



RAPID COMMUNICATION

Elevation of TRIM44 potentiates propagation of gastric cancer stem cells



Gastric cancer stem cells (CSCs), which refer to treatment-refractory and self-renewal cell populations, are critically involved in the initiation and progression of gastric cancer (GC).¹ Although gastric CSCs populations have been validated across multiple studies,² the precise molecular mechanisms of gastric CSCs properties maintenance remain unclear. Emerging evidences have highlighted the role of tripartite-motif (TRIM) family members in regulating CSCs self-renewal and differentiation through protein ubiquitination modification.³ However, the effects of TRIM family members on GC and gastric CSCs have not been elucidated. In this study, we identified TRIM44 (one of the TRIM family members) is overexpressed in human GC and gastric CSCs, and associates with GC progression and poor prognosis. In addition, our findings revealed a crucial role of TRIM44/14-3-3 ζ / β -catenin signaling in inducing gastric CSCs properties, thus supporting their function as potential therapeutic targets in modulating GC proliferation and chemoresistance.

By analyzing the expression and prognosis of each TRIM family member in the public GC datasets, we identified TRIM44 as a potential tumor promoter whose expression was upregulated and correlated with poor prognosis in GC (Fig. S1A, B). To further identify TRIM44 expression, we analyzed a microarray containing 75 GC patient tissues by immunohistochemistry (IHC) staining (Fig. 1A, left panel), and found that TRIM44 expression was significantly elevated in tumor tissues (Fig. 1A, upper-right panel). Through survival analysis, we found that TRIM44 was correlated with a poor prognosis and was an independent risk factor for shorter overall survival (OS) durations and lower recurrence-free survival (RFS) periods (Fig. 1A, bottom-right panel, and Fig. S1C–E). Moreover, compared with the normal gastric cell line GES1, the expression of TRIM44 was significantly higher in the five GC cell lines, especially the MKN45 and AGS cell lines (Fig. S1F, G).

A series of functional assays showed that knockdown TRIM44 markedly suppressed the proliferation, migration

and invasion of GC cells *in vitro* (Fig. S2A–C). These results led us to determine the role of TRIM44 in mediating gastric CSCs properties. After enriching the gastric CSCs by inducing cell spheroid formation, we found that TRIM44 was overexpressed in spheres cells in comparison to corresponding adherent cells (Fig. S3A, B). In addition, attenuated spheroid formation was observed in TRIM44 knockdown GC cells (Fig. 1B, S3C). TRIM44 knockdown also resulted in markedly decreased CD44⁺ and CD133⁺ cells proportions in spheres derived from GC cells (Fig. S3D, E). Tumor initiation ability is another feature of CSCs. The frequency of tumor-initiating cells in TRIM44-overexpressing cells was significantly increased approximately 10-fold compared with control cells (Fig. 1C, S3F). Collectively, these results indicated that TRIM44 is a critical modulator of gastric CSCs properties.

To better understand the underlying mechanisms of TRIM44-mediated gastric CSCs properties, we explored the relationship between TRIM44 and stemness-related factors. Combined with The Cancer Genome Atlas (TCGA) database and Western blotting analysis, we found that the expressions of Lgr5, c-Myc, SOX2, SOX9 were positively correlated to TRIM44 expression (Fig. S3G, H). Since TRIM44 functions as a deubiquitinase, we suspected that its regulatory effect on stemness-related factors might be indirect. Subsequently, we performed mass spectrometry to identify 14-3-3 ζ as a potential binding partner of TRIM44 (Fig. S4A). Indeed, we observed a direct interaction between TRIM44 and 14-3-3 ζ by co-immunoprecipitation and double immunofluorescent staining assays (Fig. 1D and Fig. S4B, C). In addition, we also observed that TRIM44 inhibition significantly reduced 14-3-3 ζ protein levels, while TRIM44 overexpression markedly increased 14-3-3 ζ protein levels in MKN45 and AGS cells (Fig. 1E, S4D).

We then proceeded to confirm if the TRIM44-induced gastric CSCs properties are dependent on 14-3-3 ζ . The results showed 14-3-3 ζ transfection significantly enhanced the spheroid formation frequency in TRIM44-knockdown cells (Fig. 1F, S4E). Moreover, TRIM44 shRNA-induced decreased CD44⁺ and CD133⁺ cells proportions were

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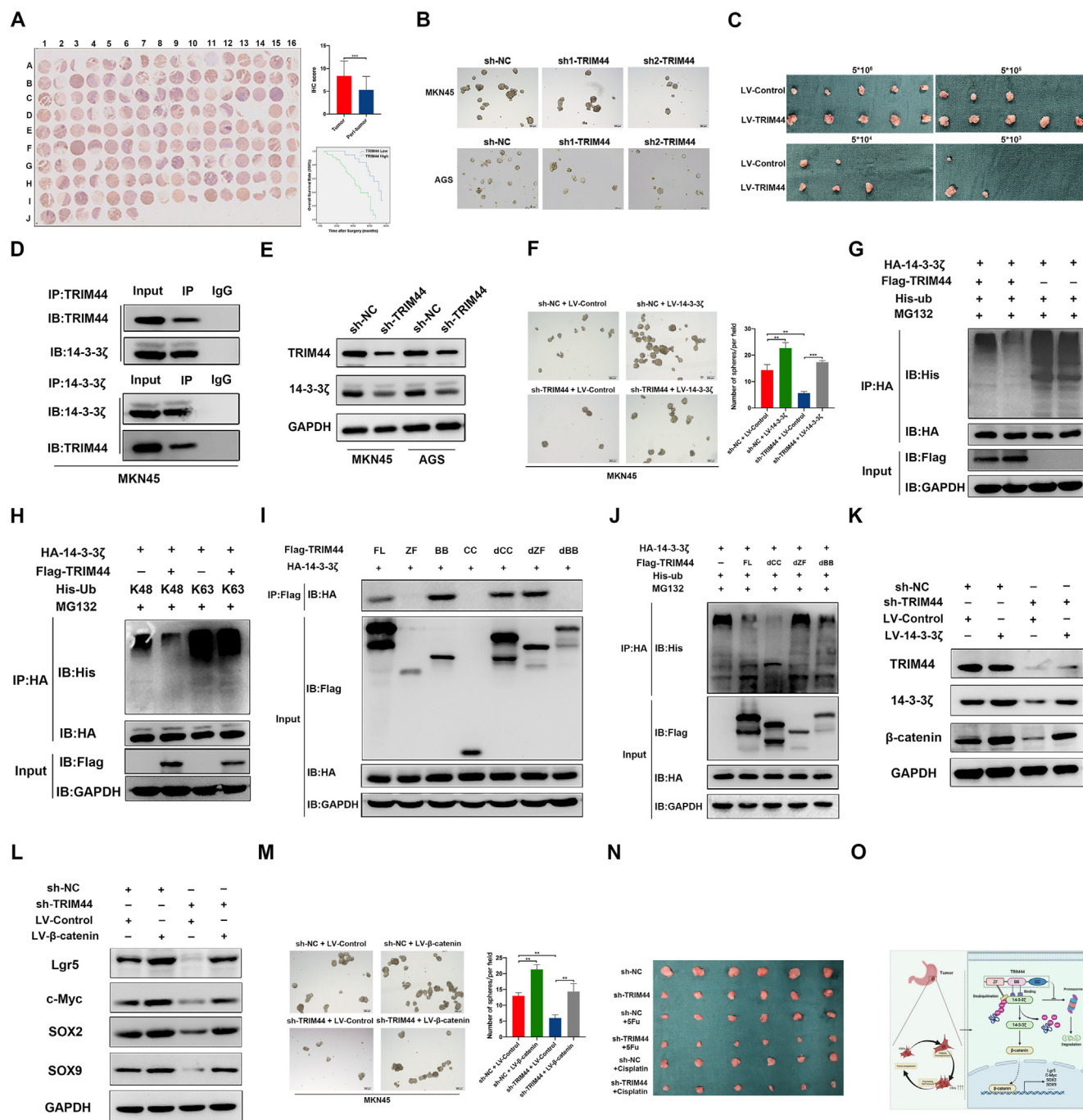


Figure 1 TRIM44 deubiquitinase activity-mediated 14-3-3 ζ upregulation promotes β -catenin-dependent CSCs properties and tumorigenesis in GC. (A) TRIM44 was overexpressed in 75 pairs human GC tissue and correlated with poor OS (** $P < 0.001$); (B) Representative images of sphere in sh-NC cells (left panel), sh1-TRIM44 cells (medium panel), or sh2-TRIM44 cells (right panel); (C) Representative images of tumors for 5×10^5 , 5×10^4 , 5×10^3 , or 5×10^2 LV-Control-MKN45 and LV-TRIM44-MKN45 cells which were implanted in nude mice; (D) TRIM44 interacts with 14-3-3 ζ in MKN45 cells. The immunoprecipitated materials by the indicated antibodies were analyzed by Western blotting; (E) Representative images showing the expression levels of TRIM44 and 14-3-3 ζ after TRIM44 knockdown in MKN45 or AGS cells by Western blotting; (F) The sphere formation assay was performed in indicated MKN45 cells, the numbers of sphere formation were measured and are shown in the bar graph (** $P < 0.01$, *** $P < 0.001$); (G) HEK293T cells which co-transfected with HA-14-3-3 ζ , Flag-TRIM44/Control and His-ub were treated with MG132 (5 μ M, 6 h), followed by immunoprecipitation (IP) and immunoblotting (IB) analysis as indicated; (H) The K48-linked ubiquitination of 14-3-3 ζ was decreased by TRIM44. HEK293T cells were co-transfected with HA-14-3-3 ζ , Flag-TRIM44/Control, His-ub-K48/K63 and were treated with MG132 (5 μ M) for 6 h, followed by IP and IB analysis as indicated; (I) HEK293T cells were transfected with HA-14-3-3 ζ and various Flag-TRIM44 truncations. Cell lysates were immunoprecipitated with anti-Flag. Immunoblotting was performed to determine the interaction between 14-3-3 ζ and individual domains of TRIM44; (J) Identification of the TRIM44 functional domain for 14-

reversed by 14-3-3 ζ overexpression in GC cells (Fig. S4F, G). Interpreted as a whole, our findings supported the idea that TRIM44 contributes to gastric CSCs characteristics through 14-3-3 ζ .

As shown in Figure S5A and B, TRIM44 exerted no significant effect on 14-3-3 ζ mRNA expression but markedly extended the half-life of the 14-3-3 ζ protein, which strongly suggests that TRIM44 may regulate 14-3-3 ζ through polyubiquitination modifications. Therefore, we performed ubiquitination assays with exogenous 14-3-3 ζ in HEK293T cell and found that TRIM44 reduced the levels of ubiquitinated 14-3-3 ζ (Fig. 1G). Through co-transfecting K48 or K63-ubiquitin plasmids, we also observed that TRIM44 decreased the 14-3-3 ζ K48-linked ubiquitination level instead of K63-linked ubiquitination level (Fig. 1H).

To identify the TRIM44 domains responsible for deubiquitinating and binding with 14-3-3 ζ , we generated truncated TRIM44 constructs which contained the zinc-finger (ZF) domain, B-box (BB) domain, coiled-coil (CC) domain, TRIM44 with a deleted CC domain (dCC), TRIM44 with a deleted ZF domain (dZF), and TRIM44 with a deleted BB domain (dBB) (Fig. S5C). Intriguingly, the BB, dCC and dZF domains possessed the ability to interact with 14-3-3 ζ , which indicates the pivotal role of the BB domain in modulating their interactions (Fig. 1I). We further investigated the functional domains for TRIM44 deubiquitinating 14-3-3 ζ . Compared to dZF and dBB domains, dCC domain could markedly reduce 14-3-3 ζ polyubiquitination (Fig. 1J). In summary, these results supported that ZF and BB domain of TRIM44 are all essential for deubiquitinating 14-3-3 ζ .

Since 14-3-3 ζ was reported as a scaffold protein to stabilize β -catenin,⁴ we analyzed the role of TRIM44/14-3-3 ζ in Wnt/ β -catenin signaling. As shown in Figure S6A, TRIM44 overexpression promoted β -catenin expression but not affected the expression of Wnt3 α and GSK3 β . Down-regulation of β -catenin induced by TRIM44 knockdown was abolished upon co-transfection with a 14-3-3 ζ -containing lentivirus (Fig. 1K), which indicated that TRIM44 upregulated β -catenin expression in an 14-3-3 ζ -dependent manner. Importantly, the suppressed expression of Lgr5, c-Myc, SOX2, and SOX9 upon sh-TRIM44 transfection could be reversed by β -catenin overexpression (Fig. 1L). Moreover, we also found TRIM44 shRNA-inhibited sphere formation capacity and CD44⁺/CD133⁺ cells percentage are reversed by β -catenin overexpression (Fig. 1M, S6B–D).

Resistance to chemotherapy is a remarkable biological feature of CSCs.⁵ Based on CCK-8 assays, TRIM44 inhibition

significantly reduced GC cell viability *in vitro* (Fig. S7A, B). We further investigated whether TRIM44 affected the chemosensitivity of GC cells *in vivo*. As shown in Figure 1N and Figure S7C, attenuated tumor volumes were observed in TRIM44-suppressed groups compared to the control groups. Moreover, the extent of necrosis was significantly increased in sh-TRIM44 and 5-FU or cisplatin treatment tumors than any individual treatment (Fig. S7D). Collectively, these results indicated that TRIM44 interference substantially improves the chemosensitivity of GC cells.

In present study, we reported that TRIM44 is overexpressed in human GC and gastric CSCs, and mediates a vital role in GC tumorigenesis and chemoresistance by intensifying gastric CSCs proliferation. Mechanistically, TRIM44 binds to 14-3-3 ζ through B-box domain and deubiquitinates 14-3-3 ζ through zinc-finger domain to block 14-3-3 ζ from ubiquitin-proteasome degradation. 14-3-3 ζ accumulation results in β -catenin protein upregulation, which goes on to enhance Lgr5, c-Myc, SOX2, and SOX9 transcription and expression in GC (Fig. 1O). Collectively, our findings demonstrated that TRIM44 promotes 14-3-3 ζ -mediated β -catenin signaling to enhance gastric CSCs survival and tumor formation, highlighting the potential of these molecules to be targeted in GC therapeutics.

Author contributions

Hai Zhu: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review and editing. **Gang Wang:** Formal analysis, Investigation, Methodology, Writing - review and editing. **Qikai Sun:** Formal analysis, Investigation, Methodology, Writing - review and editing, Funding acquisition. **Haixing Zhu:** Formal analysis, Investigation, Methodology. **Aman Xu:** Conceptualization, Funding acquisition, Supervision, Writing - review and editing.

Conflict of interests

Authors declare no conflict of interests.

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3-3 ζ deubiquitination. HEK293T cells were transfected with HA-14-3-3 ζ and indicated Flag-TRIM44 truncations, cells were treated MG132 (5 μ m) for 6 h before collection, followed by IP and IB analysis as indicated; (K) LV-Control or LV-14-3-3 ζ was transfected into TRIM44 silenced or control MKN45 cells, and IB assay was performed with indicated antibody; (L) Stable sh-NC or sh-TRIM44 MKN45 cells were co-transfected with LV-Control or LV- β -catenin, and Western blotting assay was used to determine the expression stemness-related factors; (M) The sphere formation assay was performed in indicated MKN45 cells. The numbers of sphere formation were measured and are shown in the bar graph (** $P < 0.01$); (N) Representative image of xenograft tumorigenesis and treatment with sh-NC/TRIM44, 5-Fu and cisplatin in nude mice; (O) Schematic of the underlying mechanism of TRIM44 in GC.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gendis.2021.10.008>.

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