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 mechanism 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, indicated by reduced levels of 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–}

5 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-trifluoromethoxyphenylethyl; MUTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco’s modified Eagle’s medium; AOA, N-(2-aminoethyl)-5-ethylcarboxyfluorescein succinimidyl ester; Scr., scrambled; 7AAD, 7-amino-actinomycin.


**TRPM2 in gastric cancer**

![Graph](image)

**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 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. 

To our knowledge, the functional expression of TRPM2 and TRPM2-mediated cancer cell survival is still missing (56, 57). Although inhibition of TRPM2 is advantageous in the treatment of various cancers, the underlying mechanism remains uncertain, thus limiting the benefits of the proposed therapy. Therefore, understanding the mechanism behind TRPM2-mediated cancer cell survival is crucial for the development of TRPM2-targeted cancer therapy. The published literature has attempted to explain the involvement of TRPM2 in apoptosis, autophagy, and mitochondrial function, but the link between the regulation of these biological events and TRPM2-mediated cancer cell survival is still missing (56, 57).

To our knowledge, the functional expression of TRPM2 and its role in gastric cancer have not been reported. Therefore, to decipher the possibility of TRPM2 involvement in gastric cancer, we used the shRNA lentivirus-based system to permanently knock down TRPM2 gene expression in two 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.

**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. Because of the lack of specific inhibitors for TRPM2, we used shRNA to selectively down-regulate TRPM2 in cells. Quantitative PCR (qPCR) and immunoblotting assays confirmed the efficiency of TRPM2 silencing in both cell lines (Fig. 2, A and B). Next, we tested the functionality of the channel using whole-cell patch-clamp recording. Under our experimental conditions, internal perfusion of control AGS and MKN-45 cells with 2 mM ADPR caused a rapid and stable current (Fig. 2, C and E). The linear I/V relationship aspect of the observed ADPR current, along with a reversal potential around 0 mV and high sensitivity to copper ions, represents the distinct characteristics of the TRPM2 curr-
The absence of ADPR 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 concur 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 mitochondrially 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-
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.

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

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 lipidation 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. 6, F 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 JNK-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 AGS 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.
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).

**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...
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. In this study, 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, 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 (ICR80254) 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-
Protocol for the 3rd generation lentiviral packaging system (111). 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...
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 
\[2^{-\Delta\Delta Ct}\] method and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene (112). Primer sequences are provided in Table 2.

**TRPM2 in gastric cancer**

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 ADPR.
TRPM2 in gastric cancer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPM2</td>
<td>Forward</td>
<td>AAAGTACGCTCCAGGATCCTCA</td>
</tr>
<tr>
<td>TRPM2</td>
<td>Reverse</td>
<td>GGAAAAGTGTCTCTGACGGG</td>
</tr>
<tr>
<td>ATG3</td>
<td>Forward</td>
<td>AGAAAGTGTCTCTGACGGG</td>
</tr>
<tr>
<td>ATG3</td>
<td>Reverse</td>
<td>GGAAAAGTGTCTCTGACGGG</td>
</tr>
<tr>
<td>ATG5</td>
<td>Forward</td>
<td>GTGCTTCAAGATGTGTTGTT</td>
</tr>
<tr>
<td>ATG5</td>
<td>Reverse</td>
<td>ATGCTTCAAGATGTGTTGTT</td>
</tr>
<tr>
<td>ATG6 (Beclin-1)</td>
<td>Forward</td>
<td>CCTCCGAAGTGAAGAGAAGT</td>
</tr>
<tr>
<td>ATG6 (Beclin-1)</td>
<td>Reverse</td>
<td>CACCCCTGAGTACGCTCA</td>
</tr>
<tr>
<td>ATG7</td>
<td>Forward</td>
<td>CTCGCCAGTGAAGAGAAGT</td>
</tr>
<tr>
<td>ATG7</td>
<td>Reverse</td>
<td>GGGGGAAGTAGCAGAGG</td>
</tr>
<tr>
<td>ATG12</td>
<td>Forward</td>
<td>AAGGGTATGGAAGAGGAC</td>
</tr>
<tr>
<td>ATG12</td>
<td>Reverse</td>
<td>CACCCCCGGTACGCTCA</td>
</tr>
<tr>
<td>COX4.1</td>
<td>Forward</td>
<td>GGCGGCTCCATTAGTGGT</td>
</tr>
<tr>
<td>COX4.1</td>
<td>Reverse</td>
<td>GCCGTGATGACGATGCTC</td>
</tr>
<tr>
<td>COX4.2</td>
<td>Forward</td>
<td>GAGATGAAAGCTGCTT</td>
</tr>
<tr>
<td>COX4.2</td>
<td>Reverse</td>
<td>AAATACGTAGACCCGCTGCC</td>
</tr>
<tr>
<td>BNIP3</td>
<td>Forward</td>
<td>CCTCAGCATAGAGAACAGA</td>
</tr>
<tr>
<td>BNIP3</td>
<td>Reverse</td>
<td>GCCACCCAGAATCCTACAG</td>
</tr>
</tbody>
</table>

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 (137 mM NaCl, 2.7 mM KCl, 19 mM Tris-base, 0.1% Tween 20) for 1 h at room temperature. Blots were washed three times with 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× TBST. 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; Medel 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, 1294S); 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, 12741S); anti-caspase-7 (Cell Signaling Technology, 9494S); anti-mTOR (Cell Signaling Technology, 2972S); anti-p-mTOR (Cell Signaling Technology, 2971); anti-4E-BP1 (Cell Signaling Technology, 2972S); anti-p-4E-BP1 (Cell Signaling Technology, 9455); anti-AKT (Santa Cruz Biotechnology, SC-46915); anti-p-AKT (Cell Signaling Technology, 9271); anti-JNK (Cell Signaling Technology, 9252); 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).

MTT assay

Cell viability was assessed by an MTT assay. 5 × 10³ AGS and MKN45 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% trypsin 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.

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 annexinV-fluorescein isothiocyanate (annexinV, 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 mM 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 mM oligomycin (Sigma; 75351), 1.5 mM FCCP (Sigma; C2920), 1.0 mM rotenone (Sigma; R8875), and 1.0 mM 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, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$ to 0.005; ***, $p < 0.001$.

**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.

**Acknowledgments**—We thank Dr. Paul Linsdell for constant assistance and support. We are also grateful to the encouragement and helpful comments from other members of the Department of Physiology and Biophysics at Dalhousie University.

**References**


TRPM2 in gastric cancer


TRPM2 in gastric cancer


