Dear Editor,

The intestinal epithelium is a rapidly self-renewing tissue for absorbing nutrients and providing barrier functions, and its homeostasis is orchestrated by several signaling pathways (Vermeulen and Snippert, 2014; Zhu et al., 2021). Growing evidence demonstrates the importance of cell cycle regulation in intestinal homeostatic maintenance (McKernan and Egan, 2015). Here, we report that the E3 ubiquitin ligase adaptor DDB1 (Damaged DNA Binding Protein 1) is highly expressed in the intestinal epithelium and regulates the intestinal homeostasis by preventing cell cycle arrest.

DDB1 was highly expressed throughout the intestinal epithelium, and also distributed in the lamina propria and muscularis propria (Fig. 1A and S1A). To assess DDB1 function in the intestinal epithelium, we crossed DDB1fl/fl mice with Villin-Cre mice to ablate DDB1 in the intestinal epithelium. The homozygous mice were not obtained, indicating that DDB1 plays an essential role in embryonic Villin+ cells. Next we generated the inducible DDB1 knockout (KO) DDB1fl/fl;Villin-Cre-ERT2 mice, and five-day tamoxifen (TAM) administration led to the complete ablation of DDB1 throughout the intestinal epithelium (Fig. 1A, S1A and S1B). The KO mice exhibited rapid weight loss and died before day 9 (the day with first TAM injection was regarded as day 0, Fig. S1C and S1D), indicating that DDB1 is vital for the maintenance of intestinal homeostasis. Compared to the DDB1fl/fl group (Ctrl), the small intestine of KO mice showed hemorrhage with shortened length, while the length of large intestine was unaltered (Fig. 1B and S1E). Histologically, the loss of DDB1 led to the collapse of small intestinal epithelium with deteriorating crypts (Fig. 1C and S2A), whereas the large intestinal structures exhibited moderate changes (Fig. S2B and S2C), suggesting that DDB1 plays different roles in different intestinal segments. Before the tissue collapse at day 4, the Ki67+ proliferating cells in the transient amplifying region of crypts were already decreased in the small intestine (Fig. 1D, S3A and S3B), while this change was delayed in the large intestine at day 6 (Fig. S3C and S3D). Moreover, the TUNEL assay revealed that cell death was increased in the KO small intestine (Fig. S3E and S3F). Therefore, the decreased cell proliferation and increased death would contribute to the disruption of homeostasis.

Keywords: DDB1, Intestine, Homeostasis, Cell cycle

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Next we examined whether DDB1 deficiency would affect stemness. The DDB1^fl/fl;Villin-CreERT2;Lgr5-EGFP-IRES-CreERT2 (Lgr5-EGFP) mice were used to label intestinal stem cells (ISCs) and treated with TAM as above. At day 4, the number of Lgr5^+ crypts in the KO small intestine was reduced dramatically (Fig. 1D and S4A), and the expressions of other ISC markers including Olfm4 and Ascl2 were down-regulated (Fig. S4B). Consistently, the decrease of Lgr5^+ ISCs was also observed in cultured organoids after 4-hydroxytamoxifen (4-OHT) induced knockout in vitro (Fig. S4C and S4D). We also examined differentiated cells after DDB1 deletion. The immunofluorescence staining unveiled the reduced number of Chga^+ enteroendocrine cells and Muc2^+ goblet cells at day 6, while the Lyz^+ Paneth cells were unchanged (Fig. S5).

To explore the molecular mechanism underlying the role of DDB1 in the intestinal homeostasis, the small intestinal crypts of Ctrl and KO mice at the early stage (day 2) were isolated and subjected to RNA-sequencing. The upregulated genes induced by DDB1 deficiency were enriched in the cell cycle process and p53 signaling (Fig. 1E and Table S1), reminiscent of the canonical function of DDB1 in cell cycle in other tissues (Cang et al., 2007; Zhao et al., 2020). Indeed, the immunoblotting verified the increased expression of p21 and p27 in KO small intestine (Fig. 1F), both of which are cyclin-dependent kinase (CDK) inhibitors to induce cell cycle arrest and targeted for degradation by DDB1 as an adaptor for Cul4A E3 ubiquitin ligase (Abbas et al., 2008; Bondar et al., 2006). Consistently, the cell cycle analysis of Lgr5^+ ISCs showed that DDB1 KO led to more ISCs arrested at the G1 phase (Fig. 1G). In addition, the pro-apoptotic factor Bax, downstream of p53 signaling, was also upregulated (Fig. 1F), consistent with the increased cell death. Loss of DDB1 in organoids also induced the upregulation of p21 mRNA (Fig. S6A), indicating that p21 is an important mediator of DDB1 action. Indeed, the p21 inhibitor UC2288 could partially rescue the DDB1 KO-induced death and increase budding number and ISC number in the organoids (Fig. 1H, S6B and S6C). Intestinal epithelium hyperplasia was observed in ALK3^fl/fl;Villin-CreERT2 mice, as indicated by the elongated crypts and increased proliferation zone (Qi et al., 2017) (Fig. S7A). DDB1 knockout still inhibited the proliferation in the double knockout mice (DDB1^fl/fl;ALK3^fl/fl;Villin-CreERT2) (Fig. S7B and S7C), confirming that DDB1 plays a critical role in regulation of cell proliferation.

In summary, using the genetic mouse models and organoids, we demonstrate that DDB1 plays a critical role in the fast homeostatic renewal of the intestinal epithelium, which is achieved by reducing CDK inhibitor expression, preventing cell cycle arrest in the G1 phase and thus ensuring normal cell proliferation.
Fig. 1 (See legend on previous page.)
Abbreviations
4-OHT: 4-hydroxytamoxifen; Alk3: Bone morphogenetic protein receptor, type 1A; Ascl2: Achaete-scute family bHLH transcription factor 2; Bax: BCL2-associated X protein; CDK: Cyclin-dependent kinase; Chga: Chromogranin A; Ctrl: Control; Cul4A: Cullin 4A; DDB1: Damaged DNA binding protein 1; ISC: Intestinal stem cell; KO: Knockout; Lgr5: Leucine rich repeat containing G protein coupled receptor 5; Lyz: Lysozyme; Muc2: Mucin 2; Olfm4: Olfactomedin 4; p21: Cyclin-dependent kinase inhibitor 1A; p27: Cyclin-dependent kinase inhibitor 1B; TAM: Tamoxifen; TUNEL: TdT-mediated dUTP Nick-End Labeling.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13619-022-00119-6.

Additional file 1: Supplementary Methods. Figure S1. DDB1 deficiency leads to mouse lethality with shortened small intestine. Figure S2. DDB1 deletion causes reduced crypts in the small intestine. Figure S3. DDB1 deficiency impairs cell proliferation and enhances cell death in the intestine. Figure S4. Ablation of DDB1 reduces Lgr5+ ISCs. Figure S5. Decrease of goblet cells and enteroendocrine cells in the small intestine upon DDB1 deletion. Figure S6. Inhibition of p21 with UC2288 partially rescues the phenotypes caused by DDB1 deletion in organoids. Figure S7. DDB1 deletion inhibits cell proliferation induced by ALK3 KO. Table S1. Differentially expressed genes of small intestinal crypts after DDB1 deletion at day 2.

Acknowledgements
We thank Dr. Yong Cang for DDB1fl/fl mice, Dr. Yuan Liu and Siting Wei for suggestions.

Authors’ contributions
LZ and YGC designed the experiments; LZ and HL performed the experiments; XW performed bioinformatic analysis; LZ and YGC analyzed the data and wrote the paper. The authors read and approved the final manuscript.

Funding
This work was supported by grants from the National Natural Science Foundation of China (31988101 and 31730056 to YGC) and the National Key Research and Development Program of China (2017YFA0103601 to YGC).

Availability of data and materials
Data and material are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
All mice studies were performed in accordance with the relevant guidelines and under the approval of the Institutional Animal Care and Use Committee of Tsinghua University (19-CYG).

Consent for publication
Not applicable.

Competing interests
YGC is the Editor-in-Chief of Cell Regeneration. He was not involved in the review of decision related to this manuscript.

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Received: 20 March 2022 Accepted: 18 April 2022
Published online: 01 June 2022

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