

# Tight Junction–Associated Signaling Pathways Modulate Cell Proliferation in Uveal Melanoma

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**PURPOSE.** To investigate the role of tight junction (TJ)–associated signaling pathways in the proliferation of uveal melanoma.

**METHODS.** Human uveal melanoma cell lines overexpressing the TJ molecule blood vessel epicardial substance (Bves) were generated. The effects of Bves overexpression on TJ protein expression, cell proliferation, and cell cycle distribution were quantified. In addition, localization and transcription activity of the TJ-associated protein ZO-1–associated nucleic acid binding protein (ZONAB) were evaluated using immunofluorescence and bioluminescence reporter assays to study the involvement of Bves signaling in cell proliferation-associated pathways.

**RESULTS.** Bves overexpression in uveal melanoma cell lines resulted in increased expression of the TJ proteins occludin and ZO-1, reduced cell proliferation, and increased sequestration of ZONAB at TJs and reduced ZONAB transcriptional activity.

**CONCLUSIONS.** TJ proteins are present in uveal melanoma, and TJ-associated signaling pathways modulate cell signaling pathways relevant to proliferation in uveal melanoma. (*Invest Ophthalmol Vis Sci.* 2011;52:588–593) DOI:10.1167/iov.10-5746

Uveal melanoma is the most common primary intraocular malignancy in adults.<sup>1</sup> Several histopathologic features of these tumors are indicative of disease prognosis, including epithelioid cell morphology<sup>2</sup> and the presence of vascular looping patterns.<sup>3,4</sup> Additionally, molecular profiling of tumors has revealed genes associated with uveal melanoma growth, invasion, and metastasis, such as the downregulation of cell communication and development genes<sup>5,6</sup> and the expression of matrix metalloproteinases.<sup>7</sup> Further elucidation of the molecular participants in the pathogenesis of uveal melanoma would facilitate the development of targeted diagnostic approaches and therapeutic interventions.

Tight junctions (TJs) are major components of cell junctional assemblies, which play an important role in controlling the passage of solutes across epithelial barriers, such as the cornea and gastric mucosa.<sup>8</sup> In addition to maintaining barrier function in epithelia, TJs serve as a cell signaling apparatus for multiple cell types throughout the body, contributing to the regulation of vesicular trafficking,<sup>9</sup> cell proliferation,<sup>10</sup> and cell motility.<sup>11</sup> Alterations of TJ expression and function have been associated with the development of cancer through mechanisms such as the loss of contact inhibition of cell growth.<sup>12</sup> Recently, we reported on the localization and function of a cell adhesion molecule, Bves (blood vessel epicardial substance), in corneal epithelial cells and the trabecular meshwork.<sup>13–16</sup> Bves is an approximately 360-amino acid protein expressed in many cell types from early stages of embryonic development to adulthood. It contains an extracellular amino terminus, three transmembrane domains, and an intracellular carboxyl terminus.<sup>17</sup> The C-terminal domain interacts with the TJ-associated protein zonula occludens 1 (ZO-1), a TJ-associated protein, through direct and indirect mechanisms.<sup>14</sup> Alterations in Bves expression in several cell types are associated not only with profound effects on TJ formation but also on cell morphology, motility, and proliferation.<sup>14,17–21</sup> These observations suggest that Bves may have roles that extend beyond the regulation of barrier formation in epithelia.

In cancer, the TJ complex may serve a critical role in facilitating communication between the cancer cell and the tumor microenvironment and in spatially and temporally orchestrating the mechanisms underlying tumor initiation and progression.<sup>22</sup> Although the localization and function of TJs have been partially characterized in several cancer types, including cutaneous melanoma,<sup>22</sup> their role in uveal melanoma is unclear. In this study, we investigated the role of Bves in the proliferation of uveal melanoma. Three human uveal melanoma cell lines stably transfected to overexpress Bves were analyzed using two- and three-dimensional cell proliferation assays and cell cycle distribution assays. Additionally, cells were measured for expression of TJ proteins and for the localization and activity of the TJ-associated transcription factor ZO-1–associated nucleic acid binding protein (ZONAB).<sup>23,24</sup> ZONAB is a Y-box transcription factor that modulates cell proliferation through its interaction with the SH3 domain of ZO-1 at TJs, through its association with cell division kinase 4 (CDK4), and through its upregulation of cell cycle entry proteins.<sup>25</sup> Specifically, in proliferating cells, ZONAB is observed primarily in the nucleus, where it promotes proliferation through binding of CDK4 and upregulation of proliferating cell nuclear antigen (PCNA) and cyclin D1, resulting in cell cycle entry.<sup>25</sup> However, in slowly or nonproliferating cells, nuclear levels of ZONAB are reduced. The ZONAB protein is primarily localized in the cytoplasm, where it is sequestered from the nuclear compartment through its interaction with ZO-1.<sup>24</sup> Because Bves is associated with ZO-1 at TJs,<sup>14</sup> the association of Bves with ZO-1/ZONAB signaling in uveal melanoma was investigated.

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## MATERIALS AND METHODS

### Cell Lines

OM 431, OMM 1, and OMM 2.3 human uveal melanoma cell lines were a gift of Jerry Y. Niederkorn (University of Texas Southwestern Medical Center, Dallas, TX). OM431 cells were derived from a choroidal melanoma.<sup>26</sup> OMM 1 cells were derived from subcutaneous metastasis of a uveal melanoma.<sup>27</sup> OMM 2.3 cells were derived from a liver metastasis of a uveal melanoma.<sup>28,29</sup> Cells were maintained at culture conditions of 37°C and 5% CO<sub>2</sub>, in complete DMEM supplemented with 10% FBS (Invitrogen Corporation, Carlsbad, CA). All cell lines were verified for human origin and for the presence of characteristic melanoma antigens with the use of commercially available antibodies (#ab14203; Abcam, Cambridge, MA). An isotype-matched control IgG was used as a negative control. Short tandem repeat (STR) analysis of the three cell lines was performed at the Johns Hopkins University Fragment Analysis Facility with a profiling kit (Powerplex 1.2; Promega, Madison, WI). STR profiles are shown in Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5746/-DCSupplemental>, and confirm that cell lines were not cross-contaminated with one another before analysis, in accordance with guidelines suggested by the uveal melanoma research community.<sup>30</sup>

Cells were transfected with full-length Bves cDNA with a FLAG tag, as previously described,<sup>14</sup> or an empty vector control incorporating the same cytomegalovirus promoter using a transfection reagent (FuGENE 6; Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Cells were then maintained in medium containing selective antibiotic (200 µg/mL G418; MP Biomedical, Irvine, CA) for 2 weeks before experiments were conducted.

### Immunofluorescence and Western Blot Analysis

Cell cultures were grown to either 50% or 90% confluence, then fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Inc., Hatfield, PA) in PBS, pH 7.2, for 10 minutes at room temperature. Cells were permeabilized in 0.1% Triton X-100 (Sigma-Aldrich Corp., St. Louis, MO) in PBS for 5 minutes at room temperature, rinsed three times in PBS, and blocked for 30 minutes in PBS with 10% goat serum (Sigma-Aldrich Corp.). Primary antibody staining was conducted overnight at 4°C with the following antibodies at 1:200 dilution: Bves 846 anti-sera,<sup>15</sup> anti-ZO-1, anti-FLAG (M2 clone; Sigma-Aldrich), anti-Ki67 (Vector Laboratories, Burlingame, CA), and anti-ZONAB (Zymed-Invitrogen Corp., Carlsbad, CA), with DAPI used for a nuclear counterstain. Cells were imaged using an inverted fluorescence microscope (TE2000U Eclipse; Nikon, Tokyo, Japan) with imaging software (Image Pro Plus 5.1; Media Cybernetics, Silver Spring, MD). Distinct fluorescence channels were imaged sequentially using spectrally matched bandpass excitation and emission filters to avoid spectral overlap in colocalization analysis. Isotype-matched nonspecific primary antibodies were used as negative controls.

Cells were assayed for expression of Bves, occludin, and ZO-1 using immunoblotting techniques as previously described.<sup>15</sup> Relative band densitometry was performed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).<sup>31</sup> Ki67<sup>+</sup> cells were quantified using imaging software (Image Pro Plus; Media Cybernetics), and the ratio of Ki67<sup>+</sup> cells to total cells was used to calculate the Ki67 index.

### Cell Proliferation Assays

Uveal melanoma cell lines were assayed by the XTT/PMS cell proliferation test as directed by the manufacturer (Sigma-Aldrich Corporation) at 24, 48, and 72 hours after seeding (initial density, 10<sup>4</sup> cells/cm<sup>2</sup>). Absorbance was read at 450 nm after subtracting a reference absorbance at 690 nm on a plate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA). Colony-formation assays were performed on basement membrane matrix (Matrigel; Sigma-Aldrich) as previously published,<sup>32</sup> using a seeding density of 10<sup>4</sup> cells per well of a 24 well microplate.

### Cell Cycle Analysis

Cell cycle distribution of cells was quantified using a propidium iodide-based method on synchronized cell populations as previously described.<sup>33</sup> At least 20,000 cells were analyzed per sample on a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ), and distributions were measured using the Watson pragmatic algorithm within a software suite (FlowJo 7; Treestar Software, Ashland, OR).

To evaluate the effect of transfection on apoptosis and necrosis, a flow cytometric assay was used according to the manufacturer's instructions (LIVE/DEAD Annexin V/Propidium Iodide kit, Invitrogen).

### Dual Luciferase ZONAB Reporter Assay

A dual luciferase reporter assay used to quantify ZONAB activity was a gift of Karl Matter (University College London, London, UK) and was used as previously reported.<sup>24</sup> In this assay, an ErbB2 promoter containing a ZONAB-binding site is used to regulate firefly luciferase expression. As a control, a mutated promoter of similar length, which is incapable of binding ZONAB, was used to regulate *Renilla* luciferase expression. Both plasmids were cotransfected into OMM2.3 cells using transfection reagent (FuGENE 6; Roche) according to the manufacturer's instructions. Forty-eight hours after transfection, cell lysates were measured ratiometrically for dual luciferase activity using the Promega dual luciferase reporter assay kit according to the manufacturer's instructions on a dual-injection microplate luminometer (Lumimark Plus; Bio-Rad Inc., Hercules, CA).

Results were compared using one-way ANOVA (Sigmaplot 11; Systat) with Dunnett's posttest.  $P < 0.05$  was considered significant.

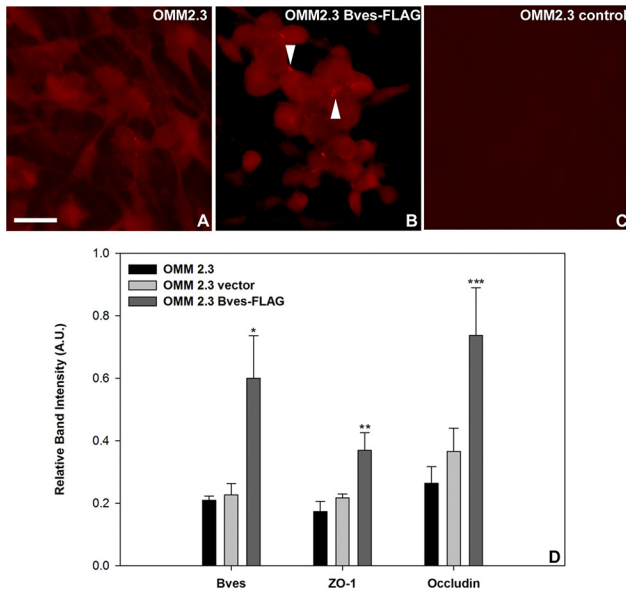
## RESULTS

### Expression and Localization of Bves in Uveal Melanoma Cell Lines

Bves overexpression in uveal melanoma was observed to alter trafficking of the protein and the formation of cell-cell contacts. Immunofluorescence analysis indicated that in wild-type uveal melanoma cells, Bves was cytoplasmically distributed regardless of cell density, and few cell-cell contact sites were observed (Fig. 1A). However, in cells transfected with a Bves-FLAG construct, increased localization of Bves toward sites of cell-cell contact was observed (Fig. 1B, arrowhead). Minimal immunoreactivity to an isotype-matched nonspecific control antibody was observed (Fig. 1C). Bves-FLAG, when expressed in melanoma cell lines, was trafficked to sites of cell-cell contact similarly to endogenous Bves. Results were consistent across all uveal melanoma cell lines tested. These data are consistent with previous localization studies of Bves in other ocular cell types, including trabecular meshwork<sup>15</sup> and corneal epithelium.<sup>15,16</sup>

### Effect of Bves Overexpression on Expression of Tight Junction Molecules

Melanoma cells stably transfected with Bves constructs exhibited an increase in Bves compared with wild-type cells (Fig. 1D). The negative control cells stably transfected with empty vector exhibited similar levels of Bves compared with wild-type cells. This increase in Bves levels was associated with significantly increased occludin and ZO-1 proteins compared with wild-type and empty vector transfected cells (Fig. 1D). Immunofluorescence analysis indicated that the TJ protein ZO-1 colocalized with Bves at sites of cell-cell contact (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5746/-DCSupplemental>), similar to previous studies in other cell types.<sup>15</sup> These results indicate that Bves overexpression modulates cell-cell adhesion and TJ formation in uveal melanoma cells in vitro as seen in previous studies using various cell types, including trabecular meshwork cells.<sup>15</sup>



**FIGURE 1.** Immunofluorescence localization of Bves and densitometric analysis of tight junction protein immunoblots in OMM2.3 cells. (A) Bves protein (red) is localized in the cytoplasm of wild-type OMM2.3 cells. (B) In OMM2.3 cells that overexpress Bves, increased localization of Bves is observed at sites of cell-cell contact (arrowheads). (C) Immunoreactivity to an isotype-matched negative control antibody was minimal. (D) Quantification of immunoblots indicates that occludin and ZO-1 proteins are upregulated in OMM2.3 cells overexpressing Bves. Data are reported as mean  $\pm$  SEM ( $n = 4$ ; \* $P = 0.018$ , \*\* $P = 0.027$ , \*\*\* $P = 0.04$ ). Results are representative of those observed for other wild-type and Bves-transfected uveal melanoma cell types. Scale bar, 20  $\mu$ m.

### Effect of Bves Overexpression on Cell Proliferation and Cell Cycle Progression

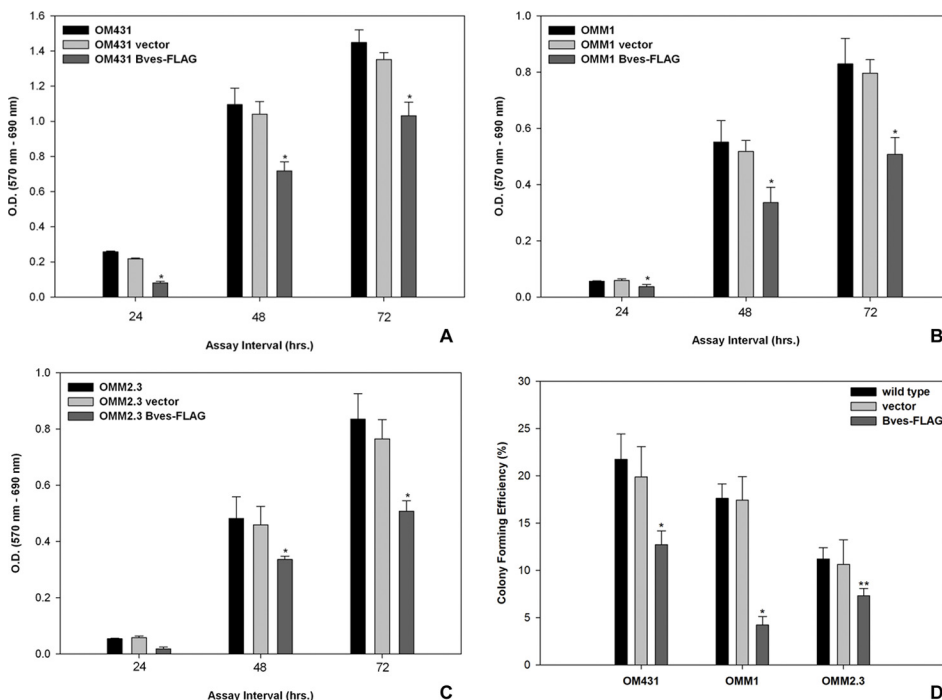
Uveal melanoma cell lines overexpressing Bves exhibited significantly-lower cell proliferation rates than wild-type cell lines (Figs. 2A-C;  $P < 0.001$ ). Vector control-transfected cells ex-

hibited proliferative capacities that were similar to those of wild-type cells. When cells were grown on a basement membrane matrix (Matrigel; Sigma-Aldrich) substrate, three-dimensional proliferative capacities measured by colony formation efficiency correlated with trends observed for two-dimensional assays (Fig. 2D). Results were similar across all three cell lines, including those transfected with empty vector as a control. Quantification of Ki67<sup>+</sup> cells using immunofluorescence microscopy exhibited results reflecting this trend (Supplementary Fig. S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5746/-/DCSupplemental>).

Because there were no differences in apoptotic or necrotic cell fraction attributable to transfection vehicle or overexpression of Bves as determined by flow cytometry (Supplementary Fig. S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5746/-/DCSupplemental>), cell cycle distribution of uveal melanoma cell lines was quantified. Cell cycle analysis revealed that a greater fraction of Bves-FLAG-transfected cells were arrested in the G<sub>1</sub> phase than wild-type cells (Fig. 3). However, a significantly larger fraction of wild-type melanoma cells was distributed in S and G<sub>2</sub>/M phases compared with Bves-overexpressing melanoma cells (Fig. 3D). These findings suggest that reduced proliferative capacity of Bves-overexpressing cells may be due to cell cycle arrest.

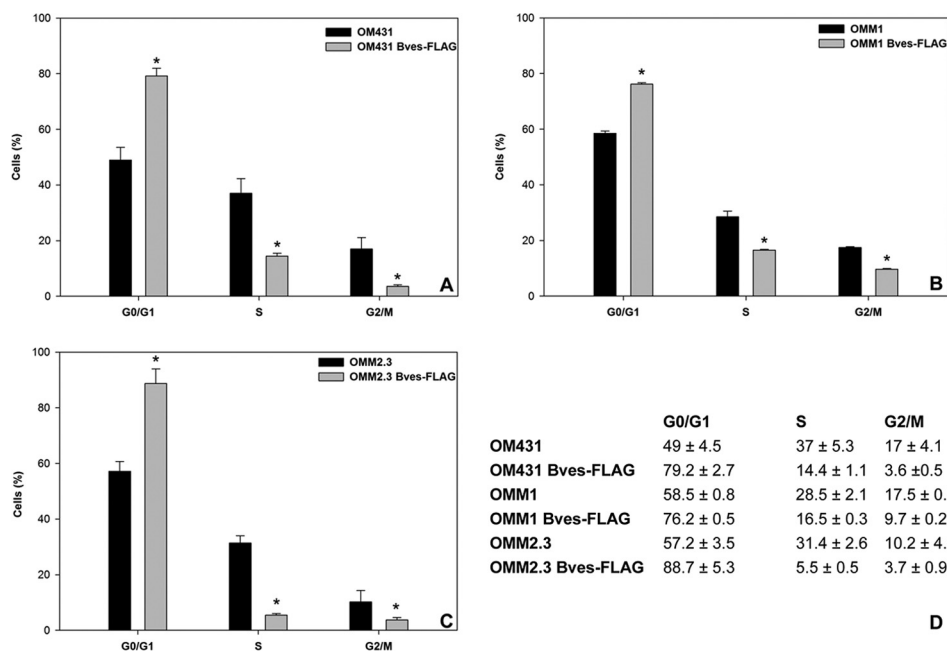
### Association of Bves with ZONAB Transcription Activity

Based on immunofluorescence analysis of wild-type and empty vector-transfected cells, ZONAB was associated primarily with the nucleus or nuclear membrane, irrespective of cell density (Figs. 4A-F), whereas in Bves-overexpressing uveal melanoma cells, ZONAB was primarily localized within the cytoplasm and was excluded from the nuclear region, suggestive of reduced ZONAB-mediated transcriptional activity (Figs. 4G-I). These observations are consistent with the understanding that Bves overexpression reduces cell proliferation by increasing TJ formation and accumulation of ZO-1 at TJs, where sequestration of ZONAB inhibits the upregulation of proliferative factors. To confirm this finding, a reporter assay was used to detect ZONAB nuclear activity.<sup>24</sup> In this assay, ZONAB interaction



**FIGURE 2.** Analysis of cell proliferation and three-dimensional colony-forming ability of uveal melanoma cell lines. (A-C) Cell lines transfected with Bves exhibit reduced cell proliferation compared with empty vector-transfected or wild-type-matched cell lines as measured by XTT assay. (D) Similarly, colony-forming capacity of Bves-transfected cell lines in basement membrane matrix is reduced. (A) OM431, (B) OMM1, (C) OMM2.3. Data are reported as mean  $\pm$  SEM ( $n = 4$ ; \* $P < 0.001$ , \*\* $P = 0.031$ ).





**FIGURE 3.** Flow cytometric analysis of cell cycle progression in uveal melanoma cell lines. In all three cell lines tested, cell cycle distribution in S and G<sub>2</sub>/M phases were reduced for Bves-transfected cells (A–C). (A) OM431, (B) OMM1, (C) OMM2.3. (D) Table of cell cycle distribution percentages for each cell type. Data are reported as mean ± SEM (*n* = 3; \**P* < 0.001).

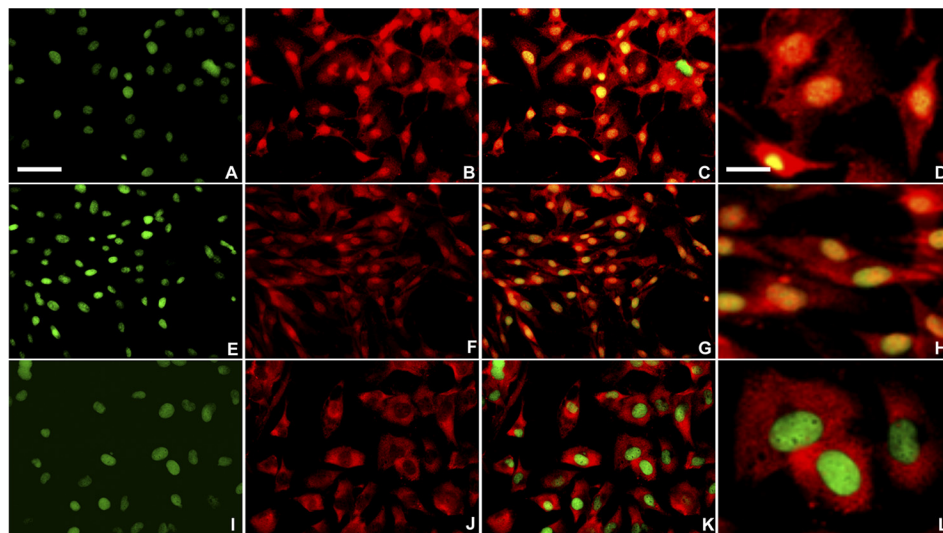
with the ErbB2 promoter results in transcriptional repression firefly luciferase. Thus, high luminescence compared with baseline levels is indicative of lower nuclear ZONAB activity. Consistent with immunofluorescence analysis of ZONAB, it was observed that Bves overexpression is associated with significantly reduced nuclear ZONAB activity (Fig. 5). These data are consistent with the hypothesis that Bves exerts control on cell proliferation through its participation in the ZO-1/ZONAB signaling pathway.

**DISCUSSION**

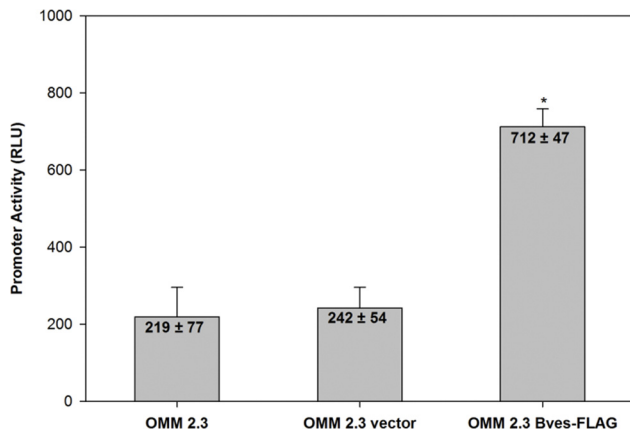
Our findings suggest that the TJ protein Bves reduces proliferative activity in uveal melanoma cells through its regulatory effects on TJ formation, which, in turn, modulate ZONAB transcriptional activity. In uveal melanoma cell lines overexpressing Bves, but not controls, an increase in TJ protein expression was observed (Fig. 1), as were reduced cell proliferation in two and three dimensions (Fig. 2) and reduced G<sub>0</sub>/G<sub>1</sub> to S cell cycle phase transition (Fig. 3). Both ZONAB

nuclear/cytoplasmic localization and transcriptional activity were shifted in response to overexpression of Bves in uveal melanoma cell lines. Specifically, overexpression of Bves resulted in a reduction of nuclear ZONAB (Fig. 4) and a reduction of ZONAB-associated transcriptional activity, as measured by dual luciferase reporter assay (Fig. 5).

The motivation underlying our study of the role of TJs in uveal melanoma stems from several reports suggesting that aberrant TJ signaling promotes cancer formation. Immunohistochemical studies demonstrated that the expression of TJ molecules ZO-1 and occludin are reduced or absent in several cancers and are associated with poor prognosis.<sup>34–37</sup> Reduced expression of ZO-1, occludin, and claudin-4 was correlated with poorly defined differentiation, higher metastatic frequency, and lower survival rates in gastric cancer.<sup>38</sup> The down-regulation of TJ proteins in cancer has been linked to the hypothesis that TJs are suppressors of cancer formation and progression.<sup>39</sup> Although the role of TJs in the progression of uveal melanoma has not been extensively investigated, we hypothesized that increasing Bves expression in uveal mel-



**FIGURE 4.** Localization of ZONAB in the OMM2.3 uveal melanoma cell line. Wild-type (A–D), empty vector control (E–H), or Bves-overexpressing cell lines (I–L) were analyzed for ZONAB nuclear association. Red: ZONAB; green: nucleus; yellow: nuclear colocalization of ZONAB. (D, H, L) High-magnification insets of overlay. Bves-overexpressing cells exhibit reduced colocalization of ZONAB with the nuclear compartment, as indicated by green nuclear fluorescence. Scale bars: 50 μm (A), 10 μm (D).



**FIGURE 5.** Dual luciferase ZONAB reporter assay. A luminometer was used to quantify ZONAB nuclear activity in wild-type, empty vector control, or Bves-overexpressing cell lines according to the dual luciferase reporter assay described in Methods. The relatively higher luciferase activity in Bves-overexpressing cells is indicative of reduced transcriptional repression by ZONAB in these cells, and therefore, reduced nuclear activity of ZONAB. Data are reported as mean  $\pm$  SEM ( $n = 4$ ; \* $P < 0.001$ ).

noma reduces the aggressiveness of cancer cells by enhancement of cell-cell contact and TJ-associated signaling mechanisms that inhibit proliferation.

We found that Bves reduces cell proliferation in uveal melanoma by modulating the nucleo-junctional localization of the protein ZONAB, according to a mechanism previously reported.<sup>40</sup> Specifically, Bves interacts with ZO-1 to form TJs, which in turn serve as a docking site for cell signaling proteins that drive cell polarization, morphogenesis, differentiation, and proliferation. One of the signaling proteins that bind to ZO-1 at TJs is ZONAB, a Y-box transcription factor that, when in the nucleus, drives transcription of PCNA and cyclin D1 genes for the promotion of cell proliferation.<sup>23-25,41-44</sup> These genes are involved in regulating cell cycle progression and may be involved in the alteration in cell cycle distribution observed on overexpression of Bves in uveal melanoma cells. The regulatory effect of Bves on TJ proteins (Fig. 1D) consequently increases extranuclear sequestration of ZONAB by ZO-1 (Fig. 4), thus inhibiting its accumulation in the nucleus, which is required for reduced proliferation.<sup>23</sup> By increasing the expression of Bves in uveal melanoma cells, the ZO-1/ZONAB interaction, initially recognized for its role in contact-inhibited cell proliferation in epithelial cells,<sup>23</sup> is activated. It is important to note, however, that the exact mechanism of ZONAB sequestration by TJs remains to be elucidated. Specifically, on overexpression of Bves in uveal melanoma cells, ZONAB appears to be associated with the nuclear region, but it is unclear whether ZONAB is in the nucleus or is merely bound to the nuclear membrane. To confirm the exact localization of ZONAB in cells overexpressing Bves, further ultrastructural analysis of ZONAB in cells is needed using confocal or electron microscopic techniques.

These studies establish a role of TJs in the proliferation of uveal melanoma. Ongoing studies in the laboratory are focused on the characterization of Bves function in uveal melanocytes and the role of Bves in cancer transformation of these cells. We hypothesize that Bves is responsible for maintenance of the melanocyte phenotype by establishing contact between the cell and microenvironment. Aberrant Bves signaling may be disrupted on cancer transformation in these cells. Restoration of Bves reduces proliferation rates in both primary and metastatic cell lines through a ZO-1/ZONAB signaling axis, suggesting that throughout disease progression, Bves signaling can partly reverse aggressiveness. On the other hand, Bves, as an adhesion mole-

cule, may play a role in facilitating metastatic cell seeding in distant organs; therefore, the complete role of Bves in melanoma must be elucidated to understand stage-specific functions of the protein. More studies are warranted to completely reveal the role of ZONAB in cancer initiation and progression. In normal cell types, ZONAB regulates cell proliferation in a density-dependent manner.<sup>23,45</sup> However, nuclear association of ZONAB in uveal melanoma cells occurred irrespective of cell density, suggesting that ZONAB trafficking and function are aberrant in cancer.

Current work is focused on the analysis of Bves expression in primary and metastatic uveal melanoma clinical specimens to investigate a potential diagnostic or therapeutic value for Bves. The interaction of Bves with other signaling pathways may reveal additional distinct roles for this protein in tumor cell migration, extravasation, and survival.

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