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Dissecting Roles of Ubiquitination in the p53 Pathway

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Abstract. Posttranslational modification of proteins by mono- or polyubiquitination represents a central mechanism to modulate a wide range of cellular functions like protein stability, intracellular transport, protein interactions, and transcriptional activity. Analogous to other posttranslational modifications, ubiquitination is a reversible process counteracted by deubiquitinating enzymes (DUBs), which cleave the isopeptide linkage between protein substrate and the ubiquitin residue. The p53 tumor suppressor is a sequence-specific DNA-binding transcriptional factor that plays a central role in regulating growth arrest and apoptosis during the stress response. Notably, recent studies indicate that both the stability and the subcellular localization of p53 are tightly regulated by ubiquitination; p53 is mainly ubiquitinated by Mdm2 but other ubiquitin ligases such as ARF-BP1/HectH9/MULE are also involved in p53 regulation in vivo. Moreover, a deubiquitinase HAUSP was initially identified in p53 deubiquitination but more recent studies showed that both Mdm2 and Mdmx are also bona fide substrates of HAUSP. In this article, we review our latest understanding of ubiquitination in modulating the p53 tumor suppression pathway.

1 Introduction

Tumor development is a multistep process that depends upon the successive activation of oncogenes and inactivation of tumor suppressor genes (Vogelstein et al. 2000). Numerous studies demonstrate that inactivation of the p53 pathway is a pivotal event in tumorigenesis of all kinds of human cancers (Prives and Hall 1999; Vousden and Lane 2007). Indeed, the germline p53 mutations of Li-Fraumeni patients confer a high risk of cancer, and more than 50% of the tumors have also been shown to contain somatic p53 mutations. The p53 tumor suppressor exerts antiproliferative effects, including growth arrest, apoptosis, and cell senescence, in response to various types of stress (Brooks and Gu 2003, 2006). p53 is also critical for maintenance of genomic stability; aberrant ploidy, gene amplification, increased recombination, and centrosomal dysregulation have been observed in cells lacking functional p53. Wild-type p53 has been called the guardian of the genome, as p53 responds to DNA damage or checkpoint failure by either arresting the cell in the G1 phase for damage repair or through the initiation of an apoptotic pathway to eliminate the damaged cell entirely (Vousden and Lane 2007). The molecular function of p53 that is required for tumor suppression involves its ability to act as a transcriptional factor in regulating endogenous gene expression (Prives and Hall 1999). A number of genes that are critically involved in either cell growth arrest or apoptosis have been identified as p53 direct targets, including $p21^{CIP1/WAF1}$, Mdm2, GADD45, Cyclin G, 14-3-3σ, Noxa, p53AIP1, PUMA and others (Vogelstein 2000). Accumulating evidence further indicates that in cells that retain wild-type p53, other defects in the p53 pathway also play an important role in tumorigenesis (Chen et al. 2005). For example, mutations of the ARF tumor suppressor are observed in tumor cells that retain wild-type p53 (Sherr 2006; Lowe and Sherr 2003). While the precise mechanisms of p53 activation are not fully understood, they are generally thought to involve posttranslational modifications of the p53 polypeptide. Ubiquitination regulates a diverse spectrum of cellular processes by providing a specific signal for intracellular protein degradation as well as some degradation-independent functions. It is well accepted that the ubiquitin-proteasome pathway plays a major part in the scope of p53 regulation; however, it is becoming more appar-

ent that the role of ubiquitination in the balance of p53 is not as simple as once thought.

2 Ubiquitination of p53 Is a Pivotal Event for Its Regulation

Protein ubiquitination, including both mono- and polyubiquitination, is involved in a broad spectrum of cellular processes. While polyubiquitination can serve to target proteins for degradation by providing a recognition signal for the 26S proteasome, monoubiquitination has been implicated in a number of degradation-independent processes, including endocytosis, virus budding, and transcriptional regulation (Hicke and Dunn 2003). p53 is a short-lived protein whose activity is maintained at low levels in normal cells. Tight regulation of p53 is essential for its effect on tumorigenesis as well as maintaining normal cell growth. The cellular functions of p53 are rapidly activated in response to stress. Ubiquitination of p53 was first discovered in papillomavirus-infected cells, where p53 degradation is mediated by the viral E6 protein and a cellular ubiquitin ligase called E6-AP. In normal cells, Mdm2 acts as a specific E3 ubiquitin ligase for p53, which, if malignantly activated, has the potential to counteract the tumor suppressor functions of p53 (see Fig. 1). The oncoprotein Mdm2 physically interacts with the N-terminus of p53 and counteracts the tumor suppressor activity of p53. The binding strongly induces p53 ubiquitination both in vitro and in vivo (Michael and Oren 2003). Importantly, by acting as p53-specific E3 ligase, Mdm2 promotes both degradation and nuclear export of monoubiquitinated p53 (Li et al. 2003). Notably, the p53 activity is downregulated in many human tumors by overexpressing the Mdm2 protein. For example, the Mdm2 gene is amplified in 30% of osteosarcomas and in 20% of soft tissue tumors in general. Interestingly, transcription of the Mdm2 gene is activated by p53, setting up an autoregulatory loop in which increased Mdm2 production limits p53 induction in response to a variety of cell stresses (Michael and Oren 2003; Prives and Hall 1999). The critical role of mdm2 in inhibiting p53 is best illustrated by studies carried out in mice where inactivation of p53 was

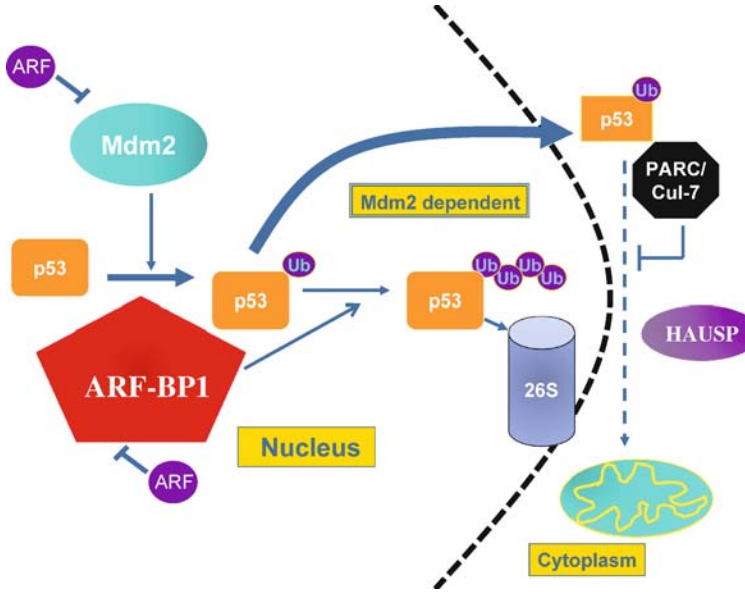

Fig. 1. Critical roles of ubiquitination in the regulation of the p53 pathway. p53 is ubiquitinated by Mdm2 and ARF-BP1/HectH9/MULE. Polyubiquitination of p53 leads to protein degradation by the 26S proteasome; monoubiquitination of p53 by Mdm2 promotes its nuclear export. *Ub* ubiquitination, *26S* 26S proteasome

shown to completely rescue the embryonic lethality caused by the loss of Mdm2 function.

Although the importance of Mdm2 in p53 regulation is well established, the precise mechanisms of ubiquitination-mediated effects remain unclear. We found that Mdm2 differentially catalyzes monoubiquitination and polyubiquitination of p53 in a dosage-dependent manner (Li et al. 2003; Brook and Gu 2006). As a consequence, low levels of Mdm2 activity induce monoubiquitination and nuclear export of p53, whereas high levels promote polyubiquitination and nuclear degradation of p53 (Fig. 1). It is likely that these distinct mechanisms are exploited under different physiological settings. For example, Mdm2-mediated polyubiquitination and nuclear degradation may play a critical

role in suppressing p53 function during the later stages of a DNA damage response or when Mdm2 is malignantly overexpressed. On the other hand, Mdm2-mediated monoubiquitination and subsequent cytoplasmic translocation of p53 may represent an important means of p53 regulation in unstressed cells, where Mdm2 is maintained at low levels. These results, together with other developments in the field (Vousden and Lane 2007), suggest the Mdm2-p53 pathway is regulated in a dynamic fashion during the DNA damage response. Nevertheless, our study also raises several critical questions. First, what is the molecular role of p53 ubiquitination in nuclear export? Does monoubiquitination act as a specific signal for nuclear export? Can we identify monoubiquitination-dependent factors that are required for nuclear export of p53?

3 ARF-BP1 Is a Potential Therapeutic Target in Tumors Regardless of p53 Status

ARF was originally identified as an alternative transcript of the *Ink4a/ARF* tumor suppressor locus (Sherr 2006; Lowe and Sherr 2003). Numerous studies indicate that ARF suppresses aberrant cell growth in response to oncogene activation, mainly by inducing the p53 pathway. The ARF induction of p53 appears to be mediated through Mdm2, since overexpressed ARF interacts directly with Mdm2 and inhibits its ability to promote p53 degradation. Interestingly, ARF also has tumor suppressor functions that do not depend on p53 or Mdm2 (Sherr 2006). For example, ARF can induce cell growth arrest in tumor cells that lack a functional *p53* gene. To elucidate novel factors and the mechanisms in ARF-mediated tumor suppression, we isolated naturally formed ARF-containing nuclear complexes from human cells and identified a novel 500-kDa ubiquitin ligase ARF-BP1, as a major ARF binding partner in human cells (Chen et al. 2005; Zhong et al. 2005). ARF-BP1 harbors a signature HECT (homolog to E6-AP C-terminus) motif and its ubiquitin ligase activity is inhibited in the presence of ARF. Notably, inactivation of ARF-BP1, but not Mdm2, suppresses the growth of p53-null cells in a manner reminiscent of ARF induction. Surprisingly, in p53 wild-type cells, ARF-BP1 directly binds and ubiquitinates p53 (Fig. 1). Thus, our study modifies the current view of ARF-mediated p53 activa-

tion and reveals that ARF-BP1 is a critical mediator of both the p53-independent and p53-dependent tumor suppressor functions of ARF. However, it also raises more general questions regarding the role of ARF-BP1 in ARF-mediated tumor suppression function. For example, (1) what are additional targets mediating p53-independent cell growth arrest and (2) how are the ARF–ARF-BP1 and ARF-BP1–p53 interactions regulated? Further analysis of this process should clarify the precise role of ARF-BP1 and yield broader insights into the mechanisms of ARF-mediated tumor suppression. First, we test whether the ARF-BP1–p53 and ARF–ARF-BP1 interactions are regulated upon oncogene activation or other types of stress. Second, to precisely understand how ARF mediates its tumor suppression effects in p53-null cells, we have isolated cellular factors that specifically interact with and mediate p53-independent functions of ARF-BP1. Finally, to define the physiological role of ARF-BP1 in normal development and tumorigenesis, we have established a knock out mouse model of ARF-BP1 to dissect its roles in vivo.

4 Ubiquitination of p53 Is Reversible

Originally, the ubiquitin-proteasome pathway was thought to have a one-way direction from substrate ubiquitination to degradation by the 26S proteasome. However, the discovery and emergence of deubiquitination enzymes (DUBs) changed the global view of the enzymatic process and quickly showed the incredible dynamics of this pathway. Our early finding that the herpesvirus-associated ubiquitin-specific protease (HAUSP) interacts and stabilizes p53 by deubiquitination (Li et al. 2002; Hu et al. 2002) was one of the first indications that DUBs exhibited a specific role in the p53 pathway (Fig. 2). Surprisingly, the simple linear model was obscured, however, with the subsequent findings that HAUSP deubiquitinates Mdm2 and is essential for controlling the Mdm2 stability in vivo (Li et al. 2004; Cummins and Vogelstein 2004; Meulmeester et al. 2005). In addition to ubiquitinating p53, Mdm2 elicits high levels of self-ubiquitination which makes Mdm2 itself very liable in cells. Our studies demonstrate that HAUSP expression can rescue Mdm2 from self-ubiquitination and is required for maintaining Mdm2-mediated func-

tion. Moreover, SiRNA-mediated inactivation of endogenous HAUSP leads to unmanageable self-ubiquitination and destabilization of Mdm2, which indirectly results in p53 activation. These findings were further supported by the study of somatic HAUSP knockout human cells (HCT116-HAUSP$^{-/-}$) in Bert Vogelstein's lab (Cummins and Vogelstein 2004) and more recently confirmed in mouse HAUSP ($^{-/-}$) embryos by our lab (N. Kon and W. Gu, unpublished data). In summary, these studies identify HAUSP as a critical regulator involved in p53 activation and implicate a dynamic role of the HAUSP deubiquitinase in regulating the p53/Mdm2 pathway (Hu et al. 2006; Brooks et al. 2007). These studies also suggest that HAUSP is a potential therapeutic target for activating p53 function by downregulating both Mdm2 and MdmX in cancer cells (Fig. 2). However, it also raises more interesting questions regarding the precise function of HAUSP in vivo. For example, what are the precise molecular mechanisms by which p53 is stabilized during the DNA damage response? Is deubiquitination the most efficient way to stabilize p53? How are the p53–HAUSP and Mdm2–HAUSP interactions regulated by DNA damage? To answer these questions, we have used biochemical methods to characterize the composition, stoichiometry, and subcellular localization of p53–HAUSP and HAUSP–Mdm2 complexes upon DNA damage, as well as the posttranslational modifications of their polypeptide components. By defining the status of these complexes with respect to these parameters during, for example, different stages of the stress response, we expect to learn when and where these complexes function and how their activities are regulated.

5 Identification of Novel Deubiquitinases in Cancer Pathways

A growing number of substrate-specific mammalian deubiquitinases (DUBs) involved in tumorigenesis are continually being revealed (Russell and Wilkinson 2005; Nijman et al. 2005; Amerik and Hochstrasser 2004; D'Andre and Pellman 1998). Considering the enzymatic process of deubiquitination does not require the cascade of enzymes needed for ubiquitination (e.g., E1, E2, and E3), DUBs may be simpler and better targets for therapeutic purpose. The deubiquitination enzyme fam-

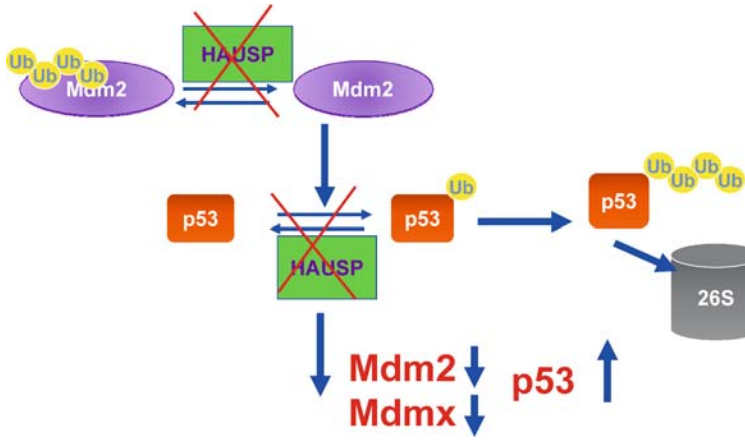

Fig. 2. A model for a dynamic role of HAUSP in regulating Mdm2 and p53. HAUSP can induce p53 deubiquitination; however, Mdm2 is also highly self-ubiquitinated and very unstable. HAUSP is required for rescuing Mdm2 from self-ubiquitination. Moreover, Mdmx stability is also tightly regulated by HAUSP. Thus, inactivation of HAUSP by RNAi or potential small molecular inhibitors will induce downregulation of the functions of both Mdm2 and Mdmx, which indirectly leads to p53 activation

ily (DUBs) falls within two classes of proteases—the metalloproteases and cysteine proteases—though most DUBs fall within the latter. Further classification subdivides the cysteine proteases into four subclasses: ubiquitin C-terminal hydrolases (UCH), ubiquitin-specific proteases (USP), Machado-Joseph disease proteases (MJD), and Otubain proteases (OUT). There are a total of 89 deubiquitinase genes in the data base (Nijman et al. 2005; Amerik and Hochstrasser 2004). Our work on HAUSP in the p53 pathway clearly validates the role of deubiquitinases in modulating cancer pathways. Recent studies also show important roles of ubiquitination in modulating other cancer pathways such as PTEN, c-Myc, Ras, and EGFR; however, specific deubiquitinases for modulating these proteins remain unknown. We will use two different approaches to identify specific deubiquitinases in these pathways. The first one is the RNAi-base screen assays. Moreover, since most of Dubs

are stable proteins, the levels of Dubs cannot be sufficiently knocked down by RNAi-base screen assays. To compensate this, the second approach is the protein-based screen assay. We have cloned and expressed 80 members out of the 89 deubiquitinase library. We will first use in vitro deubiquitination assays to identify candidates and then use these candidates to confirm the biological consequence with in vivo assays. Indeed, our preliminary studies have identified several interesting candidates as specific deubiquitinase for PTEN, cyclin D1, r-H2AX, BRCA1, and c-Myc. Further characterizations of these findings will elucidate crucial roles of these novel deubiquitinases in tumorigenesis.

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