Inhibition of USP14 and UCH37 deubiquitinating activity by b-AP15 as a potential therapy for tumors with p53 deficiency

Dear Editor,

Tumor-suppressor protein p53 is important for cell function and genome integrity. The decrease of p53 protein is a common feature of human malignant tumors, which leads to the deficiency of cell cycle detection point control and apoptosis induction. An enormous amount of research effort goes into small molecules that regulate p53, including wild-type repair of mutant p53 gene and interruption of the binding between p53 and an E3-ubiquitin ligase Mdm2 to prevent ubiquitination degradation of p53 and rescue the protein level of p53. However, it is not clear whether it is necessary to continue to inhibit the ubiquitin activity of proteasome in order to restore the p53 protein level.

To explore this issue, we utilized p53 knockout mice, in which the p53 gene of germ line is disrupted and induces the deficiency of p53 in all organs and tissues of adult mice, to screen specific small molecule inhibitors for p53-deficient tumors. Most of the adult p53−/− mice died of malignant lymphomas of thymus and heterozygous p53+/− mice develop sarcoma (including soft tissue sarcoma and osteosarcoma) at a late age (between 10 and 12 months) (Fig. 1a, b).

We previously confirmed that a specific USP14 and UCH37 inhibitor b-AP15 inhibited tumor cell growth and induced apoptosis and in vitro (data not shown). Hence, in this study, we further investigated the effect of b-AP15 on inhibiting tumor growth in heterozygous p53+/− mice in vivo. A significant prolong of overall survival in p53+/− mice was observed after b-AP15 administration (P < 0.0001) (Fig. 1a). Weight of mice in the control group was observed to significantly decrease. In contrast, the body weight and main organ weights (e.g., liver and lung) of mice in the b-AP15-treated group restored to normal weight. The number of mice with tumors was found to obviously decrease (Fig. 1b), and the tissue morphology was partially restored in heterozygous p53+/− mice group after b-AP15 treatment (Fig. 1c).

Treatment with b-AP15 significantly increased the protein levels of p53 (Fig. 1d) and p21. However, the protein levels of G2/M phase cell cycle regulatory cell division cycle (CDC) 25C, its downstream protein cyclin B1 and CDC2 decreased obviously (Fig. 1e). Moreover, the expression of the p53 regulator MDM2 is not affected in tumors following mice treatment with b-AP15 (Fig. 1e).

The protein levels of senescent markers, including the cyclin-dependent kinase inhibitors p16-Ink4a, p15-Ink4b, and decoy receptor 2, increased in senescent pre-neoplastic lesions in p53+/− mice with b-AP15 treatment (Fig. 1e). The protein levels of cell apoptosis markers, including BCL-2, BAX, and cleaved caspase-3, are induced after p53 restoration in p53−/− mice in vivo (Fig. 1e).

Deubiquitination enzyme UCH37 interacts with substrate proteins and then deubiquitinates substrates to inhibit their ubiquitination degradation in 19S regulatory particle by ubiquitin–proteasome pathway (UPP). Next, the candidate interacting proteins and potential substrates of UCH37 was explored through a yeast two-hybrid system and mass spectrometry after immunoprecipitation (Supplementary Tables S1 and S2). A few previously reported proteasome regulatory proteins, including HAUS augmin-like complex subunit 7 (HAUS7), non-ATPase 13 (RNPN13), and RPN10, were identified. Besides, COP55, a master regulator in the cells, was identified as an candidate interacting protein of UCH37 (Supplementary Tables S1 and S2).

The interaction of UCH37 and COP55 in 293T cells, in which both proteins were overexpressed, was confirmed using immunoprecipitation analysis by either anti-HA (Fig. S1a) or anti-Flag antibody (Fig. S1b). Moreover, the endogenous UCH37 interaction with endogenous COP55 in U2OS cells (Supplementary Fig. S1c, d) was also demonstrated. Cell immunofluorescence and co-focusing experiment further strengthened the evidence linking UCH37 with COP55 (Supplementary Fig. S1e). Moreover, the UCH domain and UCH with a 21-residue active-site crossover loop (ASCL) region interacted with COP55; however, neither ASCL nor the C-terminal domain alone interacted with COP55 (Supplementary Fig. S1f, g).

We detected the COP55 protein level in various types of tumors in p53−/− mice and found a significant increase. However, the protein level of COP55 decreased after b-AP15 treatment, and there is a remarkable negative correlation between COP55 and p53 protein level (Supplementary Fig. S1I, m).

We further found that UCH37 significantly upregulated the protein level of COP55, but there is no obvious change for other candidate interacting protein, including RPN10, HAUS7, and RPN13 (Supplementary Fig. S1h, i). Moreover, the COP55 protein level significantly decreased after knockdown of UCH37 and USP14 or b-AP15 treatment (Supplementary Fig. S1j, k).

UCH37 overexpression decreased the ubiquitination level of COP55 (Supplementary Fig. S2a); in contrast, ubiquitination COP55 significantly increased after treatment with b-AP15 (Supplementary Fig. S2b) in vitro and the protein level of COP55 decreased in vivo in heterozygous p53+/− mice (Supplementary Fig. S2c).

COP55 was reported to induce protein ubiquitination degradation by factors including cell cycle inhibitor p21 and tumor-suppressor p53 to induce an increase in cell proliferation. We found that p53 protein level was rescued after knockdown of COP55 and induced by UCH37 and USP14 overexpression (SupplementaryFig. S2d). Similarly, knockdown of UCH37 and USP14 (Supplementary Fig. S2e) or b-AP15 treatment (Supplementary Fig. S2f) rescued p53 protein, induced by COP55 overexpression, and enhanced the activity of transfected luciferase reporter plasmids for p53, Bax, and p21 expression (Supplementary Fig. S2g) and protein levels (Supplementary Fig. S2h) of p53 downstream target genes BAX and p21.

The nuclear and cytoplasmic co-localization between COP55 and p53 (Supplementary Fig. S2i, j) suggests a mechanism of COP55 and p53 interaction and relocation of p53 from the nucleus to the cytoplasm, and treatment of MG132, a potent proteasome

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inhibitor, significantly inhibited proteasome-dependent protein degradation of p53 and enhanced its levels in cytoplasm, which indicated the degradation of p53 induced by COPS5 (Supplementary Fig. S2k). Treatment of leptomycin B (Supplementary Fig. S2l, m) inhibited signal-mediated nuclear export by direct binding to chromosome region maintenance 1 (CRM1), RNA interference of CRM1 (Supplementary Fig. S2n), or expression of COPS5 without nuclear export signal (NES) (COPS5 ΔNES) (Supplementary Fig. S2o), arrested p53 nuclear export, and induced nuclear accumulation. Rpn13, the proteasomal receptor for Uch37 in the proteasome 19S regulatory particle, can activate UCH37 by disrupting dimerization.9 USP14 binds to the regulatory particle Rpn1 to release its catalytic USP domain and polyubiquitin chains of substrate protein.10 Overexpression of Rpn13 or Rpn1 upregulated the COP5 protein levels and downregulated the p53 protein levels (Supplementary Fig. S2p). In contrast, knockdown of Rpn13 or Rpn1 upregulated the p53 protein levels and induced its nuclear accumulation (Supplementary Fig. S2q, r). Moreover, b-AP15 treatment rescued the p53 protein level induced by Rpn13 or Rpn1 overexpression (Supplementary Fig. S2p).

In conclusion, our results showed that treatment of b-AP15 rescued the protein level of p53 and blocked its nuclear export and ubiquitination degradation induced in UPP by Rpn13- and Rpn1-mediated and UCH37-dependent COPS5 deubiquitylation.

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AUTHOR CONTRIBUTIONS
Y.-S.M., X.-F.W., Z.-Y.J., Q.-L.L., and D.F. designed the study. Y.-S.M., X.-F.W., T.-M.W., J.-B.L., Y.-J.Z., Z.-Y.J., and D.F. performed the cytological experiments. Y.-S.M., X.-F.W., F.Y., J.-B.L., Y.-J.Z., Q.X., Q.-L.L., and D.F. performed the animal experiments. Y.-S.M.,
F.Y., T.-M.W., Y.-J.Z., Z.-Y.J., Q.-L.L., and D.F. performed the statistical data analyses. Y.-S.M., X.-F.W., F.Y., T.-M.W., J.-B.L., Q.X., Z.-Y.J., Q.-L.L., and D.F. contributed to study materials. Y.-S.M., X.-F.W., Y.-J.Z., Z.-Y.J., Q.-L.L., and D.F. wrote the manuscript. All authors contributed to the final version and approved the final manuscript.

ADDITIONAL INFORMATION
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