EPHB4 inhibition activates ER stress to promote immunogenic cell death of prostate cancer cells

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Abstract
The EPHB4 receptor is implicated in the development of several epithelial tumors and is a promising therapeutic target, including in prostate tumors in which EPHB4 is overexpressed and promotes tumorigenicity. Here, we show that high expression of EPHB4 correlated with poor survival in prostate cancer patients and EPHB4 inhibition induced cell death in both hormone sensitive and castration-resistant prostate cancer cells. EPHB4 inhibition reduced expression of the glucose transporter, GLUT3, impaired glucose uptake, and reduced cellular ATP levels. This was associated with the activation of endoplasmic reticulum stress and tumor cell death with features of immunogenic cell death (ICD), including phosphorylation of eIF2α, increased cell surface calreticulin levels, and release of HMGB1 and ATP. The changes in tumor cell metabolism after EPHB4 inhibition were associated with MYC downregulation, likely mediated by the SRC/p38 MAPK/4EBP1 signaling cascade, known to impair cap-dependent translation. Together, our study indicates a role for EPHB4 inhibition in the induction of immunogenic cell death with implication for prostate cancer therapy.

Introduction
Prostate cancer (PC) is the most common cancer in men worldwide. An estimated 174,650 new cases and 31,620 new deaths will be reported in the United States in 20191. While therapy targeted at the androgen receptor pathway shows initial efficacy, a majority of patients with advanced PC go on to develop resistance2–4. Thus, there is a need for novel therapies that target additional pathways important for the growth and survival of prostate cancer cells. For example, tyrosine kinase inhibitors (TKIs) are being investigated as an additional strategy to treat prostate cancer5–8. Receptor tyrosine kinases (RTKs) regulate of cell proliferation, migration, apoptosis, and survival, and plays a role in the progression and development of multiple cancers9,10. Targeting RTKs enzymatic activity is emerging as a therapeutic strategy in many cancer. However, this approach is facing some constraints due to development resistance against these inhibitors11,12. Combination therapy approaches have to be developed to overcome this problem. EPHB4 receptor tyrosine kinase is involved in many cellular processes such as cell growth, survival, angiogenesis, vasculogenesis, and neural development13. EPHB4 specifically binds to Ephrin B2 ligand and evokes bidirectional signaling13. Several reports have shown EPHB4 overexpression in various cancers including those of the prostate, breast, colon, head and neck, and lung14–18. Xi et al.19 showed that EPHB4 expression is increased in 66% of prostate tumor samples. EPHB4 is induced by the key molecular alterations implicated in prostate cancer and castrate-resistant prostate cancer development, including loss of tumor suppressors PTEN and TP53, and activation of the PI3K pathway19. Another report demonstrated that EPHB4 knockdown by siRNA resulted in a decrease in invasion and migration of prostate cancer cell lines20. However, neither the underlying mechanisms nor the
functional consequences of EPHB4 inhibition in advanced prostate cancer are well understood. Here, we investigate the mechanisms by which EPHB4 inhibition affects cell viability. EPHB4 receptor inhibition in PC cells inhibited a SRC/MAPK/MYC pathways leading to reduced glucose transporter GLUT3 levels, decreased glucose uptake and intracellular ATP levels. Consequently, cells showed elevated ER stress and death with features of immunogenic cell death (ICD).

**Results**

**High EPHB4 expression correlates with advanced prostate cancer stage and poor outcome**

Previous studies have shown that EPHB4 promotes tumor cell survival and is overexpressed in prostate cancer\(^1\). We examined *EPHB4* mRNA expression in published human prostate cancer expression datasets and observed higher *EPHB4* expression in metastatic castration-resistant prostate cancer (mCRPC) as compared to the corresponding normal or primary tumor tissues (Fig. 1a). We also observe a positive correlation between *EPHB4* expression levels and biochemical relapse-free survival in both the Cancer Genome Atlas (TCGA) and Ross-Adams\(^{21}\) datasets (Fig. 1b). Collectively, these results indicate that EPHB4 is a valuable prognostic biomarker and raises the hypothesis that it could be a therapeutic target in prostate cancer patients.

**EPHB4 inhibition decreases cell viability and induces apoptosis**

To determine the functional significance of EPHB4 overexpression in prostate cancer, we knocked down *EPHB4* with specific siRNAs targeting EPHB4 (Fig. 2a). EPHB4 knockdown in human (PC-3, 22Rv1 and LNCaP) and mouse (Myc-CaP & Myc-CaP; Pten KO) prostate cancer cell lines resulted in a decrease in cell viability measured after 72 h (Fig. 2b). Similar results were seen after treatment with small molecule EPHB4 inhibitor, NVP-BHG712 on the viability of Myc-CaP, Myc-CaP Pten KO, PC-3, 22Rv1, and LNCaP cells (Fig. 2d). In addition we observed similar effects after the knockdown of EPHB4 ligand EFNB2 (Fig. 2c). We next examined the effects of EPHB4i on the viability of organoids generated from the neuroendocrine prostate
Fig. 2 (See legend on next page.)
cancer cell line, NCI-H660, and found a decrease in organoid viability and size after EPHB4 (Fig. 2e). The reduced viability caused by EPHB4 inhibition occurred through apoptosis, as indicated by increased caspase-3/7 activation (Fig. 2f). Collectively, our results show that inhibition of the EPHB4 receptor or its ligand EFNB2 decreases cell viability and induces apoptosis in prostate cancer cells.

EPHB4 inhibition induces immunogenic cell death

Some therapeutic agents are known to cause cell death by immunogenic cell death that can be exploited in immunotherapy. We examined whether EPHB4 inhibition induced immunogenic cell death (ICD) by assessing changes in the hallmark of ICD, including cell surface levels of calreticulin, non-histone nuclear protein high-mobility group box 1 (HMGB1) release, and ATP release from Myc-CaP cells transfected with control (siCNT) or EPHB4-targeting siRNA (siEPHB4–3). EPHB4 inhibition resulted in increased cell surface localization of calreticulin and the release of HMGB1 and ATP into the extracellular space (Fig. 3a). Similar results were obtained in Myc-CaP cells treated with NVP-BHG712 (EPHB4i) compared to Vehicle (Veh) (Fig. 3b). These results indicate that EPHB4 inhibition induces cell death consistent with ICD.

EPHB4 inhibition induces endoplasmic reticulum stress through metabolic changes

Previous studies have suggested that endoplasmic reticulum (ER) stress plays a major role in intracellular signaling pathways that induce ICD. The ER stress response is initiated through phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which has been proposed as a characteristic marker for ICD. We therefore analyzed the phosphorylation status of eIF2α in PC-3 and 22Rv1 cells upon EPHB4 knockdown by siRNA (siEPHB4–3 or −4) compared to control siRNA (siCNT). EPHB4 inhibition increased phosphorylation of eIF2α at serine 51 (Fig. 4a). Similar results were seen in PC-3 and Myc-CaP;Pten KO cells treated with small molecule EPHB4 inhibitor (EPHB4i) (Fig. 4b). In addition to eIF2α, inositol-requiring enzyme 1 (IRE1) represents another ER protein that serves as ER stress sensor and mediates the unfolded protein response (UPR). Like eIF2α activation, IRE1 phosphorylation increased after EPHB4 knockdown by EPHB4-specific siRNA in PC-3 and 22Rv1 cells (Fig. 4a). Similar results were observed in PC-3 and Myc-CaP;Pten KO cells treated with EphB4i compared to vehicle control (Veh.) (Fig. 4b). To investigate whether EPHB4 mediated effect on ER stress is ligand-dependent, we analyzed the effect of EPHB4 ligand -EFNB2 knockdown on phosphorylation of eIF2α at serine 51 and IRE1 at serine 724. Consistent with our observations above, inhibition of EFNB2 resulted in a similar increase in phosphorylation of eIF2α at serine 51 and IRE1 at serine 724 in PC-3 cells (Fig. 4c). These data indicate that EPHB4 inhibition triggers the ER stress response in prostate cancer cells, which is associated with ICD. Cellular processes in the ER require energy in the form of ATP and perturbation of these processes induce ER stress. Therefore, we sought to determine whether the ER stress induced by EPHB4 inhibition is related to ATP depletion in cancer cells. We measured the intracellular ATP level after EPHB4 inhibition in PC-3 cells transfected with siEPHB4–3 or siCNT. The levels of intracellular ATP were significantly lower in siEPHB4-transfected PC-3 cells (Fig. 4d). Similar results were obtained with EPHB4i-treated PC-3 and 22Rv1 cells (Fig. 4e). These results point to a decline in cellular ATP levels upon EPHB4 inhibition as a possible cause of ER stress. The ER depends on extrinsic energy sources via oxidative phosphorylation or glycolysis. We tested whether the decrease in intracellular ATP was associated with changes in glucose metabolism. The first essential step in glucose metabolism is glucose uptake. Both PC-3 and Myc-CaP cells demonstrated significantly reduced glucose uptake after EPHB4 knockdown by siEPHB4–3 (Fig. 4f).
EPHB4 knockdown mediated decline in glucose uptake could be due to several steps of metabolic dysregulation including reduced glucose transport. To assess the impact of EPHB4, we monitored the levels of glucose transporters, in response to EphB4 knockdown. We observed that EPHB4 depletion in PC-3 cells resulted in a significant decrease in GLUT3 protein levels (Fig. 4g). Collectively, these data suggest that EPHB4 inhibition induces ER stress through ATP deficiency, arises due to decrease levels of glucose transporter, leading to decreased glucose uptake.

**EPHB4 inhibition decreases c-MYC level and P38 MAPK pathway**

The c-MYC oncoprotein, a known regulator of glucose metabolism and has previously been linked to EPHB4 in colon carcinoma cells. Therefore, we postulated that the decrease in the glucose transporter and glucose uptake after EPHB4 inhibition could be a result of changes in MYC levels. We therefore quantified MYC at protein level and expression after EPHB4 knockdown by siRNA (siEPHB4–3) compared to control siRNA (siCNT). EPHB4 depletion decreased c-MYC protein in LNCaP, PC-3 and 22Rv1 cells (Fig. 5a). In addition, we also observed decreased MYC protein expression after EPHB4 knockdown in PC-3 cells by immunofluorescence staining (Fig. 5b). Similar to EPHB4 inhibition, we observed a decreased in MYC protein in PC-3 cells transfected with siEFNB2 (Fig. 5c). Thus decreased MYC levels after EPHB4 inhibition may lead to decreased GLUT3 expression. To show this directly, we analyzed the effect of MYC inhibition on GLUT3 expression and phosphorylation of eIF2α through MYC downregulation.

How EPHB4 inhibition may affect MYC levels is not known. Previous reports have demonstrated that c-MYC expression is controlled by multiple signaling pathways including the MAPK pathway, while EPHB4 is linked to MAPK signaling in a context-dependent manner. To investigate whether MAPK signal transduction pathways are involved in MYC downregulation after EPHB4 inhibition, we analyzed the phosphorylation of p38 MAPK in PC-3 and Muc-CaP cells upon EPHB4 knockdown by siRNA (siEPHB4–2 or –3) compared to control siRNA (siCNT). We observed that the phosphorylation of p38...
**Fig. 4** (See legend on next page.)
MAPK at Thr180/Tyr182 decreased after EPHB4 knockdown in both cell lines (Fig. 5c). As a result, we hypothesized that decrease in phosphorylation of p38 MAPK leads to decrease in phosphorylation of 4EBP1, which is known as translation repressor protein. 4EBP1 regulates the cap-dependent translation of many oncogenic proteins, including MYC, via eukaryotic translation initiation complex eIF4E [39,40]. When hypophosphorylated, 4EBP1 binds to eIF4E and blocks cap-dependent translation. Thus phosphorylation of 4EBP1 which is induced by p38 MAPK leads to an increase in cap-dependent translation [40,41]. We next examined the phosphorylation status of 4EBP1 upon EPHB4 knockdown by siRNA (siEPHB4–3) compared to control siRNA (siCNT). As expected, EPHB4 inhibition decreased phosphorylation of 4EBP1 at Thr37/46 in PC-3 and Myc-CaP cells (Fig. 5f). Collectively, these data suggest that EPHB4 inhibition decreases the levels of glucose transporter, leading to decreased glucose uptake, as well as metabolic perturbations through MYC modulation which is regulated by p38 MAPK-4EBP1.

**EPHB4 regulates p38 MAPK through SRC kinase**

It has been shown that EPHB4 binding stimulates SRC kinase in ovarian carcinoma model [62] and SRC kinase is known to play a role in regulating MAPK signaling pathways [35,44]. So, we hypothesized that the decrease in phosphorylation of p38 MAPK upon EPHB4 inhibition could be explained by changes in the SRC activity. We therefore analyzed the activity of SRC via phosphorylation status after EPHB4 knockdown by siEPHB4–3 compared to siCNT in PC-3 and Myc-CaP cells. EPHB4 inhibition decreased phosphorylation of SRC at Tyr416 in both cell lines (Fig. 6a). We also observed decreased phosphorylation of SRC at Tyr416 after EFNB2 knockdown (Fig. 6b). In addition, we have also checked the available human prostate cancer patient dataset for EPHB4 correlation with SRC and found that EPHB4 was positively correlated with SRC kinase in prostate cancer patients (Fig. 6c). This result suggests that inhibition of EPHB4/EFNB2 decreases phosphorylation of P38 MAPK by reducing SRC kinase activity.

**Discussion**

In this study, we show that targeting the receptor tyrosine kinase EPHB4 could sensitize prostate cancer to immune checkpoint blockade therapy. We describe for the first time our knowledge that targeting EPHB4 induces ICD associated with ER stress, decreased glucose transporter and glucose uptake. EPHB4 is overexpressed in the majority of prostate cancers, particularly in advanced disease and is associated with a poor outcome. The possible mechanisms for increased expression of EPHB4 include loss of the tumor suppressors, PTEN and TP53, and activation of the PI3K pathway [47]. Thus, targeting EPHB4 could represent a viable modality for treating advanced prostate cancer.

Our results indicate that EPHB4 inhibition decreases prostate tumor cell proliferation and increases cell death in vitro. Apoptotic cell death has been documented to be immunologically silent and incapable of stimulating the immune response [45]. However, certain chemotherapeutic agents can induce cell death through the ICD mechanism [46]. We have found that EPHB4 knockdown or inhibition induces ICD by release of specific molecules belonging to the DAMP family, including exposure of calreticulin on the cell surface, release of HMGB1 into the extracellular space, and secretion of ATP. DAMPs are known to alert the immune system, resulting in the activation of the innate and adaptive immune response [47]. The process of ICD induction is well documented, and ER stress plays an important role in eliciting ICD [47]. ER stress is mainly accompanied by three sensors: PKR-like ER-localized eIF2α kinase (PERK), which is pathognomonic for ICD, activating transcription factor 6 (ATF6), and inositol-requiring 1 (IRE1) [23–25]. We demonstrate that EPHB4 knockdown or inhibition increased the...
Fig. 5 (See legend on next page.)
phosphorylation of eIF2α and IRE1. ATP depletion can lead to ER stress since energy is required for protein folding, assembly, and glycosylation, and we found that inhibition of EPHB4 led to intracellular ATP depletion most likely due to decreased glycolysis as a result of reduced glucose uptake. The decreased glucose uptake after EPHB4 inhibition or depletion could be attributed to the decreased expression of the glucose transporter, GLUT3. Additionally, the reduced glycolysis in EPHB4 depleted cells can be explained by the decrease in MYC levels. MYC is known to enhance glycolysis, regulating glucose transporters and uptake in many cells. Consistent with the observations in colon carcinoma cells, we found that EPHB4 knockdown resulted in decreased MYC protein level and expression in prostate cancer cells. However, the underlying mechanism of EPHB4 mediated regulation of MYC has not yet been explored. The SRC family kinase has been reported to regulate the PI3K/AKT and ERK pathway in prostate cancer and also known to upregulate MYC level through cap-dependent translation via the ERK pathway in a breast cancer model. It has been reported that SRC is activated by EPHB4 in ovarian carcinoma models suggesting the role of SRC kinase in regulation of MYC. Our data demonstrated that EPHB4 inhibition decreased the level of SRC and also positively correlated with SRC kinase in prostate cancer patients. Phosphorylation of eIF2α and IRE1, thereby increasing sensitivity to mycetoplasma free. Cells were maintained at 37 °C in a humidified incubator and 5% CO2 atmosphere in RPMI 1640 medium (Gibco, Thermo Fisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (FBS; Corning), 1% of Penicillin-Streptomycin (10,000 U/ml; Life Technologies). For EPHB4 inhibition, inhibitor NVP-BHG712 (Selleck Chemicals) was dissolved in DMSO (Sigma). Cell viability was assessed by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) and Cell Counting Kit-8 (Dojindo Molecular Technologies). Cells were seeded at 5000 cells/well in 96-well plates (Corning) and allowed to adhere overnight. Cells were then transiently transfected with siRNA specific for EPHB4 or non-targeting control (Dharmacon). For EPHN2 siRNA, cells were transiently transfected with siRNA specific for EPHN2 or non-targeting control (Dharmacon). Absorbance was measured at 72 h at 490 nm in a microplate reader. For apoptosis, Caspase-3/7

**Materials and methods**

**Cell culture**

Human prostate cancer cell lines PC-3, 22Rv1, and LNCaP were from ATCC and mouse Myc-CaP cells were the kind gift of Charles Sawyers (Memorial Sloan-Kettering Cancer Centre). Myc-CaP; Pten-KO cells are Myc-CaP cells with deletion of Pten by CRISPR/Cas9 and have been described. Cells were authenticated and verified to be mycoplasma free. Cells were maintained at 37 °C in a humidified incubator and 5% CO2 atmosphere in RPMI 1640 medium (Gibco, Thermofisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (FBS; Corning), 1% of Penicillin-Streptomycin (10,000 U/ml; Life Technologies). For EPHB4 inhibition, inhibitor NVP-BHG712 (Selleck Chemicals) was dissolved in DMSO (Sigma). Cell viability was assessed by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) and Cell Counting Kit-8 (Dojindo Molecular Technologies). Cells were seeded at 5000 cells/well in 96-well plates (Corning) and allowed to adhere overnight. Cells were then transiently transfected with siRNA specific for EPHB4 or non-targeting control (Dharmacon). For EPHN2 knockdown, cells were transiently transfected with siRNA specific for EPHN2 or non-targeting control (Dharmacon). Absorbance was measured at 72 h at 490 nm in a microplate reader. For apoptosis, Caspase-3/7

**Fig. 5 EPHB4 inhibition decreases Myc and phosphorylation of p38 MAPK and 4EBP1.** a Western blot with indicated antibodies on LNCaP, PC-3, and 22Rv1 cells transfected with the indicated siRNAs for 72 h. Data are presented as mean ± standard deviation (n = 3) from three different independent experiments. Statistical significance was analyzed by two-tailed Student’s t-test (treated group is compared to control group). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. b c-Myc expression was analyzed by Immunofluorescence in PC-3 cells transfected with indicated siRNAs for 72 h. Image was visualized at ×40 and scale bar represents 50 μm. c Western blots with indicated antibodies on extracts from PC-3 cells transfected with control (siCNT) or with EphrinB2-specific siRNA (siEFNB2) after 72 h. Data are presented as mean ± standard deviation (n = 3) from three different independent experiments. d PC-3 cells were transfected with siMYC or siCNT for 72 h and p-eIF2α at ser51, GLUT3, and EPHB4 level were measured by western blot. Data are presented as mean ± standard deviation from at least three different independent experiments. Statistical significance was analyzed by two-tailed Student’s t-test (treated group is compared to control group). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Open triangles indicate specific bands.
activities were evaluated by Caspase-Glo 3/7 Assay (Promega) according to the manufacturer’s recommendation.

Organoid culture
NCI-H660 cells (ATCC) were seeded at 5000 cells/well density in ultra-low attachment 96-well plates (Corning) and cultured in Hepatocyte growth media (Corning) supplemented with 10 ng/ml epidermal growth factor (Corning), 5% heat inactivated charcoal stripped FBS, 1X Glutamax (Gibco), 5% Matrigel (BD Biosciences), 10 µM ROCK inhibitor (Y-27632, STEMCELL Technologies), and 1X Gentamicin/Amphotericin (Lonza), as described previously. At day 8, organoids were treated with NVP-BHG 712 (EPHB4i) from Selleck Chemicals or DMSO (Veh.), and viability was assessed at day 15 as per manufacturer’s protocol (Cell Titer Glo-3D viability assay, Promega).

RNA interference
Cells were transiently transfected with 25 nM of human siGENOME EphB4 siRNA (Set of four EPHB4 siRNA, Dharmacon Catalog no. # MU-003124-02-0002) or

Fig. 6 EPHB4 inhibition decreases phosphorylation of Src kinase. a Western blots with indicated antibodies on extracts from PC-3 and Myc-CaP cells transfected with control (siCNT) or with EphB4-specific siRNA (siEphB4) after 72 h. Data are presented as mean ± standard deviation (n = 3 or 4) from three different independent experiments. Open triangles indicate specific bands. b Western blots with indicated antibodies on extracts from PC-3 cells transfected with control (siCNT) or with EphrinB2-specific siRNA (siEFNB2) after 72 h. Data are presented as mean ± standard deviation (n = 3) from three different independent experiments. Statistical significance was analyzed by two-tailed Student’s t-test (treated group is compared to control group). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. c EPHB4 correlation with SRC was analyzed in MSKCC and SU2C dataset from Cbioportal.org
mouse siGENOME EphB4 (Dharmacon Catalog no. # MU-06046901–0002) or Non-targeted siRNA (Dharmacon Catalog no. D-001210–01–05), human siGENOME EFNB2 siRNA, Dharmacon Catalog no. # M-003659–02–0005), siGENOME Human MYC siRNA, Dharmacon catalog no. M-003282–07–0005 and DharmaFECT transfection reagent (Dharmacon), according to the manufacturer’s protocol for 72 h. After 20 min of incubation, the mixture was added to the suspended cells and these were plated in dishes for each assay. Cells were analyzed for all experiments after 72 h.

Immunogenic cell death assay

Myc-CaP cells were transfected with siEPHB4 or siCNT, or treated with EphB4 inhibitor for 72 h. After the indicated times, supernatants were collected and cell counts performed for quantifying secreted ATP (Bioluminescent Assay Kit, Sigma) and high-mobility group protein B1 (HMGB1; Elisa, Techan Trading). For detection of surface Calreticulin, cells were incubated with rabbit anti-Calreticulin (1:1000, Abcam ab2907) for 60 min and then incubated with Alexa Fluor 488 anti-rabbit secondary antibody (Invitrogen A11008 1 µg/ml) and analyzed by flow cytometry.

qRT-PCR

Total RNA extraction from PC-3 transfected cells were performed using the TRizol reagent (Life Technologies, Rockville, MD), according to the manufacturer’s instructions. The reverse transcriptase polymerase chain reaction (RT-PCR) was performed using Moloney murine leukemia virus reverse transcriptase (MMLV; Invitrogen, Carlsbad, CA) as per manufacturer’s instructions. qRT-PCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) and following primers: EFNB2 (F: TATGCAGAACTGCGATTTCCAA, R: TGGGTA TAGTACCAGTCCTTGTC) or the housekeeping gene 18S rRNA (F: GTAACCCGTTGAACCCCATT, R: CCATCCAATCGGTAGTAGCG) (Integrated DNA Technologies).

Western blot

This was performed as described\(^{56,57}\). The following primary antibodies were used: EPHB4 (D1C7N) (1:1000, Cell Signaling #14960), EPHB4 (1:500, Abcam #ab73259), phospho-eIF2α S51 (1:1000, Cell Signaling #9721 S), eIF2α (1:000, Cell Signaling #9722), phospho-IRE1(S724) (1:1000, Abcam #ab48187), c-Myc (Y69) (1:5000, Abcam.
of interest. The authors declare that they have no conflict of interest.

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Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 14 June 2019 Revised: 9 September 2019 Accepted: 3 October 2019
Published online: 22 October 2019
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