2a – USP2 androgen-regulated

VCPIP1 – Valosin Containing Protein Interacting Protein 1

VEGF – Vascular Endothelial Growth Factor

VHL – Von Hippel Lindau

XPC – Xeroderma Pigmentosum Complementation Group C

Introduction

Cancer is an important public health problem, representing the second most deathly pathology in the world (leading to around 9 million deaths per year, accordingly to the World Health Organization, 2018). There is so an unmet need to understand of the cellular and molecular mechanisms that can lead to the establishment and progression of this disease, in order to allow the discovery and development of new and effective therapeutic strategies.

The relation between protein synthesis and degradation is essential to maintain cellular homeostasis [1]. The two principal cellular mechanisms responsible for protein degradation are the autophagy-lysosome and ubiquitin-proteasome [2]. Ubiquitination and deubiquitination are posttranslational modifications (PTM) linked to a plethora of cellular processes, such as signal transduction, stress response, DNA repair and apoptosis which results from (in)activation, (re)localization of protein and/or modulation of protein-protein interactions [3, 4] Due to this major implication in many different cellular processes, the dysregulation of ubiquitination or deubiquitination PTM can be involved in the development and progression of cancer [5]. Frequently, the expression levels of several deubiquinating enzymes is altered in diverse cancers, and can exert an important oncogenic and tumour suppressors effect, as will be discussed in this review.

1. Ubiquitin and ubiquitination

The ubiquitination process mainly consists in the connection between of an ubiquitin molecule(s) to a target protein. For the establishment of an isopeptide bond between ubiquitin C-terminal carboxyl group and the amino group of a lysine on target protein, there are needed the participation of 3 families of enzymes: UBA1 (ubiquitin activating enzymes); E2 (ubiquitin conjugating enzymes) and E3 (ubiquitin ligase) (Figure 1A) [6]. Since UBA1, E2 and E3 represent families of enzymes encompassing several members, here we will use the general and accept designation E1, E2 and E3 for sake of simplicity, referring to specific family members, when adequate, along the text.

E1 enzymes catalyses the ubiquitin activation through the adenylation of the ubiquitin C-terminal and originate an ubiquitinadelynate intermediate, which is then transferred to an active site cysteine residue. Next, through a thioester bond, E2 enzymes conjugates with the ubiquitin. Finally, E3 catalyses the transfer of ubiquitin from E2 cysteine to a lysine residue of the target protein, besides to helping in the recognition between the protein substrates and the E2 [7]. As a monomer, ubiquitin can attach to an amino group (monoubiquitination) or to various amino groups (multiubiquitination) of the protein. Also, the polymerization of ubiquitin can lead to the formation of ubiquitin chains attached to the protein (polyubiquitination) (Figure 1B). Ubiquitin can connect between themselves by 7 different ways, due to the existence of 7 lysine residues (K6, K11, K27, K29, K33, K48 and K63) in each one, allowing the generation of linear or branched chains. The lysine, to which an ubiquitin connects to another, is important in the establishment of the target protein fate (Figure 1C). As an example, the polyubiquitination made through K11 or K48 linkages results in proteasome degradation. In contrast, the polyubiquitination made through K63 linkages leads to DNA damage resistance, kinase activation and endocytosis [8, 9].

Various E3 assume a fundamental role in carcinogenesis. Overexpression of these E3 were associated to a decrease of patient survival and poor prognosis in some cancers such as lung and breast [10-12]. Some of these E3 ligases are Mdm2 (Mouse double minute 2 homolog), FBW7 (F-box/WD repeat-containing protein 7), VHL (Von Hippel Lindau) and CBL (Casitas B-lineage Lymphoma). For instance, Mdm2 is responsible for the ubiquitination of the tumour suppressor p53, leading to its degradation via proteasome [13]. The upregulation Mdm2 provokes p53 depletion, and was seen to be involved in the development of several human cancers, such as soft tissue sarcoma and

lung cancer [14]. FBW7 polyubiquitylates various oncogenes and growth regulators, such as c-Myc, c-Jun, Notch homolog 1 translocation-associated (Notch1), transforming growth factor beta 1 (TGFβ1), Krueppel-like factor 5 (Klf5), and others. Following their polyubiquitination, these proteins are driven to degradation. Several studies have been showing that the loss of FBW7 function leads to the accumulation of the above mentioned substrates and, thereby is connected to the oncogenic process [15]. The downregulation of FBW7 was observed in gastric, colon, breast, lung and other solid tumours [16-19]. Additionally, it was shown that inactivation of FBW7 in mouse T-cells led to lymphomas formation, validating its role as tumour suppressor [15, 20, 21]. Mutations causing loss of function in VHL result in tumoral development, especially renal cell carcinoma [22, 23], as a result of the essential role of VHL in regulation of the Hypoxia-inducible factor 1 (HIF1) levels. When the oxygen levels in a cell are normal, the VHL-mediated by ubiquitin pathway induces HIF1 degradation via proteasome. In situations of loss of VHL function (similar to what happen in hypoxia conditions), HIF1α suffers conformational changes and migrates to the nucleus, where it interacts with HIF1β and is responsible for the transcription of several genes that favour tumoral growth. One of these genes is Vascular Endothelial Growth Factor (VEGF) that is involved in angiogenesis, leading to an increase of the vascularization that promote the oxygen levels rise and potentiate tumoral growth. [24]. CBL is another E3 ligase involved in carcinogenesis. This E3 is responsible for the negative regulation of some receptor tyrosine kinases (RTKs; high-affinity cell surface receptors for many polypeptide growth factors, cytokines and hormones). It was identified dysregulated in acute myeloid leukaemia, lymphoma and gastric cancers [25, 26].

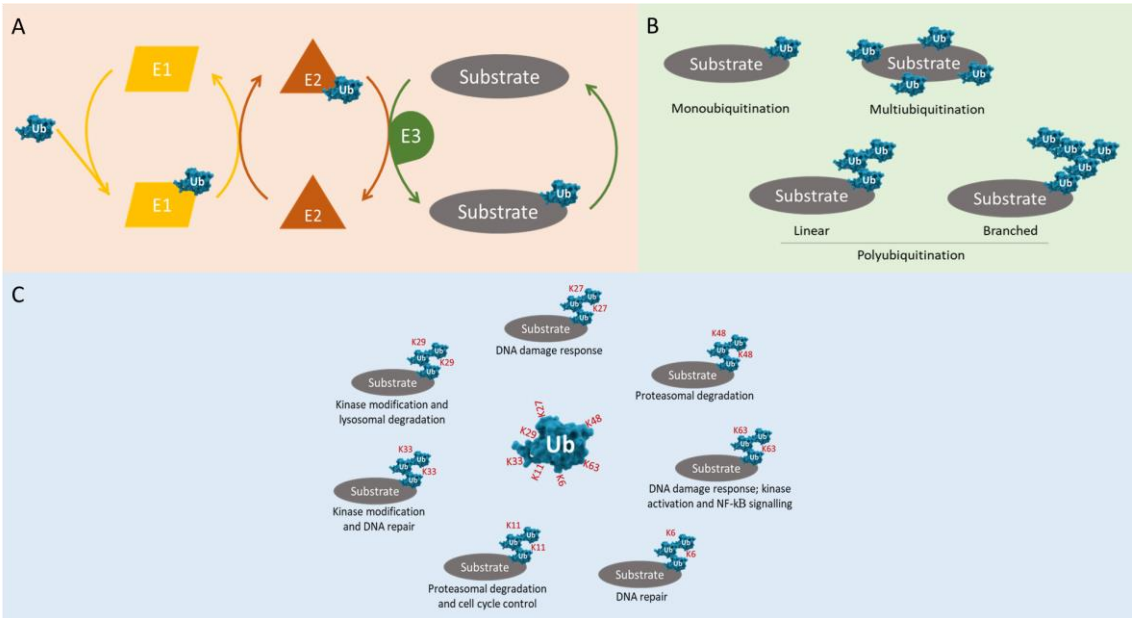

Figure 1| Overview of the ubiquitination process and its functional implications. (A) Process that allows the binding of ubiquitin (ub) to the substrate: Activation of ubiquitin by E1, transfer of ubiquitin to E2 conjugation enzymes, and finally interation between E2 and E3 ubiquitin ligase, allows the connection between ubiquitin and the substrate. (B) Different types of ubiquitination**:** Binding of a ubiquitin monomer at a single site (monoubiquitination) or several monomers at different sites (multiubiquitination) of the substrate, and ubiquitin-ubiquitin linkage in substrate, leading to the formation of linear or branched chains (polyubiquitination). (C) Different modes of polyubiquitination lead to different substrate fates. The ubiquitin molecule possesses seven different lysine residues, K6, K11, K27, K29, K33, K48 and K63, where other ubiquitins can be linked. Generally, polyubiquitination through K6 or K33 linkages leads to DNA repair, while K11 or K48 are mainly associated with proteasomal degradation. K27 or K63 linkages are commonly associated with DNA damage response and K29 or K33 a kinase modification. In addition to this already mentioned, K11, K29 or K63 linkage are associated respectively with cell cycle control, lysosomal degradation and NF-kβ signalling.

2. Deubiquitinating enzymes

The ubiquitination is a PTM responsible for regulation of protein cellular localization, for protein targeting to proteasomal degradation and for the interactions between proteins. However, this process is reversible through the action of deubiquitinating enzymes (DUBs), proteases that remove the mono or polyubiquitination signals and are involved in ubiquitin maturation (removal of nonessential ubiquitin molecules), recycling, editing and rearranging [27] (Figure 2). DUBs are relevant, not only in the rescue of targeted proteins, but also in the maintenance of the levels of free ubiquitin [28]. Ubiquitination and deubiquitination play so an important balance in the protein dynamic. The measure for protein renewal and function is defined by the relation between ubiquitylation and deubiquitylation [29].

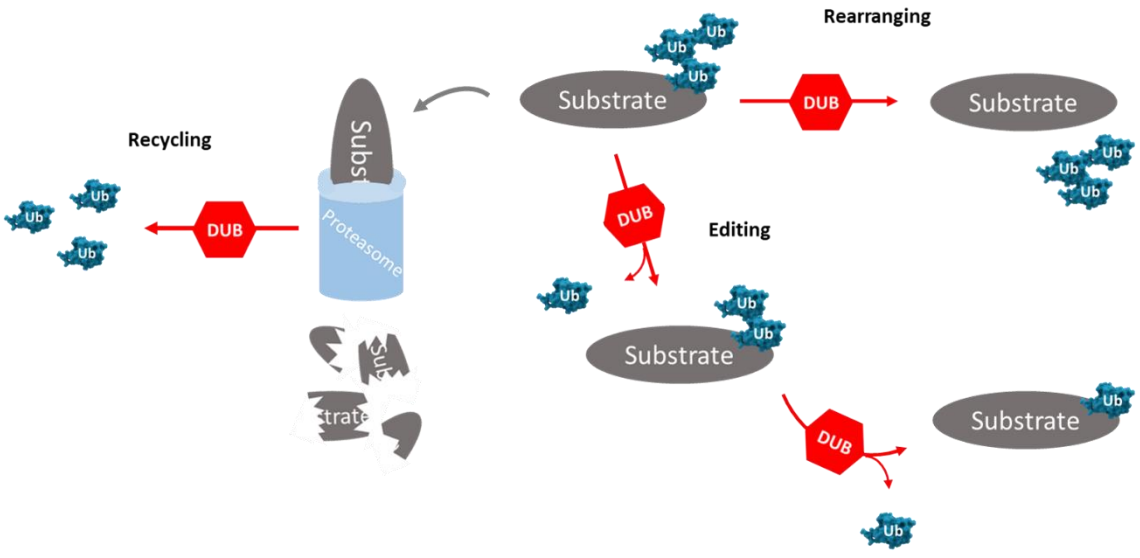

Figure 2| Role of deubiquitinating enzymes (DUBs) in ubiquitin-ubiquitin and ubiquitin-substrate linkages. DUBs can cleave the ubiquitin-substrate bond, rendering the substrate free of ubiquitin's (rearranging); modify the structure of the polyubiquitin chain by removing ubiquitin-ubiquitin linkages (editing), and recycling the ubiquitin associated with proteasomal degradation.

Furthermore, DUBs are implicated in a large variety of cellular functions, such as cell cycle progression, apoptosis, stem cell differentiation, protein turnover, DNA repair, chromosome segregation and cell signalling (kinase activation, gene expression, and localization or degradation of signalling intermediates) [4, 30, 31].

In contrast to the ubiquitination process, which requires the processing of three enzymes (E1, E2, E3), the deubiquitylating enzymes act as single ones that counterpoint both the E3 auto-ubiquitination and the substrate ubiquitination, preventing their degradation. Even so, DUBs are not exempt of being targeted by ubiquitination mediated by E3 ligase [32].

Overall, the cooperation between DUBs and E3 ligase is essential to define the protein ubiquitination balance. Besides to the interaction with ubiquitin system components, DUBs also interact with scaffold proteins which affect and influence the DUBs catalytic activity and facilitate the substrate´s recognition [33].

DUBs are classified based in their catalytic domain in six main families: ubiquitin-specific proteases (USPs), ubiquitin carboxyl-terminal hydrolases (UCHs), otubain proteases (OTUs), Machado-Joseph disease protein domain proteases (MJDs), monocyte chemotactic protein-induced proteins (MCPIPs) and JAMM/MPN domain-associated metallopeptidases (JAMMs) [6] (Fig.2). In these six families, two main enzymatic mechanisms exist. The first five families are cysteine-dependent proteases, and so, related with an nucleophilic attack of the isopeptide bond between the ubiquitin C-terminal and the lysine substrate; while the last ones, the JAMM metalloproteases, are zinc-dependent, meaning that the attack of isopeptide bond is made through the water molecules activated by zinc ions [34, 35].

Similarly to the ubiquitinating enzymes, DUBs participate and regulate several intracellular processes associated to the pathogenesis of various human disorders, including cancer. Changes in DUBs expression were correlated with immune diseases and human cancers, among other diseases. For example, high expression levels of OTUD6B, UCH37, VCPIP1, USP7 and COPS5 was reported in breast cancer. In the same way, UCHL1 and COPS5 was reported to be upregulated in lung cancer. The UCHL1 expression was directly associated with the lung tumour size [36]. The overexpression of COPS5 was related with decrease in patient survival with lung cancer

[37]. In a therapeutic level, DUBs are very attractive target due to their capacity to modulate protein fate in specific or selective manner, allowing directing the cell to death or recovering her from the pathological state.

3. Ubiquitin-specific proteases (USPs)

USPs are the biggest family of DUBs, presenting 58 described members that possess several conserved domains and similar catalytic sites among them [38]. These proteases regulate diverse cell functions important in a cancer context such as cell-cycle; mechanisms of DNA damage repair; chromatin remodelling and a variety of signalling pathways. So far, more than 40 were direct or indirectly associated within relevant cancer processes [39]. Due to this, USPs are categorized as cancer-associated proteases to which several studies have been addressing the development of anti-cancer therapies [40]. Next, we will summarize some of the most relevant functions in which USPs have been demonstrated to play a role in cancer and succinctly describe some of the most promising therapeutics that target USPs activity in cancer.

3.1. Association of USP´s with processes relevant to cancer

3.1.1. Interactions between USPs and targets involved in cell cycle progression

The cell cycle is characterized by the main stages, G1, S, G2 and M. The cell transition among these stages is regulated by Cyclin-dependent kinases (CDKs), Cyclin-dependent kinase inhibitor proteins (CDKIs), cyclins and aurora kinases [41]. Contrarily to normal cells, some cancers cells are not controlled by any inhibitor signal, and overtake cell cycle stages and acquire proliferative advantages, which represents one of the cancer hallmarks [42]. By regulation of many cell cycle checkpoints, USPs have an important function in cancer. Their targeting can therefore be tested as a potential therapeutic approach.

It was observed an high expression of USP17 in samples of colon, lung, gastric and cervical cancers that promote G1/S transition and cell proliferation [43]. It was demonstrated that USP17 specifically deubiquitinates K63 lysine linked ubiquitin chains from the suppressor of defective silencing 3 (SDS3) and negatively regulated histone deacetylase 1 and 2 (HDAC1 and HDAC2) resulting in cell proliferation inhibition [44-46]. In HeLa cells, USP17 knockdown led to the increase of the levels of the CDK inhibitor

p21 due to the block of Ras and RhoA (Ras homolog family member A) activation. As consequence, G1/S transition is blocked and cell cycle stopped [46]. In MCF7 and MDA-MB231 breast cancer cell lines, USP17 knockdown also led to a decrease of SET [Su(var)3-9, Enhancer-of-zeste and Trithorax] domain containing protein 8 (SET8) levels resulting in the suppression of cell proliferation due to the increase of p21 levels [45, 47]. In HEK293T cells, it was observed that USP17, ectopically expressed, deubiquitinated the ELK1 transcription factor, involved in ERK (Extracellular signal-regulated kinase) signalling. This deubiquitination suppresses the nuclear export of ELK1, allowing its binding to DNA and increasing the expression of ELK1 target genes (c-Fos, EGR1, EGR2 and IER2). This expression of mitogenic agents leads to an increase in the levels of cyclin D1, which promotes the G1/S transition and cell proliferation. The same study also showed that knockdown of USP17 reduces cell proliferation and stops the G1/S transition [44].

A delay in the progression of the S phase and an accumulation of DNA breaks (caused by the non-rescue of H2A and H2B), which promoted the activation of cell cycle checkpoint pathways was observed in U2OS (Human Bone Osteosarcoma Epithelial Cells) upon USP3 knockdown [48]. Also in HGC27 gastric cancer cells, the decrease of USP3 expression led to a delay in G1/S cell cycle progression by decreasing cyclins D1 and E1 levels [49]. USP3 knockdown in HeLa cells led to a reduction in CDC25A phosphatase levels, causing delay in cell cycle progression and reduction of tumour growth in mice bearing tumour xenografts from HeLa cells [50]. CDC25A phosphatase essentially activates CDK2 and CDK4, allowing the G1/S transition. In addition to this, CDC25A (similarly to the action of CDC25B and CDC25C) also dephosphorylates CDK1, allowing the entry into M phase [51]. Also, USP3 is responsible for the stabilization of Klf5, which inhibits cell cycle inhibitor p27, promoting cellular proliferation in breast cancer HCC1937, HCC1806 and SUM149PT cells. The Klf5 is highly expressed in breast cancer, possibly indicating the USP3 regulator role in cancer proliferation [52, 53].

USP50, through the interaction with Hsp90, prevent the Wee1 degradation and neutralize mitotic inducing activity of CDC25B. Wee1 phosphorylates CDK1 to inhibit the entrance in mitosis, while CDC25B degrades Wee1 leading to the CDK1 activation and allowing the cell cycle progression. In U2OS cells, knockdown of USP50 led to a reduction of Wee1 protein levels, taking to the arrest of the cell cycle in the G2/M phase [54].

USP7 (also denominated HAUSP) can stabilize the histone demethylase PHF8 (Plant homeodomain finger protein 8), resulting in the upregulation of cyclin A2 and

regulation of the transcription of some genes required for the entrance in S phase. Thus, USP7 also controls cell cycle progression. In breast cancer, there was seen a functional link between USP7 and PHF8 through their interaction and through a positive feedback mechanism. In MCF7 breast cancer cells it was observed that USP7 deubiquitinates and stabilizes PHF8, during DNA damage response, contributing for the activation of DNA repair mechanisms. Interestingly, in human breast tumour samples, USP7 and PHF8 protein levels correlate with tumour grade. The same authors observed that, in its turn, PHF8 led to the increase in the expression of USP7 [55]. It was also shown that loss of expression of USP7 in U2OS stabilize Mdm2 leading to an accumulation of p53 and to an increase of CKI p21 levels, stopping the cell cycle in G1 [56].

USP22 regulates cyclin B1 during G2/M. During G2/M transition, USP22 levels are increased in comparison with other cell cycle phases. In colon cancer tissues, it was seen an increase of USP22 and cyclin B1 levels compared to adjacent normal tissues. It was also seen that the knockdown of USP22 inhibited cell growth in HCT116 cells and tumoral growth in mouse embryonic fibroblastic cells (MEFs) [57-59].

Table 1. Summary of cellular consequences resulting from interaction between different USPs and target proteins associated with cell cycle progression.

USPs	Targets	Cell model association	USPs effects	References
USP2	Cyclin A1	Bladder carcinoma	USP2a overexpression induces cellular invasion and proliferation in T24 cells	[60]
	Cyclin D1	Colorectal carcinoma	In HCT116 cells, knockdown of USP2a inhibits cellular growth by G1/S arrest	[61]
USP3	CDC25A	Cervical adenocarcinoma	USP3 knockdown provokes a delay in cell cycle progression and reduces the tumour growth in mice bearing tumour xenografts from HeLa cells	[50]

	Klf5	Breast carcinomas	Knockdown of USP3 leads to a decrease in cellular proliferation and invasion in HCC1937, HCC1806 and SUM149PT cells	[53]
USP7	PHF8 - cyclin A2	Breast carcinoma	Knockdown of USP7 reduces cellular proliferation in MCF7 cells	[55]
		Breast carcinoma xenografts in mice	Knockdown of USP7 reduces tumoral growth in mice bearing tumour xenografts from MCF7 cells	
	PLK1	Prostate adenocarcinomas	Knockdown of USP7 in DU145 and VCaP cells leads to a decrease cellular proliferation and viability and interruption of the G2/M cell cycle	[62]
USP17	ELK1	Kidney	Knockdown of USP17 inhibits cellular proliferation and stops the G1/S cell cycle in HEK293T cells	[44]
	SET8 - p21	Breast carcinoma	Knockdown of USP17 induces G1 phase arrest and apoptosis in MCF7 cells	[45]
USP22	Cyclin B1	Colorectal carcinoma	USP22 knockdown prevent G2/M cell cycle progression and inhibits cellular proliferation in HCT116 cells	[59]
		Colorectal carcinoma xenografts in mice	Knockdown of USP22 decreases tumoral growth in mice bearing tumour xenografts from HCT116 cells	

	p15, p21 and cyclin D2	Hepatocellular carcinoma	Knockdown of USP22 reduces cellular viability and promotes the G0/G1 cell cycle progression and apoptosis in HEPG2 cells	[63]
USP39	CDK1 and cyclin B1	Ovarian carcinomas	USP39 knockdown induces the arrest of G2/M cell cycle and inhibits cellular proliferation in HO-8910 and SKOV3 cells	[64]
		Thyroid carcinoma	Knockdown of USP39 inhibits cellular proliferation and induces G2/M arrest in TT cells	[65]
	Unknown	Hepatocellular carcinoma	USP39 knockdown inhibits cellular proliferation, stops the G2/M cell cycle transition in SMMC-7721 cells	[66]
		Colorectal carcinoma	Knockdown of USP39 blocks cellular proliferation and G2/M phase in SW1116 and HCT116 cells	[67]
USP42	Cyclin D1 and Cyclin E1	Gastric adenocarcinomas	Suppression of USP42 provokes G0/G1 cell cycle arrest and inhibits cellular proliferation in AGS and MKN-45 cells	[68]
		Gastric adenocarcinoma xenografts in mice	Knockdown of USP42 also suppresses tumoral growth in mice bearing tumour xenografts from AGS cells	
USP50	Hsp90	Bone osteosarcoma	Knockdown of USP50 blocks G2/M cell cycle in U2OS cells	[54]

3.1.2. Importance of USPs in DNA damage repair mechanisms

DNA damage repair (DDR) comprehends an amount of diverse signalling pathways that are activated to repair DNA damage that occur due to natural cellular activity (e.g. ROS production that reacts with DNA) or as consequence of extracellular insults. When DNA errors occur, they are detected by ATM (ataxia-telangiectasia mutated) or ATR (ATM-and Rad3-Related), depending on whether they are double-strand DNA breaks (DDB) or single-strand DNA breaks (SDB), respectively. In turn, ATM and ATR activate DNA damage checkpoints, trough phosphorylation of different targets. ATM causes the activation of ChK2, H2AX and MDC1 while ATR is responsible for activating ChK1 [69, 70]. In this way, DDR is activated triggering a delay in cell cycle to allow the repair or, if it is not possible, DNA damaged cells undergo apoptosis [71]. This process is important to avoid tumour formation and progression [72]. Ubiquitination (and USPs) is also important in the regulation of DDR mechanisms through PTM alterations of several proteins involved in these mechanisms [73].

ChK1 (previously mentioned) when activated, inhibits CDC25 and activates Wee1, preventing cell cycle progression, and activates PCNA (Proliferating Cell Nuclear Antigen) and FANCD2 (Faconi Anemia Protein) to DDR [74]. Recently it was observed that after phosphorylation of ChK1, it undergoes ubiquitination and migrates to the nucleus, where it binds to chromatin and activates targets involved in DDR. USP3 knockdown in HCT116 colon cancer cells led to an increase in chromatin-bound ChK1 levels, and caused a high delay in the S phase of the cell cycle along with a decrease in cell apoptosis [64].

The control of DDR through USP1 is achieved by its association with UAF1 (USP1 associated factor 1 WDR48) which leads to deubiquitination of essential proteins involved in DDR, such as, FANCD2 and PCNA. The complex USP1/UAF1 is responsible for the maintenance of FANCD2 levels during a normal cellular growth. However, when DNA damage occur, the transcription of USP1 is shut off, allowing the increase of monoubiquitinated FANCD2 and its accumulation in the cell. This process is fundamental for the maintenance of the genomic stability and avoid errors that could lead to a carcinogenesis [57].

USP9X and USP20 are also important regulators of DNA damage checkpoints. These USPs deubiquitinate and stabilize Claspin (a protein responsible for the replication conclusion during DNA repair). The downregulation of USP9x in U2OS cells and USP20

in MGC-803 cells led to the reduction of Claspin levels, resulting in a block in DNA synthesis and a consequent decrease in cell survival. In contrast, Claspin overexpression was associated to primary colon and breast carcinomas [75-77].

USP11 can also target γH2AX (H2AX phosphorylated) to deubiquitination. As a response to DDB, diverse response proteins to DNA damage (namely BRCA1, RAD50, NB31, and others) accumulate in the nucleus where they interact with γH2AX. This allows the DDBs to repair and maintenance of genomic stability [78, 79]. This way, USP11 can modulate the recruitment of proteins to DDB and mark XPC (Xeroderma pigmentosum complementation group C) protein for deubiquitination. XPC is necessary to regulate nucleotide excision repair mechanisms [80, 81]. Besides USP11, also USP7 deubiquitinates XPC and avoid its degradation. Due to the fact that XPC is a critical damage recognition factor which associates to DNA lesions, allowing the repair by nucleotide excision, it is possible to say that USP7 has an important role in DNA repair due to the elimination of ubiquitination mark present in XPC [82].

Table 2. Summary of cellular consequences resulting from interaction between different USPs and target proteins associated with DNA damage repair.

USPs	Targets	Cell model association	USPs effects	References
USP1	FANCD2; PCNA	Multiple myeloma	Knockdown of USP1 reduces the viability of MM.1S cells	[83]
		Kidney	Knockdown of USP1 in HEK293T cells leads to cellular protection towards chromosomal aberrations	[57]
USP3	ChK1	Colorectal carcinoma	Knockdown of USP3 decreases apoptosis in HCT116 cells	[84]
USP7	MDC1	Cervical adenocarcinoma	Knockdown of USP7 prevented cellular proliferation in HeLa cells	[85]

		Cervical carcinoma xenografts in mice	Knockdown of USP7 supressed tumoral growth in mice bearing tumour xenografts from SiHa cells	
USP9x	Claspin	Bone osteosarcoma	USP9x loss of expression leads to accumulation of DNA damage in U2OS cells	[76]
USP11	XPC	Skin	Knockdown of USP11 inhibits DNA damage repair in HaCaT cells	[80]
	SPRTN	Lung adenocarcinoma and Bone osteosarcoma	USP11 cells is required for survival upon DNA – protein crosslinks in A549 and U2OS cells	[86]
USP20	Claspin	Gastric adenocarcinoma	Knockdown of USP20 promotes cellular proliferation in MGC-803 cells	[77]
USP21	BRCA2	Hepatocellular carcinoma	USP21 knockdown decreases tumoral growth in mice bearing tumour xenografts from HuH1 cells	[87]
USP51	H2A	Bone osteosarcoma	Knockdown of USP51 increases DNA damage in U2OS cells	[88]

3.1.3. Role of USPs in chromatin remodelling

The chromatin structure (which is usually changed in cancer) is controlled by post-translational modifications (PTMs) in histones. Although the most frequent studied PTMs in histones involved in chromatin remodelling are acetylation and methylation, also the ubiquitination (and deubiquitination) affects the chromatin structure, through the

modification in histones, mainly H2A and H2B. The ubiquitination and deubiquitination of histones, affects not only the cell cycle progression and the DDR mechanisms as mentioned above, but also the activation or repression in gene transcription [89, 90]. Recently, it was observed that the H2B monoubiquitination (specifically H2Bub1) leads to the chromatin opening, which facilitate the binding of transcription factors to DNA [91]. On the other hand, it was also shown that the monoubiquitination of the H2A histone by Polycomb Repressive Complex 1 (PRC1) lead to the compaction of the chromatin, repressing gene transcription [92]. Some USPs are involved in the process of histones deubiquitination.

USP22 interacts with both H2Aub1 and H2Bub1, removing the monoubiquitin present in the lysines 119 and 120, respectively [93, 94]. Through this interaction, the USP22 promote modifications at the chromatin structure, which are important in the genetic regulation. The overexpression of USP22 is frequently found in tumours “with a stem cell – like gene expression” and was correlated with worse prognosis in colon, pancreatic, gastric and breast cancers [95, 96].

USP7 is a significant regulator of PRC1, complex responsible to compact and inhibit chromatin remodelling [97] It was demonstrated in HCT116 cells that through its interaction with PRC1, USP7 targets the p16 *locus,* inducing apoptosis [98]. Besides to the regulation of PRC1, USP7 also control the EZH2 which is a functional enzymatic component of the PRC2 [99].

Table 3. Summary of cellular consequences resulting from interaction between different USPs and target proteins associated with chromatin remodelling.

USPs	Targets	Cell model association	USPs effects	References
USP7	EZH2 - PRC2	Prostate adenocarcinomas	Overexpression of USP7 increases migration, invasion and inhibits apoptosis in DUI145 and PC3 cells	[100]
		Prostate adenocarcinoma xenografts in mice	USP7 knockdown inhibits tumoral growth in mice bearing tumour xenografts from PC3 cells	

USP16	H2A	Bone marrow	USP16 knockdown leads to an increase of cellular quiescence in hematopoietic stem cells (HSC); USP16 regulates haematopoiesis and HSC functions	[101]
USP21	EZH2	Bladder carcinoma	USP21 overexpression promotes proliferation, invasion and migration in 5637 and T24 cells	[102]

3.1.4. Consequences of the interaction between USPs and proteins associated with several signalling pathways

a) p53 signalling pathway

p53 is a tumour suppressor, fundamental for cellular homeostasis (DNA repair, apoptosis and cell cycle arrest) and its mutations are commonly found in several human cancers [103]. Various USPs regulate p53 stability and may so play a role in tumorigenesis [104].

The USP7, USP10, USP29 and USP42 positively regulate p53, this is, they can deubiquitinate p53 and avoid its degradation by the 26S proteasome. Contrarily, the USP2 and USP4 negatively regulate p53. USP2 and USP4 stabilize the E3 ligases (Mdm2, UBE3A, ARF-BP1, Pirh2 and CARP) that are responsible for p53 inhibition [105, 106].

USP7 maintains the levels of diverse polyubiquitinated substrates and modify the p53 levels through the stabilization of Mdm2. This USP7 presents a dual role in the p53-Mdm2 pathways, since it can deubiquitinate p53 as well as Mdm2 [107, 108]. The USP7 overexpression, neutralize Mdm2-mediated p53 ubiquitination, stabilizing the p53, and inducing apoptosis. However, USP7 can also inhibit the degradation of the Mdm2 protein, led to accumulation and the suppression of p53 [104]. By this way, USP7 can be considered an oncogene or even a tumour suppressor, consonant whether it predominately deubiquitylates Mdm2 or p53, respectively [109]. The drivers or modifications governing these dual functions remain elusive.

USP10 also deubiquitinates Mdm2, independently of p53 ubiquitination. The ubiquitination of p53 by Mdm2 promote its migration from the nucleus to the cytoplasm, where it will be degraded [110]. In normal cell conditions, USP10 localizes in the cytoplasm, but in stress situations, it translocates to the nucleus where it affects p53 translocation to the nucleus. Thus, USP7 (in the nucleus) and USP10 (in the cytoplasm) modify the ubiquitination of p53 initiated by Mdm2, regulating homeostasis of p53. Similarly to USP10, also the USP29 and USP42 deubiquitylate the p53 under hypoxia conditions to stabilizing this protein, leading to the stop of the cell cycle and to the control of the cell proliferation and apoptosis [111, 112]. In gastric cancers, the USP42 was found upregulated and the staining intensity correlated with the tumour size and aggressiveness (staging, lymph node metastasis and the overall survival of patients) [68].

Contrarily, USP4 represses the p53 expression by acting as a negative regulator. It deubiquitinates and allows the accumulation of ARF-BP1 which is an E3 ligase that modulates the p53 degradation. Together with the fact that USP4 deficient MEF demonstrated retarded growth, premature senescence and resistance to oncogene transformation, these studies allow to consider USP4 as a potential oncogene [106]. Recently, it was been noticed the upregulation of USP4 in melanoma, avoiding cellular apoptosis and facilitating tumour metastasis [113]. USP2, as USP4, negatively control the p53 levels, through the exclusive regulation of an E3 that modulates p53 degradation, that in the case of USP2 is Mdm2. An upregulated USP2 leads to an increase of Mdm2 levels, and of p53 degradation [114]. In prostate adenocarcinoma, the USP2a (USP2 androgen-regulated) levels are very high, what connected with the positive regulation of USP2, avoid the death of cancer cells [115].

USP39 was seen overexpressed in ovarian cancer tissues in comparison to normal tissues. In ovarian cancer cells (HO-8910 and SKOV3), the silencing of USP39 inhibited the proliferation of these cell lines *in vitro* and led to a decrease of tumoral growth of USP39 silenced HO-8910 cells xenografted in nude mice. USP39 knockdown also increased p53/p21 levels. p53 together with p21 (CDK1 inhibitor) is responsible for the regulation of CDK1/B1 (complex that regulates the G2/M transition). Thus, p53/p21 inhibition lead to CDK/B1 suppression blocking cell cycle [64, 116]. Similarly, in thyroid and liver cancer cells (TT and SMMC-7721 cell lines, respectively), it was described that silencing of USP39 reduced cellular proliferation and induced G2/M arrest [65, 66].

Table 4. Summary of cellular consequences resulting from interaction between different USPs and target proteins associated with p53 signalling pathway.

USPs	Targets	Cell model association	USPs effects	References
USP2	Mdm2 - p53	Testicular embryonal carcinoma	USP2a knockdown induces apoptosis in NTERA-2 cells	[105]
		Prostate adenocarcinomas	Knockdown of USP2a induces apoptosis in LNCaP and DU145 cells	[115]
		Mouse embryonic fibroblasts	Overexpression of USP2a increases tumoral growth in mice bearing tumour xenografts from NIH3T3 cells	
		T-cell lymphoma	Knockdown of USP2a promotes apoptosis in MyLa2000 cells and decreases p53-dependent apoptosis in Hut-78 cells	[117]
USP3	p53	Osteosarcoma and lung	Knockdown of USP3 decreases p53 levels and increases cellular proliferation in U2OS and IMR90 cells	[118]
USP4	p53 – ARF-BP1	Mouse embryonic fibroblasts	USP4 silencing leads to early senescence, retarded growth and resistance to oncogene transformation in USP4 deficient-MEF cells	[106]
	p53	Melanoma	USP4 knockdown reduces invasion, migration and apoptosis in A2058 and 451Lu cells	[113]

			Upregulation of USP4 increases invasion, migration and apoptosis in A2058 and 451Lu cells	
USP7	Mdm2 - p53	Human fibroblasts	Slight reduction of USP7 levels destabilizes p53 levels in NHF-1 and IMR90	[56]
		Bone osteosarcoma	Severe reduction of USP7 levels stabilizes p53 levels in U2OS cells	
USP10	Mdm2 - p53	Renal carcinomas	Increase in USP10 levels inhibits colony formation and cell proliferation in CAKI-1 and CAKI-2 cells	[110]
USP29	p53	Colorectal carcinoma, Cervival adenocarcinoma and Bone osteossarcoma	USP29 provokes p53 accumulation and apoptosis in HCT116, HeLa and U2OS cells	[119]
USP39	p53	Ovarian carcinomas	Knockdown of USP39 increases p53 levels and stops the G2/M cell cycle phase in HO-8910 and SKOV3 cells	[64]
USP42	p53	Bone osteosarcoma	Downregulation of USP42 reduces p53 levels during the initial stress response phases in U2OS	[120]

b) Wnt signalling pathway

The Wnt pathway play an important role during the embryonic development and organogenesis, controlling stem cell self-renewal and cell migration, proliferation and differentiation. The end-up of the canonical pathway of Wnt is the stabilization and the

nuclear translocation of β-catenin. In the absence of Wnt ligands, β-catenin is phosphorylated by APC/GSK3/CK1 complex, becoming target of ubiquitination, with its consequent degradation [121]. β-catenin-dependent Wnt signalling is involved in various cellular functions being widely implicated in malignancy, particularly in colon cancer [122].

Some USPs were described to control of Wnt/β-catenin stabilization in cancer. Wnt is negatively regulated by USP4, through the interaction of the later with Nemo-like kinase (suppressor of the Wnt signalling pathway). On the other hand, USP15 is responsible for targeting β-catenin for degradation through the APC (Adenomatous Polyposis Coli), which is a stabilizer of a Wnt´s negative regulator [123, 124]. USP4 and β-catenin levels are positively associated in colon cancer tissues. It was demonstrated also *in vitro* that the knockdown of USP4 expression, led to decrease in the invasion potential and proliferation of HCT116 cells [125, 126]. In colon cancer, USP14 also present a direct correlation with β-catenin levels, what suggests an important oncogenic role in this pathway [127].

USP5 can activate this signalling pathway. USP5 deubiquitylates β-catenin, do not allowing its degradation, and promoting nuclear accumulation and signalling. Recently, it was seen that the expression of USP5 is higher in non-small lung cancer compared to normal tissues. This higher USP5 expression correlated with large tumour sizes, poor differentiation, advanced stage and decrease survival [128].

The expression levels of USP39 are higher in colon cancer tissues than in adjacent normal tissues and correlated with poor patient overall survival. In addition, USP39 is overexpressed in colon cancer cells (LoVo, Caco, SW480 and HT29). In two of these cell lines, SW480 and HT29, USP39 knockdown inhibited cell migration and invasion, and led to a decrease expression of β-catenin and of two matrix metalloproteinases (MMP2 and MMP9). MMP2 and MMP9 are direct transcription targets of Wnt/β-catenin signalling pathway. These MMPs are associated to cancer migration and metastization due to its role in the extracellular matrix degradation to allow cancer cells migration to out of the primary tumour and invasion of other tissues [129-131].

In ovarian cancer, USP39 expression is higher in comparison to normal ovarian tissues. It associated with TNM stages, presenting higher expression in advanced stages. The USP39 knockdown in two ovarian cancer cell lines (HO-8910 and SKOV3) led to inhibition of cell migration and blockage of epithelial-to-mesenchymal transition.

Although the mechanisms are not completely stablished, it seems that USP39 knockdown induced an increase of E-cadherin levels (generally, loss of function or expression of this protein is associated to cancer progression and metastasis) through Wnt/β-catenin signalling pathway, avoiding cellular migration [64, 132].

Table 5. Summary of cellular consequences resulting from interaction between different USPs and target proteins associated with Wnt signalling pathway.

USPs	Targets	Cell model association	USPs effects	References
USP3	MMP2	Gastric carcinoma	Knockdown of USP3 suppresses proliferation, migration and promotes G1 cell cycle arrest in HGC27 cells	[49]
		Gastric carcinoma xenografts in mice	Knockdown of USP3 decreases tumour growth in mice bearing tumour xenografts from HGC27 cells	
		Gastric adenocarcinoma	Overexpression of USP3 leads to increased migration and invasion in SK-GT-2 cells	
USP4	β-catenin	Colorectal carcinoma	USP4 knockdown decreases proliferation and invasion in HCT116 cells	[125]
USP5	β-catenin	Lung adenocarcinoma	USP5 overexpression increases cellular proliferation in A549 cells	[128]
			Knockdown of USP5 decreases cellular proliferation in H1299 cells	

		Lung adenocarcinoma xenografts in mice	USP5 knockdown decreases tumoral growth in mice bearing tumour xenografts from H1299 cells	
		Lung adenocarcinoma and Lung carcinoma	Knockdown of USP5 reduces β-catenin transcriptional activity and inhibits invasion and migration in H1299 and 95-D cells	[133]
USP7	Axin1	Kidney	Knockdown of USP7 decreases Axin levels leading to an increase in β-catenin levels and Wnt signalling activation in HEK293T cells	[134]
USP14	Dishevelled (Dvl)		Inhibition of USP14 increases polyubiquitination of Dvl hindering the progression of Wnt signalling in HEK293T cells	[127]
USP15	APC	Colorectal adenocarcinoma	Knockdown of USP15 decreases APC levels and increases β-catenin levels in HeLa cells	[135]
USP21	TCF7	Pancreas cancer *in vitro* and in vivo (xenografts in mice)	USP21 overexpression promotes cellular proliferation in hTERT-HPNE E6/E7 cells and tumoral progression in mice bearing tumour xenografts from hTERT-HPNE E6/E7 cells	[136]

USP34	Axin1	Kidney	Knockdown of USP34 decreases Axin1 levels and increases levels of β-catenin in HEK293T cells	[137]
USP39	β-catenin	Ovarian carcinomas	USP39 knockdown decreases β-catenin levels and inhibits cellular migration and invasion in HO-8910 and SKOV3	[64]
	β-catenin; TCF4; MMP2 and MMP9	Colorectal adenocarcinomas	USP39 knockdown reduces the expression of β-catenin, TCF4, MMP2 and MMP9, and prevents migration and invasion in HT29 and SW480 cells	[129]
USP44	Axin1	Colorectal adenocarcinoma and Colorectal carcinoma	USP4 overexpression increases Axin1 levels and decreases β-catenin, c-Myc and cyclin D1 levels, and also inhibits cellular proliferation and promoting apoptosis in HT29 and HCT116 cells	[138]

c) Receptor tyrosine kinase (RTK) signalling pathway

The RTK activation regulate the cellular growth and DNA repair, being important in oncogenesis and has been used as target of anticancer therapeutics. The relevance of RTK signalling in cancer is reflected by diverse abnormalities in RTK-dependent pathway [139]. Several evidences indicate that USPs ae very involved in the regulation of several RTKs (Table 6) but particularly in the regulation of EGFR (Epidermal Growth Factor Receptor), a specific type of RTKs relevant in several cancer and an important therapeutic target [140]. The USP8, USP17 and USP18 can stabilize the levels of EGFR, through the deubiquitination and regulation of the subcellular localization. The upregulation of EGFR levels is associated to cell cycle dysregulation and to cancer development. In cervical squamous cell carcinoma, the USP8 expression is increased in tumours than in adjacent normal tissues, and its expression is associated with high recurrence risk and advanced tumour stage [141]. USP17 and USP18 regulate the

expression of EGFR, due to a transcriptional activation and mRNA stabilization of miR-7 (EGFR regulator). Through EGFR control, USP17 and USP18 were shown to play a role in cancer cell survival [142]. The USP18 levels of expression are also higher in lung cancer tissues in comparison with normal adjacent tissues, stimulating invasion and metastasis [143].

Table 6. Summary of cellular consequences resulting from interaction between different USPs and target proteins associated with Receptor tyrosine kinases signalling pathway.

USPs	Targets	Cell model association	USPs effects	References
USP8	EGFR; ERBB3 and c-Met	Mouse embryonic fibroblasts	Inhibition of cellular proliferation in USP8 deficient-MEF	[144]
	EGFR	Lung adenocarcinoma	Overexpression of USP8 increases EGFR activity and cellular proliferation in PL16T cells	[145]
USP18	miR-7	Glioblastoma	USP18 knockdown increases miR-7 activity, decreases EGFR levels and cellular proliferation, and induces apoptosis in T98G and HeLa cells	[142]

d) Transforming growth factor-beta (TGFβ) signalling pathway

The TGFβ signalling pathway play a role in tumoral suppression. By one hand, it affects cancer cell proliferation through inhibition of cell cycle progression, and controls apoptosis. On the other hand, it can also promote evasion from the immune system and metastization through modulation of EMT mechanisms and tumour proliferation by microenvironment modulation [146].

The TGFβ signalling pathway is initiated when TGFβ binds to TGFβ Receptor II (TGFβRII). This linkage leads to activation of TGFβ Receptor I (TGFβRI), which in turn causes recruitment of two R-Smads (Receptor activated Smads), Smad2 and Smad3.

These bind to Smad4 and form the Smad2/Smad3/Smad4 complex. After formed, this complex migrates to the nucleus and binds to the target DNA, regulating gene expression [147]. TGFβR and Smads are regulated by reversible processes of ubiquitination [148]. Several USPs are involved on the regulation of this signalling pathway.

USP4 deubiquitinates TGFβ receptor I and activates TGFβ signalling to induce R-Smads phosphorylation, allowing the importation of R-Smads to the nucleus where they regulate the expression of target genes. In the human breast cancer MCF10A cells, it was observed the promotion of EMT by USP4, while in MDA-MB231cells it promotes migration and invasion [149] . USP4 knockdown in MDA-MB231 inhibits migration and invasion via TGFβ signalling. Also, in the breast T47D cells, the ectopic expression of USP4 led to an increase in migration and invasion [150].

USP15 remove the R-Smads monoubiquitination signal, allowing its subsequent binding to the target DNA [151]. USP15 overexpression leads to the malfunction of TGFβ pathway, and is found in breast, glioblastoma and ovarian cancers [152].

USP9x activates TGFβ through the Smad4 control. The deubiquitination of Smad4 by USP9X leads to an increase of TGFβ signalling in colon and breast cancer. In line with this, it was demonstrated that USP9X is essential for TGFβ-dependent pathway in HCT116 cells and TGFβ induced migration in MDA-MB231 cells [153].

Another USP involved in regulation of the TGFβ signalling pathway is USP26. Contrarily to the previous ones, USP26 is a negative regulator of the TGFβ signalling pathway. USP26 deubiquitinates Smad7 (antagonist of TGFβ receptor I), which results in degradation of the TGFβ receptor I. It was shown that the knockdown of USP26 in breast cancer MCF7 and MDA-MB231 cells increase TGFβ activity promotes an increase in migration and invasion of cells [154].

Table 7. Summary of cellular consequences resulting from interaction between different USPs and target proteins associated with TGFβ.

USPs	Targets	Cell model association	USPs effects	References

USP4	TGFβR1	Breast carcinoma	USP4 knockdown supresses cellular migration and invasion in MDA-MB231 cells	[149]
	Relaxin – TGFβ – Smad2 - MMP9	Breast carcinoma	Knockdown of USP4 inhibits migration and invasion in MDA-MB231 cells	[150]
		Breast carcinoma	USP4 overexpression promotes migration and invasion in T47D cells	
USP11	ALK5 – Smad7	Kidney	USP11 Knockdown inhibits epithelial mesenchymal transition in HEK293T cells	[155]
USP15	TGFβR1; Smad7	Glioblastoma xenografts in mice	USP15 knockdown reduces tumoral growth in mice bearing tumour xenografts from T98G cells	[152]
USP9x	Smad4	Breast carcinoma	USP9x knockdown increases migration in MDA-MB231 cells	[153]
USP22	TGFβR1	Lung adenocarcinoma	Knockdown of USP22 decreases cellular proliferation and invasion in H1650 cells	[156]
		Lung adenocarcinoma	Overexpression of USP22 increases cellular growth and migration in A549 cells	
USP26	Smad7	Breast carcinoma	USP26 knockdown increases cellular migration and invasion in MCF7 and MDA-MB231 cells	[154]

3.2. USPs inhibition as a therapeutic approach

Given the evidences that USPs are involved in the development of diverse human cancers, the inhibition of USPs emerge as a novel therapeutic opportunity. Several small molecules that can inhibit USPs were identified, and some of them have demonstrated antitumorigenic potential (Table 8).

Table 8. List of some inhibitors reported to target USPs and their cellular effects.

USP	Inhibitor	Cellular effects	References
USP1	Pimozide	Increases the ubiquitination levels of PCNA and FANCD2, and inhibits growth and viability in U2OS and MCF10A cells	[157]
	ML323	High specificity for USP1/UAF1; Increases the ubiquitination levels of PCNA and FANCD2 in HEK293T and H596 cells	[158]
		Inhibits (synergistic effect with cisplatin) proliferation in H596 and U2OS cells	[159]
		Inhibits migration/invasion ability of MCF7, MDA-MB231 and 4T1 cells; Suppresses lung metastatic in mice harboring 4T1 tumours	[160]
	SJB2-043	Induces apoptosis of K562 cells	[161]
	SJB3-019A	A potent USP1 inhibitor, 5 times more potent than SJB2-043 in inducing of apoptosis in K562 cells	
	C527	Degrades ID1 in human U2OS cells and increases the levels of Ub-FANCD2 and Ub-FANCI in HeLa cells	
USP2	AM146		[162]

	RA-9 RA-14	Provokes cell cycle checkpoint arrest and apoptosis in MDA-MB231 and MDA-MB468 cells	
	ML364	Increases Cyclin D1 degradation, blocks G1/S transition and inhibits cellular proliferation in colorectal cancer HCT116 and Mino (Mantle Cell Lymphoma) cells	[163]
USP4	Vialinin A	A natural compound isolated from Thelephoravialis with the ability to inhibit the enzymatic activity of USP4, USP5 and UCHL1	[164]
USP5	WP1130	Induces ubiquitination of p53 and Mcl-1 in Z138 cells	[165]
	Vialinin A	A natural compound isolated from Thelephoravialis with the ability to inhibit the enzymatic activity of USP4, USP5 and UCHL1	[164]
	EOAI3402143 (G9)	Suppresses p53 and FAS levels in A375 cells; Suppresses melanoma growth in mice harboring A375 tumours	[166]
	RA-9 RA-14	Provokes cell cycle checkpoint arrest and apoptosis in MDA-MB231 and MDA-MB468 cells	[162]
USP7	HBx19818	Stabilizes p53 and promoting G1 arrest and apoptosis in HCT116 cells	[167]
	HBx28258	Reduces HCT116 cell proliferation, induces caspase activity and PARP cleavage, and arrest HCT116 cancer cells in G1 phase	
	HBx41108	Stabilizes and activate p53, inhibits HTC116 cell growth and induces of p53-dependent apoptosis	[168]
	P5091	Induces apoptosis in MM.1S cells	[169]

	P22077	Predominantly inhibits USP7, thus regulating the apoptotic pathway of p53; Suppresses neuroblastoma growth in mice harboring IMR32 tumours	[170-172]
	FT671	Increases p53 protein levels in HCT116 and U2OS cells and stabilizes p53 in the MM.1S; Suppresses multiple myeloma growth in mice harboring MM.1S tumours	[173]
	XL188	Promotes the accumulation of p53 and p21 in MCF7 and MM.1S cells	[169, 174]
USP8	HBx90397	Induces G1 arrest and inhibits cell growth in HCT116 and PC3 cells	[167, 175]
	RA-9	Decreases viability of HeLa, SiHa, CaSki, TOV21G1, SKOV3, ES-2, MDA-MB231, MDA-MB435A, MDA-MB468 cells	[162]
		Induces apoptosis in ES-2 cells; Suppress ovarian cancer growth and increase overall survival in mice harboring ES-2 tumours	[176]
	RA-14	Decreases viability of HeLa, SiHa, CaSki, TOV21G1, SKOV3, ES-2, MDA-MB231, MDA-MB435A, MDA-MB468 cells	[162]
	AM146		
USP9x	WP1130	Reduces levels of Mcl-1 protein and stimulates apoptosis in K562 cells	[177]
	EOAI3402143 (G9)	Induces apoptosis in MV4 11 and K562 cells	
		Reduces Mcl-1 levels and increases p53 levels and apoptosis in MM.1S cells; Decreases tumoral growth in mice bearing tumour xenografts from MM.1S cells	[178]

USP10	P22077	Degrades FLT3, leading to death of HEK293T cells	[179]
	HBx19818		
	Spautin1	Induces PI3K3C3 complex degradation and leads to apoptosis of K562 cells	[180]
USP11	Mitoxantrone	Increases apoptosis in PL5 cells	[181]
USP13	Spautin1	Induces PI3K3C3 complex degradation and leads to apoptosis of K562 cells	[180]
USP14	b-AP15	Inhibits cell growth and overcomes bortezomib resistance in MM.1S cells; Anti-cancerous effect against solid tumour and multiple myeloma in vivo	[182, 183]
	VLX1570	Analogue of b-AP15 that induces toxicity and apoptosis in OPM2, KMS11, BCWM3 and RPCI-WM1 cells	[184-186]
	WP1130	Inhibits the activity of several DUBs such as USP5, UCH-L1, USP9x, USP14, and UCH37	[165]
	IU1	Induced cell cycle arrest and apoptosis in MDA-MB153 and MDA-MB231 cells and inhibited cell proliferation in LNCaP cells; Decreases (synergistic effect with enzalutamide) tumoral growth in mice bearing tumour xenografts from MCF7 cells	[187-189]
USP24	EOAI3402143 (G9)	Reduces Mcl-1 levels and increases p53 levels and apoptosis in MM.1S cells; Decreases tumoral growth in mice bearing tumour xenografts from MM.1S cells	[178]

3.2.1. Inhibitors targeting USP7

The majority of therapies targeting DUBs lean over the USP7 due to the existence of a wide role of this USP in diverse cellular pathways, but also due to its abnormal expression in diverse human cancers. Some of the inhibitors targeting USP7 are: HBx19818, HBx28258, HBx41108, P5091, P22077, FT671 and XL188.

HBx41108 is a non-selective inhibitor of USP7. Its specificity is limited, inhibiting diverse DUBs besides USP7, namely USP5 and USP8. Given that USP7 deubiquitinates and stabilizes p53 and Mdm2 levels, it was suggested in HCT116 cells, that USP7 inhibition led to Mdm2 inactivation and p53 activation, resulting in cell cycle block and apoptosis. HBx41108 was shown to revert USP7-mediated p53 deubiquitination in HEK293T (human embryonic kidney cells) and to inhibit proliferation and induce apoptosis in HCT116 cells [168].

HBx19818, HBx28258, P22077 and small molecules inhibit specifically the USP7 [168]. The HBx19818-USP7 inhibition provoke an increase of ubiquitination of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), limiting the inflammatory response in the treatment of acute diseases and chronic inflammation [2]. This USP7 inhibitor reduce the USP7 deubiquitylate function, promoting p53 mediated apoptosis in colon cancer HCT116 cells [190]. P22077 also stabilize p53 through USP7 inhibition, inducing apoptosis and cellular proliferation inhibition in neuroblastoma IMR32 and SH-SY5Y cells. This compound can be administrated to patients suffering from neuroblastoma, since in these neoplasias the USP7 levels are increased [172].

USP7 inhibition through P5091 led to an increase of Mdm2 ubiquitination and induction of apoptosis in multiple myeloma MM.1S cells. Also, it was observed in ovarian cancer cells (Hey A8; OUCAR-84) that USP7 inhibition by P5091 deregulate Mdm2 and positive regulate p53 and p21 levels. As result, there was a cell cycle block and apoptosis induction mediated by this compound [191]. In colon cancer, mutated APC promote β-catenin deubiquitination mediated by USP7 leading to the activation of Wnt signalling. This Wnt activation promote the cellular growth in colon cancer [190]. The inhibition of USP7 by P5091 in colon cancer cells (HCT116, SW480 an Caco-2) stabilize Wnt signalling activity through β-catenin degradation and supress colon cancer cells proliferation [192].

FT671 inhibits USP7, conducting to an increase of p53 levels in HCT116 and U2OS. This p53 upregulation correlated with Mdm2 degradation. Besides this, it has

been also described the stabilization of p53 levels in MM.1S cells [173]. Similarly to FT671, also XL188 provokes the rise in p53 levels and p21 in MCF7 [193].

3.2.2. Inhibitors targeting USP14

USP14 also has an important role in cellular maintenance during metabolical stress conditions and neuronal development [194]. In some cancers (colon, lung and ovarian), USP14 is implicated as oncoprotein that can be responsible for metastization and tumoral growth [195-197]. USP14 overexpression is associated to diverse diseases, and due to this, the reduction of its levels or activity can be promising as a cancer therapy strategy. Some small molecules such as b-AP15, IU1 and WP1130 were shown to inhibit USP14.

b-AP15 is an agent that selectively inhibits DUBs associated to 19S proteasome, like UCHL5 and USP14 [183]. b-AP15 induces the polyubiquitin accumulation in cells, leading to oxidative stress, which results in apoptosis. USP14 knockdown inhibits cell proliferation and induces cell apoptosis in neuroblastoma cells. b-AP15 interrupted the proliferation in MM.S1 cells through CDC25C negative regulation and, as consequence CDC25C does not phosphorilate CDK1 and cell cycle blockage. b-AP15 also inhibited in vivo tumoral growth of b-AP15 cells xenografts [182]. Furthermore, b-AP15 showed its ability to inhibit carcinogenesis process in solid tumours mice models like lung and colon [40]. Recently, it was discovered VLX1570 inhibitor, first inhibitor to enter in clinical trials, which is functionally very similar to b-AP15. As b-AP15, VLX1570 also inhibits proteasomal activity [184, 186]. However, the clinical trial with VLX1570 was interrupted due to two deaths provoked by its severe lung toxicity [184].

In opposition to b-AP15 and VLX1570, WP1130 do not induce oxidative stress and present a huge range of action [165]. WP1130 is a DUB inhibitor that can inhibit some USPs, namely, USP5, USP9x and USP14. WP1130 induces accumulation of protein-ubiquitin conjugates, leading to the cellular apoptosis. These apoptotic effects avoid tumoral proliferation in leukaemia, glioblastoma and melanoma [198-200]. In human myelogenous leukaemia K562 cells, WP1130 inhibits some interleukins (IL-6 and IL-3) and negatively regulates Bcr-Abl, avoiding cellular growth and promoting apoptosis [198].

IU1 inhibits USP14 through the connection between the inhibitor and the catalytic domain of the USP14, blocking the interaction with the substrate [189]. Inhibition of USP14 by IU1 was shown to induce cell cycle arrest and apoptosis in MDA-MB153 and MDA-MB231 cells and to inhibit cell proliferation in human prostate adenocarcinoma

LNCaP cells. USP14 is responsible for the deubiquitination and stabilization of androgen receptor (AR) in breast and prostate cancer cells. AR is associated to the progression and development of both cancers. In LNCaP cells was observed that AR promote the transition G1/S through the positive regulation of cyclin D. Cyclin D, by connecting to CDK, inactivates retinoblastoma tumour suppressor (negative regulator of cell cycle transition). In breast cancer, the combination of enzalutamide (AR inhibitor) with IU1 (USP14 inhibitor) led to a strong reduction in tumoral growth in nude mice bearing tumour xenografts from MCF7 [187, 188].

3.2.3. Others USP inhibitors

USP2 is responsible for the deubiquitination and stabilization of cyclin D1. ML364 is an inhibitor of USP2 that led to an increase cyclin D1 cellular degradation avoiding the cell cycle progression (G1/S). USP2 is an important element to the cell cycle and tumoral growth. In breast, prostate and ovarium cancer, USP2 levels are overexpressed, being associated to poor prognosis [201, 202]. A study realized with colon cancer cell line, demonstrate the capacity of ML364 in reducing the cyclin D1 levels through the block of USP2 [163].

Another inhibitor of USPs is Mitoxantrone, which inhibits USP11. USP11 in association with BRCA2 form an important complex of DDR. These two regulate the stability of proteins associated to DNA, such as p53 and H2A. The inhibition of USP11 by Mitoxantrone avoid the DNA repair [203, 204].

Similarly to ML364 and Mitoxantrone, Spautin1 is described as an inhibitor of USPs. USP10 and USP13 are responsible for regulating Beclin1, a tumor suppressor responsible for inducing autophagy processes. In myelogenous leukemia K562 cells, Spautin1 inhibited USP10 and USP13, leading to increased Beclin1 ubiquitination and degradation and inhibiting autophagy [180].

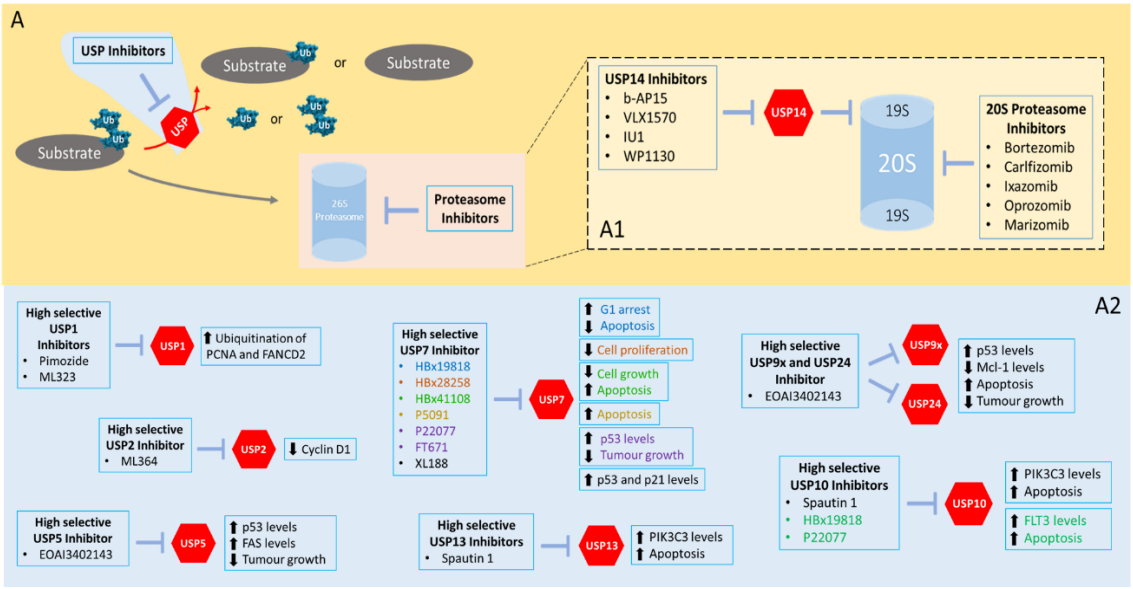

Figure 3| Interaction of USP inhibitors in the ubiquitin-proteasome pathway and cellular consequences. (A) A schematic representation about the role of USPs in the ubiquitin-proteasome pathway (image with orange and light pink background) and USP inhibitors (image with light blue background). (A1) Usp14 acts as a regulator of the 26S proteasome, due to inhibiting the 19S subunit. USP14 can be inhibited by four different inhibitors, b-AP15, VLX1570, IU1 and WP1130. (A2) Cellular consequences of specific inhibition of some USPs with inhibitors only described as USP inhibitors. (Note: The text colours serve to help the reader to associate the inhibitor with its respective cellular consequence).

General conclusion

USPs interact with a high number of proteins involved in important signalling pathways and cellular mechanisms. Through the regulation of these targets, USPs play a crucial role in the maintenance of cell homeostasis.

Due to this wide interaction network and regulation of many cellular processes, deregulation in the expression or activity of USPs have been associated with cancer development and progression.

Thus, therapeutic targeting of USPs emerges as an attractive approach for cancer management and treatment. Over the past few years, several USPs small molecule inhibitors have been developed and some demonstrated promising results.

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