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Dissenting degradation: Deubiquitinases in cell cycle and cancer.

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Abstract

Since its discovery forty years ago, protein ubiquitination has been an ever-expanding field. Virtually all biological processes are controlled by the post-translational conjugation of ubiquitin onto target proteins. In addition, since ubiquitin controls substrate degradation through the action of hundreds of enzymes, many of which represent attractive therapeutic candidates, harnessing the ubiquitin system to reshape proteomes holds great promise for improving disease outcomes. Among the numerous physiological functions controlled by ubiquitin, the cell cycle is among the most critical. Indeed, the discovery that the key drivers of cell cycle progression are regulated by the ubiquitin-proteasome system (UPS) epitomizes the connection between ubiquitin signaling and proliferation. Since cancer is a disease of uncontrolled cell cycle progression and proliferation, targeting the UPS to stop cancer cells from cycling and proliferating holds enormous therapeutic potential. Ubiquitination is reversible, and ubiquitin is removed from substrates by catalytic proteases termed deubiquitinases or DUBs. While ubiquitination is tightly linked to proliferation and cancer, the role of DUBs represents a layer of complexity in this landscape that remains poorly captured. Due to their ability to remodel the proteome by altering protein degradation dynamics, DUBs play an important and underappreciated role in the cell cycle and proliferation of both normal and cancer cells. Moreover, due to their enzymatic protease activity and an open ubiquitin binding pocket, DUBs are likely to be important in the future of cancer treatment, since they are among the most druggable enzymes in the UPS. In this review, we summarize new and important findings linking DUBs to cell cycle and proliferation, as well as to the etiology and treatment of cancer. We also highlight new advances in developing pharmacological approaches to attack DUBs for therapeutic benefit.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

Introduction

Cancer cell cycles

Cancer is a heterogenous disease. Between different disease locations and subtypes, as well as between patients with seemingly indistinguishable disease, there are a diverse set of genetic, epigenetic, metabolic and signaling aberrations that contribute to disease phenotypes and therapeutic responses. Nevertheless, cancer cells are characterized by a set of common, unifying features that are found across tumors. These are collectively referred to as the hallmarks of cancer, as first articulated by Douglas Hanahan and Robert Weinberg[1]. Among these common features, one of the most prominent, and most easily appreciated by non-scientists and cancer patients, is the uncontrolled proliferative capacity of cancer cells. This gives rise to the rapid cell divisions that result in tumor growth and the increasing cancer burden within a patient's body. The loss of proliferative control is common among all tumors. Stopping tumor cells from proliferating is the goal of most current and developing cancer therapies.

This characteristic of uncontrolled proliferation is often the result of alterations to the cell cycle machinery, which is the normal physiological process used by every organism to develop and repair tissues. The process by which one cell divides, giving rise to two identical daughter cells, has been historically studied during normal growth in yeast, as well as during the development of frogs or clams[2]. Determining how alterations to the cell cycle can produce the dramatic consequences associated with malignancy has been a driving force in cancer biology since the identification of genes, proteins and signaling pathways that provoke normal cell proliferation. Importantly, many of the proteins that coordinate normal cell cycles take on dysfunctional roles in disease, where they become "drivers", by triggering inappropriate cell divisions and thus promoting cancer. Alternatively, others which serve as "brakes" on cell cycle progression are lost in malignancy, and with them the ability to restrain cell proliferation. The loss of this latter group can give rise to inappropriate cell divisions and cancer progression.

Oscillating enzyme activities in the cell cycle

Central to cell cycle progression are the various pairs of cyclin-Cyclin dependent kinase (CDK) complexes, which direct progression through the different phases of the cell cycle[3]. The activity of cyclin-CDK is evolutionarily conserved and was first described in genetic screens done in yeast and biochemical studies in frog eggs[2]. The oscillating activity of cyclin-CDK complexes is controlled, in part, by synthesis of the cyclin subunit[4,5]. Significantly, studies in mid-1990s demonstrated that the activity of cyclin-CDK complexes is controlled by the degradation of both cyclins and CDK-inhibitors proteins (CKIs), highlighting the essential and conserved role for proteolysis in coordinating the waves of CDK activity that drive normal and cancer cell cycles[5–8]. While initial studies into CDK control focused on the cyclins which promote mitosis, cyclin A and B, later studies from the Beach, Sherr and Reed labs identified two new cyclins, D and E, which accumulate during G1 to push cells into S phase[9–11]. This new class of G1 cyclins, like the mitotic cyclins, also pairs with CDKs[12]. It was further shown that similar proteolytic events drive the temporal changes in abundance of cyclin D and E[13]. Altogether, decades of genetic and biochemical

studies provided a framework for understanding that cell cycle progression is driven by the temporally controlled activity of different cyclin-CDK pairings, whose regulation occurs at the level of its cyclin and CKI binding partners through transcriptional and proteolytic regulation[14].

A central aspect in cell cycle progression is the oscillation of these key regulators at different stages. Cell cycle transcriptional dynamics for cyclins, and many other genes, is mediated largely by the E2F, B-MYB and FoxM1 transcription factors and represents the starting point of their oscillations[15–17]. Defects in transcriptional control of cell cycle genes play on important role in tumorigenesis. This is typified by recurrent, biallelic loss-of-function mutations in the retinoblastoma gene, a vital inhibitor of E2F, in children with a rare eye cancer for which the gene is named[18]. At the other end, proteolysis through the ubiquitin proteasome system (UPS) represents an end point in a protein's life in the cell. Here we focus on proteolysis, but point interested readers to excellent reviews on transcriptional dysregulation in cancer[15–17].

The conjugation of ubiquitin onto substrates occurs through an elaborate three step enzymatic cascade which results in the covalent modification of a substrate by the small protein ubiquitin[19] (Figure 1). Historically, the UPS was revealed through elegant biochemical studies as a multi-component system required for proteolysis in an ATP-dependent manner[20–23]. This pioneering work was driven most centrally by Avram Hershko, and his collaborators Aaron Ciechanover and Irwin Rose. This led to the realization that, as is now known to occur in the vast majority of instances, ubiquitin is first activated, in an ATP-dependent manner, by an ubiquitin activating enzyme (E1), then transferred to an ubiquitin conjugating enzyme (E2), and finally ligated onto a specific substrate through the use of an ubiquitin ligase (E3), resulting in ubiquitin conjugation onto a lysine residue in the target (Figure 1). The ability of a ubiquitinated substrate to be degraded involves the generation of a ubiquitin chain, where a lysine residue in ubiquitin is subsequently conjugated to another ubiquitin. Historically, it was shown that ubiquitin chains containing at least four ubiquitin molecules and linked through lysine 48 (K48-Ub chains) are sufficient signals that are recognized by the barrel-shaped 26S proteasome, which degrades the ubiquitinated substrates, and recycles amino acids and ubiquitin molecules[24] (Figure 1). More recently, shorter chains and those linked through lysine 11 in ubiquitin (K11-linked chains) were also shown to play a role in the degradation of proteins in mitosis, and emerging evidence suggests a potential for chains with mixed linkages[25]. The highly complex nature of the cascade is further illustrated by the hundreds of human genes dedicated to ubiquitination, including two E1s, approximately 40 E2s and hundreds of E3s[26].

E3 ubiquitin ligases specify substrates for ubiquitination and play a particularly important role in cell cycle progression, where the temporal and accurate degradation of cell cycle regulators must be ensured for genome stability. The temporal activity of the CDKs is controlled by the destruction of cyclins and CKIs through the UPS. The most prominent ubiquitin ligases involved in cell cycle are the giant, 1.2 MDa, multi sub-unit ubiquitin ligase Anaphase Promoting Complex/Cyclosome (APC/C) and the modular SKP1-CULLIN1-F-box (SCF) family of E3s. The APC/C was first identified by the Hershko and Kirschner labs

using cell free extracts as the ubiquitin ligase responsible for the degradation mitotic cyclins, and thus, a main driver of chromosome segregation[27,28]. The APC/C is active from mitosis through the ensuing G1 where it restrains S-phase entry, before being turned off by myriad mechanisms to allow cells to progress to the next round of mitosis[29]. The SCF-family of E3s use F-box proteins as substrate receptors to target specific proteins for ubiquitination[30]. Skp2 is among the 70 human F-box proteins and a key cell cycle regulator[31]. SCF^{Skp2} functions to degrade the CKIs p21 and p27, which restrain progression through G1[32]. Highlighting the interplay between ubiquitin cascades, Skp2 levels are kept low in early G1 through APC/C-dependent degradation[32]. Then, once APC/C is inactivated in late G1-phase, Skp2 accumulates and triggers the degradation of the CKIs p21 and p27. This releases their inhibition on G1-S CDKs to promote S-phase entry. Taken together, these results demonstrate that the UPS is a mandatory signaling apparatus in cell cycle progression. Many E3s, as well as some E2s, have been implicated and well-studied in cell division. However, the contribution of enzymes deconjugating ubiquitin from proteins remains less well-studied by comparison and represents an emerging field where much work remains to be done.

Deubiquitinases

Overview of DUB function

In addition to defining the enzymatic basis for ubiquitin conjugation, Hershko and colleagues postulated that an “amidase” would exist to remove ubiquitin from substrates[23]. These amidases turned out to be proteases, which were discovered and first characterized by Cecile Pickart and Irwin Rose[33,34]. Indeed, analogous to other post-translational modifications, ubiquitination is a reversible process mediated by deubiquitinases or DUBs, 99 of which are encoded in our genome[35,36] (Figure 1). In comparison to our current understanding of E3 ubiquitin ligases, our knowledge of DUB function and regulation is still lagging[37]. Nevertheless, many labs have provided important insights into these fascinating enzymes, at the structural, enzymatic and physiological level. There are several excellent reviews on DUB enzymology, structure and general specificity, and interested readers are pointed towards these seminal reviews[38,39].

DUBs deconjugate ubiquitin from proteins thus antagonizing E3 ligases (Figure 1). This can result in different outcomes, although the most obvious is to protect proteins from degradation. In the case where ubiquitination is non-proteolytic, DUBs can switch off a signaling event triggered by ubiquitin conjugation. Altogether, DUBs can be seen as the other side of the coin of protein ubiquitination, and they contribute to the dynamic interplay between ubiquitin addition and removal from the whole proteome[40]. The main characteristic of DUBs is that they are proteases, and that among the ~500 genes encoding proteases in our genome, DUBs accounts for ~20% of them, making it the largest family of proteases in humans. Based on their enzymatic activity, they can be divided in two groups: cysteine-based or metalloprotease DUBs. The cysteine based DUBs include six families, based on their different enzymatic domains that can be found across this group: ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), ubiquitin carboxyterminal hydrolases (UCHs), the Machado–Joseph disease proteases (MJDs), and the recently identified motif interacting with ubiquitin-containing novel DUB family (MINDY)[41] as well as ZUP1[42–45].

The metalloprotease DUBs include only the zinc-dependent metalloproteases (JAMMs) family. The most prominent function of DUBs is to antagonize ubiquitination to contribute to the balance between conjugation/deconjugation of substrates to regulate cellular pathways. Their role is therefore analogous to phosphatases in kinase signaling pathways.

Another important and often underappreciated feature of DUBs is their role in enabling ubiquitination. The ubiquitin protein is encoded by four genes in humans, and interestingly, they are translated as inactive precursors. The first two genes, UBA52 and UBA80 consist of a single ubiquitin molecule fused through its C-terminus to a ribosomal protein, L40 and S27A, respectively. The other two genes, UBB and UBC encode ubiquitin polymers (3–4 and 7–10, respectively, depending on the organism) linked in a “head-to-tail” fashion followed by a C-terminal extension which varies depending on the gene. After translation of ubiquitin encoding mRNAs, DUBs cleave these protein polymers into active ubiquitin monomers. Five DUBs have been shown to perform this function: UCHL3, USP9X, USP7, USP5 and Otulin[46]. Interestingly, USP5 is one of four DUBs that was found to be essential across more than 400 cell lines, through multiple genome-wide CRISPR–Cas9 and RNAi screens[39]. Despite some progress, most notably during the past decade, the biology of DUBs is still in its infancy, especially compared to our vast understanding of E3 ubiquitin ligases. In the sections below, we will summarize to the best of our knowledge the findings that have linked DUBs to control of cell cycle progression, proliferation and malignant progression.

BAP1 – The most frequently mutated DUB in human cancers

BAP1, which stands for BRCA1 associated protein 1, is a DUB with tumor suppressive function[47,48]. BAP1 is a UCH family deubiquitinase that was originally described as being exclusively nuclear[49], although more recent studies have shown that it has important functions in the cytoplasm as well[50,51]. BAP1 was identified in 1998 through a yeast two hybrid screen looking for BRCA1 interacting proteins[49]. BRCA1 is a well-known tumor suppressor, involved in DNA repair via homologous recombination, and whose loss of function because of inactivating mutations has been correlated with a high risk of hereditary breast and ovarian cancer[52]. BRCA1 contains a RING domain, which is found in many E3 ubiquitin ligases, and by associating with a second RING-domain containing protein, BARD1, functions as a ubiquitin ligase for DNA-associated proteins[53]. BRCA1 is involved in a myriad of different DNA repair mechanisms, which have been extensively reviewed, thus explaining its tumor suppressive activity through its function as an effector of the DNA Damage Response (DDR)[54,55].

BAP1 was originally shown to potentiate the antiproliferative effects of BRCA1, leading to the idea that BAP1 also has tumor suppressive functions[49]. Further studies identified BARD1 as being deubiquitinated by BAP1, confirming the notion that BAP1 reinforces the activity of the BRCA1/BARD1 heterodimer[56,57]. However, in the decade following its discovery, BAP1 was shown to regulate proliferation by modulating cell cycle progression through its interaction with the transcriptional co-factor host cell factor 1 (HCF-1)[58,59]. HCF-1 is a chromatin-associated and nuclear co-activator, highly conserved in animals, which binds proteins through an HCF-1-binding motif (HBM), leading to the recruitment of a variety of epigenetic regulators, including the tri-thorax-related mixed-lineage leukemia

(MLL) and the Set1 histone H3 lysine 4 methyltransferases (H3K4 HMTs) to modulate gene expression[60,61]. HCF-1 also interacts with the E2F1 transcription factor at E2F-responsive promoters. Mechanistically, HCF-1 recruits MLL and H3K4 HMTs at the G1/S transition to induce histone methylation and transcriptional activation, thereby promoting cell proliferation[62].

The connection between HCF-1 and BAP1 was made by two different groups in 2009[58,59]. In complementary papers, BAP1 was used as a bait for IP/MS experiments and HCF-1 was identified as an interacting protein. In addition to interacting with HCF-1, BAP1 deubiquitinates and protects it from degradation. Both groups showed that BAP1 depletion inhibits proliferation[58,59], although the precise contribution of its interaction with HCF-1 is still unclear, highlighting the need for more studies to improve our understanding of how BAP1 contributes to this important oncogenic barrier[29]. In addition, the Wilkinson lab showed that following DNA damage, BAP1 becomes phosphorylated at serine 592[63], which matches the consensus sequence for the ATM/ATR DDR kinases. Mechanistically, this phosphorylation event displaces BAP1 from chromatin, presumably to remove it from specific promoters. Interestingly, BAP1 phosphorylation is cell cycle regulated, with a peak of modification at the G1/S transition. However, the importance of this phosphorylation towards BAP1 enzymatic activity or regulation of binding to substrates is still lacking.

The link between BAP1 and tumor suppression dates back more than a decade. The Wilkinson lab investigated the potential tumor suppressive effect of BAP1 because the gene locus of BAP1 had been observed to undergo frequent loss of heterozygosity in cancers[64]. They further characterized the effect of a catalytically dead version, and a mutant form of BAP1 which abrogates its nuclear localization, and found that both impaired its ability to exert tumor suppressive functions in nude mice[65]. Surprisingly, this effect was independent of BRCA1, highlighting the fact that BAP1 may be involved in additional tumor suppressive pathways. It is now well known that BAP1 is recurrently mutated in a variety of malignancies, including uveal melanoma, mesothelioma, cholangiocarcinoma and others. Remarkably, approximately 20% of cholangiocarcinoma and more than 80% of highly metastatic uveal melanoma tumors harbor inactivating mutations in BAP1, highlighting its importance in tumor suppression[66,67].

More recently, the Dixit lab showed that BAP1 knock out in mice is embryonic lethal. However, loss of BAP1 in adult mouse hematopoietic cells leads to a myeloproliferative/ myelodysplastic disorder with features of human chronic myelomonocytic leukemia (CMML)[68]. CMML-like disease is due to BAP1 loss specifically in the hematopoietic compartment, as demonstrated by bone marrow transplantation experiments. Further proteomic experiments identified O-linked N-acetylglucosamine transferase (OGT), as well as the polycomb group of proteins ASXL1 and ASXL2, as BAP1 substrates. Importantly, ASXL1 is also mutated in CMML diseases, thus suggesting that the BAP1/ASXL1 axis is central to suppression of CMML-like disorders[69]. These in vivo studies highlight the role of BAP1 in restraining cell proliferation during normal cell cycle progression, as well as contributing to tumor progression. More studies are needed to fully understand the contributions of BAP1 through its interaction with different binding partners such as BRCA1, OGT or ASXL1.

USP7 – The superstar DUB in cancer

USP7/HAUSP (herpesvirus-associated ubiquitin-specific protease, hereafter referred to as USP7) is a member of the USP family of DUBs, and by far the one that has garnered the most attention in cancer biology. The reason USP7 is so well known and studied, both by academic labs and pharmaceutical companies, is because of its central role in the p53/Mdm2 pathway. The p53 tumor suppressor is the most frequently inactivated tumor suppressor in all human cancers[70]. Indeed, its role as "guardian of the genome" allows cells to sense and trigger a complex network of molecular signals to respond to DNA damage, as well as myriad other stressors and insults, that ultimately halt proliferation by inducing a cell cycle arrest or triggering apoptosis[71]. Accordingly, more than 50% of human tumors have either lost p53 function through mutation and inactivation, or by developing oncogenic mechanisms to constitutively degrade p53 through the UPS. Among them, the most well-known is the oncogenic RING E3 ubiquitin ligase Mdm2, which ubiquitinates p53 under normal physiological conditions[72]. Thus, in nearly half of the cases where p53 is inactivated, this is due to overexpression of Mdm2, which in turn triggers constitutive p53 degradation. Therefore, the rationale of targeting the p53/Mdm2 interaction holds significant therapeutic promise for restoring p53 function in tumors where it is not mutated or lost.

The development of Mdm2 inhibitors, termed Nutlins, showed that it is possible to restore p53 function in tumors that retain wild type p53, leading to an antiproliferative effect and tumor regression. However, another way of targeting Mdm2 to restore p53 function would be through the destabilization of Mdm2 itself. In that scenario, inhibiting the DUB responsible for Mdm2 stability could hold similar therapeutic potential. This is precisely why USP7 has attracted so much interest.

USP7 was cloned in 2002 by the Gu lab, after it was found through IP/MS as a p53 interacting protein[73]. In this study, it was found that USP7 overexpression stabilized p53, suggesting that USP7 was a positive regulator of p53. However, in 2004 the Vogelstein lab observed the opposite phenotype[74]. That is, by knocking down USP7 expression through targeted homologous recombination, Vogelstein and colleagues found that rather than stabilizing p53, USP7 instead protects Mdm2 from auto-ubiquitination, thus indirectly triggering p53 degradation. Importantly, around the same time, the Gu lab, who initially identified USP7, similarly reported that USP7 loss destabilizes Mdm2, and this is concomitant with an accumulation of p53 and an induction of cell cycle arrest in G1 through subsequent upregulation of the CKI p21[75]. These complementary studies suggested the existence of a feedback loop and a more complex regulation than what was initially appreciated. A now extensive body of work has established USP7 as a negative regulator of p53 by virtue of its ability to stabilize Mdm2. This suggested the promising possibility that pharmacological inhibition of USP7 in cancer would trigger Mdm2 degradation, increasing p53 and reawakening its tumor suppressive function.

The identification of potent and selective USP7 inhibitors was recently achieved by several groups, including the Wertz, Buhrlage, and Komander labs[76–78]. Using a fluorescent reporter substrate assay, the Komander lab performed a high throughput screen with a library of more than 500,000 compounds, ultimately isolating two that specifically inhibited USP7 activity. Crystallographic studies revealed that these compounds block ubiquitin recognition by the

active cysteine of the DUB, which differs between USP7 and other DUBs. The Wertz lab started with an NMR-based screen looking at compounds binding the catalytic domain of USP7. They identified two compounds and similar structural approaches revealed inhibition of a dynamic region near the active site cysteine. Finally, the Burlage lab scoured the patent literature for USP7 inhibitors and then used structure guided design to improve the activity of one selective inhibitor by more than 100-fold. In addition, the Bezsonova lab used NMR-based studies together with proteomics to report on the mechanism of action of two previously identified USP7 inhibitors[79].

The Komander, Wertz and Burlage groups showed cell-based phenotypes consistent with inhibition of USP7, destabilization of MDM2 and upregulation of p53. The Komander and Wertz groups validated their small molecules by assessing their anti-proliferative effects in cancer cell lines in vitro and in mouse models of tumorigenesis. In both cases, a strong reduction of proliferation as well as tumor growth was observed, concomitant with p53 stabilization and p21 induction. Furthermore, the Wertz group demonstrated that combination treatment with their USP7 inhibitor and other chemotherapeutic drugs showed a synergistic effect in mouse models.

Altogether, USP7 best exemplifies the promise held by targeting DUBs for clinical benefit. These enzymes all contain an active site that can be targeted, and since their inhibition indirectly triggers degradation of their substrates, it opens new possibilities in therapeutic intervention. Potentially, any oncogenic protein which was thought to be undruggable could be now destabilized by inhibiting the DUB controlling its ubiquitination. It is notable, however, that unique features of the USP7 active site likely contributed to the ability to identify highly selective small-molecule inhibitors to this specific DUB. Due to the paucity of selective DUB inhibitors, it remains to be seen if these efforts have opened the door to the identification of selective inhibitors to other critical deubiquitinases involved in cancer and other diseases.

USP10, USP29, USP42, USP49 and OTUB1: Multiple DUBs involved in p53 regulation

As mentioned previously, p53 is the most frequently mutated gene in all human cancers, and its tremendous importance in cell physiology is outlined by its implication in many signaling networks. The importance of p53 is further illustrated by its many different layers of regulation[80]. Thus, numerous reports have shown that p53 stability is regulated by several DUBs[81]. USP10 was identified through a yeast two hybrid screen as an interacting protein of Ras-GTPase activating protein (G3BP), a protein involved in the Ras pathway[82]. Interestingly, further reports showed that the yeast ortholog of USP10, Ubp3p, together with Bre5, the yeast ortholog of G3BP, are involved in a secretion pathway through the control of transport between the endoplasmic reticulum and the Golgi apparatus[83], and similar results were observed in human cells[84]. Later, the Lou lab reported a role for USP10 in regulating p53 stability[85]. In unstressed cells, it was shown that USP10 binds, deubiquitinates and stabilizes p53 in the cytoplasm, antagonizing Mdm2-mediated p53 nuclear export and degradation. Additionally, it was found that USP10 also has an important role in regulating p53 activity after DNA damage. Mechanistically, in response to DNA double-strand breaks, the DDR kinase ATM (ataxia-telangiectasia mutated) becomes activated and phosphorylates

a multitude of proteins, including p53 and USP10. Phosphorylation of USP10 on Thr42 and Ser337 triggers its translocation, enabling the deubiquitination, stabilization and activation of p53. The exact mechanism by which phosphorylation impacts USP10 subcellular localization remains unclear, however these findings point to a potential tumor suppressive function of USP10. Importantly, this notion has been supported by additional reports and extends beyond p53 regulation, since USP10 has also been linked to the control of the transcription factor KLF4, as well as the tumor suppressors p14ARF and SIRT6, in lung and colon cancers[86–88].

In addition to USP10, three other DUBs of the USP family, USP29, USP42 and USP49, have been shown to regulate p53 stability and activity[89,90]. USP29 was found as a gene regulated by the protein JTV1, a binding partner of the far upstream element (FUSE) binding protein (FBP) which activates *c-Myc* expression[91]. JTV1 was found to translocate to the nucleus and associate with FBP to induce USP29 transcription, a DUB which was uncharacterized at the time. Interestingly, JTV1 stabilizes p53 by preventing Mdm2-mediated ubiquitination after DNA damage[92]. In a complementary study, the Vousden lab identified USP42 as a DUB regulating p53 under stress conditions[90]. This study used a cell line stably expressing a p53-dependent luciferase promoter where p53 expression could be induced by doxycycline. They used this system to screen a DUB siRNA library to identify candidates whose inhibition would impair p53 accumulation by measuring luciferase activity. USP42 was identified as a nuclear DUB, which binds, deubiquitinates and stabilizes p53. The main function of USP42 is to contribute to p53 stabilization in response to several stresses, such as actinomycin D or doxorubicin, to allow p53 to activate a subset of target genes such as p21 and trigger cell cycle arrest. Importantly though, USP42-dependent activation of p53 does not lead to apoptosis, suggesting that its main function is to contribute to the repair and recovery of cells from mild or transient damage. Finally, using a similar p53-dependent luciferase promoter, USP49 was identified among 80 DUBs to positively regulate p53 activity[93]. USP49 binds, deubiquitinates and stabilizes p53 following DNA damage, leading to p53 activation and apoptosis. It was also found that in vivo, USP49 contributes to sensitizing cells to DNA damaging agents. Interestingly, USP49 is a downstream target of p53 since its expression was also induced after DNA damage in a p53-dependent manner. This suggests the existence of a positive feedback loop, although the exact molecular mechanism still remains unclear.

The Dai lab identified OTUB1 as another DUB positively regulating p53 stability and activity[94]. By looking for non USP DUBs involved in p53 regulation, they found that a member of the OTU family of DUBs, OTUB1, but not its paralog OTUB2, could stabilize p53 and counteract Mdm2-mediated ubiquitination and degradation. OTUB1 interacts with p53 and is required for p53 induction in response to DNA damage and p53-dependent apoptosis as well as cell growth inhibition. Interestingly, OTUB1 acts through a non-canonical mechanism, in the sense that OTUB1 catalytic activity is not required for its ability to regulate p53. Instead, OTUB1 prevents Mdm2-mediated p53 ubiquitination by inhibiting the Mdm2 cognate E2 enzyme UbcH5. Importantly, Asp88 was found to be the key residue mediating OTUB1 interaction with UbcH5, since mutation of this residue into alanine prevented their binding and abolished OTUB1 effect on p53[94]. This unique mechanism on how OTUB1 suppresses the activity of an E2 has been characterized by

complementary biochemical and structural studies[95–97]. Mechanistically, OTUB1 has two ubiquitin-binding motifs, one in its C-terminal part which binds free ubiquitin and triggers OTUB1 conformational changes allowing binding to an Ub-charged E2 (such as UbcH5 or Ubc13, which has also been reported). Consequently, this interaction blocks the transfer of ubiquitin from the E2 to the substrate.

Despite the vast literature on ubiquitin dependent regulation of p53, and the now many DUBs which have been implicated, how they are integrated into the myriad stress responses, and their respective importance in different cells, tissues or diseases remains largely unknown. Due to the importance of p53 in cancer pathology, these remain important future questions in understanding how p53 is regulated in normal and pathological conditions.

CYLD – A disease causing gene turns out to be a tumor suppressor DUB

The *CYLD* gene was named after cylindromatosis, a familial condition involving multiple benign skin tumors that develop almost exclusively from hair follicles and sweat glands of the head and neck. Individuals with cylindromatosis occasionally develop other types of tumors, called spiradenomas and trichoepitheliomas, originating from sweat glands and hair follicles respectively. *CYLD* was identified as the disease causing gene from two families affected by cylindromatosis, however its function as a DUB was not known at that time[98]. CYLD is therefore a bona fide tumor suppressor gene and a member of the USP family, whose tumor suppressive function has been reported in several human cancers, including colon, lung, melanoma and multiple myeloma[99–103].

The main mechanism by which CYLD acts as a tumor suppressor was reported in 2003 in three back-to-back papers published in Nature[104–106]. CYLD negatively regulates the NF-κB pathway, which controls inflammation, oncogenesis and protection against apoptosis. NF-κB is a part of a transcription factor which is sequestered in the cytoplasm by the IκB complex. Upon stimulation by a ligand, such as Tumor Necrosis Factor (TNF) alpha or Interleukin 1B, TNF receptors recruit adapter proteins called TRAFs which in turn activate the IκB kinase (IKK) complex, which comprises IKK-α, IKK-β and NEMO. IKK subsequently phosphorylates IκB, which triggers its ubiquitination and degradation, releasing NF-κB to translocate into the nucleus and regulate the expression of various genes. These three studies found that CYLD can deubiquitinate both NEMO and TRAF2, thus negatively regulating NF-κB signaling. Phenotypically, activation of the pathway inhibits apoptosis, thus linking the tumor suppressive function to negative regulation of the anti-apoptotic function of NF-κB signaling.

A key feature of CYLD lies in its ability to cleave a certain type of ubiquitin chains. A seminal advancement in our understanding of ubiquitin biology has been the realization that it is an incredibly elaborate and sophisticated PTM, now referred to as the ubiquitin code[107]. This code has been used to describe how ubiquitin can be conjugated as a monomer on a single residue of a protein (monoubiquitination), several monomers on several residues (multiubiquitination), or as a chain of different topologies. Indeed, in addition to the well-studied, K48-linked ubiquitin chains, ubiquitin can form 7 other and structurally different chains, depending on the lysine residue within ubiquitin being used for chain elongation. Because of these different modes of ubiquitin conjugation, and the complexity of possible

combinations, the ubiquitin code can confer specialized outcomes in the sense that different signaling pathways can be associated with a particular linkage of ubiquitination[108]. This notion has been greatly advanced by the labs of Michael Rape and David Komander. Besides K48-linked ubiquitin chains, the best characterized ubiquitin chains are linked through lysine 63 (K63-linked). In contrast to the proteolytic function of K48-linked chains, K63-linked chains act as non-proteolytic signals and serve mostly as scaffolding platforms when attached to particular substrates, recruiting additional proteins and acting as signaling hubs in a variety of physiological contexts, such as the DDR[107].

Importantly, the function of CYLD in NF-κB signaling involves removal of K63-linked ubiquitin chains from both TRAF2 and NEMO. At the structural level, studies from the Barford lab demonstrated that K63-linked ubiquitin chains adopt open conformations in comparison to the closed structure of K48-linked chains[109]. An additional study by the same group determined CYLD specificity towards these types of linkages[110]. They found that CYLD can internally cleave K63-linked chains (endodeubiquitinase activity), a feature which is conferred by the atypical presence of a B box domain in its catalytic domain, but which is normally found in E3 ubiquitin ligases. This differs from the typical USP domain of other USP-family DUBs, and remarkably, the B box domain is not important for specificity or enzymatic activity but subcellular localization. While $CYLD^{WT}$ shows exclusive cytoplasmic localization, a $CYLD^{\Delta B\ Box}$ mutant shows both nuclear and cytoplasmic localization. This observation fits the described role for CYLD in regulating the NF-KB pathway and highlights how multidisciplinary approaches are needed to inform our understanding of DUB function. An interesting question raised by this observation is what factors contribute to this exclusive cytoplasmic distribution of CYLD and under which conditions is CYLD imported to the nucleus.

Interestingly, cell cycle functions for CYLD were also reported in two different studies. A study by the Elledge lab showed that CYLD is required for entry into mitosis[111]. In this study, the authors performed a genetic screen to identify genes whose knock down lead to a mitotic entry delay or defects in spindle checkpoint function. CYLD was identified as a regulator of mitotic progression, which is itself cell cycle regulated and degraded at mitotic exit. Interestingly, the authors performed IP/MS analysis using CYLD as a bait and found Polo Like Kinase 1 (Plk1) as an interacting protein. However, Plk1 protein levels were not affected by CYLD, which might be explained by CYLD specificity for K63-linked ubiquitin chains. An interesting question raised by this study is why such a potent tumor suppressor would favor cell division. As is often the case with studies describing cell cycle functions of DUBs, more work is needed to fully address this apparent discrepancy.

Another study, by the Massoumi lab, found that CYLD negatively regulates cell cycle progression by virtue of its association with microtubules (MT) and histone deacetylase-6 (HDAC6)[112]. CYLD contains three cytoskeleton-associated protein glycine-conserved (CAP-Gly) domains in its N-terminus. Previous work by the same lab showed that CYLD could accumulate in perinuclear spaces[113], thus the authors aimed at understanding if the mechanism involved MT regulation. It was found that CYLD interacts with MTs and tubulin, and leads to an increase in acetylated α-tubulin. Interestingly, this effect of CYLD on tubulin acetylation depends on its interaction with the histone deacetylase-6 (HDAC6)

through its N-terminus, which contains the CAP-Gly domains and not the catalytic domain. Furthermore, the authors showed that CYLD overexpression induced a delay in the G1/S transition while knock down promoted S-phase entry. Moreover, CYLD can deubiquitinate Bcl-3 which prevents its nuclear translocation, inhibiting the transcriptional activity of NF-κB leading to reduction in cyclin D1 expression, a master cell cycle regulator in G1 progression. Thus, this study linked the roles of CYLD in NF-κB signaling, cyclin D1 regulation and cell cycle progression into a well-coordinated pathway.

The discrepancy observed between the positive and negative effect of CYLD on cell cycle progression remains to be elucidated. A possible explanation would be that different tissues might use CYLD for different functions. Nevertheless, more studies are needed to have a clearer understanding of the contribution of CYLD to cell cycle progression in the context of its tumor suppressive function.

USP2 – At the crossroads of the p53/Mdm2 pathway and Cyclin D1 stability

USP2 is a member of the USP family and has been shown, by several groups, to have important functions both in normal cell cycle control as well as in cancer cell proliferation. USP2 was originally identified in mouse and was shown to be expressed in a wide range of adult and embryonic tissues, including testis, heart, skeletal muscle, diaphragm, brain, kidney, liver, pancreas, lung, and skin, suggesting that its function was not restricted to a particular organ[114]. The first reports implicating USP2 in cell proliferation came from the Saville lab, who found USP2 as a new regulator of the p53 pathway[115]. Using a yeast two hybrid screen with Mdm2 as a bait, USP2 was identified as an Mdm2-interacting protein. Subsequent analysis demonstrated that USP2 can bind and deubiquitinate Mdm2. In contrast to USP7, which can also deubiquitinate p53, USP2 only regulates Mdm2. Accordingly, ectopic expression of USP2 stabilizes Mdm2, thereby triggering p53 degradation, while silencing USP2 causes the opposite effect and leads to p53 accumulation and activation. In a subsequent report, USP2 was found to bind MdmX[116], another p53 negative regulator, which shares high sequence homology with Mdm2[117]. However, in contrast to Mdm2, MdmX exerts its inhibitory effect towards p53 by inhibiting its transcriptional activation domain rather than degrading it[118]. As an additional layer of regulation, MdmX stability is also regulated by Mdm2, through an interaction via its RING domain. In this subsequent study, USP2 was shown to deubiquitinate MdmX and protect it from Mdm2-mediated degradation, leading to inhibition of p53 activity. Importantly, cisplatin treatment of testicular embryonal carcinoma cells triggered a decrease in USP2 mRNA levels, concomitant with a decrease in MdmX protein levels and induction of the cell cycle inhibitor p21 as well as the pro-apoptotic protein Bax. In addition to USP2, USP48 has also been shown recently to contribute to Mdm2 stability to enhance Mdm2-mediated degradation of p53[119]. Surprisingly, it appears that USP48 exerts its function independent of its catalytic activity, suggesting a non-canonical, deubiquitination-independent mechanism to promote Mdm2 stability. While it is not surprising that an important regulator such as Mdm2 can be regulated by different DUBs, it remains unclear what are the relative contributions of USP7, USP2 and USP48, and if this differs between cell types or in different cancer types and subtypes. Alternatively, USP7, USP2 and USP48 could regulate different pools of Mdm2.

This is an important area for future investigation with clear implications for cancer therapeutic development.

The Gu lab identified USP2 as a specific DUB for Cyclin D1[120]. In this study, the authors devised an experimental system to isolate a mono-ubiquitinated form of cyclin D1 (Ub-Cyclin D1) from cultured cells. In order to identify DUBs targeting cyclin D1, they expressed, purified and tested 76 human DUBs using in vitro assays towards Ub-cyclin D1 and found USP2 as a cyclin D1 DUB. Biochemical experiments demonstrated that USP2 binds, deubiquitinates and regulates cyclin D1 stability. Phenotypic experiments showed that depletion of USP2 impairs cell proliferation as well as progression through G1 phase in cells whose growth depends on cyclin D1. Importantly, despite the role of USP2 in regulating the p53 pathway, none of these effects were p53 dependent.

Altogether, these studies show that USP2 possesses oncogenic properties and that targeting USP2 with small molecules could be beneficial to halt proliferation of cancer cells. It remains unknown if USP2 is amplified, overexpressed or is otherwise activated in human cancers, and analysis of TCGA datasets indicates that it is not recurrently upregulated at the mRNA or genomic level in breast, prostate, lung or pancreatic malignancies[121]. Interestingly, small molecule inhibitors of USP2 have been recently identified and characterized using an NMR-based fragment screen, with the most potent inhibitor having an IC50 of 3.3 μM[122]. While promising, further studies are needed. First, these assays were done using in vitro enzymatic measurements, and further cell biology-based assays are necessary to test if this inhibitor can exert anti-proliferative effects in cells. Second, the authors tested the specificity of this inhibitor by showing that it does not affect USP7. However, since the catalytic domain of USP DUBs is highly homologous, it is important to have a better understanding of cross reactivity among other USP members. Finally, it is notable that, at least in vitro, USP2 has been shown to be non-specific and can deubiquitinate many proteins[123]. Thus, this raises the concern of how specific such an inhibitor would be. While these are concerns for many inhibitors and could be similar for other DUBs, they highlight the point that finding inhibitors of DUBs is still in its infancy and will require significant effort in the years to come.

USP22 – The SAGA of an oncogenic DUB regulating c-Myc, p21, Cyclin D1 and Cyclin B

Another member of the USP family has been shown to have oncogenic properties by regulating important cancer-related, proliferative drivers. USP22 was identified bioinformatically in 2006 and cloned and characterized from mice tissues[124]. It was found to be highly abundant in heart and skeletal muscle, and moderately expressed in brain, placenta, kidney and pancreas. Shortly after its discovery, the Devys and McMahon labs reported that USP22 was a component of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex[125,126]. The SAGA complex was first discovered in yeast, and its role as a coactivator complex involved in both chromatin regulation as well as gene expression serves as a paradigm of transcriptional control[127]. Biochemical and genetic studies defined SAGA as a 1.8 MDa multi-protein complex with subunits having gene-specific activating functions, including RNA polymerase as well as histone acetylation activity[128]. USP22 is the human ortholog of yeast Ubp8p, and before the connection between USP22 and SAGA was made in

humans, studies reported on the function of Ubp8p in the yeast SAGA complex[129–131]. USP22 deubiquitinates both histone H2A and H2B in vitro, and the importance of the deubiquitinating activity of the SAGA complex in chromatin remodeling has been shown in the drosophilia eye, as well as the recruitment of hSAGA to promoters by the androgen receptor, whose full activation is regulated by USP22[125]. The USP22 conferred deubiquitinating activity in the hSAGA complex acts as a transcriptional co-activator, since it is recruited to specific genes by activators such as c-Myc, where it is necessary for activator-driven transcription. Importantly, USP22 depletion compromises anchorage-independent growth as well as cell cycle progression, further strengthening its connection with a cancer related gene expression program.

The observation that USP22 promotes cell cycle progression proved particularly important, as USP22 was later found to regulate crucial cell cycle regulators, including p21, cyclin D1 and cyclin B1. One study reported that USP22 had elevated expression in the human HepG2 hepatic cancer cell line compared to normal hepatocytes, and that knock down of USP22 impaired cell growth, induced a G1 cell cycle arrest and led to down regulation of cyclin D2, concomitant with p21 accumulation[132]. However, no molecular mechanisms were provided in order to explain how USP22 exerted its function. One clue came from the Dent lab who made the similar observation that USP22 could affect the expression of p21[133]. By applying a proteomic strategy, they purified ubiquitinated proteins from nuclear extracts of cells depleted for USP22 and compared it to control cells. Peptides identified following USP22 knock down would represent candidate substrates, and the majority of the identified proteins were transcriptional regulators. One of the identified transcriptional regulators was FBP1, which is known to control the expression of oncogenes such as c-Myc and p21[91,134]. USP22 binds and deubiquitinates FBP1, however, the stability of FBP1 was not affected by USP22 activity. Instead, USP22 specifically removes K63-linked chains, suggesting that FBP1 deubiquitination is important for its function. FBP1 binds to FUSE on target genes and recruits FBP-interacting repressors (FIR) to repress target genes[135]. In light of such a mechanism, it was found that USP22 knock down leads to an increase in p21 transcription concomitant with a lower occupancy of FBP1 on the p21 FUSE, suggesting that K63-linked ubiquitin chains conjugated to FBP1 inhibits its activity. Finally, depletion of either FBP1 or USP22 impaired cell proliferation and remarkably, this effect could be rescued by co-depletion of p21.

The importance of USP22 in cell cycle progression was further established by the McMahon lab, who performed a proteome-wide screen for USP22-dependent ubiquitination[136]. Similar to the strategy employed by the Dent lab, this screen relied on USP22 knock down to look for enriched ubiquitinated proteins. However, they performed affinity capture of ubiquitinated proteins by using an antibody specific for the di-glycine tag which is left on ubiquitinated lysine residues after trypsin digestion[137]. USP22 was found to bind, deubiquitinate and protect cyclin D1 from proteasome-mediated degradation in several cell lines. In agreement with previous reports, USP22 knock down led to a G1 arrest which could be genetically rescued by ectopic expression of cyclin D1. Moreover, experimentally increasing USP22 expression led to an increase in cyclin D1 and a more rapid progression through G1, an effect which could be blocked by treating cells with a CDK4/6 inhibitor. Finally, USP22 protein levels directly correlated with cyclin D1 levels in tumor tissue

samples from patients with colon and lung cancer, illustrating the potential of pharmacologically inhibiting USP22. Several other reports demonstrated the oncogenic nature of USP22 in colorectal and colon cancers, as well as in HCT116, HeLa and non-small cell lung cancer (NSCLC) cells[138–142]. Remarkably, all of these studies reported a similar molecular mechanism to explain how USP22 regulate cell proliferation, through upregulation of the c-Myc, Cyclin D and BMI-1 axis.

Finally, and paradoxically, USP22 has also been found to regulate cyclin B1 during G2/M[143]. By performing IP/MS-MS analysis using cyclin B1 as bait, USP22 was found among a dozen high confidence interacting protein. Analysis of colon cancer tissues compared to adjacent normal ones revealed that both USP22 and cyclin B1 protein levels are significantly increased in cancer tissues, suggesting that the potential relationship between USP22 and Cyclin B1 might be associated with human colon cancer development. In accordance with this observation, knocking down USP22 inhibited tumor growth both in vitro and in vivo, even though it was not clearly demonstrated that this effect was due to the control of cyclin B1. However, this report clearly demonstrates that USP22 binds and deubiquitinates cyclin B1, leading to its stabilization. USP22 itself is cell cycle regulated, with highest expression during G2/M, establishing it as one of the few DUBs whose expression is known to oscillate during cell division. Moreover, cyclin B1-Cdk1 leads to USP22 phosphorylation on residues Thr147 and Ser237, which in turns promotes its deubiquitinase activity. Finally, USP22 is degraded by the APC/C during mitotic exit. Interestingly, USP14 has also been shown to deubiquitinate and regulate the degradation of cyclin B[144]. USP14 is a proteasome associated deubiquitinase and it is therefore unclear if this is specific for cyclin B or a more general activity relevant to many other proteasome substrates. Nevertheless, characterizing mechanisms by which phosphorylation impacts USP22, and whether or not a similar process impacts its function as a subunit of the SAGA complex remains to be investigated. Altogether, these reports establish USP22 as a DUB with multiple oncogenic properties. Thus, together with USP7, USP22 might represent the most promising DUB which could be pharmacologically targeted for therapeutic benefit, even though no small molecule inhibitors have been described yet.

USP37 – APC/C antagonism, mitotic spindle regulation and cancer relevance

Another member of the USP family, USP37, also has functions in both cell cycle control and cancer proliferation, and importantly, these have been reported in several studies by different teams. Originally, the first documented function of USP37 came from Dixit and colleagues, who identified USP37 in a proteomic screen looking at interacting proteins of the Anaphase Promoting Complex/Cyclosome (APC/C)[145]. The APC/C is the major E3 ubiquitin ligase involved in completion of mitosis and progression through G1. It is a massive 1.2 MDa multi sub-unit protein complex, which targets myriad cell cycle regulators for degradation, and whose activity depends on activating sub-units that recognize substrates, Cdc20 and Cdh1/Fzr1. While Cdc20 is only associated with the APC/C at anaphase, Cdh1 promotes protein degradation from late mitosis until late-G1, when the APC/C is turned off to allow S phase entry. Dixit and colleagues performed IP/MS analysis using both Cdc20 and Cdh1 as bait. Remarkably, USP37 was identified among other known APC/C interactors and subunits only with Cdh1 but not Cdc20. Subsequently, the authors showed that in addition to binding to

the APC/C, USP37 levels fluctuate during cell cycle progression and that knock down of USP37 impact G1/S progression. Mechanistically, they found that USP37 counteracts APC/C-mediated ubiquitination of cyclin A, and that USP37 itself is phosphorylated by cyclin A-Cdk2 to increase its DUB activity and allow S-phase entry. Interestingly, DUB3/USP17 was recently found to also regulate cyclin A stabilization[146]. Similar to USP37, DUB3 binds cyclin A, leading to its deubiquitination and stabilization, and regulates the G1/S transition. Indeed, knock down of DUB3 led to a moderate G1 arrest which could be rescued by forced cyclin A expression. DUB3 also promotes proliferation of NSCLC through regulation of cyclin A protein levels. However, whether DUB3 is cell cycle regulated and subjected to cyclin A-Cdk2 phosphorylation to modulate its activity, like USP37, remains unknown.

Yeh and colleagues also uncovered a complementary role of USP37 in mitotic progression[147]. Central to cell division is the duplication of genetic material during S-phase and the equal segregation of chromosomes into two daughter cells. Thus, sister chromatids must remain paired until anaphase is initiated to avoid aneuploidy. To this end, sister chromatids are held together by the cohesin complex. When the APC/C becomes active during mitosis to trigger anaphase, it destroys Securin, an inhibitor of Separase, the protease which removes cohesin to trigger chromosome segregation[148]. By performing an RNAi screen, Yeh et al. identified USP37 as a regulator of mitotic progression and chromosome segregation, whose knock down led to spindle defects. Additional IP/MS analysis identified cohesin, as well as negative regulators of cohesin, as USP37 interacting proteins and substrates. It is noteworthy that in this screen, the authors also identified several APC/C subunits, thus strengthening the finding of the Dixit lab. The authors further demonstrated that USP37 interacts with both cohesin and a negative regulator WAPL, which is required for releasing cohesin from chromatid arms in prophase. Mechanistically, USP37 deubiquitinates WAPL which reinforces its negative regulation on cohesin during prophase. However, it is still not clear how USP37 regulates cohesin, and whether it is affecting other WAPL-dependent processes. Since USP37 is regularly found as an APC/C interactor (including unpublished data from our own lab), more studies are needed to better understand how USP37 functions during cell cycle progression. As is the case with USP2, it remains unknown if USP37 is upregulated or activated in cancer to promote malignant progression, or if targeting USP37 could be therapeutically beneficial in cancer.

USP44 and USP9X – Regulators of the Mitotic Checkpoint Complex

The mitotic checkpoint complex (MCC) is a multiprotein complex whose function is to sense the orientation of sister chromatids on the mitotic spindle before anaphase can be triggered. The MCC is the main effector of the spindle assembly checkpoint (SAC), which ensures that following anaphase and chromosome segregation, each sister cell will receive the same genetic material[148]. The main function of the MCC is to inhibit the APC/C ubiquitin ligase until all chromosomes are correctly bioriented on the mitotic spindle. Interestingly, the activation of APC/C in mitosis is driven by auto-ubiquitination of its substrate receptor Cdc20[149]. When the checkpoint is on, Cdc20 is sequestered in the MCC and APC/C activity is low. The APC/C drives non-proteolytic ubiquitination of Cdc20 to dissociate it from the MCC, thus activating itself and initiating anaphase[149].

The DUB USP44 emerged from an RNAi-based screen looking for regulators of the mitotic spindle checkpoint[150]. USP44 reverses Cdc20 ubiquitination, thereby preventing full activation of the APC/C and implicating USP44 as a component of the mitotic spindle checkpoint. However, USP44 is unique among spindle checkpoint proteins in that it is non-essential, and USP44 knockout mice are viable. Interestingly, mouse USP44 plays a role in centrosome stasis, with a more minor role in mitotic checkpoint function[151].

More recently, the DUB USP9X was shown to serve a similar function as USP44[152]. USP9X has been described to both suppress and promote tumorigenesis, suggested a complicated and context dependent role in tumorigenesis. It has been implicated in controlling the apoptotic protein MCL1, YAP1 in the Hippo pathway, cyclin D1 expression via the forkhead box transcription factor FOXO3A, and the F-box substrate receptor protein FBXW7[153–155].

Focusing on USP9X and cell cycle progression, Skowyra et al. observed that depletion of USP9X impacted the stability of several mitotic and APC/C substrates. Rather than antagonizing APC/C-mediated ubiquitination, the authors showed that USP9X served a similar function as the one for USP44 described above. Briefly, USP9X reinforces the SAC by antagonizing Cdc20 release from the MCC. The effects of USP9X depletion include shortened mitosis, elevation of chromosome segregation defects and enhancement of chromosomal instability by virtue of premature activation of the APC/C. It is important to note that most of these observations were made through time lapse microscopy in U2OS, whereas most of the observations related to USP44 function were made in HeLa cells through the use of biochemical approaches. While complementing each other, these studies also raise the question of whether one can compensate for another, or if other mechanisms are involved, such as cell line or tissue specificity. Given the importance of the SAC in faithful cell division, and frequency of aneuploidies in most solid tumors, further studies are needed, including ones looking at the combined depletion of USP9X and USP44, to better understand the contribution of DUBs in the regulation of the MCC and mitotic checkpoint function.

USP21 – A nuclear DUB involved in epigenetic regulation and proliferation

Human USP21 was cloned in 2000 from an infant brain library, and the mouse ortholog was found in the same study[156]. Confusingly, human USP21 was originally named USP23, and was also found to be an ortholog of USP2, known as Ubp41 at the time[157]. Nevertheless, early characterization of this gene showed that it was ubiquitously expressed in different tissues. It was not until 2008 that the first report described a role for USP21 in epigenetic control[158]. Using microarray-based expression dynamics in resting hepatocytes compared to regenerating ones, while simultaneously looking at the modification profile of nucleosomes, it was found that ubiquitinated H2B (Ub-H2B) was deubiquitinated in proliferating hepatocytes, concomitant with an increase in di- and trimethylation of H3K4. Accordingly, USP21 was identified as a protein whose mRNA was induced during regeneration. Mechanistically, this study elegantly shows that Ub-H2B represses di- and trimethylation of H3K4, which leads to inhibition of transcription initiation of certain genes. Interestingly, USP21 specifically deubiquitinates Ub-H2B thus regulating transcription in vivo by modulating the epigenetic status of nucleosomes. Importantly, Ub-H2B has been implicated

in controlling the expression of proto-oncogenes, and loss of the Ub-H2B E3 ligase BRE1, increased transformation and genome instability[159,160]. Interestingly, USP21 subcellular distribution was found to be almost entirely nuclear in this study, because of the presence of a nuclear import signal. This is notable in light of a subsequent study by Urbe and Clague who systemically analyzed 66 DUBs tagged with GFP to survey their cellular distribution[161]. They found USP21 to be exclusively cytoplasmic, with USP21 co-localizing with microtubules and associating with microtubules in vitro. Moreover, USP21 also co-localized with centrosomes, the microtubule organizing organelle, whose importance is illustrated by their function in forming the two poles of the bipolar spindle necessary for moving the chromosomes during mitosis. While the enzymatic activity was dispensable for USP21 co-localization with centrosomes, it was necessary for microtubule regrowth from the centrosome following depolymerization. In a similar fashion, USP21 was also found to be important for cilium formation and neurite outgrowth, two processes that also require microtubules. Therefore, it is interesting that while USP21 was first implicated in epigenetic control and nuclear signaling, it can also exert its function in the cytoplasm. Whether such differences in USP21 function are tissue specific or that different pools of USP21 at any given time are implicated in these different processes remain to be elucidated.

More recently, several studies, including one from our lab, implicated USP21 in cell cycle control as well as oncogenic and proliferative processes[162–164]. By using an RNAi-based screen to identify new regulators of double strand breaks (DSB) through the regulation of homologous recombination (HR) regulators, Liu et al. found USP21 as a positive effector of HR[162]. Mechanistically, USP21 interacts and deubiquitinates BRCA2, which in turn allows RAD51, an important mediator of HR, to be loaded onto DNA at DSB sites. Phenotypically, USP21 depletion leads to a decrease in HR efficiency and impairs tumor cell proliferation, both in vitro as well as in animal models. Moreover, TCGA analysis revealed that USP21 is overexpressed in hepatocellular carcinoma where it correlates with poor patient prognosis. In line with this finding, our lab has recently described another function for USP21 in promoting tumor growth[163].

By performing an RNAi-based screen focusing on nuclear DUBs, we identified USP21 as a DUB regulating FoxM1 stability. The transcription factor FoxM1 is a master cell cycle regulator, which is well known for controlling the dynamics of mitotic genes such as Aurora A, cyclin B1, Cdk1 and Plk1, thus allowing entry and progression through mitosis. The FoxM1 gene signature corresponds to a highly oncogenic transcriptional program, whose overexpression is observed in the majority of basal-like breast cancers, the most aggressive form of breast cancer which lacks any targeted or precision therapy. In this study, we showed that USP21 binds and regulates FoxM1 stability, and accordingly, modulates its transcriptional program. Importantly, knock out of USP21 causes a delay in cell cycle progression and also sensitizes cells to paclitaxel, in both mouse xenograft tumors as well as cell proliferation in vitro. More recently, the DePinho lab similarly identified USP21 as an oncogenic enzyme in pancreatic cancer[164]. By performing a bioinformatics analysis of TCGA data, they found that USP21 overexpression correlated with pancreatic ductal adenocarcinoma (PDAC) progression. In vitro and in vivo experiments showed that USP21 promotes cell growth, while its knock down had the opposite effect. Mechanistically, this effect was mediated by the nuclear function of USP21 in modulating the Wnt/β-catenin

pathway and the stability of the transcription factor TCF7, which maintains cancer cell stemness. This study further establishes the oncogenic nature of USP21 and highlights its potential as a drug target. Importantly, given USP21 nuclear function in controlling the stability of transcription factors and epigenetic markers, it illustrates the promising rationale held by targeting DUBs, insofar as many of what were previously thought to be "undruggable" targets could be indirectly targeted through inhibition of their cognate DUB.

Cezanne and USP35 – The rising importance of DUBs during mitosis

The Rape lab showed that ubiquitin chains linked through lysine 11 (K11-linked chains) are instrumental for cell cycle dependent degradation of mitotic regulators by the APC/C[165]. Using a synchronized human cell extracts, it was demonstrated that degradation of mitotic regulators such as cyclin B and Securin relied on K11-linked chains assembled by the APC/C[165]. The same group later expanded on this observation by showing that branched chains are also formed, that is, a single ubiquitin molecule modified at two different lysines simultaneously[166–168]. Nevertheless, this represented the first example of a particular cellular process, that is the degradation of mitotic regulators, to be associated with K11-linked ubiquitin chains. Around the same time, the lab of David Komander made the surprising discovery that the OTU family of DUBs (16 members) showed different linkage specificity[169]. Among them, OTUD7B/Cezanne (hereafter referred to as Cezanne) displayed very high specificity and activity towards K11-linked chains[169,170]. Follow up studies by the same group solved several crystal structures of Cezanne with di-ubiquitin chains linked through K11, but remarkably, the significance of that specificity in cells remained elusive[171]. However, this particular ubiquitin linkage specificity of Cezanne was the first clue to its cellular function.

Our group showed recently that Cezanne is a cell cycle regulated DUB, which is expressed during mitosis, when the APC/C becomes active[172,173]. The APC/C triggers degradation of mitotic regulators through the generation of K11-linked chains, and Cezanne can antagonize this process on some substrates, thus contributing to the kinetics of degradation. Importantly, Cezanne knock down leads to mitotic defects which ultimately impairs cell proliferation, further establishing its role in cell cycle control. It remains to be determined if Cezanne has additional and non-APC/C dependent functions during mitosis. Similarly, establishing the profile of mitotic substrates controlled by Cezanne will allow us to obtain a better idea of its contribution to mitotic progression. Remarkably, Cezanne and USP21 are co-located on chromosome 1q and both are recurrently amplified and overexpressed in numerous cancers, including breast, ovarian and pancreatic malignancies. It remains to be seen if small-molecules targeted Cezanne and/or USP21 could prove therapeutically beneficial in cancers harboring these alterations.

USP35 is another DUB with a newly defined role during mitosis. Originally, this DUB was found in a study which defined a new breast cancer subtype called high-grade Estrogen Receptor-negative tumors, characterized by a low genomic instability index[174]. By using an oligo-array-based high-resolution analysis of copy number alterations in 171 primary breast tumors of relatively small size, USP35 was found in a "hotspot" on chromosome 11q14.1. This "hotspot" corresponds to a genomic region showing the strongest coordinate expression

changes associated with this cancer subtype, however, the function of USP35 was not defined. Recently, a study by the Song lab demonstrated the importance of USP35 during mitosis by regulating the stability of the Aurora B kinase[175,176]. In a previous report, the authors analyzed the role of DUBs in cell cycle control by using a siRNA library targeting approximately 70 human DUBs before treating cells with Taxol, nocodazole, or monastrol to inhibit mitotic progression[177]. This approach identified DUBs whose depletion resulted in either pre-mitotic arrest or spindle check-point bypass in drug-treated cells and led to the identification of USP35 as a top candidate. In order to confirm the results of their screen, the authors found that USP35 depletion results in several mitotic defects such as chromosome misalignments, multipolar spindles, lagging chromosomes, chromatin bridges, and micronuclei formation. These observations pointed to an important role of USP35 in mitotic progression, and importantly, they looked similar to defects observed following depletion of the Aurora B kinase. Since depletion of a DUB of interest leads to destabilization of a bona fide substrate, the relationship between USP35 and Aurora B was further investigated. The Aurora B kinase is part of the chromosomal passenger complex (CPC) which regulates chromosome condensation and segregation, as well as cytokinesis, by helping spindle microtubules and membrane movements during mitosis. It was found that USP35 interacts, deubiquitinates and stabilizes Aurora B, thus establishing a function for USP35 during mitosis. Interestingly, USP35 transcript levels were found to be controlled by the FoxM1 transcription factor, and interestingly Cdh1 also interacts with USP35 even though it was shown to not affect its stability. Whether USP35 expression and activity dynamics are cell cycle regulated still remains unclear. Importantly, these different aspects of DUB regulation outlined here represent a major area of investigation, especially in the case of cell cycle regulated enzymes such as Cezanne and USP35, thus highlighting the need to characterize what is downstream and upstream of each DUB.

Conclusions

The past decade has seen a steady increase in the number of studies focusing on the role of DUBs in cell biology and cancer. Since DUBs control protein stability and can be targeted by small molecule inhibitors, this opens new possibilities for indirectly attacking otherwise undruggable targets. That is, drugs targeting a specific DUB could trigger the ubiquitination and subsequent destruction of specific substrates. Thus, harnessing the innate degradative machinery to reshape the cancer proteome landscape could have an enormous, and yet unrealized impact on cancer outcomes. The interest in and success of PROTACs in modulating E3 specificity to destroy proteins of interest might be an indicator of the potential future of targeting DUBs[178].

It is noteworthy that much of the research done in the DUB field has focused on biochemical analyses of the enzymes involved. This has illuminated mechanisms of DUB enzymatic activity and specificity for particular types of ubiquitin chains. Many of these discoveries also generated new analytical tools to study and characterize ubiquitin biology, like the ability to interrogate ubiquitin chain topologies and unveil fundamental rules of the ubiquitin code. In contrast, the involvement of DUBs in specific physiological processes remains a work in progress, as illustrated by the involvement of just a few DUBs in cell cycle progression (Figure 2 and 3). As illustrated, several DUBs have been shown to exert

important functions at different stages of mitosis (Figure 2) or during the rest of the cell cycle (Figure 3). We anticipate that additional studies are needed to fully appreciate their individual contributions, mechanisms governing their cell cycle activity, and difference in their roles among different cell, tissue and cancer types. Moreover, we predict that more DUBs will be discovered to have an impact on cell cycle progression and cancer proliferation. Altogether, we anticipate that the biochemical tools and concepts established by the DUB field will enable future interrogation of DUBs roles in cell cycle and their contribution to substrate deubiquitination and degradation dynamics.

Several other questions remain to be addressed and have only begun to be touched. One of the most important, and related to the previous point, is the importance of determining which DUBs are involved in cell cycle through phenotypic analysis. Importantly, determining their specific contributions to cancer proliferation also remains an important open question.

Second, it is to understand how DUB activities might be regulated during cell cycle progression or during tumor development. Very little is known regarding DUB activation mechanisms, and no clear rules exist which are common among all enzymes. By comparison, the activity of cullin-RING E3s, which are the most abundant E3s in humans, are controlled by a common post-translational modification termed neddylation. No similar activating modification is known to exist in DUBs. It is tempting to imagine that DUBs have an intrinsic activity and that they are simply always active. However, how changes in cell cycle progression or proliferative cues could influence their activity, abundance specificity or ability to recognize substrates is almost entirely unknown.

Third, it is unknown how DUBs select substrates for deubiquitination. The mechanism by which many E3s bind substrates has been analyzed. It is well-established that most E3s recognize a specific amino acid motif in their targets, termed degrons. In addition, many but not all, E3-substrate interactions can be modulated by post-translational modifications, most notably by substrate phosphorylation. In striking contrast, no such mechanisms have been well-established for DUBs.

Fourth is the extent of redundancy among DUB enzymes. Determining if and how DUBs can substitute for each other will be of tremendous importance to understand the different layers of regulation and their contributions to cell physiology and disease. For example, it will be important to know how many DUBs contribute to antagonizing substrate degradation at the mitotic exit, as has been suggested for several of those discussed above.

Finally, for most DUBs, very few if any substrates are known. Moreover, for the substrates that have been described, there has remarkably little cross-validation of substrates between different studies. This “dark” aspect of the DUB landscape precludes a deep understanding of how each might contribute in complex ways to physiological and pathological phenotypes.

A growing body of evidence suggests significant dynamism in transcriptional, post-transcriptional and enzymatic regulation of DUB enzymes, and that unveiling such mechanisms will facilitate a better and in-depth appreciation of the many different layers of DUB biology. This should ultimately lead to a better understanding for how dysfunctional

activity and expression of DUBs contributes to diseases like cancer, and how harnessing DUBs by targeting them pharmacologically could reshape the protein landscape for therapeutic benefit.

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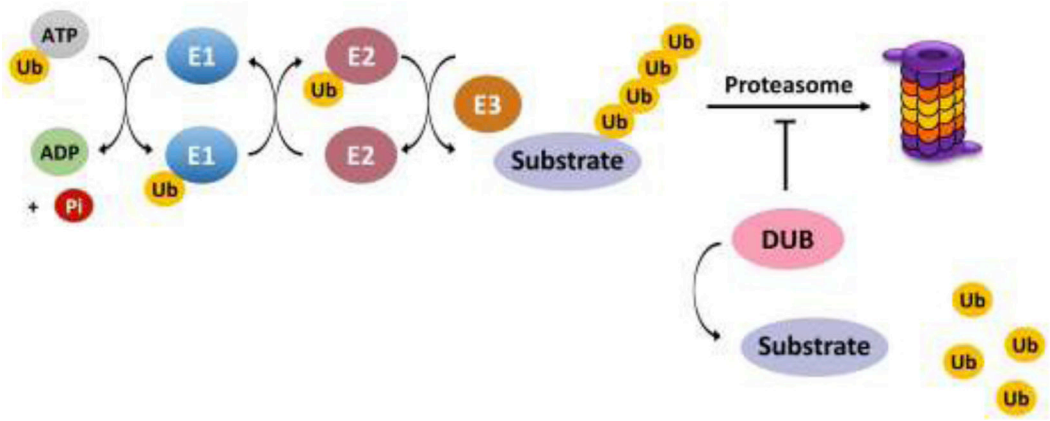

Figure 1. The role of DUBs in the ubiquitin-proteasome system.

Ubiquitin conjugation starts by its activation, in an ATP-dependent manner, by an ubiquitin activating enzyme (E1, two in the human genome). Ubiquitin is then transferred to an ubiquitin conjugating enzyme (E2, ~40 in the human genome) and then, an ubiquitin ligase (E3, more than 600 in the human genome) brings a substrate in close proximity to an ubiquitin-charged E2, leading to the covalent modification of a substrate by ubiquitin. Deubiquitinases (DUBs, 99 members in the human genome) can reverse ubiquitination and protect proteins from degradation or from ubiquitin-dependent regulation.

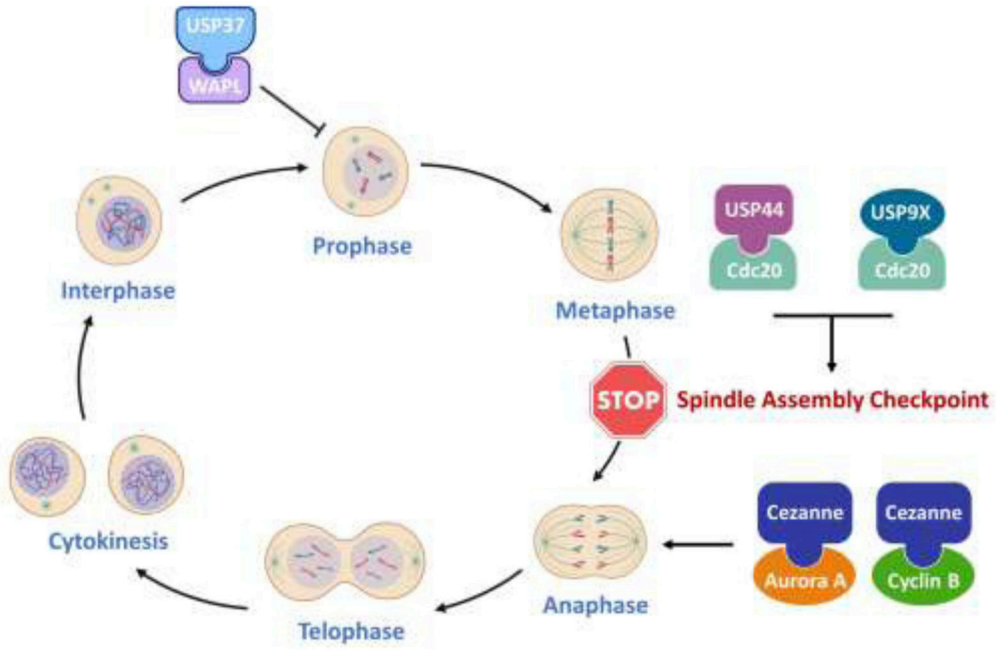

Figure 2. The role of DUBs during mitotic progression.

USP37 deubiquitinates WAPL, which inhibits cohesion during prophase thus contributing to chromosome segregation. USP44 and USP9X have been described to deubiquitinate the APC/C co-activator Cdc20, leading to stabilization of the mitotic checkpoint complex (MCC) to prevent premature anaphase initiation. Finally, OTUD7B/Cezanne antagonizes APC/C-mediated ubiquitination of a few mitotic regulators, such as Aurora A and Cyclin B, thus controlling their degradation kinetics and contributing to faithful cell division.

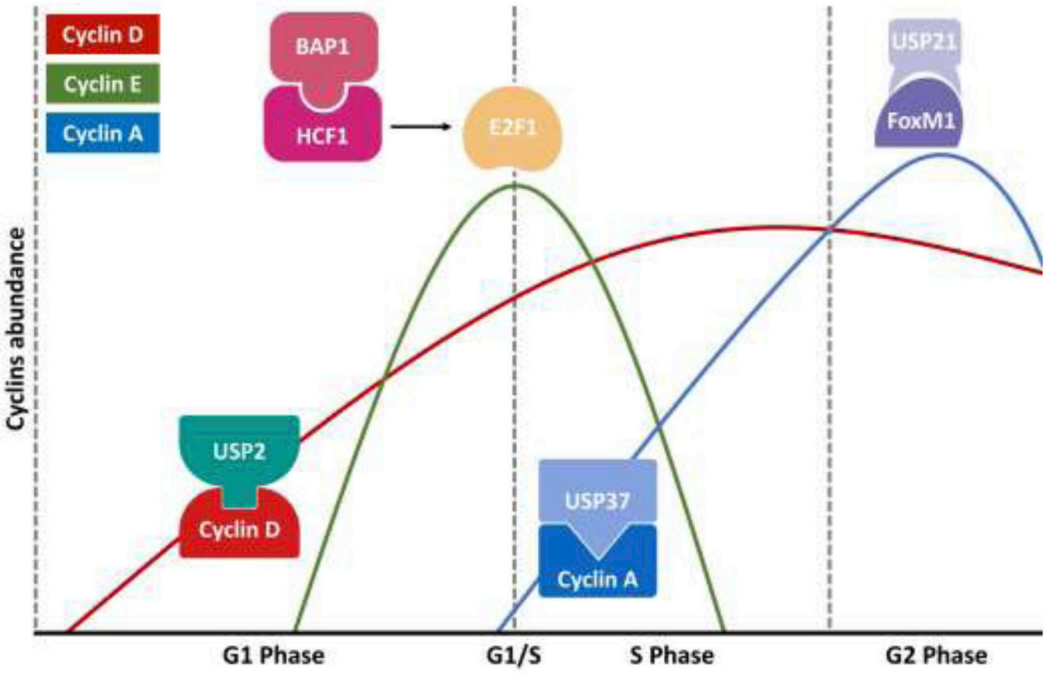

Figure 3. The role of DUBs during the G1, G1/S and G2 phases of the cell cycle.

USP2 deubiquitinates and stabilizes Cyclin D, allowing cells to progress through G1. BAP1 regulates HCF1 stability, which associates with the activating E2F1 transcription factor, allowing progression through G1/S. USP37 is a cell cycle regulated DUB, whose transcription is controlled by E2F1. USP37 is phosphorylated by CDK2, stabilizes Cyclin A, and promotes progression into the cell cycle. USP21 is nuclear DUB, which deubiquitinates and stabilizes the FoxM1 transcription factor. FoxM1 regulates Aurora A, Cyclin B1, Cdk1 or Plk1 genes, thus USP21 contributes to entry and progression through mitosis.