TRPM2 channel–mediated regulation of autophagy maintains mitochondrial function and promotes gastric cancer cell survival via the JNK-signaling pathway

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A lack of effective treatment is one of the main factors contributing to gastric cancer–related death. Discovering effective targets and understanding their underlying anti-cancer mechanisms are key to achieving the best response to treatment and to limiting side effects. Although recent studies have shown that the cation channel transient receptor potential melastatin-2 (TRPM2) is crucial for cancer cell survival, the exact mechanism remains unclear, limiting its therapeutic potential. Here, using molecular and functional assays, we investigated the role of TRPM2 in survival of gastric cancer cells. Our results indicated that TRPM2 knockdown in AGS and MKN-45 cells decreases cell proliferation and enhances apoptosis. We also observed that the TRPM2 knockdown impairs mitochondrial metabolism, indicated by a decrease in basal and maximal mitochondrial oxygen consumption rates and ATP production. These mitochondrial defects coincided with a decrease in autophagy and mitophagy–associated proteins (i.e. ATGs, LC3A/B II, and BNIP3). Moreover, we found that TRPM2 modulates autophagy through a c-Jun N-terminal kinase (JNK)-dependent and mechanistic target of rapamycin-independent pathway. We conclude that in the absence of TRPM2, down-regulation of the JNK-signaling pathway impairs autophagy, ultimately causing the accumulation of damaged mitochondria and death of gastric cancer cells. Of note, by inhibiting cell proliferation and promoting apoptosis, the TRPM2 down-regulation enhanced the efficacy of paclitaxel and doxorubicin in gastric cancer cells. Collectively, we provide compelling evidence that TRPM2 inhibition may benefit therapeutic approaches for managing gastric cancer.

Gastric cancer is the fifth most common type of cancer worldwide, affecting millions each year (1–4). The 5-year survival rate is estimated at ~30% (5) making it one of the deadliest malignancies in the world and the second leading cause of cancer–related mortality in Eastern Asia (6, 7). Currently, surgery is the most effective available therapy against gastric cancer; however, its efficacy is limited to the early-stage gastric cancer patients (8, 9). For patients with late-stage tumors, surgery is not an option, and despite systemic chemotherapy, the disease is deemed incurable (10–12). Considering the poor efficacy of current anti-cancer agents, the increasing resistance to chemotherapy drugs and the lack of treatment options for late-stage patients, the development of novel and effective therapeutic approaches is of critical importance.

Over the last decade, transient receptor potential (TRP) channels have gained considerable attention in the field of cancer-targeted therapy (13–16). TRP channels are often altered in cancer cells, and disruption in their normal function can affect various signaling pathways, ultimately leading to cancer progression and growth (17, 18). The TRP family is divided into seven subfamilies consisting of a total of 28 members. Some members, including the second member of the melastatin subfamily, TRPM2, are now considered as a potential therapeutic target in several types of cancer (19). As a non-selective tetrameric cation channel, TRPM2 is widely expressed in human tissues and cells (20, 21). TRPM2 is naturally activated by ADP-ribose (ADPR) (22, 23), a mitochondrial metabolite generated by oxidative stress (24), whereas AMP (25, 26) and acidic pH (27, 28) negatively regulate its function. Currently, there is growing evidence demonstrating the key role of TRPM2 function in many cellular events, including insulin secretion (29–

The abbreviations used are: TRP, transient receptor potential; mTOR, mechanistic target of rapamycin (OCR), oxygen consumption rate; JNK, c-Jun N-terminal kinase; ADPR, ADP-ribose; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco’s modified Eagle’s medium; ACA, N-(p-amylcinnamoyl)anthranilic acid; 2-APB, 2-aminoethoxydiphenyl borate; FFA, fluafenac acid; KD, knockdown; TRP, transient receptor potential; qPCR, quantitative PCR; CFSE, carboxyl fluorescein succinimidyl ester; Scr., scrambled; 7AAD, 7-amino-actinomycin.

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Figure 1. Expression level of TRPM2 is negatively correlated with the overall survival rate of gastric cancer patients. The expression of TRPM2 was analyzed according to the Kaplan-Meier method using a median cutoff. Patients with TRPM2 mRNA levels higher than the median value were considered “high,” and patients with mRNA expression lower than the median were classified as “low.” Survival curves show the correlation between high TRPM2 expression and low patient survival. A, all patients; B, patients with stage I and II cancer and patients with stage III and IV gastric cancer. The hazard ratios generated are greater than 1 suggesting that patients with high TRPM2 expression will die at a higher rate in a given period of time than those with low TRPM2.

Results

TRPM2 expression is negatively correlated with the overall survival of gastric cancer patients

To determine whether TRPM2 expression correlates with patient outcome, we used online databases to establish the role of TRPM2 as a potential biomarker. Kaplan Meier survival analysis of gastric cancer patients was performed using an online database accessed through KM Plot. Patients were segregated into two groups: low- and high-TRPM2 expression as determined by a median cutoff. The median is an independent classifier with low intrinsic bias that splits the patient group into equally sized groups based on their expression of TRPM2. Patients with mRNA levels below the median were assigned to the “low TRPM2” group, whereas those with expression levels higher than the median were considered as “high TRPM2.” Using the Kaplan-Meier analysis method, we found that TRPM2 expression is negatively associated with the overall survival of gastric cancer patients ($n = 876; p = 0.0071$) (Fig. 1A). Furthermore, given that the highest mortality rate occurs in late-stage cancer patients, we divided patients into early (stages I and II) and advanced (stages III and IV) gastric cancer subgroups. Following patient stratification, Kaplan-Meier survival analysis revealed a relationship between TRPM2 expression and poor patient survival at advanced stages, suggesting a role for TRPM2 as a prognostic marker for late stage gastric cancer rather than the early stage (Fig. 1B).

TRPM2 is functionally expressed in gastric cancer cells

For identifying the role of TRPM2 in gastric cancer cells, we first examined the expression and activity of this channel in two gastric cancer cell lines, AGS and MKN-45. Our results showed the following. 1) TRPM2 is functionally expressed in gastric cancer cells and acts as a cation channel, 2) TRPM2 knockdown (KD) inhibits proliferation and enhances the rate of apoptosis in gastric cancer cells. 3) The absence of TRPM2 alters mitochondrial function and decreases ATP production. 4) TRPM2 KD inhibits autophagy, which in turn plays a key role in gastric cancer cell survival and mitochondrial bioenergetics. 5) Selective down-regulation of TRPM2 increases the efficacy of chemotherapy for gastric cancer. Overall, our data illustrate the importance of TRPM2 in gastric cancer progress and its potential as a new therapeutic target to improve current treatment options.
rent (Fig. 2, D and F). As expected, in control cells, the absence of ADPR (Fig. 2, C and E, open circles) produced a small current. Interestingly, the presence of ADPR in TRPM2 KD (red and blue inverted triangles) cells resulted in the same small-scale current we detected in control cells without ADPR (Fig. 2, C–F). Together, these data demonstrate that TRPM2 is functionally expressed in gastric cancer cells and acts as a plasma membrane ion channel.

TRPM2 down-regulation decreases cancer cell survival and enhances apoptosis in gastric cancer cells

Having confirmed the functional expression of TRPM2, we aimed to examine the biological role of TRPM2 in gastric cancer cells. For this purpose, we measured cell proliferation in both scrambled and TRPM2 KD cells using trypan blue cell counting, MTT viability, and CFSE proliferation assays. As shown in Fig. 3, the three assays concede to the fact that TRPM2 KD cells grew slower than control cells, evidence which hints at the potential key role of TRPM2 in gastric cancer cell proliferation. To determine whether the proliferative effect of TRPM2 is also concomitant with cell death in these cells, we looked at the apoptosis level in TRPM2 KD cells. Apoptosis was assessed by annexin V/7AAD staining and analyzed by using flow cytometry. Our results indicate that TRPM2 down-regulation increases the percentage of apoptotic cells, as shown by the shift of the cell population from left to the right along the annexin V axis (Fig. 4, A and B). To confirm the apoptotic effect of TRPM2, we measured the protein level of cleaved caspase-7, an established apoptosis marker. In accordance with our flow cytometry results, the level of cleaved caspase-7 was elevated in TRPM2 KD cells as compared with scrambled cells (Fig. 4, C and D), further emphasizing the role of TRPM2 in gastric cancer cell apoptosis.

TRPM2 down-regulation hampers mitochondrial function

Previous research has demonstrated the importance of mitochondrial function in cancer cell survival, growth, and progression, most of which revolves around altered energy production and enhanced cellular metabolism (58–62). To evaluate whether the antiproliferative effect of TRPM2 KD is associated with an alteration in mitochondrial function, we examined the mitochondrial oxygen consumption rate (OCR) and ATP production level. As shown in Fig. 5B, both basal and maximal OCRs were significantly reduced in TRPM2-depleted cells as compared with scrambled cells. Likewise, the reduction in OCR by oligomycin suggests decreased mitochondrial generated ATP levels in TRPM2-deficient cells (Fig. 5C). Upon close examination of mitochondrion-related gene expression using RT-qPCR and Western blotting, we found a statistically significant decrease in the expression of cytochrome c oxidase sub-
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Autophagy is a catabolic degradation system that plays a housekeeping role in almost all mammalian cells. It is responsible for the degradation and recycling of long-lasting proteins, aggregates, and damaged organelles (66). Autophagy is crucial for protecting the mitochondria against oxidative stress, removing damaged mitochondria (mitophagy), and maintaining mitochondrial integrity; hence, the function of the mitochondria is heavily reliant on autophagy (67–69). Considering our results thus far and the link between mitochondrial function and autophagy, we proposed that the low metabolic activity of the mitochondria in TRPM2 KD cells is due to mitochondrial dysfunction that was caused by defective autophagy machinery. To determine whether TRPM2 affects the autophagy pathway in gastric cancer cells, we examined the level of autophagy flux in TRPM2 KD and control cells using an autophagy detection kit. Indeed, we found that TRPM2 down-regulation is associated with a reduction in autophagy flux (Fig. 6A), and it causes a significant reduction in the mRNA and protein levels of autophagy-related markers (ATG3, ATG5, ATG6, ATG7, and ATG12) along with a remarkable decrease in the lipiddation of LC3A/BII to LC3A/BII (Fig. 6, B and C). Additionally, to confirm the activation of autophagy in control cells and establish a correlation between the decreased expression of autophagy genes and autophagy flux in TRPM2 KD cells, we measured LC3A/BII level in the presence and absence of chloroquine (inhibitor of autophagy). As predicted, our results showed that treatment with chloroquine is associated with a significant elevation in LC3A/BII level in scrambled cells with a moderate increase in TRPM2 KD cells (Fig. 6D). These results confirm the presence of active and functional autophagy machinery in control cells, which has been hampered in the TRPM2 KD. Here, we show for the first time that TRPM2 is a key modulator of autophagy in gastric cancer cells.

Thus far, we have established the role of TRPM2 in mediating the autophagy pathway in gastric cancer cells; as such, we next examined whether direct autophagy inhibition alone can impact gastric cancer cell metabolism and/or survival. To achieve this objective, we generated ATG5 and ATG7 knockdowns in AGS cells, and we confirmed the inhibition of autophagy machinery by visualizing the decreased levels of LC3A/BII (Fig. 6E). Similarly, ATG5 and ATG7 silencing caused a significant decrease in cell growth rate, mitochondrial OCR, and ATP production (Fig. 6F and G). These data confirm the results from established literature showing the role of autophagy in gastric cancer cell survival (70, 71), and for the first time we demonstrate that direct inhibition of autophagy through the down-regulation of ATGs affects mitochondrial function in AGS cells. Therefore, our findings support our hypothesis that TRPM2-mediated modulation of autophagy leads to mitochondrial dysfunction.

**TRPM2 regulates autophagy in a JNK-dependent and mTOR-independent manner**

We next sought to understand the mechanism through which TRPM2 controls autophagy. Considering the central role of the mTOR signaling pathway in controlling the autophagy machinery (72–75), we examined the levels of phospho-mTOR (Ser-2448) as well as its upstream regulator, phospho-AKT (Ser-473), and its downstream target, p-4E-BP1 (Thr-37/46). As shown in Fig. 7A, TRPM2 down-regulation resulted in a decrease in p-AKT. This suggests that p-AKT could play a role in the TRPM2-mediated cell growth inhibition. However, no change was detected in p-mTOR and p-4E-BP1 indicating that TRPM2 induces autophagy through an mTOR-independent pathway. Previous studies have shown the involvement of c-Jun NK-signaling pathway in the regulation of autophagy (76–79), which led us to the next objective, determining whether TRPM2-mediated regulation of autophagy involves the JNK-signaling pathway. Our results show higher levels of p-JNK (Thr-183/Tyr-185) in control cells as compared to TRPM2 KD cells (Fig. 7B). These data confirm the presence of active and functional autophagy machinery in control cells, which has been hampered in the TRPM2 KD. Here, we show for the first time that TRPM2 is a key modulator of autophagy in gastric cancer cells.

**TRPM2 down-regulation alters the autophagy process in gastric cancer cells**

**Figure 3. TRPM2 KD inhibits proliferation in AGS and MKN-45 cells.** A and C, trypan blue counting of Scr. and TRPM2 knockdown cells at 24, 48, and 72 h after being seeded (n = 4). B and D, MTT assay was used to quantify viable cells at 24, 48, and 72 h after being seeded (n = 5). E and G, CFSE proliferation assay after 4 days of cell culturing. In the corresponding histograms, the x axis represents the CFSE fluorescent signal intensity, and the y axis shows the number of events. F and H, bar graphs representing CFSE mean of data from n = 3; ***, p < 0.001; **, p < 0.01.

unit 4 (COX4.1 and −4.2) and Bcl2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3), a key regulator of mitophagy (Fig. 5, D and E) (63–65). Our results suggest that TRPM2 is involved in the maintenance of mitochondrial function and control bioenergy production.

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with TRPM2 KD cells, demonstrating the possibility of a constitutive biological function for JNK in these cells. However, the decrease in the levels of p-JNK in TRMP2 KD cells indicates a potential role for TRPM2 in the regulation of the JNK-signaling pathway (Fig. 7B). We subsequently investigated whether direct changes in JNK function can modulate autophagic events in AGS cells. Using a JNK inhibitor (SP600125), we found a significant decrease in ATG5, ATG7, and BNIP3 protein levels (Fig. 7C). Furthermore, these effects were concomitant with a decrease in LC3A/B II lipidation, alluding to the involvement of the JNK-signaling pathway in the TRPM2-mediated autophagy control (data not shown). This result is supported by published literature showing the link between JNK activation and the expression of ATGs and BNIP3 (65, 79–81).

TRPM2 down-regulation sensitizes gastric cancer cells to paclitaxel and doxorubicin

Although paclitaxel and doxorubicin are widely used for the treatment of gastric cancer (9, 82, 83), their efficacy is limited due to low bioavailability and high toxicity (84, 85). Based on our results on the role of TRPM2 in gastric cancer cell survival, we aimed to investigate the effect of TRPM2 down-regulation on the efficacy of chemotherapy drugs in gastric cancer cells. For this purpose, we first estimated the IC50 value for both paclitaxel and doxorubicin in AGS and MKN-45 cells. As shown in Fig. 8, treating AGS or MKN-45 cells with paclitaxel/doxorubicin resulted in a significant dose-dependent inhibition of cell proliferation. Interestingly, exposure of TRPM2 KD cells to paclitaxel or doxorubicin resulted in a further reduction in cell viability. Furthermore, because paclitaxel and doxorubicin are known for their apoptotic effect in cancer cells (86, 87), we assessed whether TRPM2 down-regulation is associated with an increase in apoptosis following paclitaxel and/or doxorubicin treatment. Consistently, the apoptotic effects of paclitaxel and doxorubicin were enhanced in TRPM2 KD cells when compared with control cells (Fig. 9, A–D). Our findings illustrate the benefits of targeting TRPM2 in combination with chemotherapy drugs as a strategy to enhance the efficacy of current gastric cancer treatments.

Discussion

Each year, 1 million new patients are diagnosed with gastric cancer, 700,000 of whom will lose the battle with this devastating disease, making gastric cancer one of the deadliest cancers
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Figure 5. Silencing TRPM2 alters mitochondrial function in gastric cancer cells. Mitochondrial respiration rate of AGS cells was measured using the XF-24 extracellular flux analyzer. A, metabolic flux; B, basal and maximal respiration rates; C, ATP production rates were quantified by the Seahorse Wave 2.3 software. OCR was obtained from both basal condition and following treatment with 1 µM oligomycin (A), 1.5 µM FCCP (B), 1 µM etomoxine (C), and 1 µM antimycin A (D) (n = 3). D, mRNA expression level of mitochondrial membrane protein (COX4.1/4.2 and BNIP3) (n = 4). E, Western blot analysis of mitochondrial membrane proteins (n = 3) (* test versus Scr. *** p < 0.001; **, * p < 0.01; *, p < 0.05).

in the world (88). Additionally, gastric cancer is highly malignant, and current therapies are mostly ineffective in late-stage cancer patients (6). Gastric cancer remains a major burden to individuals worldwide, highlighting the importance of understanding the mechanisms behind it and finding new treatments in the hopes of improving patient survival. In this study, we characterized the function of TRPM2 in gastric cancer cells to provide an overview of its potential role in cancer cell survival. Throughout this study, we intentionally avoided the use of several non-specific or short-term inhibitors that have been previously used to block the TRPM2 channel (e.g. flufenamic acid (FFA), imidazole antifungal agents (clotrimazole and econazole), N-(p-amylcinnamoyl)anthranilic acid (ACA), 2-aminoethoxydiphenyl borate (2-APB), and clotrimazole (89) or 8-Br-ADPR (90)). For example, 2-APB and FFA work as general inhibitors of most TRP channels (91–93), whereas clotrimazole is known as an inhibitor of calcium-activated potassium channels (94–96). For these reasons, we opted for the use of shRNA lentiviral technology to specifically down-regulate TRPM2. We have shown that TRPM2 knockdown significantly decreases gastric cancer cell survival mainly through the inhibition of autophagy, mitochondrial function, and ATP production. We also demonstrated that targeting TRPM2 improves the effectiveness of the anti-cancer drugs, paclitaxel and doxorubicin. Our findings are in accordance with previous research, illustrating the importance of TRPM2 in survival and growth of many types of cancer cells. Hence, finding a specific inhibitor for TRPM2 holds significant clinical potential and may serve as a new anti-cancer agent.

The novelty of this study lies with the identification of the mechanism behind TRPM2-mediated gastric cancer cell survival. Here, we proposed that TRPM2 operates by modulating autophagy to maintain mitochondrial energy metabolism and shape the fate of gastric cancer cells. Our hypothesis was supported by our data showing that TRPM2 KD cells have a defective autophagic response and mitochondrial metabolism, as well as decreased growth. The role of autophagy on mitochondrial function and cell growth was further confirmed by knocking down ATG5 and ATG7 in AGS cells. These findings are consistent with previous studies confirming the importance of ATGs in gastric cancer cell growth and tumor progression (70, 71); however, we show for the first time that autophagy is a key element in the maintenance of mitochondrial integrity in AGS cells. The effect of TRPM2 on mitochondrial function could be partly explained through a decrease in the expression of COX4.1/4.2, essential proteins in mitochondrial membrane electron transport chain, in TRPM2 KD cells (63, 64). The other possible explanation is that a decrease in the expression level of the mitophagy regulator, BNIP3, resulted in accumulation of damaged mitochondria.

In addition, we identified a JNK-dependent and mTOR-independent signaling pathway responsible for the regulation of autophagy in AGS cells. The mTOR-independent regulation of autophagy has been previously reported in other studies using different cancer cells such as the HT1080 (fibrosarcoma cells), MCF7 (breast cancer cells), and primary human hepatocytes, which further validates our results (98). Our results showed that autophagy in TRPM2 KD gastric cancer cells was inhibited through the down-regulation of the JNK-signaling pathway, an event that has been validated in various cancers (99, 100). Moreover, the expression level of ATGs and BNIP3 has been consistently demonstrated to be directly regulated by the activated JNK pathway (65, 78). This is consistent with our finding showing that inhibition of JNK in AGS cells resulted in a decrease in protein level of ATG5 and ATG7, and BNIP3. Altogether, our results confirm that TRPM2 regulates autophagy/mitophagy via the JNK-signaling pathway in AGS cells.

The JNK pathway also plays a key role in promoting cell survival in many cancers, including gastric cancer (101–104). Studies have shown that specific antisense oligonucleotides against JNK lead to decreased cell growth by promoting apoptosis in gastric, lung, and prostate cancer cells (104, 105). However, the observed decrease in proliferation of TRPM2 KD cells could be due to a decrease in p-AKT (106, 107); cross-talk between JNK- and AKT-signaling pathways has been established and shown to inhibit apoptosis as a means of promoting cancer cell survival (108). Altogether, our study provides new evidence that TRPM2 triggers both the AKT- and JNK-signaling pathways to promote gastric cancer cell survival.
Finally, we have demonstrated that TRPM2 knockdown significantly enhances gastric cancer cell sensitivity to paclitaxel and doxorubicin, which validates its therapeutic potential as an anti-cancer target. These results are consistent with reports on the benefits of targeting TRPM2 in the treatment of neuroblastoma (50) and breast cancer (55). Additionally, the impact of anti-cancer drugs on autophagy (109, 110) may explain the synergistic effect seen in TRPM2 KD cells. Given the negative correlation between the TRPM2 expression level and patient survival, we suggest that combination of chemotherapeutics and TRPM2-targeted drugs may lead to an increase in treatment effectiveness and improve patient outcome.

Experimental procedures

Cell culture

Human gastric adenocarcinoma cell lines, AGS (ATCC; CRL-1739) and MKN45 (JCRB0254) were cultured in DMEM/F-12 (Ham’s) and RPMI 1640 medium (Gibco, Life Technologies, Inc.), respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Inc.) and 20 μg/ml penicillin and streptomycin. Cells were grown at 37 °C and 5% CO₂.

Generation of stable TRPM2 knockdown cell lines

TRPM2-shRNA clones were purchased from Dharmacon. PLKO-LV-shTRPM2 plasmids were used according to the pro-
Briefly, lentiviral particles were generated in HEK 293 cells by co-transfection with PPAX2 (6 μg), MD2G (3 μg), and PLKO-LV-shTRPM2 (6 μg) plasmids in the presence of polyethyleneimine transfection reagent (Sigma). The lentivirus was collected at 24 and 48 h post-transfection, filtered (Millex-GS; 0.22-μm sterile filter), and stored at −80 °C.

For transduction, AGS and MKN-45 cells were seeded in 6-well plates and cultured for 24 h. Medium containing 200 μl of lentivirus and 8 μg/ml of Sequebrene (Sigma) was added to the cells and allowed to incubate for 48 h. Puromycin (concentration varied based on cell type) was used to select transduced cells. Knockdown efficiency was assessed with RT-qPCR and Western blotting (shRNA sequences Table 1).

**RT-qPCR**

RNA from AGS and MKN-45 samples was isolated using the standard TRIzol procedure and the RNA purification kit from Invitrogen. The purified RNA was quantified using a spectrophotometer. Following quantification, 2 μg of RNA was used for the synthesis of complementary DNA (cDNA) according to the SuperScript II first-strand synthesis system (Invitrogen). Gene expression was quantified by real-time PCR using the CFX96 touch real-time PCR instrument (Bio-Rad) and gene-

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**Figure 7. TRPM2 modulates autophagy via a JNK-dependent and mTOR-independent signaling pathway.** A and B, Western blot analysis of the protein levels of AKT, p-AKT, mTOR, p-mTOR, 4E-BP1, p-4E-BP1, JNK, and p-JNK in Scr. and TRPM2 KD cells (n = 4). C, protein level of autophagy and mitophagy markers in AGS cells after treatment with 50 μM JNK inhibitor (SP600125) for 24 h (n = 3).

**Figure 8. TRPM2 down-regulation enhances the efficacy of paclitaxel and doxorubicin in a dose-dependent manner.** A–D, MTT cell viability assay in AGS and MKN-45 cells after treatment with various concentrations of paclitaxel or doxorubicin for 24, 48, and 72 h. Data were represented as a dose-response curve with the corresponding IC_{50} dose of the drugs after 72 h of treatment (IC_{50} for paclitaxel was 7.4 and 26.2 nM in AGS and MKN-45 cells, respectively; IC_{50} for doxorubicin was 28 and 44.8 nM in AGS and MKN-45 cells, respectively) (n = 3). E and F, comparison between the viability of Scr. and TRPM2 KD cells 72 h after treatment with the IC_{50} dose of the two chemotherapeutics. All experiments were performed in triplicate and analyzed for statistical significance using t tests (n = 3, ***, p < 0.001; **, p < 0.01).
specific primers (Life Technologies, Inc.): TRPM2, COX4.1, COX4.2, BNIP3, ATG3, ATG5, ATG6, ATG7, and ATG12. Data were analyzed based on the Livak and Schmittgen’s 2−ΔΔCT method and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene (112). Primer sequences are provided in Table 2.

**Electrophysiology**

TRPM2 currents were measured in TRPM2 knockdown and scrambled cells using whole-cell patch clamp at 21–25 °C with voltage ramp (−80 to 80 mV). Cells were kept in standard extracellular saline: 140 mM NaCl, 2.8 mM KCl, 1 mM CaCl2, 2 mM MgCl2, 10 mM glucose, and 10 mM HEPES-NaOH (pH 7.2 adjusted with NaOH). Pipette-filling solution contained: 140 mM cesium glutamate, 8 mM NaCl, 1 mM MgCl2, and 10 mM HEPES cesium KOH (pH 7.2, adjusted with cesium KOH). ADPR (2 mM) was included in the pipette solutions to activate

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**Table 1**

Specific shRNA clones used to permanently knock down gene expression

| Target gene | Clone ID     | Sequence (5’ to 3’) |
|-------------|--------------|---------------------|
| TRPM2       | TRCN0000044152 | AAGTAGGAGAGGATGTTCAGG |
| TRPM2       | TRCN0000044154 | ATCCCTCAATCAATGAAGCTT |
| ATG5        | TRCN0000150940 | AAGCAAATAGTATGGTTCTGC |
| ATG5        | TRCN0000151963 | TTAAAGAGAGTCACTACTG |
| ATG7        | TRCN0000007585 | AAGAGAAGAGAAAACCTCTC |
| ATG7        | TRCN0000007587 | TAAATGTTCCAAATAGCTG |

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**Figure 9.** Knockdown of TRPM2 improves the apoptotic effect of paclitaxel and doxorubicin in gastric cancer cells. A and C, annexin V/7AAD staining of Scr. and TRPM2 KD cells 72 h after treatment with IC50 dose of paclitaxel and/or doxorubicin (n = 3). B and D, bar graph represents quantification of the annexin V/7AAD staining result. The data are represented as a mean of three different experiments. (n = 3, t test versus non-treated cells *** p < 0.001; ** p < 0.01).
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Table 2

| Gene   | Primer       | Sequence 5’ to 3’ |
|--------|--------------|-------------------|
| TRPM2  | Forward      | AGTATACCTTGGATCCTCCA |
| TRPM2  | Reverse      | CCGAAAAATGTTTCTGGCA |
| ATG3   | Forward      | AGCTTACCCTTGTGGCC |
| ATG3   | Reverse      | AGAAGATGTTTCTGGCC |
| ATG5   | Forward      | GTCTCATTCAAGTGGATGTT |
| ATG5   | Reverse      | ATGCATTTTCATTGTTG |
| ATG6s  (Beclin-1) | Forward | CTTCGAGGAGAGAGGAGGCTC |
| ATG6s  (Beclin-1) | Reverse | CAGGCTGTCAGGTCCTC |
| ATG7   | Forward      | CTCGAGGAGAGAGGAGGCTC |
| ATG7   | Reverse      | CAGGCTGTCAGGTCCTC |
| ATG12  | Forward      | AAAGGGAGGAGAGGAGGAGGAC |
| ATG12  | Reverse      | CGCTGTCAGGTCCTC |
| COX4.1 | Forward      | GGCGATCTTACATCTGTTG |
| COX4.1 | Reverse      | GCCATGTTACAGTGGATG |
| COX4.2 | Forward      | GAGATGAACCGTCGCTCCAA |
| COX4.2 | Reverse      | GCCACCCCAGGATCTAACAG |
| BNIP3  | Forward      | CTCGACATGAGGAAACAG |
| BNIP3  | Reverse      | GCCACCCCAGGATCTAACAG |

TRPM2 channels. In other experiments, ADPR was withheld to show specificity of TRPM2 currents (88).

Western blotting

To examine protein expression in gastric cancer cell lines, cells were lysed with 1× RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Nonidet P-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin). Lysates were then quantified, and protein concentrations were calculated according to the BCA assay protocol from Thermo Fisher Scientific. For Western blot analysis, protein samples (20 μg of each protein was used) were separated using SDS-gel electrophoresis and then transferred onto a 0.45-μm nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% milk powder dissolved in 1× TBST and incubated with specific primary antibodies overnight at 4 °C. All primary antibodies were diluted in an antibody dilution buffer consisting of 5% BSA and 0.01% Tween 20 in 1× TBS. Following incubation with primary antibodies, membranes were washed three times with 1× TBST and incubated with the appropriate secondary antibody (1:5000; goat anti-mouse, goat anti-rabbit; Mandel Scientific) for 1 h at room temperature. Membranes were scanned using the Li-Cor Odyssey 9120 infrared imager, and band intensity was quantified with the ImageJ 1.48v software. The following are the primary antibodies used in this work: anti-TRPM2 (Bethyl, A300-414A-2); anti-ATG3 (Cell Signaling Technology, 3415s); anti-ATG5 (Cell Signaling Technology, 12741s); anti-ATG7 (Cell Signaling Technology, 8558s); anti-ATG12 (Cell Signaling Technology, 4180s); anti-beclin-1 (Cell Signaling Technology, 3495s); anti-LC3A/B (Cell Signaling Technology, 2972s); anti-p-mTOR (Cell Signaling Technology, 2971s); anti-4E-BP1 (Cell Signaling Technology, 2972s); anti-p-4E-BP1 (Cell Signaling Technology, 9455s); anti-AKT (Santa Cruz Biotechnology, SC-1294s); anti-p-AKT (Cell Signaling Technology, 9271s); anti-JNK (Cell Signaling Technology, 9252s); anti-p-JNK (Cell Signaling Technology, 4668); anti-COX4.1 (Millipore, AB10526); anti-COX4.2 (Abcam, ab70112); anti-BNIP3 (Abcam, ab109362); anti-GAPDH (Santa Cruz Biotechnology, sc-365062); and anti-β-actin (Santa Cruz Biotechnology, SC-8432).

Trypan blue cell counting

AGS and MKN-45 cells were seeded in 6-well culture plates. At 0, 24, 48, and 72 h, cells were detached using 0.05% trypsin and resuspended in 1 ml of 1× PBS. Cells were then mixed with a 0.4% trypan blue solution at a 1:1 ratio and counted using the Bio-Rad TC20 automated cell counter. The total number of viable cells was represented with a line graph.

MTT assay

Cell viability was assessed by an MTT assay. 5 × 104 AGS and MKN45 cells were seeded in 96-well plates and allowed to incubate for 24, 48, and 72 h. At the respective time points, cells were treated with 200 μl of the MTT solution (5 mg/ml MTT in phosphate-buffered saline) and incubated at 37 °C for 3 h. Cells were then incubated with 100 μl of dimethyl sulfoxide (DMSO) to dissolve the formazan product. Absorbance was measured at 570 nm using the Beckman Coulter AD340 plate reader.

Cell proliferation assay

Cells suspended in 1 ml of 1× PBS were incubated with 100 μl of 2.5 μM CFSE (Sigma) for 15 min in the dark at 37 °C. CFSE-treated cells were seeded in 12-well plates and grown at 37 °C and 5% CO2 for 4 days. Cytometric analysis was performed using the BD FACSCalibur™ (Spectron Corp.) at a wavelength of 488 nm. A decrease in the level of CFSE is indicative of a high proliferation rate. Data were quantified using the Flowing software 2.5.1.

Annexin V/7AAD-binding assay

To assess the percentage of apoptosis and necrosis in gastric cancer cell lines, the flow cytometry-based annexin V/7AAD-binding assay was utilized. Cells were incubated with 12.5 μg/ml annexin V-fluorescein isothiocyanate (annexin V, Alexa Fluor 488; Invitrogen) and 20 μg/ml 7AAD solution (Biologend) for 15 min in the dark at room temperature, followed by a wash with 1× annexin buffer (0.1 M HEPES/NaOH, pH 7.4, 1.4 mM NaCl, 25 mM CaCl2). Cells were then resuspended in 1 ml of 1× annexin buffer and quantified using the BD FACScalibur™ (Spectron Corp.). The acquired data were processed using the FCS Express 30 Plus software.

Autophagy assay

To visualize changes in autophagy in the control and TRPM2 KD cells, an autophagy detection kit (Abcam; ab139484) was used as per the manufacturer’s instructions. Cells were cultured overnight, and negative control cells were incubated with 100 μM chloroquine (Abcam) for 24 h. Samples were centrifuged at 500 rpm, resuspended in 100 μl of FACS buffer (1× PBS, 1% FBS, and 1% 0.5 M EDTA) containing the autophagy green stain (1:1000 dilution), and incubated for 30 min in the dark at 37 °C. Cells were then washed with FACS buffer, pelleted, and resuspended in 500 μl of FACS buffer. Prepared samples were ana-
lyzed using the above-mentioned flow cytometer. Acquired data were graphically represented using the Flowing software.

**Extracellular flux analysis**

Mitochondrial function was assessed using the Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA). Cells were cultured at a density of $1 \times 10^5$ in a 24-well plate purchased from Seahorse Bioscience. The OCR of AGS and MKN-45 cells was measured in XF media (unbuffered DMEM containing 10 mm glucose) under basal conditions and after the administration of mitochondrial inhibitors (1.0 $\mu$M oligomycin (Sigma; 75351), 1.5 $\mu$M FCCP (Sigma; C2920), 1.0 $\mu$M rotenone (Sigma; R8875), and 1.0 $\mu$M antimycin (Sigma; A8674)). OCR and ECAR were normalized to the final cell number calculated after the completion of the assay. Basal OCR was calculated by subtraction of the residual rate after antimycin A treatment; maximal rate was calculated by subtraction of the residual rate after antimycin A treatment from FCCP-induced OCR. ATP production was calculated by subtraction of OCR after oligomycin treatment from basal OCR.

**Survival curves**

Online gastric cancer data bases were accessed through the KM Plot online visualization tool and analyzed according to the pre-established Kaplan Meier method (97, 113, 114). Patients were segregated into high and low groups based on a median cut-off. Data were plotted using the GraphPad Prism 6 software.

**Calculation of IC$_{50}$**

AGS and MKN-45 cells were treated with paclitaxel and doxorubicin for 24, 48, and 72 h. MTT viability assay was used to calculate the percentage of viable cells. The corresponding IC$_{50}$ was calculated using the non-linear regression analysis method in GraphPad Prism 5.0.

**Reagents**

Cell culture media, FBS, PBS, and penicillin/streptomycin antibiotics were acquired from Invitrogen/Thermo Fisher Scientific. MTT, doxorubicin, paclitaxel, oligomycin, FCCP, rotenone, and antimycin were purchased from Sigma.

**Statistical analysis**

All experiments were executed at least three times with one biological replicate being represented in each figure. Statistical significance was calculated using the Student’s $t$ test as indicated in the figure legends. Asterisks above each graph represent the degree of significance and correspond to the following $p$ values: n.s, $p \geq 0.05$; *, $p < 0.01$ to 0.05; **, $p < 0.001$; and ***, $p < 0.001$.

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**Author contributions**—S. A. performed experiments, analyzed data, and wrote the paper. B. E. K. performed and analyzed the experiments shown in Fig. 5. M. E. A. provided technical assistance for all experiments. A. M. S. performed and analyzed the experiments shown in Fig. 1. S. G. provided assistance and contributed to the preparation of Figs. 6 and 7. S. P. S. provided assistance and contributed to the writing of the paper. Y. E. H. conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.
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