Cellular Physiology and Biochemistry

Cell Physiol Biochem 2016;40:1141-1152
DOI: 10.1159/000453168
Published online: December 14, 2016
Accepted: November 23, 2016

Original Paper

Chorein Sensitive Orai1 Expression and Store Operated Ca^{2+} Entry in Rhabdomyosarcoma Cells

Willi Yu[a] Sabina Honisch[a] Sebastian Schmidt[a] Jing Yan[a] Evi Schmid[b] Saad Alkahtani[c,d] Abdullah A. AlKahtane[c] Saud Alarifi[c] Christos Stournaras[d] Florian Lang[a,e]

[a]Department of Cardiology, Vascular Medicine and Physiology, [b]Department of Pediatric Surgery and Pediatric Urology, University of Tuebingen, Germany; [c]Department of Zoology, Science College, King Saud University, Riyadh, Saudi Arabia; [d]Department of Biochemistry, University of Crete Medical School, Heraklion, Greece; [e]Department of Molecular Medicine II, Medical Faculty, Heinrich Heine University, Duesseldorf, Germany

Key Words
VPS13A • Orai1 • NFκB • SGK1 • SOCE • Cancer

Abstract
Background: Chorein, a protein encoded by VPS13A (vacuolar protein sorting-associated protein 13A), is defective in chorea acanthocytosis, a rare disease characterized by acanthocytosis of red blood cells and neuronal cell death with progressive hyperkinetic movement disorder, cognitive dysfunction, behavioral abnormalities and chronic hyperkalemia. Chorein is highly expressed in ZF rhabdomyosarcoma cells and counteracts apoptosis of those cells. Chorein is effective in part by interacting with and fostering stimulation of phosphoinositide-3-kinase (PI3K)-p85-subunit. PI3K dependent signaling includes the serum and glucocorticoid inducible kinase SGK1. The kinase activates NFκB with subsequent up-regulation of the Ca^{2+} channel subunit Orai1, which accomplishes store operated Ca^{2+} entry (SOCE). Orai1 and SOCE have been shown to confer survival of tumor cells. The present study thus explored whether chorein impacts on Orai1 expression and SOCE. ***Methods:*** In rhabdomyosarcoma cells chorein, Orai1, NFκB and SGK1 transcript levels were quantified by RT-PCR, Orai1 protein abundance by Western blotting, FACS analysis and confocal laser microscopy, $[Ca^{2+}]_i$ utilizing Fura-2 fluorescence, and SOCE from the increase of $[Ca^{2+}]_i$ following store depletion with extracellular Ca^{2+} removal and inhibition of the sarcoendoplasmatic reticular Ca^{2+} ATPase with thapsigargin. ***Results:*** The mRNA coding for chorein was most abundant in drug resistant, poorly differentiated human ZF rhabdomyosarcoma cells. Chorein silencing significantly decreased Orai1 transcript levels and Orai1 protein expression, as well as SGK1 and NFκB transcript levels. SOCE in ZF rhabdomyosarcoma cells was significantly blunted by chorein silencing, Orai1 inhibitor 2-APB (50 μM), SGK1 inhibitor EMD638683 (50 μM, 10 h) and NFκB inhibitor wogonin (50 μM, 24 h). ***Conclusion:*** Chorein is a stimulator of Orai1 expression and thus of store operated Ca^{2+} entry. The effect may involve SGK1 and NFκB.

W. Yu and S. Honisch contributed equally and thus share first authorship.
C. Stournaras and F. Lang contributed equally and thus share last authorship.

Prof. Dr. Florian Lang
Department of Cardiology, Vascular Medicine and Physiology, Universitaet Tuebingen, Gmelinstr. 5, 72076 Tuebingen (Germany)
Tel. +49 7071 29 72194, Fax +49 7071 29 5618, E-Mail florian.lang@uni-tuebingen.de

KARGER

Introduction

The protein chorein interacts with the phosphoinositide-3-kinase (PI3K)-p85-subunit presumably leading to stimulation of PI3K-p85-subunit tyrosine phosphorylation [1-3]. Subsequent chorein sensitive signaling includes activation of Rac1 and PAK1, polymerization of cortical actin, phosphorylation of Bad, and prevention of mitochondrial depolarization [3]. Loss-of-function mutations of the chorein encoding gene VPS13A (vacuolar protein sorting-associated protein 13A) account for chorea-acanthocytosis (ChAc), an autosomal recessive genetic disease [4-9] characterized by progressive hyperkinetic movement disorder, cognitive dysfunction, behavioral abnormalities, chronic hyperkalemia and variable acanthocytosis of red blood cells [5, 10]. In mice, chorein knockout has been shown to result in erythrocyte shape changes [11], neuronal apoptosis [12] and behavioral abnormalities [12].

Chorein is expressed in diverse tissues [13-15] and participates in the regulation of platelet secretion and aggregation [15], of endothelial cell stiffness [14], of cytoskeleton rearrangement [16] and of survival of ZF rhabdomyosarcoma cells [17]. Chorein silencing in ZF rhabdomyosarcoma cells blunted phosphoinositide 3 kinase (PI3K) activation and fostered induction of apoptosis [17].

PI3K sensitive mechanisms counteracting apoptosis include the serum & glucocorticoid inducible kinase SGK1 [18]. In part by activation of the transcription factor NFκB, SGK1 up-regulates the store-operated Ca^{2+} entry (SOCE) or Ca^{2+} release-activated Ca^{2+} channel I_{CRAC} [19-21], which are accomplished by the pore-forming Ca^{2+} channel subunits Orai1, Orai2 and/or Orai3 [22-25], as well as their regulators STIM1 and/or STIM2 [26-30]. Orai1 is decisive for rhabdomyosarcoma cell survival [31].

The present study explored, whether chorein modifies the expression of SGK1 and Orai1 and thus impacts on SOCE. To this end, the influence of chorein silencing on SGK1 and Orai1 transcript levels and protein expression as well as SOCE were determined in ZF rhabdomyosarcoma cells.

Materials and Methods

Cells

The rhabdomyosarcoma cell lines ZF (established at the Children's Hospital Tuebingen from a multifocal, alveolar rhabdomyosarcoma of an eight year old girl), RD and A204 (ATCC, USA) were cultured in DMEM high glucose (Gibco) containing 10% FBS and 1% penicillin/streptomycin (Sigma-Aldrich, USA) under standard culture conditions (37°C, 5% CO_2). The cells were seeded at $3x10^5$ cells/ml for 48 h before RNA isolation.

Silencing of chorein

To silence chorein, ZF rhabdomyosarcoma cells were seeded in 6 well plates 24h before transfection. Then cells were transfected with validated siRNA for VPS13A (chorein) (ID# s23342, Ambion, Darmstadt, Germany) or with a negative control siRNA (ID#4390843, Ambion) using siPORT amine transfection agent (Ambion) according to the manufacturer's protocol. The efficiency of silencing was checked by RT-PCR.

RT-PCR

Transcript levels of the respective genes were determined by real-time PCR. To this end total RNA was isolated 48h after transfection using the Trifast Reagent (Peqlab, Erlangen, Germany). cDNA was generated by reverse transcription of two µg RNA and GoScript Reverse Transcriptase Kit (Promega) according to the manufacturer's protocol. Quantitative real-time PCR was performed with the BioRad iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories) using GoTaq Sybr Green Master Mix (Promega). The reaction was applied in a final volume of 20 µl containing 2 µl of cDNA under following conditions: an initial incubation at 95°C for 5 min, 40 cycles at 95°C for 15 s, 59°C for 20 s and 72°C for 30s. Specificity of the PCR products was verified by melting curve analysis. The subsequent primers were used (5'→3' orientation):

VPS13A fw: AGTTCTCATCTTCTGGCTTCAG

VPS13A rev: AGTGGGACGACGTCTGTACAC
SGK1 fw: AGATTCACAGGGACACATTGTCC
SGK1 rev: TCGTACATTTCAGCTGTGTTTCG
ORAI1 fw: AGCCTCAACGAGCACTCCAT;
ORAI1 rev: CTGATCATGAGCGCAAACAGG;
NFκB1 fw: GCC CGA AAC GCC GAA TAT
NFκB1 rev: CCG TGG TTC GTG GCT CTC
GAPDH fw: TGAGTACGTCGTGGAGTCCACTG
GAPDH rev: GGTGCTAAGCAGTTGGTGGTG

The gene expression levels were normalized to the expression levels of GAPDH in the same cDNA sample. Relative quantification was calculated according to the ΔCt method.

Ca^{2+} measurements

Fura-2 fluorescence was utilized to determine intracellular Ca^{2+} measurements. Therefore cells were loaded with Fura-2/AM (2 μM, Invitrogen, Goettingen, Germany) for 15 min at 37°C. Afterwards the cells were excited alternatively at 340 nm and 380 nm through an objective (Fluor 40×/1.30 oil) built in an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Emitted fluorescence intensity was recorded at 505 nm. Data were acquired using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA). Cytosolic Ca^{2+} activity was estimated from the 340 nm/380 nm ratio. SOCE was determined by extracellular Ca^{2+} removal and subsequent Ca^{2+} re-addition in the presence of thapsigargin (1 μM, Invitrogen) [32]. For quantification of Ca^{2+} entry, the peak (delta ratio) and slope (delta ratio/s) were calculated following re-addition of Ca^{2+} [33].

Experiments were performed with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 $CaCl_2$, 2 Na_2HPO_4, 32 HEPES, 5 glucose, pH 7.4 (NaOH). To reach nominally Ca^{2+}-free conditions, experiments were performed using Ca^{2+}-free Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 Na_2HPO_4, 32 HEPES, 0.5 EGTA, 5 glucose, pH 7.4 (NaOH). Where indicated the cells were treated with Orai1 inhibitor 2-APB (50 μM), SGK1 inhibitor EMD638683 (50 μM, 10 h) and NFκB inhibitor wogonin (50 μM, 24 h) prior to and during the experiment.

Confocal laser scanning microscopy

For confocal laser scanning microscopy negative and chorein silenced ZF cells were seeded on glass chamber slides (Sarstedt, Germany). After washing twice with PBS, cells were fixed with 4% PFA for 15 min and blocked with 3% BSA in PBS for 1 hour at room temperature. Then, the cells were exposed to anti-Orai1 primary antibody (1:200, Abcam) at 4 °C overnight. After three washing steps with PBS the cells were incubated with CF™ 488A-labeled anti–rabbit secondary antibody (1:250, Sigma, USA) and with DRAQ-5 dye (1:3000, Biostatus, Leicestershire, UK) for 1 h at room temperature. Following three washes with PBS all slides were mounted with ProLong Gold antifade reagent (Life Technologies, USA). Images were subsequently taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss, Germany) with a water immersion Plan-Neofluar 40/1.3 NA DIC [14, 34].

FACS measurement

To determine Orai1 surface abundance, negative and chorein silenced ZF cells were detached, washed two times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min at room temperature. Then the cells were blocked for 30 min with 3% BSA in PBS and incubated for 60 minutes (37°C) with anti-Orai1 primary antibody (1:100, Abcam). After two washes with PBS the cells were stained with a CF™ 488A-labeled anti–rabbit secondary antibody (1:200, Sigma, USA) for 30 minutes (37°C). After two washing steps samples were analyzed on a FACS Calibur flow cytometer (BD Biosciences).

Western Blotting

Orai1 protein abundance was further detected by western blotting. Negative and chorein silenced ZF cells were washed twice with ice cold PBS and suspended in 200 μl ice-cold RIPA lysis buffer (Thermo Fisher Scientific, USA) containing Halt Protease and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, USA). Protein concentration was determined using the Bradford assay (BioRad, München, Germany). Thirty μg of protein were solubilized in sample buffer at 95°C for 5 min. The proteins were separated by a 10%

SDS-PAGE in a Glycine-Tris buffer and electro-transferred onto PVDF membranes for 70 min. After blocking with 5% BSA in TBST at room temperature for 1 h, the membranes were incubated with primary anti-Orai1 antibody (1:1000, Cell Signaling) and anti-GAPDH antibody (1:2000, Cell Signaling) at 4°C overnight. After washing (TBST), the blots were incubated with secondary anti-rabbit (1:2000, Cell Signaling) antibody for 1 h at room temperature. Protein bands were detected after additional washes (TBST) with an ECL detection reagent (Amersham, Freiburg, Germany) and quantified with Quantity One Software (BioRad, München, Germany). To assign the right protein size we used Protein-Marker VI (Peqlab, Erlangen, Germany).

Statistics

Data are expressed as arithmetic means ± SEM. Statistical analysis was made by unpaired t-test. A $p<0.05$ value was considered statistically significant.

Results

The present study addressed the impact of chorein on Orai1, SGK1 and NFκB expression as well as store operated Ca^{2+} entry (SOCE) in rhabdomyosarcoma cells. RT-PCR was employed in order to quantify the transcript levels of chorein and Orai1 in various rhabdomyosarcoma cell lines. As illustrated in Fig. 1A, B, chorein and Orai1 transcripts were most abundant in poorly differentiated ZF rhabdomyosarcoma cells. Accordingly, these cells were used throughout this study. As illustrated on Fig. 1C, Orai1 transcript levels were significantly decreased by chorein silencing (Fig. 1C).

We further analyzed the effect of chorein silencing on expression of Orai1 protein in ZF rhabdomyosarcoma cells. According to confocal laser scanning microscopy (Fig. 2A), Western Blotting (Fig. 2B), and FACS analysis (Fig. 2C), chorein silencing was followed by a significant downregulation of Orai1 protein expression levels.

Fura-2 fluorescence of Fura-2-AM loaded ZF rhabdomyosarcoma cells was taken as a measure of cytosolic concentration ($[Ca^{2+}]_i$). Store operated Ca^{2+} entry (SOCE) was triggered by depletion of intracellular Ca^{2+} stores which was accomplished by extracellular Ca^{2+} removal and addition of sarcoendoplasmatic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin. As illustrated in Fig. 3, chorein silencing did not appreciably modify Ca^{2+} release from intracellular stores but significantly decreased the peak of SOCE.

A further series of experiments tested whether SOCE is sensitive to Orai1 blocker 2-APB. As shown in Fig. 4, the Orai1 blocker 2-APB significantly blunted the peak increase of $[Ca^{2+}]_i$ following intracellular Ca^{2+} release and virtually abrogated SOCE (Fig. 4).

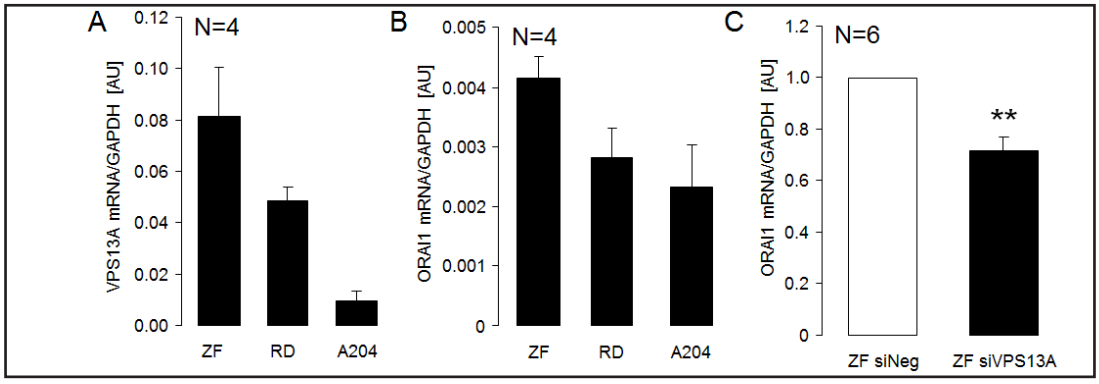

Fig. 1. Chorein and Orai1 expression in various rhabdomyosarcoma cell lines as well as chorein sensitive Orai1 transcript levels in ZF rhabdomyosarcoma cells. A, B. Arithmetic means ± SEM (n = 4) of (A) chorein, and (B) Orai1 transcript levels in ZF, RD and A204 rhabdomyosarcoma cells. C. Arithmetic means ± SEM (n = 6) of Orai1 transcript levels in ZF rhabdomyosarcoma cells prior to (white bar) and following (black bar) chorein silencing. **($p<0.01$) indicates statistically significant difference following chorein silencing.

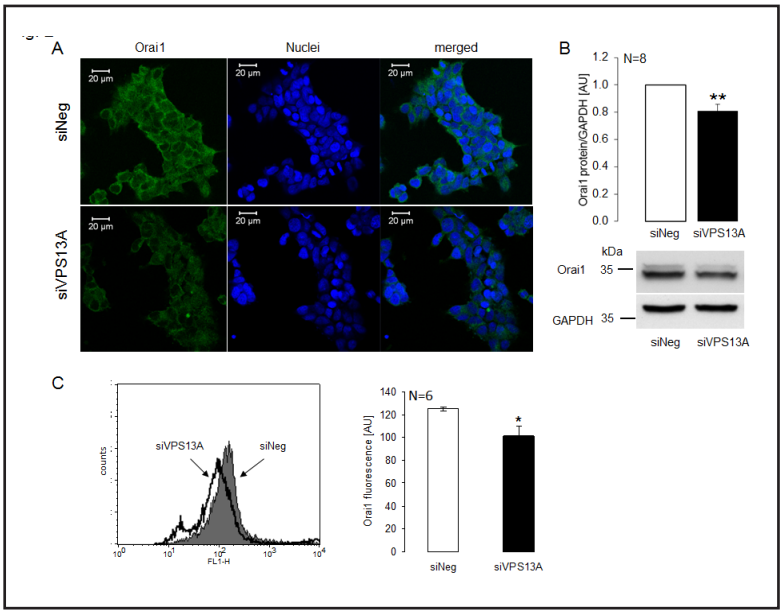

Fig. 2. Chorein sensitive Orai1 protein expression. A. Original confocal microscopy of ZF rhabdomyosarcoma cells following siNeg or chorein silencing and stained with anti-Orai1 antibody (green) and DRAQ-5 (blue) for nuclei. B. Original Western blot and arithmetic means ± SEM (n = 8) of protein abundance in ZF rhabdomyosarcoma cells prior to (white bar) and following chorein silencing (black bar) **(p<0.01) indicates statistically significant difference following chorein silencing. C. Original histogram of anti-Orai1 fluorescence in ZF rhabdomyosarcoma cells prior to and following chorein silencing and arithmetic means ± SEM (n = 6) of the Orai1 protein abundance in ZF rhabdomyosarcoma cells prior to (white bar) and following chorein silencing (black bar). *(p<0.05) indicates statistically significant difference following chorein silencing.

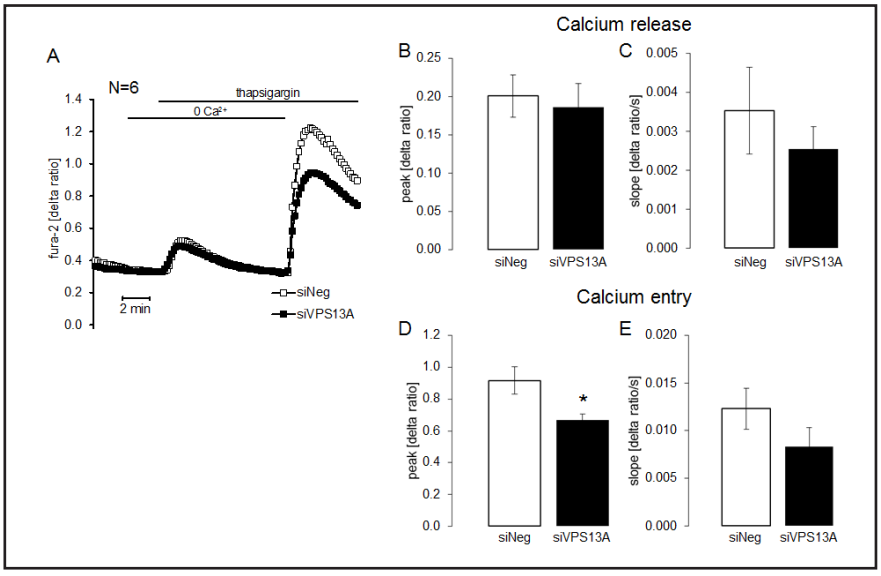

Fig. 3. Chorein sensitive intracellular Ca^{2+} release and store-operated Ca^{2+} entry (SOCE) in ZF rhabdomyosarcoma cells. A. Representative tracings of Fura-2 fluorescence-ratio in fluorescence spectrometry before and following extracellular Ca^{2+} removal and addition of thapsigargin (1 µM), as well as re-addition of extracellular Ca^{2+} without (white symbols) and with (black symbols) prior chorein silencing. B, C. Arithmetic means (± SEM, n = 6) of peak (B) and slope (C) increase of fura-2-fluorescence-ratio following addition of thapsigargin (1 µM) in ZF rhabdomyosarcoma cells without (white bars) and with (black bars) prior chorein silencing. D, E. Arithmetic means (± SEM, n = 6) of peak (D) and slope (E) increase of fura-2-fluorescence-ratio following re-addition of extracellular Ca^{2+} in ZF rhabdomyosarcoma cells without (white bars) and with (black bars) prior chorein silencing. *(p<0.05) indicates statistically significant difference from untreated cells (two-tailed unpaired *t*-test).

A next series of experiments explored whether SOCE is sensitive to SGK1 signaling. As shown in Fig. 5A, chorein silencing of ZF rhabdomyosarcoma cells decreased SGK1 transcript levels. Pharmacological inhibition of SGK1 with the SGK1 blocker EMD638683 (50 µM, 10 h) significantly decreased the peak increase of $[Ca^{2+}]_i$ following intracellular Ca^{2+} release and significantly blunted SOCE (Fig. 5B-F).

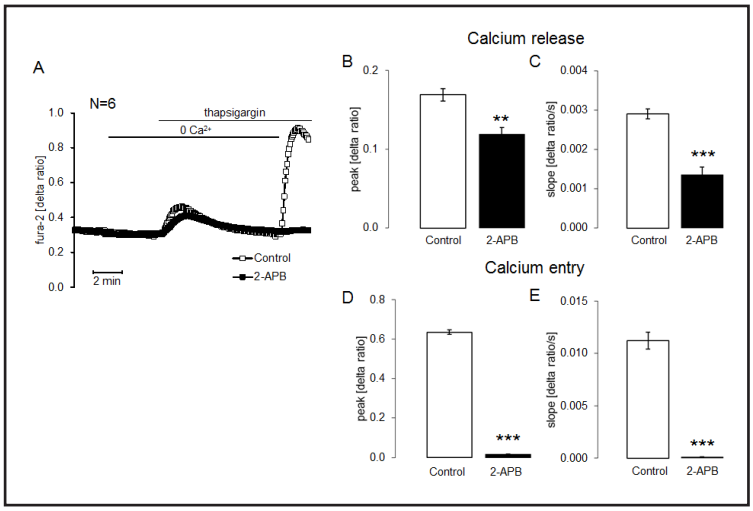

Fig. 4. 2-APB sensitive intracellular Ca^{2+} release and store-operated Ca^{2+} entry (SOCE) in ZF rhabdomyosarcoma cells. A. Representative tracings of Fura-2 fluorescence-ratio in fluorescence spectrometry before and following extracellular Ca^{2+} removal and addition of thapsigargin (1 μM), as well as re-addition of extracellular Ca^{2+} without (white symbols) and with (black symbols) presence of 2-APB. B, C. Arithmetic means (± SEM, n = 6) of peak (B) and slope (C) increase of fura-2-fluorescence-ratio following addition of thapsigargin (1 μM) in ZF rhabdomyosarcoma cells without (white bars) and with (black bars) presence of 2-APB (50 μM). **(p<0.01), ***(p<0.001) indicate statistically significant difference from absence of 2-APB (two-tailed unpaired *t*-test). D, E. Arithmetic means (± SEM, n = 6) of peak (D) and slope (E) increase of fura-2-fluorescence-ratio following re-addition of extracellular Ca^{2+} in ZF rhabdomyosarcoma cells without (white bars) and with (black bars) presence of 2-APB (50 μM). ***(p<0.001) indicates statistically significant difference from absence of 2-APB (two-tailed unpaired *t*-test).

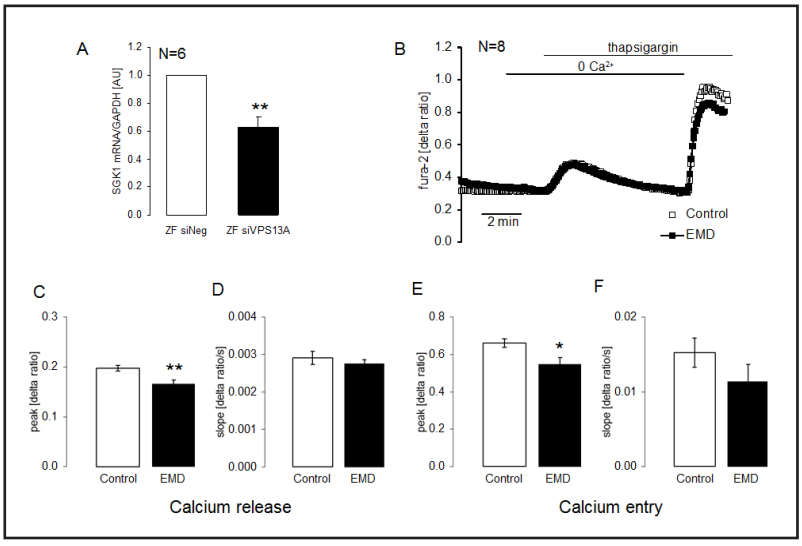

Fig. 5. Chorein sensitive down-regulation of SGK1 transcript levels as well as EMD638683 sensitive intracellular Ca^{2+} release and store-operated Ca^{2+} entry (SOCE) in ZF rhabdomyosarcoma cells. A. Arithmetic means ± SEM (n = 6) of SGK1 transcript levels prior to (white bar) and following (black bar) chorein silencing. **(p<0.01) indicates statistically significant difference following chorein silencing. B. Representative tracings of Fura-2 fluorescence-ratio in fluorescence spectrometry before and following extracellular Ca^{2+} removal and addition of thapsigargin (1 μM), as well as re-addition of extracellular Ca^{2+} without (white symbols) and with (black symbols) prior treatment with EMD638683 (50 μM, 10 h). C, D. Arithmetic means (± SEM, n = 8) of peak (C) and slope (D) increase of fura-2-fluorescence-ratio following addition of thapsigargin (1 μM) in ZF rhabdomyosarcoma cells without (white bars) and with (black bars) prior treatment with EMD638683 (50 μM, 10 h). **(p<0.01) indicates statistically significant difference from absence of EMD638683 (two-tailed unpaired *t*-test). E, F. Arithmetic means (± SEM, n = 8) of peak (E) and slope (F) increase of fura-2-fluorescence-ratio following re-addition of extracellular Ca^{2+} in ZF rhabdomyosarcoma cells without (white bars) and with (black bars) prior treatment with EMD638683 (50 μM, 10 h). *(p<0.05) indicates statistically significant difference from absence of EMD638683 (two-tailed unpaired *t*-test).

An additional series of experiments tested the impact of NFκB on SOCE upon chorein silencing. As illustrated in Fig. 6A, chorein silencing significantly decreased NFκB

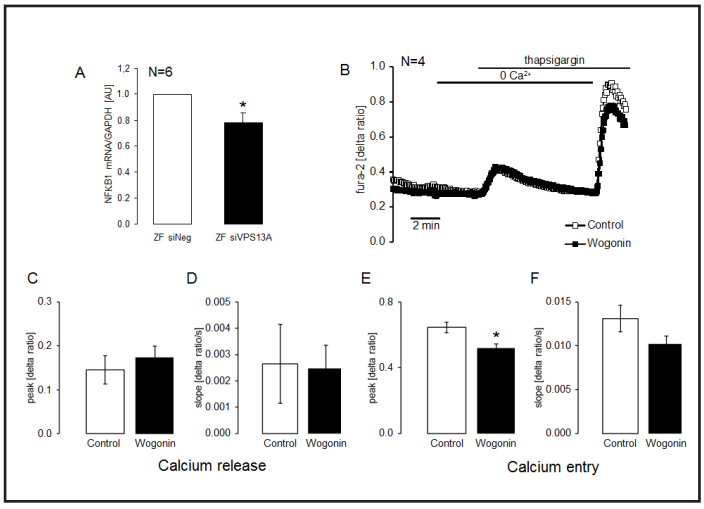

Fig. 6. Chorein sensitive down-regulation of NFκB transcript levels as well as Wogonin sensitive intracellular Ca^{2+} release and store-operated Ca^{2+} entry (SOCE) in ZF rhabdomyosarcoma cells. A. Arithmetic means ± SEM (n = 6) of NFκB transcript levels prior to (white bar) and following (black bar) chorein silencing. *($p<0.05$) indicates statistically significant difference following chorein silencing. B. Representative tracings of Fura-2 fluorescence-ratio in fluorescence spectrometry before and following extracellular Ca^{2+} removal and addition of thapsigargin (1 μM), as well as re-addition of extracellular Ca^{2+} without (white symbols) and with (black symbols) prior treatment with wogonin. C, D. Arithmetic means (± SEM, n = 4) of peak (C) and slope (D) increase of fura-2-fluorescence-ratio following addition of thapsigargin (1 μM) in ZF rhabdomyosarcoma cells without (white bars) and with (black bars) prior treatment with wogonin (50 μM, 24 h). E, F. Arithmetic means (± SEM, n = 4) of peak (E) and slope (F) increase of fura-2-fluorescence-ratio following re-addition of extracellular Ca^{2+} in ZF rhabdomyosarcoma cells without (white bars) and with (black bars) prior treatment with wogonin (50 μM, 24 h). *($p<0.05$) indicates statistically significant difference from absence of wogonin (two-tailed unpaired *t*-test).

transcription levels. As shown in Fig. 6B-F, pharmacological inhibition of NFκB with wogonin (50 μM, 24 h) significantly decreased SOCE.

Discussion

The present study uncovers a novel function of chorein, i.e. the regulation of Orai1, the pore forming Ca^{2+} channel subunit accomplishing store operated Ca^{2+} entry (SOCE) or Ca^{2+} release activated Ca^{2+} channel current (I_{CRAC}). The effect is presumably related to the influence of chorein on PI3K. Indeed, chorein interacts with PI3K and chorein silencing decreases the phosphorylation of the PI3K-subunit-p85 in ZF rhabdomyosarcoma cells [1, 2, 17]. PI3K sensitive signaling includes SGK1 [18]. As illustrated in Fig. 5A, SGK1 transcript levels are down-regulated by chorein silencing. Decreased PI3K activity would further be expected to compromise activation of expressed SGK1[18]. SGK1 in turn activates the transcription factor NFκB, which up-regulates Orai1, and thus SOCE and I_{CRAC} [19-21]. As shown in Fig. 6A, chorein silencing downregulates NFκB transcript levels. Decreased SGK1 activity would further be expected to compromise phosphorylation and thus activation of IKKβ thus affecting NFκB translocation into the nucleus [18]. Collectively, the observed decrease of SOCE following chorein silencing could at least in part be due to down-regulation of SGK1 with subsequent decrease of NFκB activity and Orai1 expression. The present observations do, however, not rule out further chorein sensitive mechanisms that may participate in the regulation of Orai1 expression and SOCE.

The up-regulation of Orai1 may contribute to the previously observed supporting effect of chorein on tumor cell survival [17]. Survival of chorein expressing rhabdomyosarcoma cells, but not of colon carcinoma cells with low levels of chorein expression is compromized by chorein silencing [17]. Chorein silencing has previously been shown to trigger apoptosis of rhabdomyosarcoma cells [17] and K562 cells [3]. The effect of chorein silencing depends on chorein expression prior to silencing [17].

The impact of chorein on tumor cell survival is presumably related to stimulation of PI3K signaling, which counteracts apoptosis of diverse cells including cancer cells [35-49] and neurons [50-53]. Along those lines PI3K inhibitors are powerful drugs in the treatment of malignancy [42, 54]. Similarly, up-regulation of SGK1 has been shown to foster tumor cell survival [18, 55]. Moreover, Orai1 and SOCE have previously been shown to support survival and therapy resistance of several tumor cell types [56-65]. Along those lines, 2-APB and wogonin inhibit proliferation of rhabdomyosarcoma cells [31]. The previous observations and present study, do, however, not rule out the contribution of further mechanisms other than Orai1 and SOCE to the influence of chorein on rhabdomyosarcoma cell survival.

On the other hand, Orai1 and SOCE may contribute to further chorein sensitive cellular mechanisms. Besides its effect on cell survival, chorein participates in the regulation of dopamine release [66, 67]. Chorein stabilizes the cortical actin filament network [3] and intermediate filament structures [16]. The cytoskeleton in turn influences cell shape, exocytosis, membrane blebbing and receptor function [3, 68-78]. Moreover, chorein sensitive Orai1 expression and function may modify the survival and function of further cell types. Chorein expression is particularly high in testis, kidney, spleen and brain [13]. The clinical course of chorea acanthocytosis reveals that survival of neurons and skeletal muscle cells are particularly dependent on chorein [5, 10].

In conclusion, the present observations reveal a novel function of chorein, i.e. the up-regulation of the Ca^{2+} channel Orai1 and thus of store operated Ca^{2+} entry. Chorein thus participates in the regulation of Ca^{2+} sensitive cellular functions.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch and Lejla Subasic. This study was supported by the International Scientific Partnership Program ISPP at King Saud University (KSU-ISPP#009) and the Deutsche Forschungsgemeinschaft, GRK 1302, SFB 773, La 315/13-3, and Open Access Publishing Fund of Tuebingen University

Disclosure Statement

All authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

References

1 IntAct DIfi: [http://www.ebi.ac.uk/intact/pages/interactions/interactions.xhtml?conversationContext=1]. EMBL EBI database 2011;
2 Wu C, Ma MH, Brown KR, Geisler M, Li L, Tzeng E, Jia CY, Jurisica I, Li SS: Systematic identification of SH3 domain-mediated human protein-protein interactions by peptide array target screening. Proteomics 2007;7:1775-1785.
3 Foller M, Hermann A, Gu S, Alesutan I, Qadri SM, Borst O, Schmidt EM, Schiele F, vom Hagen JM, Saft C, Schols L, Lerche H, Stournaras C, Storch A, Lang F: Chorein-sensitive polymerization of cortical actin and suicidal cell death in chorea-acanthocytosis. FASEB J 2012;26:1526-1534.
4 Mohiddin SA, Fananapazir L: Systolic compression of epicardial coronary and intramural arteries in children with hypertrophic cardiomyopathy. Tex Heart Inst J 2002;29:290-298.
5 Dobson-Stone C, Rampoldi L, Bader B, Velayos BA, Walker RH, Danek A, Monaco AP: Chorea-Acanthocytosis. Gene Rev 1993;updated 2010.

6 Dobson-Stone C, Danek A, Rampoldi L, Hardie RJ, Chalmers RM, Wood NW, Bohlega S, Dotti MT, Federico A, Shizuka M, Tanaka M, Watanabe M, Ikeda Y, Brin M, Goldfarb LG, Karp BI, Mohiddin S, Fananapazir L, Storch A, Fryer AE, Maddison P, Sibon I, Trevisol-Bittencourt PC, Singer C, Caballero IR, Aasly JO, Schmierer K, Dengler R, Hiersemenzel LP, Zeviani M, Meiner V, Lossos A, Johnson S, Mercado FC, Sorrentino G, Dupre N, Rouleau GA, Volkmann J, Arpa J, Lees A, Geraud G, Chouinard S, Nemeth A, Monaco AP: Mutational spectrum of the CHAC gene in patients with chorea-acanthocytosis. Eur J Hum Genet 2002;10:773-781.

7 Dobson-Stone C, Velayos-Baeza A, Filippone LA, Westbury S, Storch A, Erdmann T, Wroe SJ, Leenders KL, Lang AE, Dotti MT, Federico A, Mohiddin SA, Fananapazir L, Daniels G, Danek A, Monaco AP: Chorein detection for the diagnosis of chorea-acanthocytosis. Ann Neurol 2004;56:299-302.

8 Ueno S, Maruki Y, Nakamura M, Tomemori Y, Kamae K, Tanabe H, Yamashita Y, Matsuda S, Kaneko S, Sano A: The gene encoding a newly discovered protein, chorein, is mutated in chorea-acanthocytosis. Nat Genet 2001;28:121-122.

9 Tomiyasu A, Nakamura M, Ichiba M, Ueno S, Saiki S, Morimoto M, Kobal J, Kageyama Y, Inui T, Wakabayashi K, Yamada T, Kanemori Y, Jung HH, Tanaka H, Orimo S, Afawi Z, Blatt I, Aasly J, Ujike H, Babovic-Vuksanovic D, Josephs KA, Tohge R, Rodrigues GR, Dupre N, Yamada H, Yokochi F, Kotschet K, Takei T, Rudzinska M, Szczudlik A, Penco S, Fujiwara M, Tojo K, Sano A: Novel pathogenic mutations and copy number variations in the VPS13A Gene in patients with chorea-acanthocytosis. Am J Med Genet B Neuropsychiatr Genet 2011;156:620-631.

10 Saiki S, Sakai K, Murata KY, Saiki M, Nakanishi M, Kitagawa Y, Kaito M, Gondo Y, Kumamoto T, Matsui M, Hattori N, Hirose G: Primary skeletal muscle involvement in chorea-acanthocytosis. Mov Disord 2007;22:848-852.

11 Walterfang M, Looi JC, Styner M, Walker RH, Danek A, Niethammer M, Evans A, Kotschet K, Rodrigues GR, Hughes A, Velakoulis D: Shape alterations in the striatum in chorea-acanthocytosis. Psychiatry Res 2011;192:29-36.

12 Tomemori Y, Ichiba M, Kusumoto A, Mizuno E, Sato D, Muroya S, Nakamura M, Kawaguchi H, Yoshida H, Ueno S, Nakao K, Nakamura K, Aiba A, Katsuki M, Sano A: A gene-targeted mouse model for chorea-acanthocytosis. J Neurochem 2005;92:759-766.

13 Kurano Y, Nakamura M, Ichiba M, Matsuda M, Mizuno E, Kato M, Agemura A, Izumo S, Sano A: In vivo distribution and localization of chorein. Biochem Biophys Res Commun 2007;353:431-435.

14 Alesutan I, Seifert J, Pakladok T, Rheinlaender J, Lebedeva A, Towhid ST, Stournaras C, Voelkl J, Schaffer TE, Lang F: Chorein sensitivity of actin polymerization, cell shape and mechanical stiffness of vascular endothelial cells. Cell Physiol Biochem 2013;32:728-742.

15 Schmidt EM, Schmid E, Munzer P, Hermann A, Eyrich AK, Russo A, Walker B, Gu S, vom Hagen JM, Faggio C, Schaller M, Foller M, Schols L, Gawaz M, Borst O, Storch A, Stournaras C, Lang F: Chorein sensitivity of cytoskeletal organization and degranulation of platelets. FASEB J 2013;27:2799-2806.

16 Honisch S, Gu S, Vom Hagen JM, Alkahtani S, Al Kahtane AA, Tsapara A, Hermann A, Storch A, Schols L, Lang F, Stournaras C: Chorein Sensitive Arrangement of Cytoskeletal Architecture. Cell Physiol Biochem 2015;37:399-408.

17 Honisch S, Yu W, Liu G, Alesutan I, Towhid ST, Tsapara A, Schleicher S, Handgretinger R, Stournaras C, Lang F: Chorein addiction in VPS13A overexpressing rhabdomyosarcoma cells. Oncotarget 2015;6:10309-10319.

18 Lang F, Perrotti N, Stournaras C: Colorectal carcinoma cells--regulation of survival and growth by SGK1. Int J Biochem Cell Biol 2010;42:1571-1575.

19 Borst O, Schmidt EM, Munzer P, Schonberger T, Towhid ST, Elvers M, Leibrock C, Schmid E, Eylenstein A, Kuhl D, May AE, Gawaz M, Lang F: The serum- and glucocorticoid-inducible kinase 1 (SGK1) influences platelet calcium signaling and function by regulation of Orai1 expression in megakaryocytes. Blood 2012;119:251-261.

20 Eylenstein A, Gehring EM, Heise N, Shumilina E, Schmidt S, Szteyn K, Munzer P, Nurbaeva MK, Eichenmuller M, Tyan L, Regel I, Foller M, Kuhl D, Soboloff J, Penner R, Lang F: Stimulation of Ca2+-channel Orai1/STIM1 by serum- and glucocorticoid-inducible kinase 1 (SGK1). FASEB J 2011;25:2012-2021.

21 Lang F, Eylenstein A, Shumilina E: Regulation of Orai1/STIM1 by the kinases SGK1 and AMPK. Cell Calcium 2012;52:347-354.

22 Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A, Hogan PG: Orai1 is an essential pore subunit of the CRAC channel. Nature 2006;443:230-233.

23 Putney JW Jr.: New molecular players in capacitative Ca2+ entry. J Cell Sci 2007;120:1959-1965.
24 Vig M, Peinelt C, Beck A, Koomoa DL, Rabah D, Koblan-Huberson M, Kraft S, Turner H, Fleig A, Penner R, Kinet JP: CRACM1 is a plasma membrane protein essential for store-operated Ca2+ entry. Science 2006;312:1220-1223.
25 Yeromin AV, Zhang SL, Jiang W, Yu Y, Safrina O, Cahalan MD: Molecular identification of the CRAC channel by altered ion selectivity in a mutant of Orai. Nature 2006;443:226-229.
26 Fahrner M, Muik M, Derler I, Schindl R, Fritsch R, Frischauf I, Romanin C: Mechanistic view on domains mediating STIM1-Orai coupling. Immunol Rev 2009;231:99-112.
27 Peinelt C, Vig M, Koomoa DL, Beck A, Nadler MJ, Koblan-Huberson M, Lis A, Fleig A, Penner R, Kinet JP: Amplification of CRAC current by STIM1 and CRACM1 (Orai1). Nat Cell Biol 2006;8:771-773.
28 Penna A, Demuro A, Yeromin AV, Zhang SL, Safrina O, Parker I, Cahalan MD: The CRAC channel consists of a tetramer formed by Stim-induced dimerization of Orai dimers. Nature 2008;456:116-120.
29 Smyth JT, Hwang SY, Tomita T, DeHaven WI, Mercer JC, Putney JW: Activation and regulation of store-operated calcium entry. J Cell Mol Med 2010;14:2337-2349.
30 Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, Stauderman KA, Cahalan MD: STIM1 is a Ca2+ sensor that activates CRAC channels and migrates from the Ca2+ store to the plasma membrane. Nature 2005;437:902-905.
31 Schmid E, Stagno MJ, Yan J, Stournaras C, Lang F, Fuchs J, Seitz G: Store-operated Ca2+ entry in rhabdomyosarcoma cells. Biochem Biophys Res Commun 2016;477:129-136.
32 Bird GS, DeHaven WI, Smyth JT, Putney JW Jr: Methods for studying store-operated calcium entry. Methods 2008;46:204-212.
33 Yang W, Nurbaeva MK, Schmid E, Russo A, Almilaji A, Szteyn K, Yan J, Faggio C, Shumilina E, Lang F: Akt2- and ETS1-dependent IP3 receptor 2 expression in dendritic cell migration. Cell Physiol Biochem 2014;33:222-236.
34 Munoz C, Pakladok T, Almilaji A, Elvira B, Decher N, Shumilina E, Lang F: Up-regulation of Kir2.1 (KCNJ2) by the serum & glucocorticoid inducible SGK3. Cell Physiol Biochem 2014;33:491-500.
35 Davis NM, Sokolosky M, Stadelman K, Abrams SL, Libra M, Candido S, Nicoletti F, Polesel J, Maestro R, D'Assoro A, Drobot L, Rakus D, Gizak A, Laidler P, Dulinska-Litewka J, Basecke J, Mijatovic S, Maksimovic-Ivanic D, Montalto G, Cervello M, Fitzgerald TL, Demidenko Z, Martelli AM, Cocco L, Steelman LS, McCubrey JA: Deregulation of the EGFR/PI3K/PTEN/Akt/mTORC1 pathway in breast cancer: possibilities for therapeutic intervention. Oncotarget 2014;5:4603-4650.
36 Ding D, Wei S, Song Y, Li L, Du G, Zhan H, Cao Y: Osthole exhibits anti-cancer property in rat glioma cells through inhibiting PI3K/Akt and MAPK signaling pathways. Cell Physiol Biochem 2013;32:1751-1760.
37 Francipane MG, Lagasse E: mTOR pathway in colorectal cancer: an update. Oncotarget 2014;5:49-66.
38 Fruman DA, Rommel C: PI3K and cancer: lessons, challenges and opportunities. Nat Rev Drug Discov 2014;13:140-156.
39 Hirsch E, Ciraolo E, Franco I, Ghigo A, Martini M: PI3K in cancer-stroma interactions: bad in seed and ugly in soil. Oncogene 2014;33:3083-3090.
40 Hou J, Lam F, Proud C, Wang S: Targeting Mnks for cancer therapy. Oncotarget 2012;3:118-131.
41 Martelli AM, Chiarini F, Evangelisti C, Cappellini A, Buontempo F, Bressanin D, Fini M, McCubrey JA: Two hits are better than one: targeting both phosphatidylinositol 3-kinase and mammalian target of rapamycin as a therapeutic strategy for acute leukemia treatment. Oncotarget 2012;3:371-394.
42 Martini M, De Santis MC, Braccini L, Gulluni F, Hirsch E: PI3K/AKT signaling pathway and cancer: an updated review. Ann Med 2014;10.3109/07853890.2014.9128361-12.
43 McCubrey JA, Steelman LS, Bertrand FE, Davis NM, Sokolosky M, Abrams SL, Montalto G, D'Assoro AB, Libra M, Nicoletti F, Maestro R, Basecke J, Rakus D, Gizak A, Demidenko ZN, Cocco L, Martelli AM, Cervello M: GSK-3 as potential target for therapeutic intervention in cancer. Oncotarget 2014;5:2881-2911.
44 McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Franklin RA, Montalto G, Cervello M, Libra M, Candido S, Malaponte G, Mazzarino MC, Fagone P, Nicoletti F, Basecke J, Mijatovic S, Maksimovic-Ivanic D, Milella M, Tafuri A, Chiarini F, Evangelisti C, Cocco L, Martelli AM: Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR cascade inhibitors: how mutations can result in therapy resistance and how to overcome resistance. Oncotarget 2012;3:1068-1111.

45 Ocana A, Vera-Badillo F, Al-Mubarak M, Templeton AJ, Corrales-Sanchez V, Diez-Gonzalez L, Cuenca-Lopez MD, Seruga B, Pandiella A, Amir E: Activation of the PI3K/mTOR/AKT pathway and survival in solid tumors: systematic review and meta-analysis. PLoS One 2014;9:e95219.
46 Rodon J, Dienstmann R, Serra V, Tabernero J: Development of PI3K inhibitors: lessons learned from early clinical trials. Nat Rev Clin Oncol 2013;10:143-153.
47 Xiao M, Tang Y, Wang YL, Yang L, Li X, Kuang J, Song GL: ART1 silencing enhances apoptosis of mouse CT26 cells via the PI3K/Akt/NF-kappaB pathway. Cell Physiol Biochem 2013;32:1587-1599.
48 Zhang H, Guo M, Chen JH, Wang Z, Du XF, Liu PX, Li WH: Osteopontin knockdown inhibits alphav,beta3 integrin-induced cell migration and invasion and promotes apoptosis of breast cancer cells by inducing autophagy and inactivating the PI3K/Akt/mTOR pathway. Cell Physiol Biochem 2014;33:991-1002.
49 Zhang L, Zhou F, ten Dijke P: Signaling interplay between transforming growth factor-beta receptor and PI3K/AKT pathways in cancer. Trends Biochem Sci 2013;38:612-620.
50 Sedding DG: FoxO transcription factors in oxidative stress response and ageing--a new fork on the way to longevity? Biol Chem 2008;389:279-283.
51 Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME: Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 1997;275:661-665.
52 Arboleda G, Morales LC, Benitez B, Arboleda H: Regulation of ceramide-induced neuronal death: cell metabolism meets neurodegeneration. Brain Res Rev 2009;59:333-346.
53 Kreis P, Rousseau V, Thevenot E, Combeau G, Barnier JV: The four mammalian splice variants encoded by the p21-activated kinase 3 gene have different biological properties. J Neurochem 2008;106:1184-1197.
54 McLean BA, Zhabyeyev P, Pituskin E, Paterson I, Haykowsky MJ, Oudit GY: PI3K inhibitors as novel cancer therapies: implications for cardiovascular medicine. J Card Fail 2013;19:268-282.
55 Lang F, Stournaras C: Serum and glucocorticoid inducible kinase, metabolic syndrome, inflammation, and tumor growth. Hormones (Athens) 2013;12:160-171.
56 Chen YF, Chiu WT, Chen YT, Lin PY, Huang HJ, Chou CY, Chang HC, Tang MJ, Shen MR: Calcium store sensor stromal-interaction molecule 1-dependent signaling plays an important role in cervical cancer growth, migration, and angiogenesis. Proc Natl Acad Sci U S A 2011;108:15225-15230.
57 Courjaret R, Machaca K: STIM and Orai in cellular proliferation and division. Front Biosci (Elite Ed) 2012;4:331-341.
58 Dellis O, Arbabian A, Papp B, Rowe M, Joab I, Chomienne C: Epstein-Barr virus latent membrane protein 1 increases calcium influx through store-operated channels in B lymphoid cells. J Biol Chem 2011;286:18583-18592.
59 Kondratska K, Kondratskyi A, Yassine M, Lemonnier L, Lepage G, Morabito A, Skryma R, Prevarskaya N: Orai1 and STIM1 mediate SOCE and contribute to apoptotic resistance of pancreatic adenocarcinoma. Biochim Biophys Acta 2014;1843:2263-2269.
60 Lang F, Friedrich F, Kahn E, Woll E, Hammerer M, Waldegger S, Maly K, Grunicke H: Bradykinin-induced oscillations of cell membrane potential in cells expressing the Ha-ras oncogene. J Biol Chem 1991;266:4938-4942.
61 Liu H, Hughes JD, Rollins S, Chen B, Perkins E: Calcium entry via ORAI1 regulates glioblastoma cell proliferation and apoptosis. Exp Mol Pathol 2011;91:753-760.
62 Raphael M, Lehen'kyi V, Vandenberghe M, Beck B, Khalimonchyk S, Vanden Abeele F, Farsetti L, Germain E, Bokhobza A, Mihalache A, Gosset P, Romanin C, Clezardin P, Skryma R, Prevarskaya N: TRPV6 calcium channel translocates to the plasma membrane via Orai1-mediated mechanism and controls cancer cell survival. Proc Natl Acad Sci U S A 2014;111:E3870-3879.
63 Schmidt S, Liu G, Liu G, Yang W, Honisch S, Pantelakos S, Stournaras C, Honig A, Lang F: Enhanced Orai1 and STIM1 expression as well as store operated Ca2+ entry in therapy resistant ovary carcinoma cells. Oncotarget 2014;5:4799-4810.
64 Sobradillo D, Hernandez-Morales M, Ubierna D, Moyer MP, Nunez L, Villalobos C: A reciprocal shift in transient receptor potential channel 1 (TRPC1) and stromal interaction molecule 2 (STIM2) contributes to Ca2+ remodeling and cancer hallmarks in colorectal carcinoma cells. J Biol Chem 2014;289:28765-28782.
65 Zhu H, Zhang H, Jin F, Fang M, Huang M, Yang CS, Chen T, Fu L, Pan Z: Elevated Orai1 expression mediates tumor-promoting intracellular Ca2+ oscillations in human esophageal squamous cell carcinoma. Oncotarget 2014;5:3455-3471.

66 Hayashi T, Kishida M, Nishizawa Y, Iijima M, Koriyama C, Nakamura M, Sano A, Kishida S: Subcellular localization and putative role of VPS13A/chorein in dopaminergic neuronal cells. Biochem Biophys Res Commun 2012;419:511-516.
67 Honisch S, Fehrenbacher B, Lebedeva A, Alesutan I, Castor T, Alkahtani S, Alarifi S, Schaller M, Stournaras C, Lang F: Chorein Sensitive Dopamine Release from Pheochromocytoma (PC12) Cells. Neurosignals 2015;23:1-10.
68 Eliyahu E, Tsaadon A, Shtraizent N, Shalgi R: The involvement of protein kinase C and actin filaments in cortical granule exocytosis in the rat. Reproduction 2005;129:161-170.
69 Keller H, Rentsch P, Hagmann J: Differences in cortical actin structure and dynamics document that different types of blebs are formed by distinct mechanisms. Exp Cell Res 2002;277:161-172.
70 Malacombe M, Bader MF, Gasman S: Exocytosis in neuroendocrine cells: new tasks for actin. Biochim Biophys Acta 2006;1763:1175-1183.
71 Mitchell T, Lo A, Logan MR, Lacy P, Eitzen G: Primary granule exocytosis in human neutrophils is regulated by Rac-dependent actin remodeling. Am J Physiol Cell Physiol 2008;295:C1354-C1365.
72 Onabajo OO, Seeley MK, Kale A, Qualmann B, Kessels M, Han J, Tan TH, Song W: Actin-binding protein 1 regulates B cell receptor-mediated antigen processing and presentation in response to B cell receptor activation. J Immunol 2008;180:6685-6695.
73 Insall RH, Machesky LM: Actin dynamics at the leading edge: from simple machinery to complex networks. Dev Cell 2009;17:310-322.
74 Papakonstanti EA, Stournaras C: Cell responses regulated by early reorganization of actin cytoskeleton. FEBS Lett 2008;582:2120-2127.
75 Andrade AL, Rossi DJ: Simulated ischaemia induces Ca2+-independent glutamatergic vesicle release through actin filament depolymerization in area CA1 of the hippocampus. J Physiol 2010;588:1499-1514.
76 Stournaras C, Gravanis A, Margioris AN, Lang F: The actin cytoskeleton in rapid steroid hormone actions. Cytoskeleton (Hoboken) 2014;71:285-293.
77 Liu G, Honisch S, Liu G, Schmidt S, Alkahtani S, AlKahtane AA, Stournaras C, Lang F: Up-regulation of Orai1 expression and store operated Ca(2+) entry following activation of membrane androgen receptors in MCF-7 breast tumor cells. BMC Cancer 2015;15:995.
78 Liu G, Honisch S, Liu G, Schmidt S, Pantelakos S, Alkahtani S, Toulany M, Lang F, Stournaras C: Inhibition of SGK1 enhances mAR-induced apoptosis in MCF-7 breast cancer cells. Cancer Biol Ther 2015;16:52-59.