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RAPID COMMUNICATION

SIRT6 promotes autophagy through direct interaction with ULK1 and competitive binding to PUMA

Akt aberrant activation accelerated tumor development and metastasis, and our previous study identified silent information regulator 6 (*SIRT6*) as a novel and critical tumor suppressor in colorectal cancer (CRC) downstream of Akt.[1] However, the function of SIRT6 in cancer progression has not been fully elucidated. In the present study, we found that except for inducing apoptosis, SIRT6 could serve as a crucial regulator to initiate autophagy by directly interacting with and activating ULK1. More importantly, we also reported autophagy regulation in a SIRT6-dependent indirect manner, namely, SIRT6 competitively bound to PUMA, which led to the release of ULK1. Of note, autophagy inhibition enhanced the pro-apoptotic effect of Midostaurin both *in vitro* and *in vivo*. Summarily, SIRT6 induced apoptosis accompanied by protective autophagy, and SIRT6 promoted autophagy by directly interacting with ULK1 and competitive binding to PUMA. This new insight identified the dual function of SIRT6 on apoptosis and autophagy, which has attractive clinical applications in cancer therapy.

In the present study, Midostaurin, a multiple kinase inhibitor, inhibited CRC (HCT-116, HT29, and SW620) cell growth and induced apoptosis (Fig. 1A; Fig. S1). Akt, a key regulator of colorectal cell growth and drug resistance[2] was found to be largely inactivated by Midostaurin (Fig. S2A). We previously found that *SIRT6* expression can be regulated by Akt/FoxO3a pathways in CRC cells.[1] Here, we demonstrated the robust induction of SIRT6 and Akt inhibition after Midostaurin treatment (Fig. 1B; Fig. S2B–D). Using a constitutively active Akt expression vector (Myr-Akt), we found that activated Akt blocked Midostaurin-induced SIRT6 expression (Fig. S2E–G), suggesting Akt inhibition is responsible for the upregulation of *SIRT6*. Conversely, Akt knockdown mimics the effect of Midostaurin in triggering *SIRT6* expression (Fig. S2H–J). Taken together, these data strongly suggest that Midostaurin induced *SIRT6* expression through inhibition of Akt activation.

SIRT6 was recently shown to regulate apoptosis in several types of cancer cells.[3] However, little is known about the role of SIRT6 in autophagy. Here, we found endogenous LC3 punctate structures and the autophagic flux in expressing mRFP-EGFP-LC3 cells after Midostaurin treatment (Fig. 1C; Fig. S3A, B). Similarly, the ratio of LC3-II/I and the expression of p-ULK1 and p-Beclin1 were upregulated (Fig. 1D; Fig. S3C–F), suggesting the occurrence of autophagy induced by Midostaurin. We next assessed the effects of SIRT6 on this autophagy. As shown in Figure S3G and S3H, SIRT6 overexpression promoted the occurrence of autophagy. Importantly, *SIRT6* knockdown completely abolished Midostaurin-induced autophagy (Fig. 1E; Fig. S3I–K). These data demonstrated that Midostaurin induced SIRT6-dependent autophagy.

We then attempted to identify the detailed mechanism of SIRT6-dependent autophagy. Firstly, the level of PUMA and ULK1 was found to have a great increase after Midostaurin stimulation (Fig. 1F; Fig. S4A–C). To determine the effects of PUMA and ULK1 on autophagy, CRC cells were transfected with PUMA or ULK1 shRNA. The results showed that ULK1 knockdown reduced autophagy obviously, while PUMA interference resulted in autophagy upregulation (Fig. 1G, H; Fig. S4D-G). This finding was further confirmed by the overexpression experiments (Fig. S4H, I). Taken together, our results revealed that ULK1 triggered SIRT6-dependent autophagy, while PUMA suppressed this autophagy in CRC cells.

We further demonstrated the relationship between SIRT6, PUMA, and ULK1, as well as their potential modulation of autophagy. On one hand, co-immunoprecipitation results revealed increased binding of SIRT6 with ULK1 during Midostaurin-induced autophagy (Fig. 1I, J). Similar results were observed in SIRT6 or ULK1 overexpressed cells

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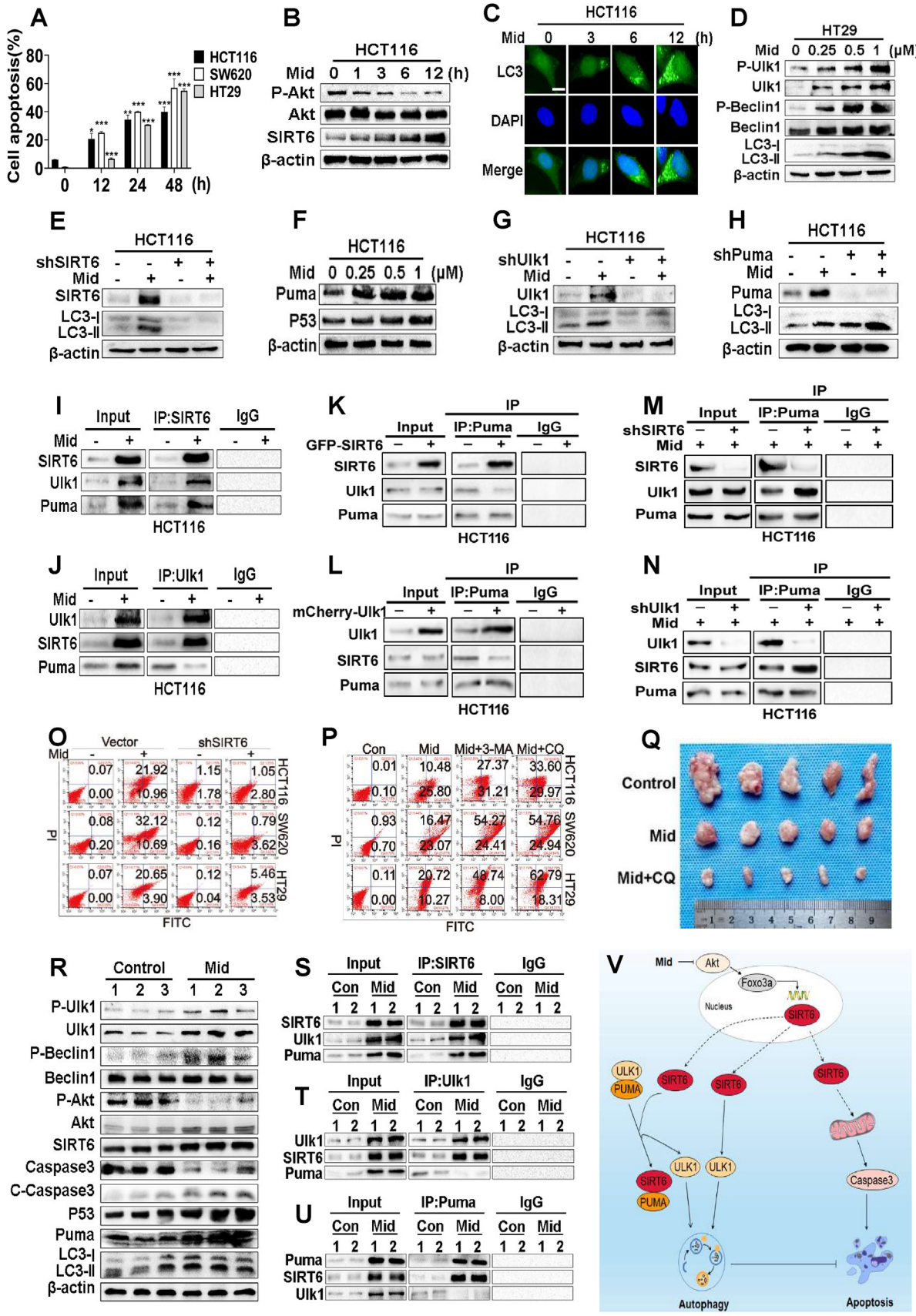

Figure 1 SIRT6 induced protective autophagy in both direct and indirect ways and promoted apoptosis in colorectal cancer (CRC). **(A)** Midostaurin induced apoptosis in CRC cells. The quantitative assay for apoptosis by Annexin V/PI Staining (shown in

(Fig. S5A, B), indicating enhanced complex conformation of SIRT6 and ULK1. Notably, SIRT6 interference abolished ULK1 phosphorylation and autophagy by Midostaurin (Fig. 1E; Fig. S3I–K, S5C). Taken together, we conclude that SIRT6 activated autophagy through the direct binding and activation of ULK1. On the other hand, we found an increase in the interaction between PUMA and SIRT6, while simultaneously a reduction in the binding of PUMA and ULK1 upon Midostaurin treatment (Fig. S6A–C). Together with the negative role of PUMA in autophagy (Fig. 1H; Fig. S4F–I) and the former conclusion that SIRT6 could bind to ULK1 to trigger autophagy directly, we hypothesized that PUMA may affect the interaction of ULK1 and SIRT6 to block autophagy. As shown in Figure 1K and S6E, over-expressing SIRT6 increased the interaction between PUMA and SIRT6 but reduced the interaction between PUMA and ULK1 (Fig. 1K; Fig. S6D). We obtained consistent results with the interference experiment (Fig. 1M). Similarly, *ULK1* overexpression enhanced the interaction of ULK1 and PUMA (Fig. 1L; Fig. S6E) with reduced binding of SIRT6 and PUMA, which was further confirmed by *ULK1* knockdown. These suggested the competition between ULK1 and SIRT6 for PUMA interaction. Altogether, SIRT6 competitively bound to PUMA, leading to the release of ULK1 from PUMA, which eventually activated autophagy (Fig. S6F).

The relationship between autophagy and apoptosis is complex[4] and we present here that SIRT6 can induce both of these two processes (Fig. 1E, O; Fig. S3G–K, S7). To further investigate the biological significance of SIRT6-mediated autophagy during Midostaurin-induced apoptosis, autophagy inhibitors 3-Methyladenine (3-MA) and Chloroquine (CQ) were used (Fig. S8). Cell apoptosis was detected by caspase-3 activation and flow cytometry by Annexin V-FITC/PI staining (Fig. 1P; Fig. S9A). Cell proliferation was significantly lower in the Midostaurin plus CQ/3-MA group than that in the Midostaurin group (Fig. S9B–G). These data demonstrated that autophagy is a cytoprotective mechanism for CRC cells in Midostaurin-induced cell apoptosis.

Finally, we used tumor xenograft models to further confirm the importance of autophagy and its detailed regulatory mechanisms of SIRT6-mediated processes. In line with our *in vitro* results, CQ significantly enhanced the antitumor activity of Midostaurin without organ-related toxicity (Fig. 1Q; Fig. S10A–D). In addition, SIRT6 was induced accompanied by autophagy and apoptosis in colorectal cancerous tissues (Fig. 1R; Fig. S10E, F). Especially, SIRT6 bound to ULK1 to activate it directly (Fig. 1S, T) or impaired its interaction with PUMA (Fig. 1T, U).

Generally, we identified a dual autophagy-apoptosis regulator SIRT6 as a potential biomarker for CRC. Firstly, Midostaurin induced autophagy and apoptosis in a SIRT6-dependent manner (Fig. 1A–H, 1O; Fig. S1–S4, S7). Secondly, a novel signaling axis consisting of SIRT6/ULK1 was found to be responsible for regulating autophagy. Specifically, SIRT6 activated ULK1 via both directly interacting with ULK1 and competitively binding to PUMA (Fig. 1I–N; Fig. 1S–U, S5, S6). Finally, the autophagy inhibitor significantly potentiated the antitumor activity of Midostaurin both *in vitro* and *in vivo* (Fig. 1P, Q; Fig. S8–S10), suggesting the presence of SIRT6-induced cytoprotective autophagy. These findings unveil the role of SIRT6 inducing apoptosis and autophagy and its intrinsic relationships (Fig. 1V). In summary, we emphasized that SIRT6 mediated autophagy via the direct way (interacting with ULK1) and the indirect way (competitively binding to PUMA) to activate the ULK1/Beclin1 signaling. More importantly, SIRT6 may serve as a potential therapeutic target for CRC, and the combination therapy of CQ (autophagy inhibitor) and Midosaurin (SIRT6 inducer) should be considered an effective strategy for the treatment of CRC.

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Fig. S1) in the three CRC cell lines (HCT116, SW620, and HT29) after 1 μM Midostaurin treatment for 0, 12, 24, and 48 h $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control group. **(B)** The activity of Akt and *SIRT6* expression was detected by Western blot in HCT116 cells after 1 μM Midostaurin treatment for 0, 1, 3, 6, and 12 h. **(C, D)** Midostaurin mediated autophagy in CRC cells. **(C)** The fluorescence images of LC3 punctate aggregation in HCT116 cells treated with 1 μM Midostaurin for the indicated time points. **(D)** Western blot analysis of p-ULK1, ULK1, p-Beclin1, Beclin1, SIRT6, and LC3-II/I in HT29 cells after 0, 0.25, 0.5 and 1 μM Midostaurin treatment for 24 h. **(E–H)** The effects of SIRT6, ULK1, and PUMA on the regulation of autophagy. The expression of LC3-II/I in HCT116 cells transiently transfected with empty vector or **(E)** shSIRT6 **(G)** shULK1, **(H)** shPUMA in the presence/absence of Midostaurin. **(F)** Western blot analysis of PUMA and P53 expression in HCT116 cells treated with various concentrations of Midostaurin for 24 h. **(I, J)** Co-immunoprecipitation (Co-IP) analysis of the interaction between **(I)** SIRT6 and ULK1/PUMA, or between **(J)** ULK1 and SIRT6/PUMA in HCT116 cells after Midostaurin treatment. **(I)** Anti-SIRT6 IP or **(J)** anti-ULK1 IP followed by Western blot with anti-SIRT6, anti-ULK1, and anti-PUMA antibody. Anti-rabbit IgG IP was used as a negative control. **(K–N)** The kinetic balance between the SIRT6-PUMA complex and the ULK1-PUMA complex. Co-IP analysis was performed with anti-PUMA antibody followed by Western blot detection of SIRT6, ULK1, and PUMA expression in HCT116 cells transfected with control vector or **(K)** GFP-SIRT6 **(L)** mCherry-ULK1, **(M)** shSIRT6 **(N)** shULK1. **(O, P)** The effects of SIRT6 and autophagy inhibitors on Midostaurin-induced autophagy. Flow cytometric analysis of CRC (HCT116, SW620, and HT29) cells **(O)** transfected with control vector or shSIRT6 or **(P)** pre-treated with 3-MA or CQ in the presence of 1 μM Midostaurin treatment. **(Q–U)** The antitumor effects of Midostaurin and CQ *in vivo*. Tumor-bearing mouses were treatment with 100 mg/kg/d Midostaurin alone or in combination with 50 mg/kg/d CQ ($n = 5$). **(Q)** Representative tumors at the end of the experiment. **(R)** The expression of autophagy- and apoptosis-related proteins in tumor tissues. **(S–U)** Co-IP analysis of the interaction between SIRT6, ULK1, and PUMA *in vivo*. Tissue lysates were immunoprecipitated with (S) anti-SIRT6 (T) anti-ULK1, or (U) anti-PUMA antibody, and Western blot analysis was performed to detect the indicated protein in tumor tissue. **(V)** Schematic diagram of the role of SIRT6-activated protective autophagy via the direct way (interacting with ULK1) and the indirect way (competitively binding to PUMA) to activate ULK1.

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Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.09.012.

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