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# The noncanonical function of borealin, a component of chromosome passenger complex, promotes glycolysis via stabilization of survivin in squamous cell carcinoma cells

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ABSTRACT

The chromosome passenger complex (CPC) is a kinase complex formed by Aurora B, borealin, survivin and inner centromere protein (INCENP). The CPC is active during mitosis and contributes to proper chromosome segregation via the phosphorylation of various substrates. Overexpression of each CPC component has been reported in most cancers. However, its significance remains unclear, as only survivin is known to confer chemoresistance. This study showed that the overexpression of borealin, a CPC component, stabilized survivin protein depending on its interaction with survivin. Unexpectedly, the accumulation of survivin by borealin overexpression did not affect the well-characterized functions of survivin, such as chemoresistance and cell proliferation. Interestingly, the overexpression of borealin promoted lactate production but not the overexpression of the deletion mutant that lacks the ability to bind to survivin. Consistent with these findings, the expression levels of glycolysis-related genes were enhanced in borealin-overexpressing cancer cells. Meanwhile, the overexpression of survivin alone did not promote lactate production. Overall, the accumulation of the borealin–survivin complex promoted glycolysis in squamous cell carcinoma cells. This mechanism may contribute to cancer progression via excessive lactate production.

## 1. Introduction

Borealin (also known as CDCA8 and DASRA) is a component of the chromosome passenger complex (CPC), which is composed of Aurora B, borealin, survivin (also known as BIRC5), and inner centromere protein (INCENP) [1]. The CPC plays an essential role in ensuring proper chromosome segregation during mitosis via the kinase activity of Aurora B. INCENP is the scaffolding protein for other components. Borealin and survivin are directly associated with INCENP in a three-helix bundle and play a critical role in CPC localization to the centromere of chromosomes [2,3]. Once cell division is completed, CPC function is terminated by triggering the degradation of borealin by anaphase-promoting complex/cyclosome $(APC/C)^{Cdh1}$ ubiquitin ligase-mediated proteolysis in the G1 phase [4]. Although the overexpression of borealin has been reported in gastric, colorectal, lung, and liver cancers [5–7], the significance of borealin overexpression in cancer remains unclear.

Survivin is a member of the inhibitor of the apoptosis protein family, which suppresses apoptosis by preventing caspase activation [8]. Many studies have shown that survivin is highly expressed in various cancers, and its overexpression leads to cancer progression by promoting apoptosis resistance and cell proliferation [9]. Therefore, survivin is considered a therapeutic target in cancer. Several inhibitors of survivin, such as YM155, have been developed and have shown activity against a broad range of cancer types in cancer cell lines and preclinical models [10].

Glycolysis is a process of oxidative glucose catabolism. During this process, glucose is degraded to lactate with adenosine triphosphate production under anaerobic conditions. Cancer cells prefer to use glycolysis for energy production even in the presence of sufficient oxygen, which is known as the “Warburg effect” [11]. Furthermore, recent studies have revealed that excessive production of lactate promotes tumor progression via extracellular acidification, which induces metastasis, inhibition of antitumor immunity, and resistance to antitumor therapy [12]. Collectively, lactate is considered a potential

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therapeutic target in cancer treatment.

Therefore, this study aimed to demonstrate the novel role of borealin in stabilizing survivin protein during the interphase and whether the borealin–survivin interaction promotes lactate production in squamous cell carcinoma cells.

## 2. Materials and methods

### 2.1. Cell culture and synchronization

HeLa cells were obtained from the American Type Culture Collection. HSC2 and OSC19 cells were obtained from the Japanese Collection of Research Bioresources. HeLa and HSC2 cells were maintained in Dulbecco's modified Eagle's medium (Wako), whereas OSC19 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with heat-inactivated 10% fetal bovine serum (Invitrogen) at 37 °C in 5% $CO_2$.

For prometaphase arrest, HeLa cells were synchronized at prometaphase by treatment with 50 ng/ml of nocodazole (Sigma-Aldrich) for 12h, followed by mitotic shake-off. Subsequently, synchronized cells were released from the mitotic arrest by washing out nocodazole. Cells were collected at the indicated time points.

### 2.2. Reagents and antibodies

Doxycycline was purchased from Takara Bio Inc. MG132 (Z-Leu-Leu-Leu-CHO) was purchased from Peptide Institute Inc. Cycloheximide, nocodazole, doxorubicin cisplatin, and staurosporine were purchased from Sigma. The following commercial antibodies were obtained: mouse monoclonal anti-borealin antibody (Santa Cruz Biotechnology), mouse monoclonal anti-p27 antibody (Transduction Laboratories), anti-INCENP, anti-FoxM1 p-T600, anti-GFP, and anti-Vinculin antibodies (Cell Signaling Technology), rabbit monoclonal anti-Aurora B antibody (Abcam), anti-Cdh1 antibody (MBL), anti-Fbxl7 antibody (Invitrogen), β-actin antibody (Sigma-Aldrich), mouse monoclonal anti-FLAG and anti-c-Myc (Wako), and anti-survivin antibody (Novus Biologicals).

### 2.3. Lentiviral gene transfer

For borealin overexpression, CSII-CMV-IRES-Bsd and packaging plasmids (pCAG-HIVgp and pCMV-VSV-G-RSV-Rev) were obtained from Dr. Miyoshi (RIKEN). pcDNA3.1 plasmid encoding full-length human borealin, deletion mutant lacking the region between amino acids 18 and 77 and substitution mutant (W70E/F74E) were subcloned into CSII-CMV-IRES-Bsd. For tetracycline-inducible expression of Aurora B, pCW-Cas9-Bsd was obtained from Addgene. pcDNA4-Xpress-His encoding full-length human Aurora B was subcloned into the pCW vector after removing Cas9 cDNA by *Nhe* I/*BamH* I digestion.

For shRNA transduction, pSIH-H1 control shRNA-Puro and packaging plasmids (psPAX2 and pMD2.G) were obtained from Addgene. Each sense and antisense oligonucleotide was annealed and cloned into the *BamH* I/*EcoR* I site of the pSIH-H1 shRNA-Puro vector. All shRNA constructs were confirmed by sequencing. The target sequences for borealin, Cdh1, and FBXL7 shRNAs are as follows: Borealin: GAATCATGTGCTATGTTCTAA, Cdh1: GGATTAACGAGAATGAGAA, and FBXL7: GGCAAATGCCCTTTGGTATCC.

Lentiviral vectors and packaging plasmids were transfected into Lenti-X 293T cells (Takara Bio Inc.) using PEI max (Polysciences). Supernatants were collected 48 h after transfection and filtered using a 0.45 μm membrane. HSC2, OSC19, and HeLa cells were infected with filtered supernatants with 4 μg/ml polybrene. After 24 h, the medium was replaced with a fresh medium containing 10 μg/ml blasticidin or 1 μg/ml puromycin.

### 2.4. siRNA transfection

For transient siRNA experiments, 30 nM of siRNA was transfected using Oligofectamine® RNAi MAX (Invitrogen) according to the manufacturer's instructions. *AurkB*, *BIRC5*, and negative control siRNAs were obtained from Horizon Discovery Ltd., and the sequences are as follows: Aurora B: TCGTCAAGGTGGACCTAAA, survivin: CAAAGGAAACCAACAATAA, and negative control: TGGTTTACATGTC GACTAA. Borealin and INCENP were obtained from GeneDesign, Inc., and the sequences are as follows: Borealin: AGGTAGAGCTGTCTGTTCA and INCENP: GGCTTGGCCAGGTGTATAT.

### 2.5. Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen) and reverse-transcribed into cDNA using PrimeScript RT Master Mix (Takara Bio). Transcript levels of target genes were determined using a LightCycler 96 System (Roche Diagnostics) with TB Green Premix Ex TaqII (Takara Bio) and the following primers: *CDCA8* (borealin): forward, 5′-CCTGACACCCAGGTTTGACT-3′ and reverse, 5′-GCAATACTGTGCCTCTGCAA-3'; *BIRC5* (survivin): forward, 5′-GGACCACCGCATCTCTACAT-3′ and reverse, 5′-GTCTGGCTCGTTCTCAGTGG-3'; *AURKB* (Aurora B): forward, 5′-GACCTAAAGTTCCCCGCTTC-3′ and reverse, 5′-ATCAGGCGACAGATTGAAGG-3'; *INCENP*: forward, 5′-CCCCAGAAAGTTGGTTCTGA-3′ and reverse, 5′-CAATCTCCGTGTCATTGTGG-3'; *FBXL7*: forward, 5′-GAAGCACCTTGGCATAGAGC-3′ and reverse, 5′-GCACTGCACAATCTCCTGAA-3'; *GLUT1*: forward, 5′-CAT CCCATGGTTCATCGTGGCTGAACT-3′ and reverse, 5′-GAAGTAGGTGAA GATGAAGAACAGAAC-3'; *GLUT4*: forward, 5′-TTTTGAGATTGGCC CTGGCCCCAT-3′ and reverse, 5′-TCAGGTACTCTTAAGAAGGTGAAG-3'; *HK2*: forward, 5′-GCCATCCTGCAACACTTAGGGCTTGAG-3′ and reve rse, 5′-GTGAGGATGTAGCTTGTAGAGGGTCCC-3'; *GPI*: forward, 5′-TATTGTGTTCACCAAGCTCACACC-3′ and reverse, 5′-TGGTAGAAGCGT CGTGAGAGGTC-3'; *PFKL*: forward, 5′-GGAGAAGCTGCGCGAGGTT TAC-3′ and reverse, 5′-ATTGTGCCAGCATCTTCAGCATGAG-3'; *ALDOA*: forward, 5′-AGGCCATGCTTGCACTCAGAAGT-3′ and reverse, 5′-AGG GCCCAGGGCTTCAGCAGG-3'; *GAPDH*: forward, 5′-TCCACCACCC TGTTGCTGTA-3′ and reverse, 5′-GCATCCTGGGCTACACTGAG-3'; *PGK1*: forward, 5′- ATGTCGCTTTCTAACAAGCTG-3′ and reverse, 5′-GCGGAGGTTCTCCAGCA-3'; *PGAM1*: forward, 5′-GGAAACGTGTACTGATTGCAGCCC-3′ and reverse, 5′-TTCCATGGCTTTGCGCACCGTCT-3'; *ENO1*: forward, 5′-GACTTGGCTGGCAACTCTG-3′ and reverse, 5′-GGTCATCGGGAGACTTGAA-3'; *ENO2*: forward, 5′-TCATGGTGAGTCATCGCTCAGGAG-3′ and reverse, 5′-ATGTCCGGCAAAGCGAGCTTCATC-3'; *PKM2*: forward, 5′-GCCCGTGAGGCAGAGGCTGC-3′ and reverse, 5′-TGGTGAGGACGATTATGGCCC-3'; *LDHA*: forward, 5′-ATG GCAACTCTAAAGGATCA-3′ and reverse, 5′-GCAACTTGCAGTTCGGGC-3'; *ACTB* (β-actin): forward, 5′-ACAGAGCCTCGCCTTTGC-3′ and reverse, 5′-GATGCCTCTCTTGCTCTGGG-3'; *TUB1A* (α-tubulin): forward, 5′-CCAAGCTGGAGTTCTCTA-3′ and reverse, 5′-CAATCAGAGTGCTCCAGG-3'. The relative mRNA expression of each transcript was normalized against β-actin or α-tubulin mRNA using the $2^{-(\Delta\Delta Ct)}$ method.

### 2.6. Cell counting kit-8 (CCK-8)

HSC2 cells were suspended at a density of 10,000 cells/well in a 96-well flat bottom plate for 24 h to evaluate cell viability. The culture medium was replaced with a fresh medium containing doxorubicin or cisplatin, and the plate was incubated for 24 (doxorubicin) or 48 h (cisplatin). Cell viability was determined by absorbance at 450 nm using the CCK-8 reagent (Dojindo) according to the manufacturer's protocol.

HSC2 cells were cultivated at a density of 2500 cells/well in a 96-well flat bottom plate for 24 h to evaluate cell proliferation. Cell proliferation was determined using the CCK-8 reagent at 24, 48, and 72 h.

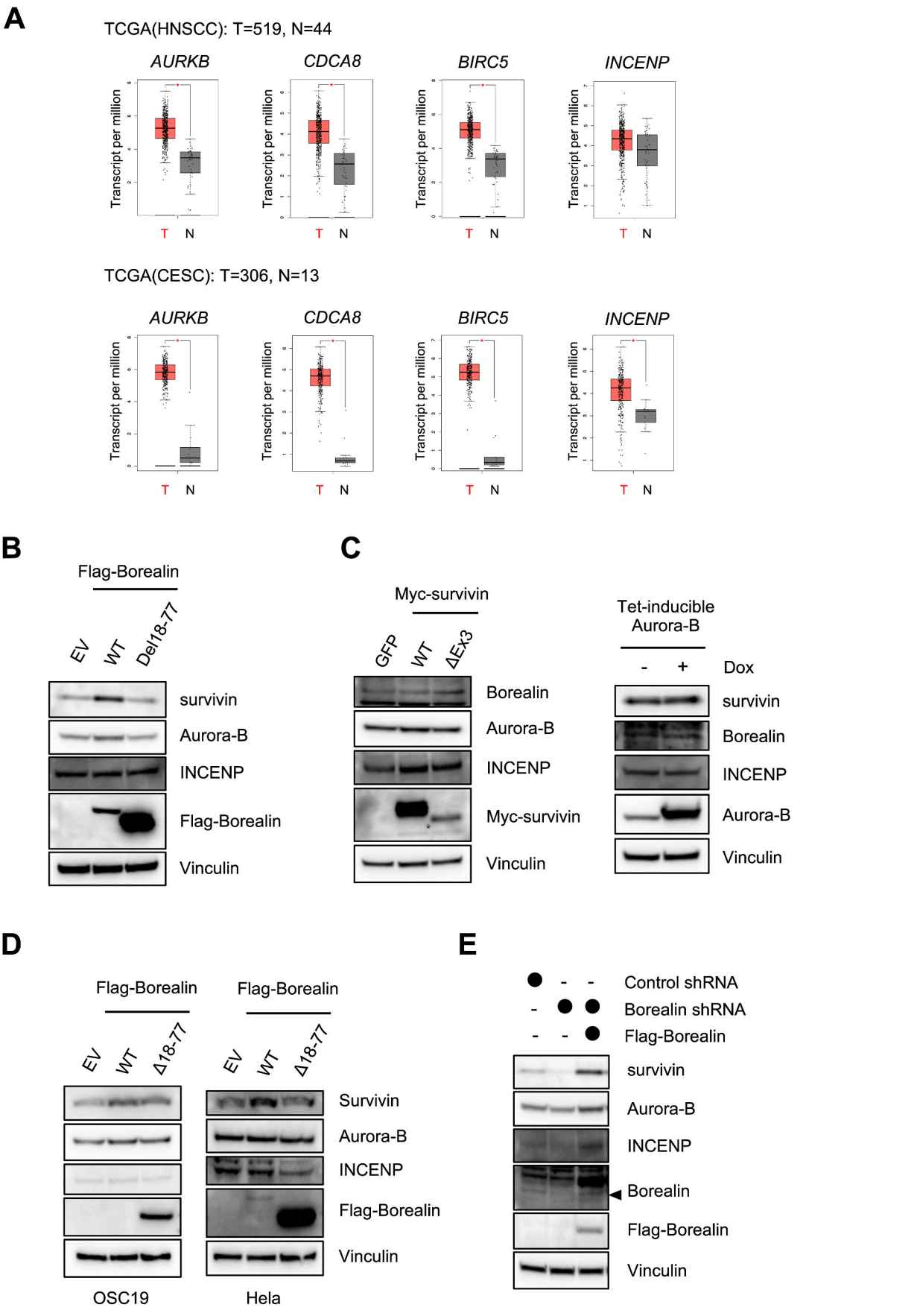


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**Fig. 1.** The overexpression of borealin induces the accumulation of survivin. (A) The expression of each chromosome passenger complex (CPC) component was analyzed using The Cancer Genome Atlas datasets of head and neck squamous cell carcinoma (HNSCC) and cervical squamous cell carcinoma (CESC) using GEPIA software (http://gepia.cancer-pku.cn). T indicates tumor, and N indicates matched normal. (B) HSC2 cells were transfected with empty vector (EV), Flag-borealin wild type (WT), and Del18-77 mutant, which lacks binding to Survivin and INCENP. Cells were collected and lysed for immunoblotting as indicated. (C) HSC2 cells were transfected with GFP, Myc-survivin WT, and ΔEx3 splicing variant, which could not form CPC (left panel). HSC2 cells were transfected with tetracycline-inducible Aurora B WT. After 48 h with doxycycline, cells were collected and lysed for immunoblotting as indicated (right panel). (D) OSC19 and HeLa cells were transfected with EV, Flag-borealin WT, and Del18-77 mutant. Cells were collected and lysed for immunoblotting as indicated. (E) HeLa cells were cotransfected with control shRNA, borealin shRNA, and Flag-borealin WT. Cells were collected and lysed for immunoblotting as indicated. Vinculin expression was used as a loading control.

### 2.7. Flow cytometry

HSC2 cells were treated with 250 nM staurosporine for 6 h and stained for 15 min in FACS buffer with 7-aminoactinomycin D (7-AAD) (eBioscience) and Apotracker Green (BioLegend) to detect apoptosis cells.

For cell cycle analysis, cells were treated with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 30 min, fixed with 70% ethanol, and stored at −20 °C before analysis. The fixed cells were denatured with 2 M hydrochloric acid with 0.5% Triton X-100 for 30 min and neutralized with 0.1 M sodium tetraborate. The cells were washed and resuspended in 1% BSA/TBS-T and stained with anti-BrdU antibody conjugated with allophycocyanin (BioLegend) for 30 min. After washing with 1% BSA/TBS-T, the cells were resuspended in a 7-AAD solution containing ribonuclease A and NP-40.

Cell populations were quantified based on each fluorescent signal using the CytoFLEX S Flow Cytometer (Beckman Coulter). Data analysis was performed using the FlowJo FACS Analysis software (BD Biosciences).

### 2.8. Western blotting

The cells were lysed using lysis buffer (25 mM Tris-HCl pH7.6, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM $MgCl_2$, 1 mM EDTA, and 10% glycerol) with protease inhibitor cocktail (Wako) and sonicated on ice. Protein concentration was determined by measuring the absorption at 595 nm on a SpectraMax i3 microplate reader (Molecular Devices) using Bio-Rad protein assay dye reagent concentrate (Bio-Rad laboratories Inc.). For immunoblotting, 30 μg of protein was subjected to 5%–20% gradient polyacrylamide gel (ATTO) electrophoresis followed by electroblotting onto a nitrocellulose membrane (Cytiva). The immunocomplex was detected using Immobilon HRP substrate (EMD Millipore). Chemiluminescence was detected using Fusion SOLO 7S. EDGE (VILBER). Band signals were quantified using the Evolution Capt software (VILBER).

### 2.9. Extracellular lactate concentration measurement

The cells were plated in a 96-well plate at $5.0 \times 10^3$ (OSC19 cells) or $1.0 \times 10^4$ cells (HSC2 and HeLa cells) per well. The cells were incubated with 5% CO2 for 24 h at 37 °C. Lactate measurement was performed using Lactate Assay Kit-WST (Dojindo) according to the manufacturer's protocols. The lactate concentration was determined by measuring the absorption at 450 nm on a SpectraMax i3 microplate reader (Molecular Devices).

### 2.10. Statistical analysis

Statistical analysis was performed using one-way analysis of variance followed by Tukey's multiple comparison tests or two-sided unpaired Student's *t*-tests with GraphPad Prism 9 (GraphPad Software). A $p$-value $<0.05$ was considered statistically significant.

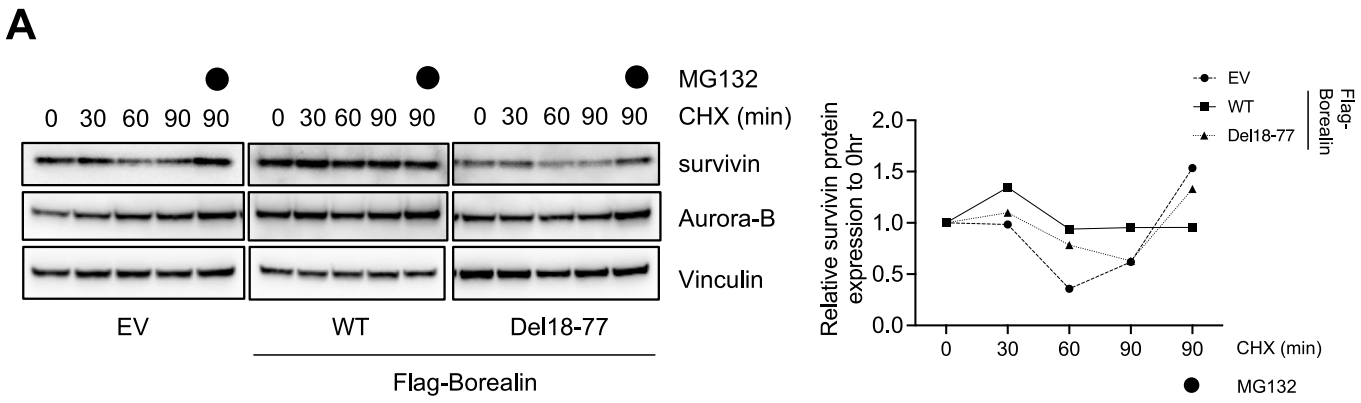
## 3. Results

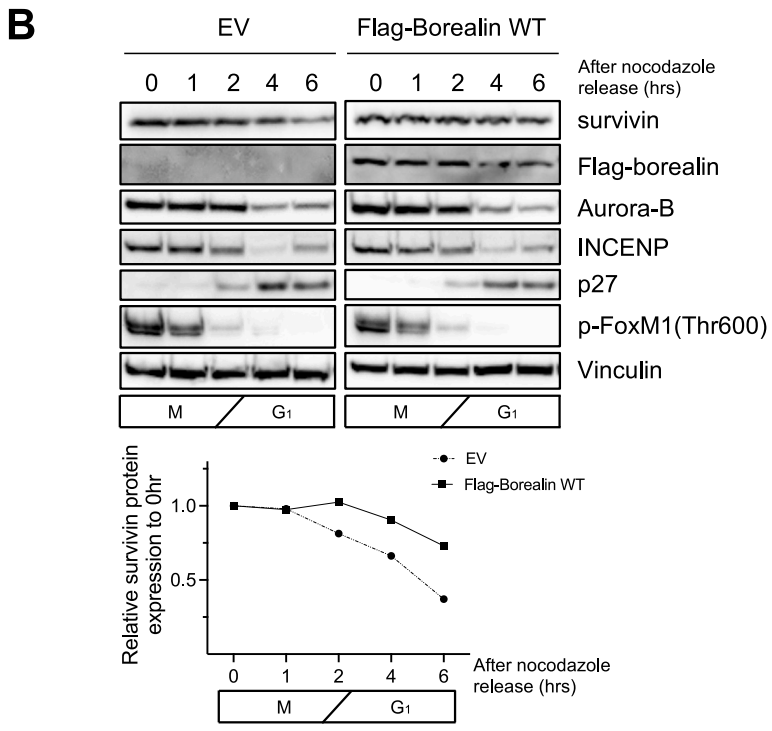
### 3.1. Overexpression of borealin induces accumulation of survivin protein but not other CPC components

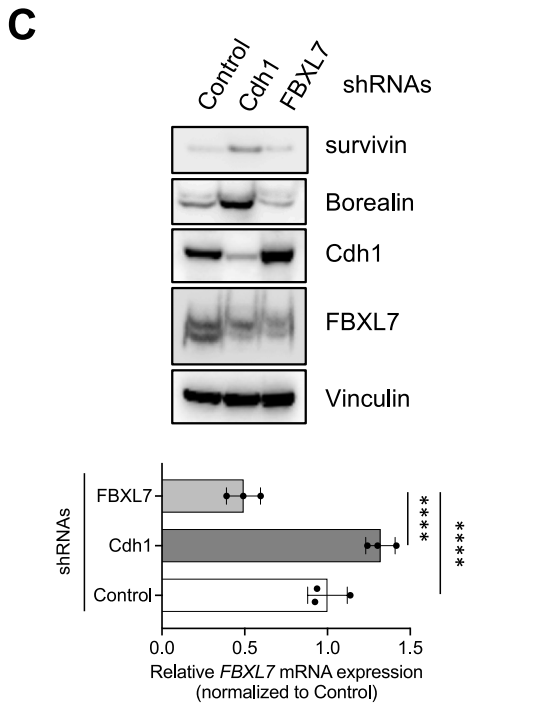
The mRNA levels of each CPC component were first analyzed using The Cancer Genome Atlas datasets of head and neck squamous cell carcinoma, cervical squamous cell carcinoma, and matched normal tissues to address the significance of the overexpression of CPC components. The mRNA levels of *AURKB* (Aurora B), *CDCA8* (borealin), and *BIRC5* (survivin), except *INCENP*, significantly increased in both cancer tissues (Fig. 1A). Knockdown of each CPC component has been reported to affect the protein level of other components [13]. Therefore, the knockdown of each CPC component was performed using HeLa cells. As expected, the knockdown of each CPC component decreased the protein level of other components (Supplementary Fig. 1A). Based on these results, we hypothesized that the overexpression of each CPC component would enhance the protein level of other CPC components and promotes carcinogenesis and cancer progression. HSC2 cells were transfected with each CPC component except INCENP in head and squamous cell carcinoma cell lines to test this hypothesis. Interestingly, the overexpression of borealin resulted in increased protein level of survivin but not Aurora B and INCENP (Fig. 1B). Unexpectedly, the overexpression of survivin and Aurora B did not affect the protein level of other CPC components, including borealin (Fig. 1C). Importantly, the accumulation of survivin protein was not observed upon the overexpression of borealin Del18-77 mutant (Fig. 1B). This phenomenon was also confirmed in other squamous cell carcinoma cell lines, such as OSC19 and HeLa cells (Fig. 1D). In addition, the protein levels of Aurora B and INCENP were decreased in borealin-depleted cells and overexpression of borealin rescued it (Fig. 1E). Furthermore, the overexpression of borealin further enhanced the protein expression of survivin in borealin-depleted cells (Fig. 1E). In CPC, the N-terminus residues 10–109 of borealin bind survivin and INCENP to form a three-helix bundle [1]. Consistent with this report, we previously showed that borealin Del18-77 mutant could not bind survivin and INCENP [4]. These results suggested that the accumulation of survivin protein induced by borealin overexpression depended on the binding to survivin and INCENP. To address whether survivin and/or INCENP are involved in this phenomenon, we utilized the borealin substitution mutant of W70E/F74E, which lacks binding to survivin but not INCENP [1]. Interestingly, overexpression of borealin W70E/F74E did not enhance the protein level of survivin in both Hela and OSC19 cells (Supplementary Fig. 3A). Taken together, the overexpression of borealin induced the accumulation of survivin protein through the formation of borealin–survivin complex.

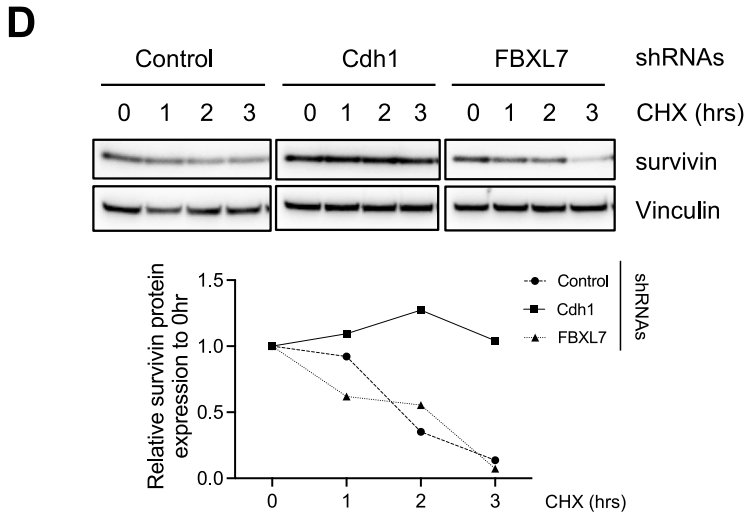
### 3.2. Overexpression of borealin stabilizes survivin at the protein level

The mRNA expression of each CPC component was tested to investigate how borealin overexpression induces the accumulation of surviving protein. The overexpression of borealin did not affect the mRNA expression of *BIRC5* (survivin) and other CPC components (Supplementary Figs. 1B and C). The results suggested that borealin affected survivin expression at the posttranscriptional level. Therefore, the protein half-life of survivin was analyzed using cycloheximide. Survivin protein decreased in a time-dependent manner in HeLa cells expressing



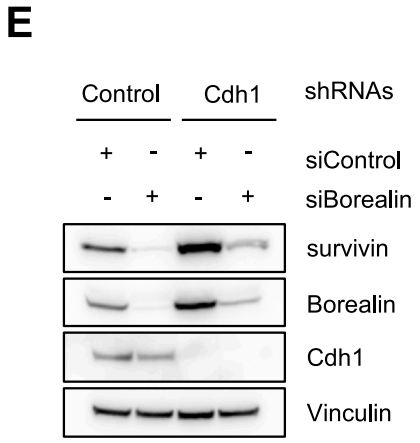




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**Fig. 2.** Overexpression of borealin stabilizes survivin protein. (A) HeLa cells were transfected with EV, Flag-borealin WT, and Del18-77 mutant. Cells were treated with 25 μg/ml cycloheximide and 10 μM MG132 for the indicated time and lysed for immunoblotting as indicated. Densitometric analysis of survivin and vinculin was performed. Based on imaging intensity, the graph shows the protein expression levels relative to the 0 h. Survivin expression was normalized with vinculin expression. (B) HeLa cells were transfected with EV and Flag-borealin WT. Cells were synchronized by mitotic shake-off with nocodazole. After being released from mitotic arrest, the cells were collected and lysed for immunoblotting at the indicated time points. Densitometric analysis of survivin and vinculin was performed. Based on imaging intensity, the graph shows the protein expression levels relative to the 0 h. Survivin expression was normalized with vinculin expression. (C) HeLa cells were transfected with control, Cdh1, and FBXL7 shRNAs and lysed for immunoblotting as indicated (upper panel). The mRNA expression level of FBXL7 was determined by quantitative reverse transcription polymerase chain reaction (lower panel). Data are presented as fold change in the Control group and the mean ± SD of triplicates from each group. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. ***$p < 0.001$, and ****$p < 0.0001$. (D) HeLa cells were transfected with control, Cdh1, and FBXL7 shRNAs and treated with 25 μg/ml cycloheximide for the indicated time. Cells were collected and lysed for immunoblotting as indicated. Densitometric analysis of survivin and vinculin was performed. Based on imaging intensity, the graph shows the protein expression levels relative to the 0 h. Survivin expression was normalized with vinculin expression. (E) HeLa cells expressing with control and Cdh1 shRNAs were transfected with control and borealin siRNA. After 48 h, cells were collected and lysed for immunoblotting as indicated.

empty vector (EV) and borealin Del18-77 mutant. However, the protein half-life of survivin was prolonged in HeLa cells expressing borealin wild type (WT) (Fig. 2A). Additionally, the decreased protein expression of survivin in HeLa cells expressing EV and borealin Del18-77 mutant was rescued by treatment with the proteasome inhibitor MG132. These findings suggest that ubiquitin-mediated proteolysis may be involved in the elongation of survivin half-life induced by the overexpression of borealin. Recently, borealin has been reported to be ubiquitylated and degraded by the APC/$C^{Cdh1}$ ligase complex after cell division [4]. The protein level of survivin during mitotic exit was tested using cell cycle synchronization. As expected, accumulation of survivin protein was observed in borealin-overexpressing cells compared with EV-expressing cells during the G1 phase (Fig. 2B). Indeed, an excessive amount of borealin during the G1 phase stabilized survivin protein. Survivin has been reported to be ubiquitylated and degraded by the $SCF^{FBXL7}$ ubiquitin ligase complex, and the knockdown of Cdh1 has been reported to induce the accumulation of survivin [14,15]. Knockdown of Cdh1 and FBXL7 in HeLa cells was performed to test the involvement of these ubiquitin ligases in the borealin-mediated accumulation of survivin protein. As expected, the accumulation of borealin and survivin was observed in Cdh1-depleted cells. Unexpectedly, the knockdown of FBXL7 did not affect the protein level of survivin (Fig. 2C. upper panel). The mRNA expression of *FBXL7* was also confirmed by quantitative PCR in the FBXL7 knockdown cells (Fig. 2C. lower panel). Furthermore, the protein half-life of survivin was analyzed using cycloheximide. Elongation of the half-life of survivin was observed in Cdh1-depleted cells but not in FBXL7-depleted cells (Fig. 2D). These results indicated that APC/$C^{Cdh1}$ directly or indirectly affected the stability of survivin protein. Additionally, knockdown of borealin in Cdh1-depleted cells was performed to clarify the relationship between APC/$C^{Cdh1}$, borealin, and survivin. The knockdown of borealin decreased the protein level of survivin in Cdh1 shRNA-transfected cells and control shRNA-transfected cells (Fig. 2E). These results revealed that the stabilization of survivin caused by Cdh1 depletion depended on the accumulation of borealin. Taken together, the ectopic expression of borealin during the G1 phase induced the accumulation of survivin protein.

### 3.3. Accumulation of survivin protein induced by borealin overexpression does not affect chemoresistance and cell proliferation

It is well characterized that the overexpression of survivin confers chemoresistance and promotes cell proliferation in several cancers [9]. Based on previous reports, chemoresistance and cell proliferation were tested using borealin-overexpressing HSC2 cells with an accumulation of survivin. Drug sensitivity was examined using doxorubicin and cisplatin. Unexpectedly, borealin WT-overexpressing cells showed no significant differences compared with EV- or borealin Del18-77 mutant-expressing cells with both doxorubicin and cisplatin treatment (Fig. 3A). Consistent with these results, borealin WT-overexpressing cells did not obtain antiapoptotic capacity compared with EV- or borealin Del18-77 mutant-expressing cells in staurosporine treatment (Fig. 3B). Additionally, cell proliferation was examined using CCK-8. Borealin WT-overexpressing cells showed no differences compared with EV- or borealin Del18-77-mutant expressing cells on any day (Fig. 3C). Consistent with this result, the cell cycle distribution showed a similar pattern between these cells (Fig. 3D). Taken together, these results showed that the accumulation of survivin induced by borealin overexpression did not affect on the well-characterized functions of survivin, such as chemoresistance and cell proliferation.

### 3.4. Accumulation of survivin prtotein induced by borealin overexpression promotes lactate production in cancer cells

The medium color of borealin WT-overexpressing cells in the culture dish changed from red to yellow earlier than that of EV- or borealin Del18-77 mutant-expressing cells (Fig. 4A). Generally, this change reflects an acidification of the culture media due to lactate production. Therefore, we hypothesized that overexpression of borealin might promote the Warburg effect, leading to significant lactate production. Extracellular lactate concentration in conditioned media from these cells was measured to test our hypothesis. Interestingly, the extracellular lactate concentration in borealin WT-overexpressing HeLa cells was significantly higher than that in EV- or borealin Del18-77 mutant-expressing cells (Fig. 4B left panel). This phenomenon was confirmed in OSC19 (Fig. 4B right panel) and HSC2 cells (Supplementary Fig. 2A). Furthermore, overexpression of the borealin W70E/F74E mutant decreased or had no effect on the extracellular lactate concentration in Hela and OSC19 cells, rather than increasing it (Supplementary Fig. 3B). Finally, the mRNA expression of 13 glycolysis-related genes including glucose transporter 1 (*GLUT1*), glucose transporter 4 (*GLUT4*), hexokinase 2 (*HK2*), glucose-6-phosphate isomerase (*GPI*), 6-phosphofructokinase, liver type (*PFKL*), aldolase A (*ALDOA*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), phosphoglycerate kinase 1 (*PGK1*), phosphoglycerate mutase 1 (*PGAM1*), enolase 1 (*ENO1*), enolase 2 (*ENO2*), pyruvate kinase M2 (*PKM2*) and lactate dehydrogenase A (*LDHA*) was examined as shown in Fig. 4C (left panel). Consistent with the results in Fig. 4B, the mRNA expression of several glycolysis-related genes such as *GLUT1*, *HK2*, *GAPDH*, *PGK1*, *PGAM1*, *ENO1*, *ENO2* and *LDHA* was significantly enhanced in borealin-overexpressing cells compared with EV- or borealin Del18-77 mutant-expressing Hela cells (Fig. 4C right panel). These enhanced mRNA expression were confirmed in OSC19 and HSC2 cells (Supplementary Fig. 2B). Consistent with these results, overexpression of borealin W70E/F74E mutant did not enhance the mRNA expression of glycolysis-related genes in Hela and OSC19 cells (Supplementary Fig. 3C). The mRNA expression of some glycolysis-related genes such as *PGK1* and *LDHA* were decreased in borealin W70E/F74E mutant overexpressing Hela cells. On the other hand, single overexpression of survivin did not promote the lactate production (Fig. 4D). These results showed that the borealin–survivin interaction might be essential for the lactate production in cancer cells via increasing the mRNA expression of glycolysis-related genes.

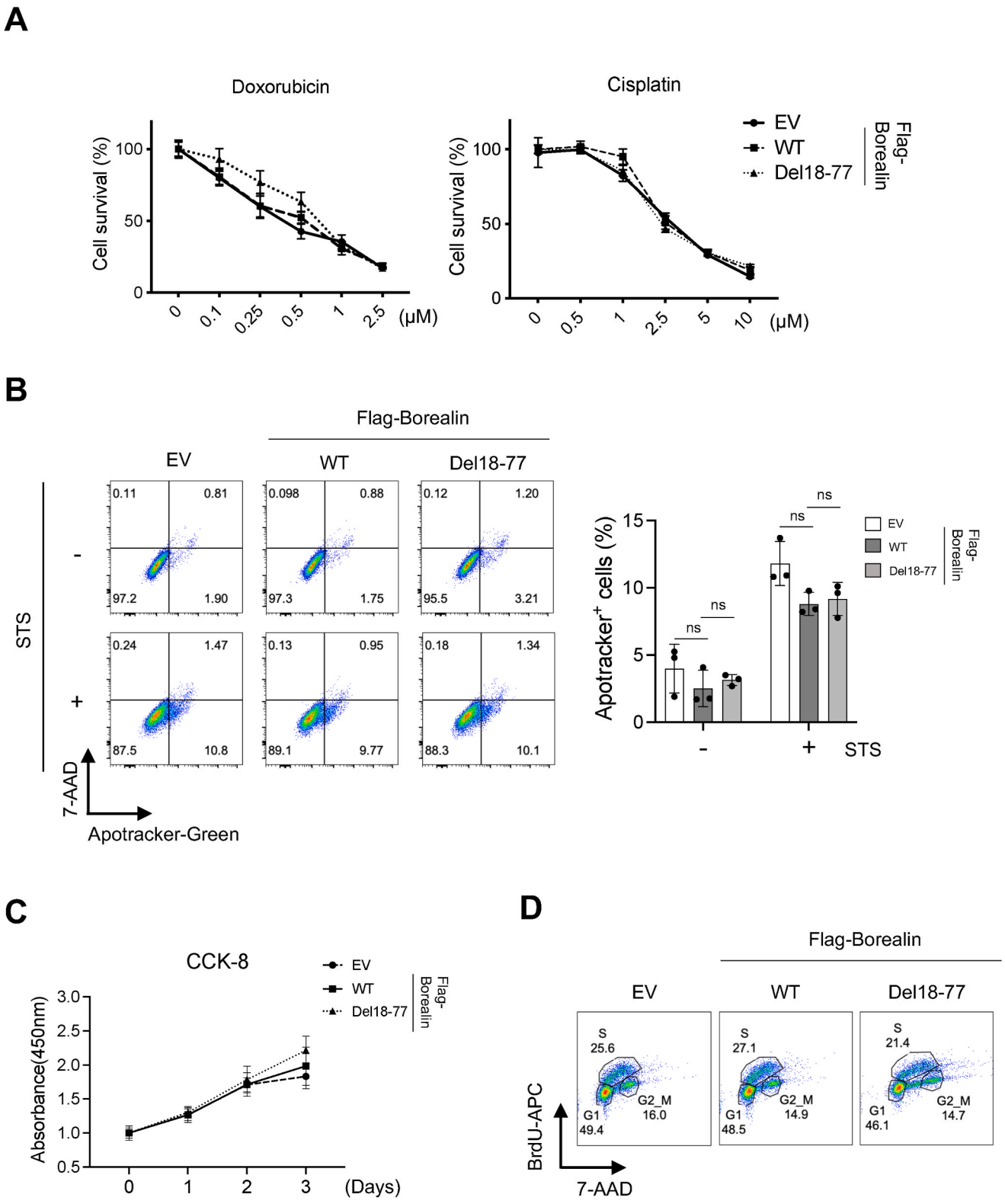


**Fig. 3.** Accumulation of survivin induced by the overexpression of borealin does not affect chemoresistance and cell proliferation. HSC2 cells were transfected with EV, Flag-borealin WT, and Del18-77 mutant. Cell phenotypes were evaluated in chemosensitivity (A), induction of apoptosis (B), cell proliferation (C), and cell cycle profile (D). (A) HSC2 cells were treated with doxorubicin for 24 h or cisplatin for 48 h. Cell viability was determined using CCK-8. Cell survival was normalized to the absorbance of untreated cells (0 μM). Data are presented as the mean ± standard deviation (SD) of six replicates from each group. (B) HSC2 cells were treated with or without staurosporine for 6 h. Apoptotic cells were determined by flow cytometric analysis staining with 7-AAD and Apotracker Green. The graph shows the percentage of Apotracker-positive cells in total cells. Data are presented as the mean ± SD of triplicates from each group. (C) Cell proliferation was measured using CCK-8 at the indicated time. Data are presented as the mean ± SD of six replicates from each group. (D) HSC2 cells were treated with 10 μM BrdU for 30 min. Cell cycle distribution was evaluated by flow cytometric analysis staining with 7-AAD and anti-BrdU antibody. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (A–C).

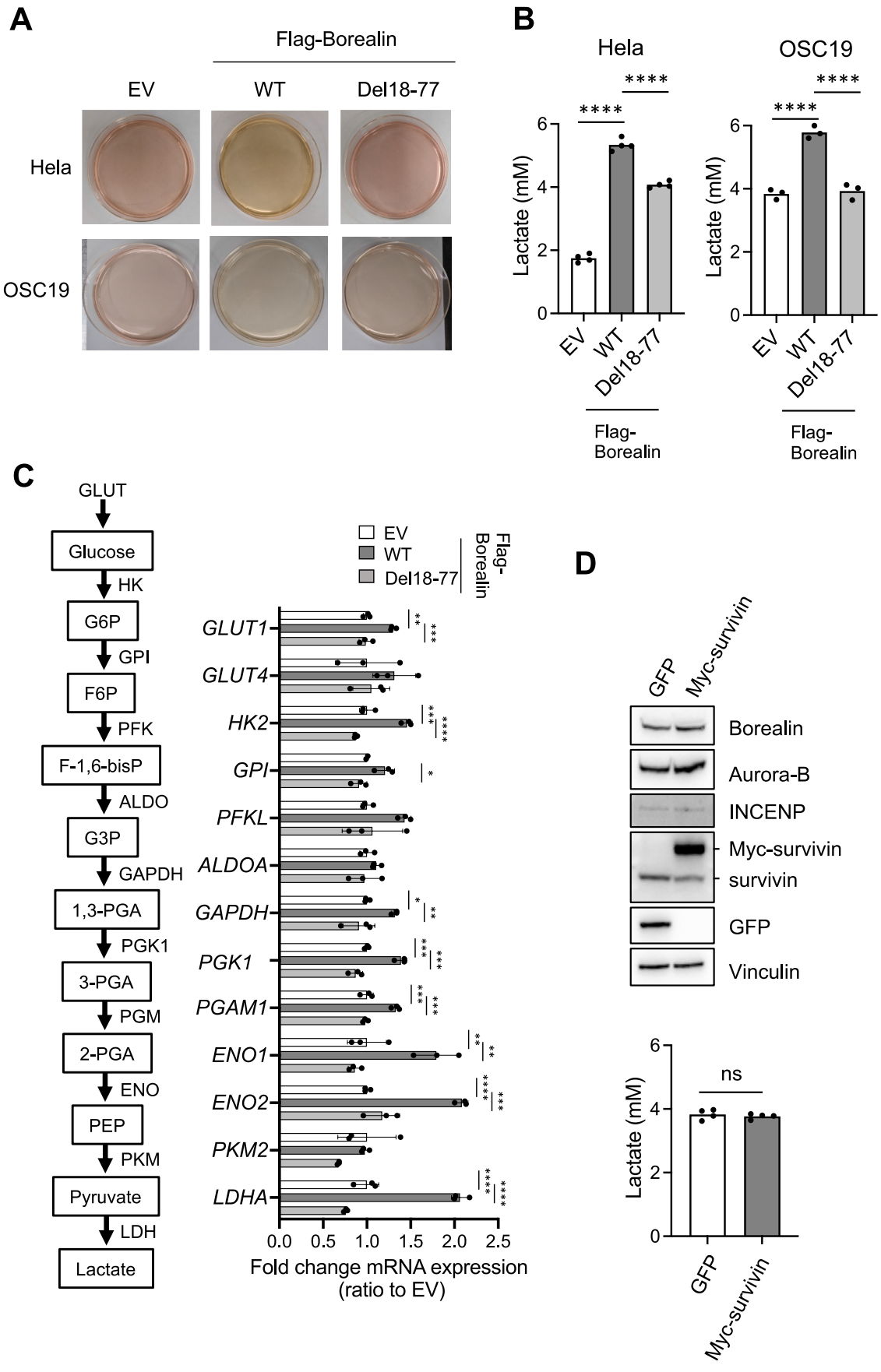


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**Fig. 4.** Accumulation of survivin induced by borealin overexpression promotes lactate production in cancer cells. (A) HeLa and OSC19 cells were transfected with EV, Flag-borealin WT, and Del18-77 mutant. Cells were suspended at equal cell numbers in 100 mm dishes and cultured for 72 h. The photograph shows the color of the conditioned media. (B) Extracellular lactate concentration in conditioned media from HeLa and OSC19 cells was determined using Lactate Assay Kit-WST. Data are presented as the mean ± SD of four or three replicates from each group. (C) As indicated, the mRNA expression levels of glycolysis-related genes were determined by quantitative reverse transcription polymerase chain reaction using HeLa cells expressing EV, borealin WT, and Del18-77 mutant. Data are presented as fold change in the EV group and the mean ± SD of triplicates from each group. (D) HSC2 cells were transfected with GFP and Myc-survivin. Cells were collected and lysed for immunoblotting as indicated (upper panel). Vinculin expression was used as a loading control. Extracellular lactate concentration in conditioned media from HSC2 cells was determined using Lactate Assay Kit-WST (lower panel). Data are presented as the mean ± SD of four replicates from each group. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests (B and C) and two-sided unpaired Student's *t*-tests (D). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

## 4. Discussion

The study results showed that the overexpression of borealin stabilized survivin protein during the interphase. Furthermore, their protein–protein interaction was essential for the borealin-induced stabilization of survivin. Unexpectedly, this stabilization did not promote the antiapoptotic function and cell proliferation known as the well-characterized function of survivin. The borealin–survivin complex enhanced lactate production in the culture supernatant of cancer cells by promoting the expression of glycolysis-related genes.

Survivin is a bifunctional protein that depends on cell cycle progression and intracellular localization. During interphase, survivin predominantly localizes cytoplasm, and cytoplasmic survivin suppresses apoptosis [8]. Once cells enter mitosis after nuclear envelope breakdown, survivin plays an essential role in ensuring proper mitotic progression as a member of the CPC [1–3]. Survivin is shuttled from the nucleus to the cytoplasm via the CRM1/exportin pathway when the nuclear envelope forms after cell division due to its active nuclear export signal [17]. A previous report showed the possibility of eliminating nuclear survivin through ubiquitin-mediated degradation by the APC/$C^{Cdh1}$ ubiquitin ligase [15]. This paper showed that nuclear survivin accelerated its proteasomal degradation using the fusion protein of survivin added to the nuclear localization signal, and the knockdown of Cdh1 prevented the degradation of survivin during the G1 phase. However, there is no direct evidence that survivin is ubiquitylated by APC/$C^{Cdh1}$. Additionally, the study results showed that the knockdown of Cdh1 induced survivin accumulation and prolonged the half-life of survivin protein (Fig. 2C and D). A recent report showed that borealin was degraded by APC/$C^{Cdh1}$ during the G1 phase [4]. Additionally, the ectopic expression of borealin during the G1 phase attenuated the degradation of survivin, and the knockdown of borealin reduced the accumulation of survivin in Cdh1-depleted cells. These results suggest that borealin contributes to the stabilization of survivin localized at the nucleus during the G1 phase.

$SCF^{FBXL7}$ ubiquitin ligase has been reported to ubiquitylate survivin using murine lung epithelial cell lines and to have a proapoptotic function [14]. However, the knockdown of FBXL7 did not induce the accumulation of survivin using several squamous cell carcinoma cell lines in this study. Recently, hypermethylation of the FBXL7 promoter has been reported in various human cancers [16]. Although we did not analyze the methylation status of the FBXL7 promoter, the mRNA expression level of FBXL7 was low in these cell lines (data not shown). Thus, the downregulation of FBXL7 at the steady state may be the reason why the knockdown of FBXL7 did not affect the expression and half-life of survivin in these cell lines.

Deregulated survivin expression in cancer cells confers resistance to chemotherapy [9]. In most cancer tissues, accumulation of survivin has been observed in the cytoplasm. Interestingly, accumulation of cytoplasm and nucleus has been reported in some cancer tissues, such as head and neck squamous cell carcinoma, bladder cancer, and lung cancer. Furthermore, nuclear accumulation of survivin has a positive relationship with clinicopathological factors and clinical stage [13,18,19]. However, the role of nuclear survivin remains unclear. The study results revealed that borealin-mediated stabilization of survivin did not promote antiapoptotic function or cell proliferation. These results are consistent with those of a previous report that showed that nuclear survivin does not have a cytoprotective role using the overexpression of survivin fused to a nuclear localization signal [15]. Interestingly, we found that the overexpression of wild-type borealin significantly promoted lactate production, whereas the deletion mutant lacking binding capacity to survivin could not enhance it. Furthermore, the overexpression of borealin increased the expression of several glycolysis-related genes. Recently, it has been reported that survivin is associated with the transcription factors IRF1 and SMAD3, which repress the mRNA expression of the metabolic checkpoint enzyme phosphofructokinase 2 gene *PFKFB3* and promote glycolysis [20]. However, the mRNA expression of *PFKFB3* was not altered in borealin-overexpressing cancer cells (data not shown). Importantly, in this study, the overexpression of survivin alone did not promote lactate production. These results indicate that nuclear survivin may play a distinct role in promoting glycolysis in a borealin-binding-dependent manner under transcriptional control. However, how the borealin–survivin complex enhances the mRNA expression of several glycolysis-related genes remains unclear. To clarify this mechanism, futher epigenomic profiling such as Chromatin immunoprecipitation with sequence (ChIP-seq), CUT&RUN and CUT&Tag are required [21]. Furthermore, it would be interesting to identify the other components involved in this non-canonical borealin-survivin complex, such as transcription factor and epigenetic factor.

In conclusion, borealin plays a novel role in stabilizing survivin during the G1 phase. Moreover, the ectopic borealin–survivin complex positively regulated lactate production by enhancing the mRNA expression of glycolysis-related genes. Interestingly, several reports have reported various effects of lactate on the tumor microenvironment (TME). These study showed that lactate secretion by cancer cells promoted TME acidification. This TME acidification induces the apoptosis of CD8 lymphocytes and natural killer cells, which are major immune cells for antitumor immunity [22,23]. Moreover, lactate-induced acidification promotes polarization to an M2-like macrophages, which induces neovascularization, cancer cell proliferation, and epithelial-mesenchymal transition [24,25]. Our findings may contribute to carcinogenesis and cancer progression by promoting lactate production and can help further understand how cancer cells shape TME through excessive lactate production. To address this issues, futher studies require to be perform *in vivo* such as tumor transplantation. In current study, we utilized human squamous cell carcinoma cell lines which could be transplated to immunodeficient mice. Therefore, it would be interesting to utilize syngenic mice model such as oral squamous cell carcinoma for focusing on TME [26].

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## CRediT authorship contribution statement

**Hiroaki Tawara:** Validation, Investigation, Formal analysis, Data curation. **Takaaki Tsunematsu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Shojiro Kitajima:** Writing – review & editing, Supervision. **Ruka Nagao:** Writing – review & editing, Formal analysis. **Shigefumi Matsuzawa:** Writing – review & editing, Formal analysis. **Kunihiro Otsuka:** Writing – review & editing, Formal analysis. **Aya Ushio:** Writing – review & editing, Formal analysis. **Naozumi Ishimaru:** Writing – review & editing, Writing – original draft, Supervision.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2024.149741.

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