

ERK contributes to B cell receptor-induced cell spreading in the A20 mouse B cell line

Victoria Peters¹, Nikola Deretic¹, Kate Choi¹, Michael R Gold^{1§}

¹Department of Microbiology & Immunology and the Life Sciences Institute, University of British Columbia, Vancouver, Canada

[§]To whom correspondence should be addressed: mgold@mail.ubc.ca

Abstract

B cells provide protective immunity by secreting antibodies. When a B cell encounters its specific antigen, B-cell receptor (BCR) signaling initiates actin remodeling. This allows B cells to spread on antigen-bearing surfaces and find more antigen, which increases BCR signaling and facilitates B cell activation. The BCR activates multiple signaling pathways that target actin-regulatory proteins. Although the extracellular signal-regulated kinases ERK1 and ERK2 regulate actin-dependent processes in adherent cells, their role in BCR-induced actin remodeling had not been investigated. Here, we show that targeting ERK with chemical inhibitors or siRNA inhibits BCR-induced spreading in a murine B cell line.

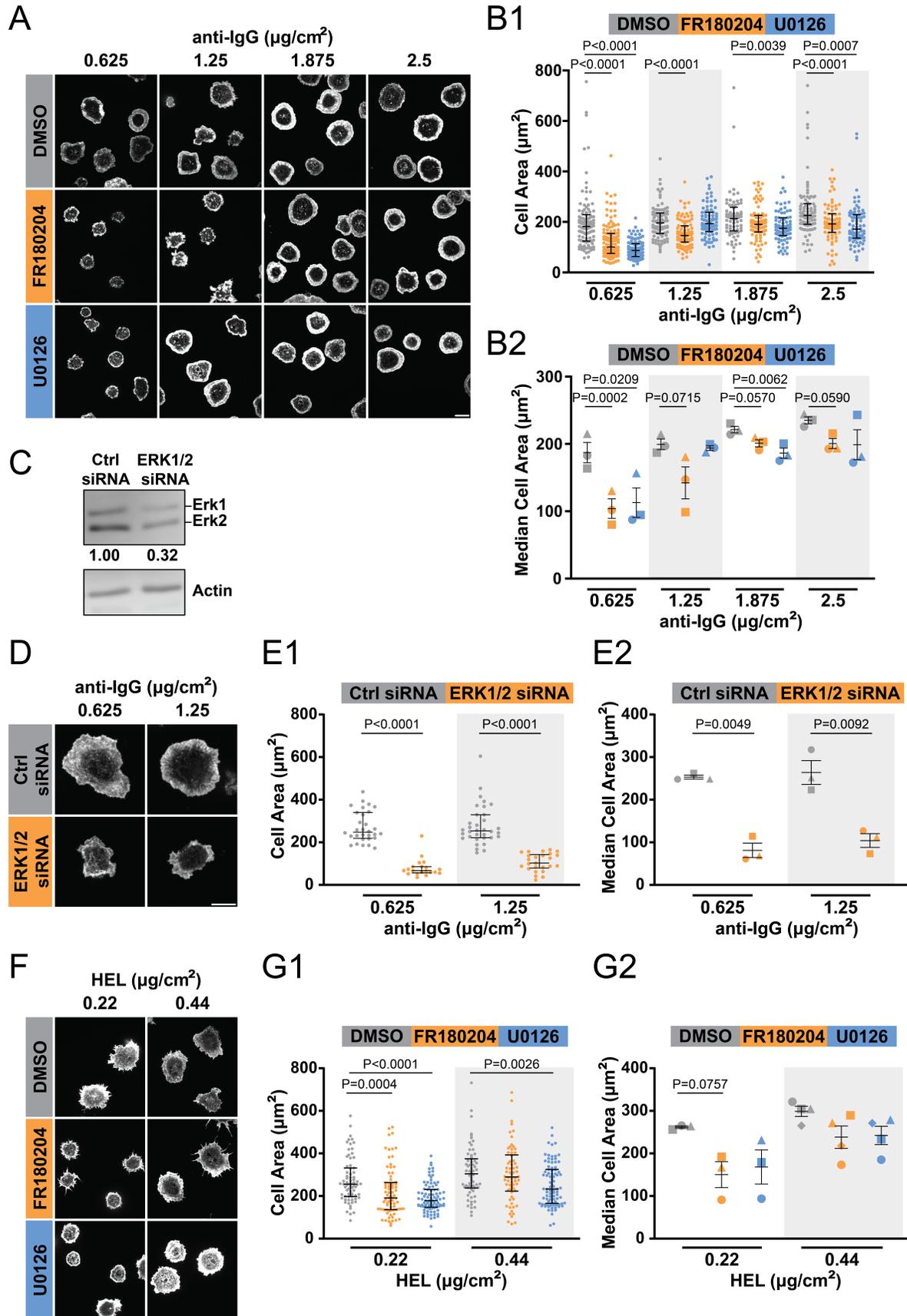


Figure 1. ERK activity is important for BCR-induced spreading

(A,B) A20 cells were pre-treated with 30 μM of the ERK inhibitor FR180204 or the MEK inhibitor U0126 for 1 hr and then allowed to spread for 30 min on coverslips coated with the indicated amounts of goat anti-mouse IgG. The cells were stained with rhodamine-phalloidin to visualize F-actin and the peripheral F-actin ring was used to define the outer edges of the cells and quantify cell areas. Representative images are shown in (A). Data from a representative experiment are shown in (B1). Each dot is one cell and the median and interquartile ranges are shown. The graph in (B2) shows compiled data from 3 independent experiments with $n = 57\text{-}134$ cells per condition. Each symbol is an individual experiment and the data are presented as the mean \pm SEM for the median values from the 3 experiments.

(C-E) A20 D1.3 cells were transfected with either control non-targeting siRNA or a combination of siRNAs directed against ERK1 and ERK2. In (C), cell lysates were analyzed by immunoblotting with an ERK1/2 antibody or with an actin antibody (loading control). ERK1 + ERK2 levels relative to the control siRNA sample are shown beneath the ERK blot. The siRNA-transfected cells were allowed to spread for 30 min on coverslips coated with the indicated amounts of goat anti-mouse IgG. Representative images are shown (D) along with data from a representative experiment (E1) and compiled data from 3 independent experiments (E2) with $n = 20\text{-}34$ cells per condition.

(F,G) A20 D1.3 cells were pre-treated with 30 μM of the ERK inhibitor FR180204 or the MEK inhibitor U0126 for 1 hr and then allowed to spread for 30 min on coverslips coated with 0.22 $\mu\text{g}/\text{cm}^2$ or 0.44 $\mu\text{g}/\text{cm}^2$ HEL. Representative images are shown (F) along with data from a representative experiment (G1) and compiled data from 3 independent experiments (G2) with $n = 40\text{-}87$ cells per condition.

All scale bars are 10 μm . P-values were calculated using the Mann Whitney U test (panels B1, E1, G1) or Student's paired t-test (panels B2, E2, G2). Only P-values <0.1 are shown.

Description

When B cells encounter immobilized antigens, or antigens that are tethered to planar lipid bilayers, they undergo radial spreading that is driven by the formation of large lamellipodia (Freeman et al., 2011). As the cells spread, a peripheral ring of F-actin forms, concomitant with clearance of F-actin from the center of the substrate contact site (Bolger-Munro et al., 2019). Branched actin polymerization nucleated by the Arp2/3 complex drives the formation of this peripheral actin ring and exerts outward force on the plasma membrane that drives cell spreading (Pollard and Borisy, 2003). This spreading response is thought to mimic the initial steps that occur when B cells encounter antigens that are displayed on the surface of antigen-presenting cells (APCs), resulting in the formation of an immune synapse (Harwood and Batista, 2010; Abraham et al., 2016).

The binding of antigens to the B cell antigen receptor (BCR) initiates the activation of multiple signaling pathways (Abraham et al., 2016), including Ras-dependent activation of the ERK1 and ERK2 kinases (Gold et al., 1992; Tordai et al., 1994; Sutherland et al., 1996). Although ERK promotes membrane protrusion and cell motility in a variety of adherent cell types (Mendoza et al., 2015; Tanimura and Takeda, 2017; Hirata and Kiyokawa, 2019; Lavoie et al., 2020), its role in actin-mediated processes in lymphocytes has not been studied.

To assess the role of ERK in BCR-induced cell spreading we used the IgG⁺ A20 murine B-lymphoma cell line and its derivative, the A20/D1.3 cell line. A20 cells have been widely used to study B cell spreading on surfaces displaying anti-IgG antibodies. Anti-Ig antibodies bind to the constant region of the membrane Ig subunit of the BCR and initiate signaling by clustering BCRs. In addition to its endogenous BCR, A20/D1.3 cells express a transfected hen egg lysozyme (HEL)-specific BCR derived from the D1.3 monoclonal antibody (Batista and Neuberger, 1998). This allows us to study B-cell spreading initiated by the binding of HEL to the antigen-binding site of the BCR.

To assess the role of ERK in B cell spreading we pre-treated A20 or A20/D1.3 cells with either FR180204, a selective inhibitor of ERK activity (Ohuri et al., 2005), or U0126, an effective inhibitor of MEK1 and MEK2 (Favata et al., 1998), the kinases that phosphorylate and activate ERK1 and ERK2 (Lavoie et al., 2020). U0126 has been used to inhibit the MEK/ERK pathway in B cells (Richards et al., 2001). Figure 1A and 1B show that pre-treating A20 cells with 30 μM FR180204 for 1 hr reduced the ability of B cells to spread on immobilized anti-IgG antibodies. Greater inhibition was observed when the coverslips were coated with 0.625 $\mu\text{g}/\text{cm}^2$ than when they were coated with 1.25 $\mu\text{g}/\text{cm}^2$. At higher anti-IgG densities, e.g., 2.5 $\mu\text{g}/\text{cm}^2$, inhibiting ERK did not have a significant impact on BCR-induced spreading. Similarly, the MEK inhibitor U0126 significantly reduced the spreading of A20 cells on coverslips coated with 0.625 $\mu\text{g}/\text{cm}^2$ anti-IgG but had much less effect at higher anti-IgG densities. This suggests that activation of the MEK/ERK pathway is more important for B cell spreading when the antigen density is low, as it might be on the surface of APCs that capture soluble antigens.

To validate the role of ERK in BCR-induced spreading we used a complementary loss-of-function approach in which we transfected A20/D1.3 cells with a combination of ERK1- and ERK2-specific siRNAs. The combined ERK1/2 siRNA

transfection reduced the levels of ERK1/2 in the cells by ~68% compared to cells transfected with control non-targeting siRNA (Figure 1C). Importantly, siRNA-mediated depletion of ERK1 and ERK2 significantly reduced the ability of the cells to spread on coverslips coated with either 0.625 $\mu\text{g}/\text{cm}^2$ or 1.25 $\mu\text{g}/\text{cm}^2$ anti-IgG (Figure 1D and 1E).

Finally, we showed that pre-treating A20/D1.3 cells with either FR180204 or U0126 reduced their ability to spread on coverslips coated with HEL (Figure 1F and 1G). As was the case for spreading on anti-IgG, the ERK inhibitor was more effective than the MEK inhibitor at reducing B cell spreading on HEL, and greater reductions in spreading area were observed at the lower density of HEL (0.22 $\mu\text{g}/\text{cm}^2$) than at a higher density (0.44 $\mu\text{g}/\text{cm}^2$). Hence, ERK activity contributes to both antigen- and anti-IgG-induced B cell spreading, especially at lower densities of these activating stimuli.

Although ERK has important roles in B cell development, survival, proliferation, and transcriptional regulation (Richards et al., 2001; Yasuda et al., 2008; O'Reilly et al., 2009), to our knowledge this is the first report that ERK is involved in BCR-induced spreading. How ERK regulates the actin remodeling that drives B cell spreading remains to be determined. The radial spreading of B cells on antigen-coated surfaces is driven by branched actin polymerization nucleated by the Arp2/3 complex (Bolger-Munro et al., 2019). In fibroblasts, ERK signaling recruits the Arp2/3 complex to the leading edge of motile cells and increases Arp2/3 complex-mediated actin polymerization that powers the outward growth of lamellipodial membrane protrusions (Mendoza et al., 2015). Whether ERK activity enhances Arp2/3 complex recruitment or activity at the peripheral actin ring of spreading B cells remains to be assessed. The Arp2/3 complex is activated by the WASp, N-WASp, and WAVE2 nucleation-promoting factors (Rottner et al., 2017). It had been reported that ERK promotes the formation of lamellipodia by phosphorylating WAVE2 and its binding partner Abi1 (Danson et al., 2007; Nakanishi et al., 2007; Mendoza et al., 2011; Mendoza, 2013). However, a recent study found that these putative ERK phosphorylation sites are not important for lamellipodia formation or cell motility in several cell types and that WAVE2 phosphorylation is ERK-independent (Singh et al., 2020). A number of other actin-regulatory proteins such as cortactin, filamin A, and paxillin are putative substrates of either ERK or the p90Rsk and Mnk1 kinases that are activated by ERK (Tanimura and Takeda, 2017; Lavoie et al., 2020). Phosphoproteomic analyses of control versus ERK-inhibited B cells spreading on immobilized antigen could identify candidate ERK effectors whose role in linking BCR engagement to actin remodeling could then be studied.

Dynamic reorganization of the actin cytoskeleton is especially important for B cells to respond to antigens that are displayed on the surface of APCs (Bolger-Munro et al., 2019). In this case, actin-dependent formation of an immune synapse amplifies BCR signaling and may lower the threshold for the amount of cell-bound antigen required to trigger B cell activation and proliferation. Further work is needed to test whether ERK activity is important for APC-induced B cell activation. B cell activation is also dependent on actin-dependent cell motility that enables B cells to migrate into and within lymphoid organs to encounter antigens and then interact with helper T cells (Cyster, 2010). Based on our findings, further studies should assess the role of ERK activity in chemokine-directed B cell migration. ERK inhibitors are currently in clinical trials for cancer therapy (Sullivan et al., 2018; Chin et al., 2019), raising the possibility that these drugs could be used to reduce aberrant B cell activation in autoimmune diseases.

Methods

Cell culture

The A20 murine IgG⁺ B lymphoma cell line (Kim et al., 1979) was obtained from ATCC (#TIB-208). The A20/D1.3 murine B cell line (Batista and Neuberger, 1998), which expresses a transfected HEL-specific IgM-containing BCR in addition to its endogenous IgG-containing BCR, was a gift from F. Batista (Ragon Institute, Cambridge, MA). The cell lines were confirmed to be mycoplasma-negative and were cultured in RPMI-1640 (Sigma, #R0883) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Gibco, #12483020), 2 mM glutamine (Fisher Scientific, #O2956-100), 1 mM pyruvate (Gibco, #11360070), 50 μM 2-mercaptoethanol (Sigma-Aldrich, #M3148), 50 U/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin (Gibco, #15140122).

Chemical inhibitors

The ERK inhibitor FR180204 (Sigma-Aldrich, #SML0320) (Tocris Bioscience, #3706) (Ohori et al., 2005) and the MEK inhibitor U0126 (Cayman Chemical, #70970) (Favata et al., 1998) were used at final concentrations of 30 μM .

siRNA transfection and immunoblotting

Using an Amaxa Nucleofector (program L-013) and the Ingenio Electroporation Kit (Mirus, #MIR50118), 3×10^6 A20/D1.3 cells were transiently transfected with 4 μg of control non-targeting siRNA (Dharmacon, #D-001810-10-20) or with 4 μg each of mouse MAPK3/ERK1-specific (Dharmacon, #L-040126-00-0005) and mouse MAPK1/ERK2-specific siRNA (Dharmacon, #L-040613-00-0005). The cells were then cultured for 24 hr prior to use. siRNA-mediated depletion of ERK1 and ERK2 was

analyzed by immunoblotting. Cell extracts were prepared, separated on 12% SDS-PAGE gels, and analyzed by immunoblotting as described previously (Bolger-Munro et al., 2021). Nitrocellulose membranes were incubated with a rabbit anti-ERK1/2 antibody (Cell Signaling Technologies, #9102, 1:1000) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, #170-6515, 1:3000) or with a mouse anti- β -actin antibody (Santa Cruz, #Sc-47778, 1:3000) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, #170-6516, 1:3000). Immunoreactive bands were visualized using an enhanced chemiluminescence detection reagent (Azure Biosystems, #AC2010), and quantified using a Li-Cor C-DiGit Scanner and Image Studio software. ERK signals were normalized to the actin band intensity for the same sample.

Cell spreading assays and cell area quantification

Cell spreading assays were performed as described previously (Bolger-Munro et al., 2021). Glass coverslips were coated with the indicated amounts of goat anti-mouse IgG (Jackson ImmunoResearch, #115-005-008) or HEL (NANOCs, #LSN-BN-1) for 1 hr at room temperature and then blocked with 2% bovine serum albumin (BSA) in PBS for 30 min at room temperature. A20 or A20/D1.3 cells were resuspended to 7.5×10^5 /mL in modified HEPES-buffered saline (25 mM sodium HEPES pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM Na_2HPO_4 , 0.5 mM MgSO_4 , 1 g/L glucose, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol) with 2% FBS. The cells were pre-treated with the ERK or MEK inhibitor, or an equivalent volume of DMSO, for 1 hr at 37°C before adding 7.5×10^4 cells in 100 μL to each coverslip. After the indicated times at 37°C, the cells were fixed by adding 100 μL of 8% PFA (4% final concentration) for 10 min and then permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. F-actin was visualized by staining with rhodamine-conjugated phalloidin (Thermo Fisher, #R415, 1:400 in dilution in PBS) for 30 min at room temperature. Coverslips were mounted onto slides using ProLong Diamond anti-fade reagent (Thermo Fisher, #P36965). Images of the B cell-coverslip interface were captured using a Zeiss Axiovert 200M spinning disk confocal microscope with a 100X NA 1.45 oil objective lens. The cell area was quantified from thresholded binary images using Fiji software (Schindelin et al., 2012). The outer face of the peripheral actin ring was used to define the cell edge and compute the total cell area.

Statistical Analysis

For individual cell spreading experiments, the data are presented as dot plots and the Mann-Whitney U test was used to compare ranked values between the samples. Two-tailed paired t-tests were used to compare mean values for matched sets of samples from multiple experiments.

Acknowledgements: We thank the LSI Imaging Facility.

References

- Abraham L, Wang JC, Bolger-Munro M, Gold MR. 2016. Structure, function, and spatial organization of the B cell receptor (BCR). *Encyclopedia of Immunobiology*, ed. By M.J. Ratcliffe 2: 40-54. DOI: <http://dx.doi.org/10.1016/B978-0-12-374279-7.05005-0>
- Batista FD, Neuberger MS. 1998. Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. *Immunity* 8: 751-9. PubMed ID: [9655489](https://pubmed.ncbi.nlm.nih.gov/9655489/)
- Bolger-Munro M, Choi K, Scurl JM, Abraham L, Chappell RS, Sheen D, et al., Gold MR. 2019. Arp2/3 complex-driven spatial patterning of the BCR enhances immune synapse formation, BCR signaling and B cell activation. *eLife* 8: 10.7554. PubMed ID: [31157616](https://pubmed.ncbi.nlm.nih.gov/31157616/)
- Bolger-Munro M, Choi K, Cheung F, Liu YT, Dang-Lawson M, Deretic N, Keane C, Gold MR. 2021. The Wdr1-LIMK-Cofilin Axis Controls B Cell Antigen Receptor-Induced Actin Remodeling and Signaling at the Immune Synapse. *Front Cell Dev Biol* 9: 649433. PubMed ID: [33928084](https://pubmed.ncbi.nlm.nih.gov/33928084/)
- Chin HM, Lai DK, Falchook GS. 2019. Extracellular signal-regulated kinase (ERK) inhibitors in oncology clinical trials. *J Immunother Precis Oncol* 2: 10-16 DOI: https://doi.org/10.4103/JIPO.JIPO_17_18
- Cyster JG. 2010. B cell follicles and antigen encounters of the third kind. *Nat Immunol* 11: 989-96. PubMed ID: [20959804](https://pubmed.ncbi.nlm.nih.gov/20959804/)
- Danson CM, Pocha SM, Bloomberg GB, Cory GO. 2007. Phosphorylation of WAVE2 by MAP kinases regulates persistent cell migration and polarity. *J Cell Sci* 120: 4144-54. PubMed ID: [18032787](https://pubmed.ncbi.nlm.nih.gov/18032787/)
- Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, et al., Trzaskos JM. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 273: 18623-32. PubMed ID: [9660836](https://pubmed.ncbi.nlm.nih.gov/9660836/)

- Freeman SA, Lei V, Dang-Lawson M, Mizuno K, Roskelley CD, Gold MR. 2011. Cofilin-mediated F-actin severing is regulated by the Rap GTPase and controls the cytoskeletal dynamics that drive lymphocyte spreading and BCR microcluster formation. *J Immunol* 187: 5887-900. PubMed ID: [22068232](#)
- Gold MR, Sanghera JS, Stewart J, Pelech SL. 1992. Selective activation of p42 mitogen-activated protein (MAP) kinase in murine B lymphoma cell lines by membrane immunoglobulin cross-linking. Evidence for protein kinase C-independent and -dependent mechanisms of activation. *Biochem J* 287 (Pt 1): 269-76. PubMed ID: [1384467](#)
- Harwood NE, Batista FD. 2010. Early events in B cell activation. *Annu Rev Immunol* 28: 185-210. PubMed ID: [20192804](#)
- Hirata E, Kiyokawa E. 2019. ERK Activity Imaging During Migration of Living Cells In Vitro and In Vivo. *Int J Mol Sci* 20: . PubMed ID: [30764494](#)
- Kim KJ, Kanellopoulos-Langevin C, Merwin RM, Sachs DH, Asofsky R. 1979. Establishment and characterization of BALB/c lymphoma lines with B cell properties. *J Immunol* 122: 549-54. PubMed ID: [310843](#)
- Lavoie H, Gagnon J, Therrien M. 2020. ERK signalling: a master regulator of cell behaviour, life and fate. *Nat Rev Mol Cell Biol* 21: 607-632. PubMed ID: [32576977](#)
- Mendoza MC, Er EE, Zhang W, Ballif BA, Elliott HL, Danuser G, Blenis J. 2011. ERK-MAPK drives lamellipodia protrusion by activating the WAVE2 regulatory complex. *Mol Cell* 41: 661-71. PubMed ID: [21419341](#)
- Mendoza MC. 2013. Phosphoregulation of the WAVE regulatory complex and signal integration. *Semin Cell Dev Biol* 24: 272-9. PubMed ID: [23354023](#)
- Mendoza MC, Vilela M, Juarez JE, Blenis J, Danuser G. 2015. ERK reinforces actin polymerization to power persistent edge protrusion during motility. *Sci Signal* 8: ra47. PubMed ID: [25990957](#)
- Nakanishi O, Suetsugu S, Yamazaki D, Takenawa T. 2007. Effect of WAVE2 phosphorylation on activation of the Arp2/3 complex. *J Biochem* 141: 319-25. PubMed ID: [17202194](#)
- O'Reilly LA, Kruse EA, Puthalakath H, Kelly PN, Kaufmann T, Huang DC, Strasser A. 2009. MEK/ERK-mediated phosphorylation of Bim is required to ensure survival of T and B lymphocytes during mitogenic stimulation. *J Immunol* 183: 261-9. PubMed ID: [19542438](#)
- Otori M, Kinoshita T, Okubo M, Sato K, Yamazaki A, Arakawa H, et al., Fujii T. 2005. Identification of a selective ERK inhibitor and structural determination of the inhibitor-ERK2 complex. *Biochem Biophys Res Commun* 336: 357-63. PubMed ID: [16139248](#)
- Pollard TD, Borisy GG. 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell* 112: 453-65. PubMed ID: [12600310](#)
- Richards JD, Davé SH, Chou CH, Mamchak AA, DeFranco AL. 2001. Inhibition of the MEK/ERK signaling pathway blocks a subset of B cell responses to antigen. *J Immunol* 166: 3855-64. PubMed ID: [11238629](#)
- Rottner K, Faix J, Bogdan S, Linder S, Kerkhoff E. 2017. Actin assembly mechanisms at a glance. *J Cell Sci* 130: 3427-3435. PubMed ID: [29032357](#)
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al., Cardona A. 2012. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9: 676-82. PubMed ID: [22743772](#)
- Singh SP, Thomason PA, Lilla S, Schaks M, Tang Q, Goode BL, et al., Insall RH. 2020. Cell-substrate adhesion drives Scar/WAVE activation and phosphorylation by a Ste20-family kinase, which controls pseudopod lifetime. *PLoS Biol* 18: e3000774. PubMed ID: [32745097](#)
- Sullivan RJ, Infante JR, Janku F, Wong DJL, Sosman JA, Keedy V, et al., Li BT. 2018. First-in-Class ERK1/2 Inhibitor Ulixertinib (BVD-523) in Patients with MAPK Mutant Advanced Solid Tumors: Results of a Phase I Dose-Escalation and Expansion Study. *Cancer Discov* 8: 184-195. PubMed ID: [29247021](#)
- Sutherland CL, Heath AW, Pelech SL, Young PR, Gold MR. 1996. Differential activation of the ERK, JNK, and p38 mitogen-activated protein kinases by CD40 and the B cell antigen receptor. *J Immunol* 157: 3381-90. PubMed ID: [8871635](#)
- Tanimura S, Takeda K. 2017. ERK signalling as a regulator of cell motility. *J Biochem* 162: 145-154. PubMed ID: [28903547](#)
- Tordai A, Franklin RA, Patel H, Gardner AM, Johnson GL, Gelfand EW. 1994. Cross-linking of surface IgM stimulates the Ras/Raf-1/MEK/MAPK cascade in human B lymphocytes. *J Biol Chem* 269: 7538-43. PubMed ID: [8125975](#)

Yasuda T, Sanjo H, Pagès G, Kawano Y, Karasuyama H, Pouysségur J, Ogata M, Kurosaki T. 2008. Erk kinases link pre-B cell receptor signaling to transcriptional events required for early B cell expansion. *Immunity* 28: 499-508. PubMed ID: [18356083](https://pubmed.ncbi.nlm.nih.gov/18356083/)

Funding: Canadian Institutes of Health Research

Author Contributions: Victoria Peters: investigation, methodology, visualization, formal analysis, writing - original draft. Nikola Deretic: investigation, methodology, visualization, formal analysis. Kate Choi: data curation, visualization, writing - review editing, validation, formal analysis. Michael R Gold: conceptualization, funding acquisition, project, supervision, validation, visualization, writing - original draft, writing - review editing, data curation.

Reviewed By: Pieta Mattila

History: Received November 3, 2022 **Accepted** November 21, 2022 **Published Online** November 23, 2022 **Indexed** December 7, 2022

Copyright: © 2022 by the authors. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International (CC BY 4.0) License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Citation: Peters, V; Deretic, N; Choi, K; Gold, MR (2022). ERK contributes to B cell receptor-induced cell spreading in the A20 mouse B cell line. *microPublication Biology*. [10.17912/micropub.biology.000665](https://doi.org/10.17912/micropub.biology.000665)