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Downregulation of ClC-2 by JAK2

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Key Words
JAK2V617F • Leptin • Erythropoietin • Cell volume regulation • Chloride channels

Abstract
JAK2 (Janus kinase-2) is activated by cell shrinkage and may thus participate in cell volume regulation. Cell volume regulatory ion channels include the small conductance Cl^- channels ClC-2. The present study thus explored whether JAK2 influences ClC-2 activity. To this end, ClC-2 was expressed in *Xenopus* oocytes with or without wild type JAK2, active ^{V617F}JAK2 or inactive ^{K882E}JAK2 and the Cl^- channel activity determined by dual electrode voltage clamp. Expression of ClC-2 was followed by a marked increase of cell membrane conductance. The conductance was significantly decreased following coexpression of JAK2 or ^{V617F}JAK2, but not by coexpression of ^{K882E}JAK2. Exposure of the oocytes expressing ClC-2 together with ^{V617F}JAK2 to the JAK2 inhibitor AG490 (40 μM) resulted in a gradual increase of the conductance. According to chemiluminescence JAK2 decreased the channel protein abundance in the cell membrane. The decline of conductance in ClC-2 and ^{V617F}JAK2 coexpressing oocytes following inhibition of channel protein insertion by brefeldin A (5 μM) was similar in oocytes expressing ClC-2 with ^{V617F}JAK2 and oocytes expressing ClC-2 alone, indicating that ^{V617F}JAK2 might slow channel protein insertion into rather than accelerating channel protein retrieval from the cell membrane. In conclusion, JAK2 down-regulates ClC-2 activity and thus counteracts Cl^- exit, an effect which may impact on cell volume regulation.

Introduction

Janus-activated kinase-2 JAK2 participates in the signaling of leptin [1], growth hormone [2], erythropoietin [3], thrombopoietin [3], granulocyte colony-stimulating factor [3] and a variety of cytokines [3, 4]. JAK2 contributes to the signaling of neoplasms and JAK2 inhibitors are considered as drugs in the treatment of myeloproliferative disorders [5-11]. The gain of function mutation ^{V617F}JAK2 is observed in the majority of myeloproliferative diseases [12]. JAK2 is activated by hyperosmotic shock and participates in the stimulation of the Na^+/H^+ exchanger [13, 14]. Accordingly, JAK2 may participate in regulatory cell volume increase following cell shrinkage.

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Anion channels, such as the ubiquitously expressed and highly conserved inwardly rectifying Cl^- channel ClC-2 [15, 16] are involved in cell volume regulatory decrease [16-18]. Cell shrinkage is known to be followed by inhibition of Cl^- channels thus decreasing cellular Cl^- loss [19, 20]. Cl^- channels further participate in the regulation of apoptosis, which is paralleled by enhanced channel activity [21-31]. At least in theory, inhibition of Cl^- channels could thus foster the survival of tumor cells.

The present study explored, whether JAK2 contributes to the regulation of ClC-2. To this end, ClC-2 induced currents were determined utilizing dual electrode voltage clamp and ClC-2 protein abundance in the cell membrane utilizing chemiluminescence in *Xenopus* oocytes expressing ClC-2 with or without wild type JAK2, active ^{V617F}JAK2 and inactive ^{K882E}JAK2. As a result, coexpression of JAK2 and active ^{V617F}JAK2 but not of ^{K882E}JAK2 decreased ClC-2 induced conductance and ClC-2 protein abundance in ClC-2 expressing *Xenopus* oocytes.

Materials and Methods

Constructs

For generation of cRNA the following cDNA constructs were used: Wild-type human ClC-2 [32-34] and wild-type human JAK2 (Imagenes, Berlin, Germany). The inactive ^{K882E}JAK2 mutant [35] and the gain of function ^{V617F}JAK2 mutant [12] were generated by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit; Stratagene, Heidelberg, Germany) as described previously [36]. The following primers were used: ^{V617F}JAK2: 5'-AGC ATT TGG TTT TAA ATT ATG GAG TAT GT**T** TCT GTG GAG ACG AGA-3'; ^{V617F}JAK2: 5'-TCT CGT CTC CAC AGA **A**AC ATA CTC CAT AAT TTA AAA CCA AAT GCT-3'; ^{K882E}JAK2: 5'-GGG AGG TGG TCG CTG TA**G** AAA AGC TTC AGC ATA GT-3'; and ^{K882E}JAK2: 5'-ACT ATG CTG AAG CTT TT**C** TAC AGC GAC CAC CTC CC-3'.

Underlined bases indicate mutation sites. Hemagglutinin (HA)-tagged ClC-2 was generated by site-directed mutagenesis [37]. The mutants were sequenced to verify the presence of the desired mutation. The mutants were used for generation of cRNA as described previously [38].

Voltage clamp in Xenopus oocytes

Xenopus oocytes were prepared as previously described [39]. Wild type JAK2 cRNA or ^{V617F}JAK2 or ^{K882E}JAK2 cRNA (10 ng) and 15 ng ClC-2 were injected on the first day after preparation of the oocytes [37]. The oocytes were maintained at 17°C in ND96 solution containing (in mM: 96 NaCl, 4 KCl, 1.8 $MgC1_2$, 0.1 $CaC1_2$, 5 HEPES, pH 7.4). The ND96 solution was supplemented with 100 mg/l gentamycin tetracyclin 50 mg/l, ciprofloxacin 1.6 mg/l theophyllin (90 mg/l) and, where indicated, with AG490 (40 μM final concentration) or brefeldin A (5 μM final concentration). Experiments were performed at room temperature 3 days after injection. The currents were determined in two-electrode voltage-clamp utilizing a pulse protocol of 10 s pulses from -140 mV to +40 mV in 20 mV increments. The intermediate holding-voltage was -60 mV. The current at the end of each voltage step was taken for data analysis. The data were filtered at 2 kHz, and recorded with MacLab digital to analog converter and software for data acquisition and analysis (ADInstruments, Castle Hill, Australia). The control bath solution (ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath solution was reached within about 10 s.

Detection of ClC-2 cell surface expression by chemiluminescence

To determine ClC-2 cell surface expression by chemiluminescence, defolliculated oocytes were injected with 10 ng cRNA encoding either wild type JAK2 or JAK2-mutant (^{V617F}JAK2 or ^{K882E}JAK2) or with water and at the same day with 15 ng cRNA encoding ClC-2-HA which contains an inserted HA epitope. Oocytes were incubated with 0.5μg/mL primary rat monoclonal anti-HA antibody (clone 3 F10, Roche, Mannheim, Germany) for 2h at room temperature and subsequently with secondary, HRP-conjugated goat anti-rat IgG (H&L) antibody (1:1000, Cell Signaling Technology, MA, USA) for 1 h at room temperature. Post-staining individual oocytes were placed in 96 well plates with 20 μL of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer, Juegesheim, Germany) by integrating the signal over a period of 1 s. The results of the experiments are given as normalized relative light units [40].

Determination of cell volume in Jurkat cells

The forward scatter in flow cytometric analysis (FACSCalibur from Becton Dickinson) was taken as measure for cell volume [41]. Human Jurkat cells [42] (10^6 cells per ml) were cultured for 16 hours in RPMI 1640 (PAA - 15-840) supplemented with 10% FBS, 1% penicillin/streptomycin, HEPES, L-glutamine, sodium-pyruvate, NEAA- non-essential amino acids and β-mercaptoethanol at 37°C in a humidified atmosphere with 5% CO_2 / air in the absence or presence of the JAK2 inhibitor AG490 (40 μM).

Statistical analysis

Data are provided as means ± SEM, n represents the number of oocytes investigated. All experiments were repeated with at least 2-3 batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA or t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored the effect of JAK2 (Janus kinase-2) on ClC-2 channel. To this end cRNA encoding ClC-2 was injected into *Xenopus* oocytes with

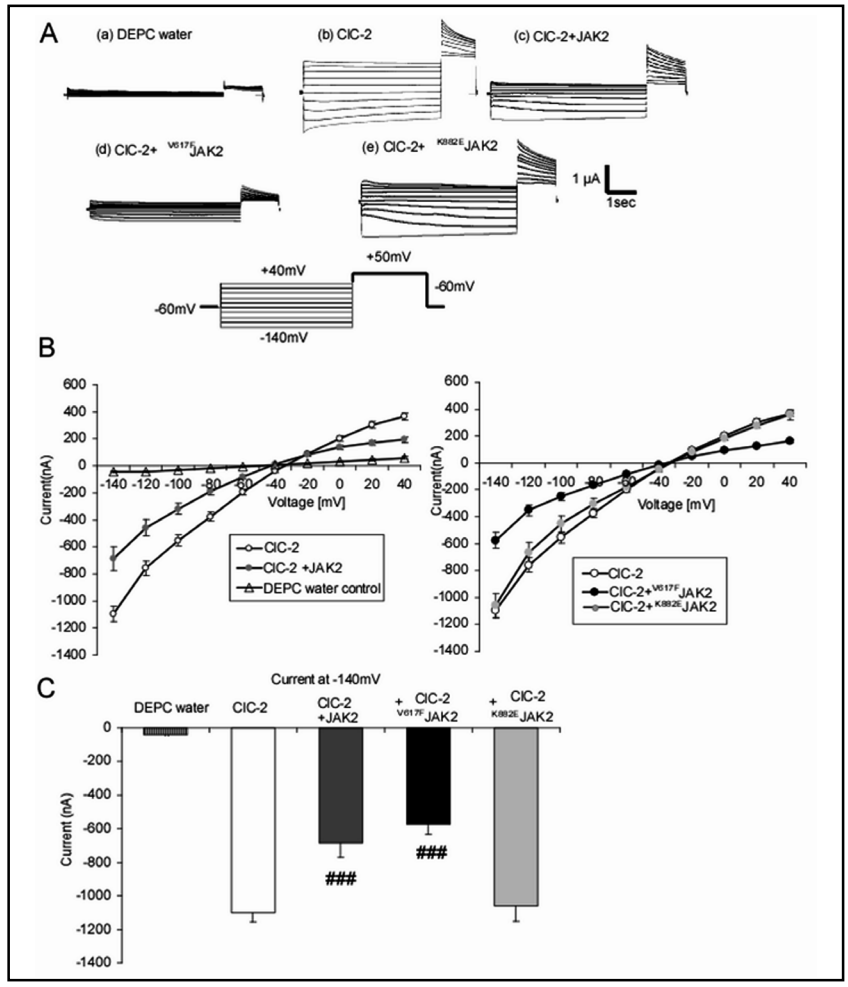

Fig. 1. Coexpression of JAK2, of active ^{V617F}JAK2, but not of inactive ^{K882E}JAK2 decreases Cl^- conductance in ClC-2-expressing *Xenopus* oocytes. A: Representative original tracings showing currents in *Xenopus* oocytes injected with water (a), as well as in oocytes expressing ClC-2 without (b) or with additional coexpression of wild type JAK2 (c), of constitutively active ^{V617F}JAK2 (d) or of inactive ^{K882E}JAK2 (e). B: Arithmetic means ± SEM (n = 15-25) of the current (I) as a function of the potential difference across the cell membrane (V) in *Xenopus* oocytes injected with water (DEPC water, white triangles), expressing ClC-2 alone (ClC-2, white circles) or expressing ClC-2 together with wild type JAK2 (ClC-2+JAK2, dark grey circles), with constitutively active ^{V617F}JAK2 (black circles) or with inactive ^{K882E}JAK2 (light grey circles). C. Arithmetic means ± SEM (n = 15-25) of the current at -140 mV in *Xenopus* oocytes injected with water (DEPC water), expressing ClC-2 alone (ClC-2, white bar) or expressing ClC-2 together with wild type JAK2 (ClC-2+JAK2, dark grey bar), with constitutively active ^{V617F}JAK2 (ClC-2+ ^{V617F}JAK2, black bar) or with inactive ^{K882E}JAK2 (ClC-2+^{K882E}JAK2, light grey bar). ### ($p<0.001$) indicate statistically significant difference to expression of ClC-2 alone

or without cRNA encoding JAK2 and the cell membrane conductance determined utilizing dual-electrode voltage-clamp. In water injected oocytes the cell membrane conductance was low (Fig.1). Expression of ClC-2 was, however, followed by a marked increase of cell membrane conductance. As illustrated in Fig. 1, the conductance was significantly decreased by additional expression of JAK2. Accordingly, coexpression of JAK2 down-regulated ClC-2 activity.

Similar to wild type JAK2, constitutively active mutant ^{V617F}JAK2 inhibited ClC-2 activity. Again, the conductance was significantly lower in *Xenopus* oocytes expressing ClC-2 together with ^{V617F}JAK2 than in *Xenopus* oocytes expressing ClC-2 alone (Fig. 1). In contrast, the inactive mutant ^{K882E}JAK2 did not significantly modify the activity of ClC-2 (Fig. 1). Treatment of ClC-2 and ^{V617F}JAK2 expressing *Xenopus* oocytes with the JAK2 inhibitor AG490 (40 µM) was followed by a gradual increase of the cell membrane conductance (Fig. 2). The effect of the inhibitor was slow and reached statistical significance only after 24 hours AG490 treatment.

The down-regulation of ClC-2 activity by JAK2 could have resulted from an influence on the protein abundance in the cell membrane. To explore that possibility, the channel protein abundance in the plasma membrane was determined by chemiluminescence. As illustrated in Fig. 3, the coexpression of JAK2 and ^{V617F}JAK2 significantly decreased the ClC-2 protein abundance within the oocyte cell membrane.

JAK2 could enhance ClC-2 protein abundance by either interference with channel insertion into the cell membrane or by accelerating channel protein retrieval from the cell membrane. To discriminate between these two possibilities, ClC-2 and ^{V617F}JAK2 expressing *Xenopus* oocytes were treated with 5 µM brefeldin A, which disrupts the insertion of new channel protein into the cell membrane. As shown in Fig. 4, the decline of conductance in the presence of brefeldin A was similar in oocytes expressing ClC-2 with ^{V617F}JAK2 and oocytes expressing ClC-2 alone. Accordingly, ^{V617F}JAK2 might not downregulate ClC-2 activity by interference with removal of channels from the cell membrane but possibly rather by interference with channel protein insertion into

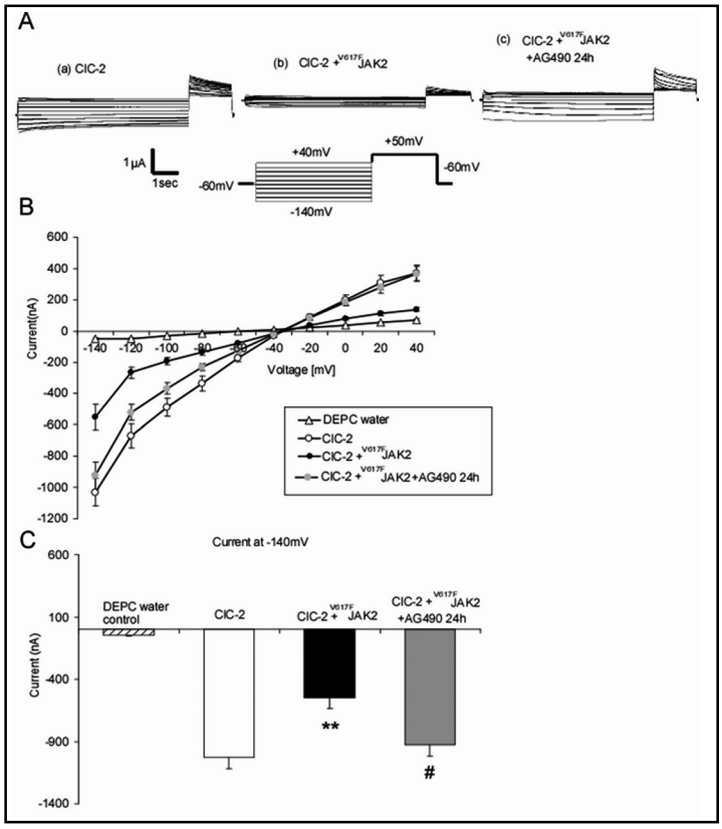

Fig. 2. The effect of JAK2 is reversed by JAK2 inhibitor AG490. A: Representative original tracings showing currents in *Xenopus* oocytes injected with ClC-2 alone (a), or coexpressing ClC-2 with constitutive active ^{V617F}JAK2 (b-c). Tracings were recorded after pretreatment with JAK2 inhibitor AG490 (40 µM) for 24 h (c). B: Arithmetic means ± SEM (n = 7-16) of current (I) in *Xenopus* oocytes injected with water (DEPC water, white triangles), expressing ClC-2 alone (ClC-2, white circles) or expressing ClC-2 together with constitutively active ^{V617F}JAK2 and incubated for 24 h in the absence (ClC-2+^{V617F}JAK2, black circles) or presence of the JAK2 inhibitor AG490 (40µM) (ClC-2+^{V617F}JAK2+AG490, light grey circles). C: Arithmetic means ± SEM (n = 7-16) of the current at -140mV in *Xenopus* oocytes injected with H_2O (DEPC water), expressing ClC-2 alone (ClC-2, white bar) or expressing ClC-2 together with ^{V617F}JAK2 and incubated for 24 h in the absence (ClC-2+^{V617F}JAK2, black bar) or presence of AG490 (ClC-2+^{V617F}JAK2+AG490 24h, light grey bar). **indicates statistically significant ($p<0.01$) difference from ClC-2 (i.e. in the absence of JAK2). # indicates statistically significant ($p<0.05$) difference from the absence of JAK2 inhibitor AG490

the cell membrane.

Inhibition of JAK2 is expected to enhance ClC-2 channel activity, thus fostering Cl^- exit and decrease of cell volume. Accordingly, an additional series of experiments was performed to elucidate whether AG490 influences cell volume of Jurkat lymphocytes. Cell volume was estimated from forward scatter in flow cytometry. Exposure of Jurkat cells for 16 hours to AG490 (40 µM) slightly but significantly ($p<0.05$) decreased forward scatter from 486 ± 6 (n = 3) to 467 ± 3 (n = 3).

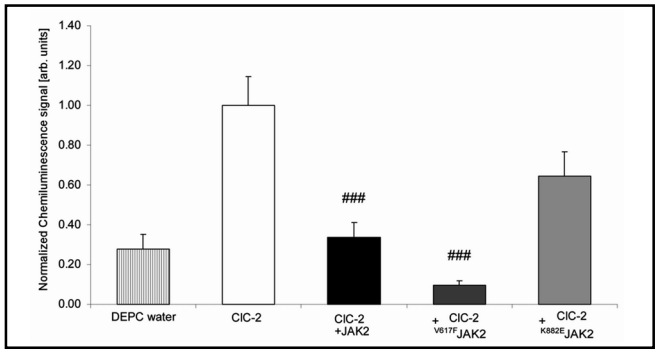

Fig. 3. Effect of JAK2 and ^{V617F}JAK2 on surface ClC-2 protein abundance in ClC-2-HA expressing *Xenopus* oocytes. Arithmetic means ± SEM (n = 33-46) of the chemiluminescence in *Xenopus* oocytes expressing ClC-2 alone (ClC-2, white bar) or expressing ClC-2 together with wild type JAK2 (ClC-2+JAK2, dark grey bar), with constitutively active ^{V617F}JAK2 (ClC-2+^{V617F}JAK2, black bar) or with inactive ^{K882E}JAK2 (ClC-2+^{K882E}JAK2, light grey bar). ### ($p<0.001$) indicate statistically significant difference to expression of ClC-2 alone.

Discussion

The present observations reveal a novel mechanism in the regulation of the Cl^- channel ClC-2. The Janus Kinase 2 decreases the ClC-2 protein abundance in the cell membrane and thus counteracts Cl^- exit, an effect resulting from decrease of channel protein abundance in the cell membrane. According to the experiments with brefeldin A, JAK2 might interfere with channel protein insertion rather than accelerating channel protein retrieval from the cell membrane.

JAK2 sensitive down-regulation of ClC-2 may participate in cell volume regulation, as ClC-2 has been shown to be sensitive to cell volume [18]. Cell volume regulatory mechanisms following cell shrinkage do include inhibition of Cl^- channels [19, 20]. As JAK2 is activated by osmotic cell shrinkage [13, 14], the kinase could contribute to or even account for Cl^- channel inhibition during osmotic cell shrinkage.

ClC-2 further participates in the regulation of intra-

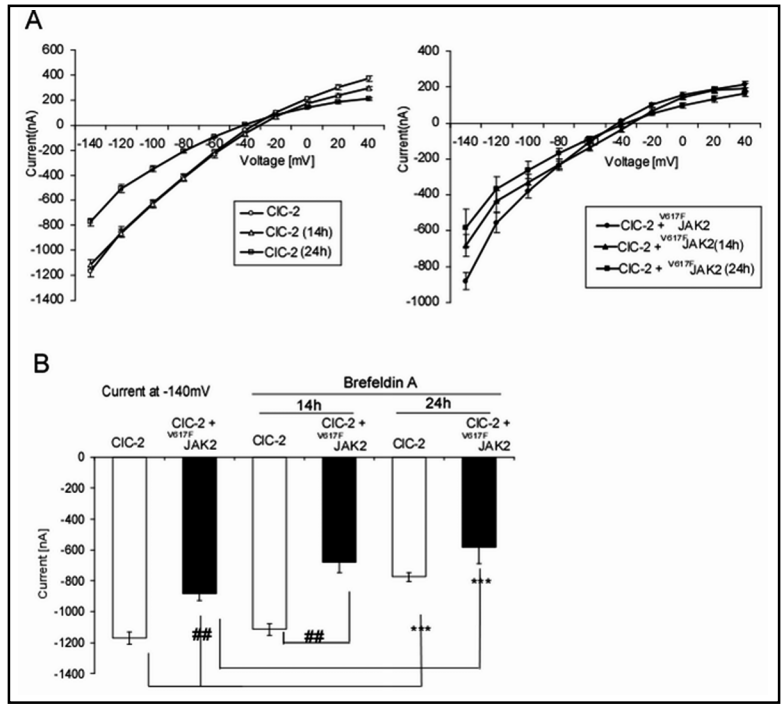

Fig. 4. Effect of brefeldin A on ClC-2 channel activity with or without coexpression of ^{V617F}JAK2. A. Arithmetic means ± SEM (n = 11-20) of current (I) in *Xenopus* oocytes injected with ClC-2 alone (ClC-2, white symbols) or expressing ClC-2 together with ^{V617F}JAK2 (ClC-2+^{V617F}JAK2, black symbols) and incubated in the absence of brefeldin A (circles) or following pre-treatment with the brefeldin A (5 µM) for 14 h (triangles) or 24 h (squares). B. Arithmetic means ± SEM (n = 11-20) of currents in *Xenopus* oocytes injected with ClC-2 alone (white bars) or expressing ClC-2 together with ^{V617F}JAK2 (black bars) prior to (left bars) and following treatment with 5 µM brefeldin for 14 h (middle bars) or 24 h (right bars). ## indicates statistically significant ($p<0.01$) difference from expression of ClC-2 alone, *** indicates statistically significant ($p<0.001$) difference from respective value prior to brefeldin A treatment.

cellular chloride concentration and thus cell membrane potential of neurons [43], in the survival of male germ cells and photoreceptors [44] as well as in the pulmonary chloride and water secretion, which is in turn necessary for fetal lung development [45]. Whether those functions are influenced by JAK2 is unknown.

Excessive JAK2 activity has been implicated in tumor cell growth [12] and JAK2 inhibitors have been shown to exert antineoplastic effects in myeloproliferative disorders [5-11]. Along those lines, the gain of function mutation ^{V617F}JAK2 is found in the majority of myeloproliferative diseases [12]. Activation of Cl^- channels may promote apoptosis [21-31]. Whether downregulation of ClC-2 enhances the resistance of lymphoid cells and/or other tumor cells against triggers of apoptosis remains to be shown.

In conclusion, JAK2 down-regulates the cell volume regulatory Cl^- channel ClC-2 and may thus contribute to regulation of cell volume and apoptosis. At least in theory, the regulation of chloride channels may play a role in neoplastic effects of the ^{V617F}JAK2 mutation.

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