The interactions of Bcl9/Bcl9L with β-catenin and Pygopus promote breast cancer growth, invasion, and metastasis

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INTRODUCTION

Aberrant canonical Wnt signaling and the resulting dysregulation of β-catenin’s transcriptional functions play key roles in the progression of inflammation, fibrosis, and tumor formation [1, 2]. Canonical TGFβ signaling also plays a key role in the development of these pathological disorders and also efficiently activates an epithelial–mesenchymal transition (EMT) program [3, 4]. EMT is a multistage and central process during malignant tumor progression. It regulates the transitions between epithelial and mesenchymal states and cell plasticity, which contributes to intra-tumoral heterogeneity [5]. The reverse process, a mesenchymal–epithelial transition (MET), appears required for the colonization of metastatic cells in distant organs [6]. In breast cancer, driver mutations in Wnt or in TGFβ signaling pathways are rare, yet downstream effectors and target genes are often induced during malignant tumor progression and metastasis formation [7, 8]. While the functional contribution of TGFβ signaling to EMT, malignant tumor progression, and metastasis formation has been amply demonstrated, the role of Wnt signaling in late-stage breast cancer progression still remains unclear.

β-catenin has two roles: it is a critical component of cadherin-mediated cell–cell adhesion complexes and it is the transcriptional co-activator in canonical Wnt signaling. It interacts with a variety of proteins, including adhesion molecules, cytoplasmic signaling regulators, and nuclear transcriptional regulators [1, 9]. B-cell CLL/lymphoma 9 (Bcl9) and its paralog Bcl9-Like (Bcl9L) affected mammary gland carcinogenesis in the MMTV-PyMT transgenic mouse model of metastatic breast cancer. Conditional knockout of both Bcl9 and Bcl9L resulted into tumor cell death. In contrast, disrupting the interaction of Bcl9/Bcl9L with β-catenin, either by deletion of their HD2 domains or by a point mutation in the N-terminal domain of β-catenin (D164A), diminished primary tumor growth and tumor cell proliferation and reduced tumor cell invasion and lung metastasis. In comparison, the disruption of HD1 domain-mediated binding of Bcl9/Bcl9L to Pygopus had only moderate effects. Interestingly, interfering with the β-catenin–Bcl9/Bcl9L–Pygopus chain of adapters only partially impaired the transcriptional response of mammary tumor cells to Wnt3a and TGFβ treatments. Together, the results indicate that Bcl9/Bcl9L modulate but are not critically required for canonical Wnt signaling in its contribution to breast cancer growth and malignant progression, a notion consistent with the “just-right” hypothesis of Wnt-driven tumor progression.
cardiac defects in zebrafish and mice by a β-catenin-dependent mechanism, yet the defective embryos still maintained a broad transcriptional activity of β-catenin [16]. Finally, the interaction of Bcl9/Bcl9L with β-catenin is important for the maintenance of intestinal epithelial stem cells by selectively modulating Wnt/β-catenin-mediated transcriptional output [17]. Yet, other transcription factors may also interact with Bcl9/Bcl9L and β-catenin to modulate their transcriptional output, as for example shown for

Fig. 1  Canonical Bcl9/Bcl9L-dependent Wnt signaling during TGFβ-induced EMT. A Tcf and Smad-promoter luciferase reporter assays (Qiagen Cignal Lenti System) were used to monitor canonical Wnt-mediated and canonical TGFβ transcriptional outputs during TGFβ-induced EMT of Py2T cells. The graph represents luciferase activity units of Tcf and Smad reporters relative to negative controls Py2T cells 7 days after TGFβ treatment. Firefly luciferase activity measurements were normalized to Renilla luciferase activity. Data are presented as mean ± SEM. Statistical analysis was performed using the unpaired t-test. ***p < 0.001. B TOPflash (TCL/LEF-Firefly luciferase) and FOPflash (Neg control, mutated Tcf-Lef-binding site) luciferase reporter assay in Py2T cells treated with Wnt3a or TGFβ or both. Firefly luciferase activity measurements were normalized to Renilla luciferase activity. Data are presented as mean ± SEM. Statistical analysis was performed using the ordinary one-way ANOVA multiple comparison test. *p < 0.05; **p < 0.01; ***p < 0.001. C siRNA-mediated depletion of Bcl9 and Bcl9L prevents TGFβ-induced EMT. NMuMG/E9 cells were transfected with control siRNA (siCtrl) or siRNAs against Bcl9 (siB9), Bcl9L (siB9L) or both (siB9/B9L) 2 days before starting the TGFβ treatment for further 4 days. Immunofluorescence microscopy analysis visualized the epithelial markers tight junction protein-1 (ZO-1) and E-cadherin and the mesenchymal marker Vimentin. Fluorescently labeled phalloidin visualized the actin cytoskeleton, and nuclei were counterstained with DAPI. Scale bar, 100 µm. D siRNA-mediated depletion of Bcl9 and Bcl9L partially reverses TGFβ-induced EMT. Mesenchymal Py2T cells previously treated for >20 days (Py2T-LT) were transfected with control siRNA (siCtrl) or siRNAs against Bcl9 (siB9), Bcl9L (siB9L), or both (siB9/B9L). Immunofluorescence microscopy analysis visualized the epithelial markers tight junction protein-1 (ZO-1) and the mesenchymal marker Vimentin. Nuclei were counterstained with DAPI. Scale bar, 100 µm. E siRNA-mediated depletion of Bcl9 and Bcl9L reduces migration of Py2T-LT cells. Untreated epithelial Py2T cells or mesenchymal Py2T cells previously treated for >20 days (Py2T-LT) were transfected with siCtrl, siB9, siB9L or both (siB9/B9L) and allowed to migrate for 18 h along a serum gradient in a Transwell migration assay. Migrated cells were stained with DAPI and quantified relative to siCtrl-transfected cells. Data are presented as mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA multiple comparison test. *p < 0.05; **p < 0.01.
the Tbx3-mediated promotion of colorectal cancer cell metastasis [18].

Enhanced Wnt signaling regulates the maintenance of a stemness state in normal and tumorigenic tissues. For example, knockout of Bcl9/Bcl9L in a mouse model of colorectal cancer has resulted in the reprogramming of cancer cells from a stemness state to differentiation manifested by the downregulation of EMT-related gene expression [19]. Importantly, the gene signature of Bcl9/Bcl9L knockout cells is negatively associated with the high stemness subtypes of colorectal cancers and positively correlates with patient overall survival [20]. In breast cancer, BCL9 has been identified as a prognostic biomarker for high-risk human ductal carcinoma in situ (DCIS) [21]. Subsequently, BCL9 has been found to form a complex with STAT3 and to enhance the expression of the genes encoding for integrin β3 and its associated metalloproteinase MMP16 [22]. Another study has shown that nuclear BCL9L is associated with high nuclear grade and the expression of ErbB2/HER-2 in both DCIS and invasive ductal carcinoma (IDC) [23]. How BCL9 and BCL9L contribute to the breast cancer progression is not clear.

Here, we have analyzed the effect of interfering with the Bcl9/Bcl9L branch of Wnt signaling on tumor cell growth and invasion in a mouse model of metastatic breast cancer (FVB/N-Tg(MMTV-PyVT)634Mul/J) [24]. MMTV-PyMT transgenic mice form multiform tumors in mammary glands which progress from hyperplasia to adenoma to invasive carcinoma and finally seed metastases to the lungs, thus recapitulating the luminal B subtype of human breast cancer with ErbB2 overexpression [25]. Cell lines derived from tumors of these mice offer suitable models for TGFβ-induced EMT in vitro and in vivo [26, 27], and the blockade of TGFβ has inhibited tumor cell viability, migration, and lung metastases [28]. We have found that the disruption of the interaction of β-catenin with Bcl9/Bcl9L proteins in tumor cells of MMTV-PyMT transgenic mice causes a significant reduction in primary tumor growth and metastasis formation. Tumor cell proliferation is reduced, tumor cell differentiation is increased and EMT is prevented. The results suggest that in breast cancer Bcl9/9L-dependent Wnt-β-catenin signaling regulates genes promoting tumor cell growth and metastasis.

**RESULTS**

Wnt/β-catenin signaling is activated during TGFβ-induced EMT

We have previously induced EMT and MET in the Py2T and 1099-PyMT murine breast cancer cell lines derived from tumors of MMTV-PyMT transgenic mice and performed RNA-sequencing [26, 27, 29]. Differential gene expression analysis revealed that expression of many components of the Wnt signaling pathway substantially changed during TGFβ-induced EMT and TGFβ withdrawal-induced MET (Supplementary Fig. 1A). Py2T cells gradually transitioned from epithelial to mesenchymal cell morphology during 10 days of TGFβ treatment (Supplementary Fig. 1B). This phenotype change correlated with the loss of the expression of the epithelial cell adhesion molecule E-cadherin and an increased expression of mesenchymal markers, such as N-cadherin and fibronectin (Supplementary Fig. 1C). With the induction of EMT biomarkers, the expression of Bcl9, integrin α5 (Itga5), and β-catenin target genes, such as Axin2, Lef1, and Wls, was increased (Supplementary Fig. 1C, D). However, repeated experiments revealed that canonical Wnt signaling, as determined by immunoblotting for non-phosphorylated (activated) β-catenin, appeared to be activated already in epithelial cells. It is then found reduced during the early stages of EMT and to re-appear at the later stages of EMT. Total β-catenin was rather decreased during EMT most likely due to the dissolutions of adherens junctions during EMT and the subsequent degradation of some of β-catenin (Supplementary Fig. 1C).

Next, we assessed whether β-catenin/Tcf transcriptional activity was increased during TGFβ-induced EMT using a Tcf motif-containing luciferase reporter construct. A construct containing Smad-binding motifs was used as a control of effective EMT induction. Treatment of Py2T cells with TGFβ for 7 days significantly increased both Smad-mediated and β-catenin/Tcf-mediated transcriptional activities (Fig. 1A). In addition, in Py2T cells transfected with the TCF reporter plasmid TOPFlash treatment with TGFβ or the canonical Wnt ligand Wnt3a comparably induced β-catenin’s transcriptional activity (Fig. 1B). These results raise the possibility that canonical Wnt signaling may contribute to TGFβ-induced EMT in murine breast cancer cells.

Bcl9 and Bcl9L contribute to TGFβ-induced EMT in vitro

To test the role of Wnt signaling in TGFβ-induced EMT, we ablated the expression of Bcl9 and Bcl9L alone (siB9 and siB9L) or together (siB9/B9L) during TGFβ-induced EMT in non-tumorigenic normal murine mammary gland epithelial cells (NMuMG/E9) and in tumorigenic Py2T cells [26, 29, 30]. Cells were transfected with control siRNA (siCtrl), siB9, siB9L or siB9/B9L 2 days before TGFβ treatment for 4 days to monitor potential effects on EMT initiation. siRNA-mediated knockdown efficiently reduced the levels of Bcl9 and Bcl9L proteins (Supplementary Fig. 2A). Morphogenic changes during EMT were assessed by immunofluorescence microscopy analysis of the expression and localization of epithelial and mesenchymal markers. Regardless of the levels of Bcl9 and Bcl9L, in the absence of TGFβ treatment the cells retained their epithelial phenotype (Supplementary Fig. 2B). In contrast, treatment with TGFβ initiated EMT in siCtrl-transfected NMuMG cells; the tight junction protein Zonula occludens-1 (ZO-1) and the adherens junction protein E-cadherin were down-regulated, mesenchymal markers, such as vimentin, were upregulated and actin stress fibers formed, as determined by phalloidin staining (Fig. 1C). Compared to siCtrl-transfected cells, transfection with siB9/B9L and to a lesser extent with siB9L inhibited TGFβ-induced EMT: these cells still formed epithelial cell clusters and expressed ZO-1 and E-cadherin at their cell membranes (Fig. 1C). Knockdown of Bcl9 alone was not sufficient to substantially affect the induction of EMT, although the protein was substantially depleted (Supplementary Fig. 2A).

To study the functional contribution of Bcl9 and Bcl9L to the maintenance of an EMT process, Py2T cells were treated for >20 days with TGFβ to undergo a full EMT (Py2T-LT) and then transfected with siCtrl, siB9, and siB9L. Cells transfected with siB9L alone or in combination with siB9 partially returned to an epithelial morphology and re-expressed ZO-1 at the cell membranes to form epithelial cell clusters (Fig. 1D).

Since EMT is strongly implicated in cancer cell migration [3], we tested the effects of siB9/B9L knockdown on the ability of Py2T cells pretreated with TGFβ for 20 days (Py2T-LT) to migrate toward a serum gradient using a Transwell migration assay. Knockdown of siB9 and siB9L, and most efficiently the combination of both, caused a significant reduction of Py2T cell migration (Fig. 1E).

In conclusion, the results show that Bcl9 and more prominently Bcl9L contribute to the initiation and maintenance of TGFβ-induced EMT and cell migration of cultured mammary tumor cells in vitro, but that they are not exclusive determinants of these TGFβ-induced processes.

Bcl9 and Bcl9L are highly expressed in invasive carcinomas

Previous studies have shown that Bcl9 and Bcl9L are expressed during the different stages of mouse mammary gland development, with a reduction of Bcl9L expression during post-lactational involution [31]. Motivated by this and the knowledge of a functional requirement of Bcl9 and Bcl9L for EMT in MMTV-PyMT-derived tumor cell lines in vitro, we next studied the expression of

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both proteins during the different stages of tumor progression in mammary glands of MMTV-PyMT transgenic mice in vivo. MMTV-PyMT mammary tumors start to form upon activation of the MMTV promoter during puberty. Tumors progress from hyperplasia to adenoma and finally to an IDC phenotype. This progression correlates with a reduced expression of hormone receptors in the advanced carcinoma stages and with an increase of lung metastasis at the age of 12–14 weeks (Fig. 2A) [24]. Immunohistochemical staining for Bcl9, Bcl9L, and β-catenin protein on paraffin sections from primary tumors of MMTV-PyMT revealed that cytoplasmic and nuclear Bcl9 and Bcl9L and nuclear β-catenin increased during the progression to IDC (Fig. 2B).
Murine and human Bcl9 and Bcil9L proteins are highly conserved (Supplementary Fig. 2C). To examine the relevance of Bcl9 and Bcil9L in tumor progression and invasion in breast cancer patients, we first analyzed the genetic alteration of both genes in breast cancer patient data (from TCGA-BRCA PanCancer Atlas data collection). These data showed a frequency of gene alterations of 11% for BCL9 and of 1.3% for BCL9L, with a frequency of 9.13% and 0.28% for gene amplification, respectively (Supplementary Fig. 3A). Heatmap analysis indicated a high expression of BCL9 and BCL9L in patients with any kind of genetic alterations in their BCL9 and BCL9L genes (cBioportal online tool) (Supplementary Fig. 3A).

The frequency of BCL9L gene amplification in different breast cancer subtypes is highest in basal and luminal B breast cancer subtypes, with less incidence in the other subtypes and none in normal-type breast cancer (Fig. 2C). BCL9 amplification correlated with poor prognosis in breast cancer patients (cBioportal online tool) (Fig. 2D). Consistent with our analysis, using a separate breast cancer data set (provisional TCGA; 959 cases) Elsarraj et al. reported significant levels of gene alteration for BCL9 (26%) and BCL9L (5%) in invasive breast cancers, mainly through gene amplification and partly by mRNA upregulation [21]. Using the same TCGA breast cancer data set we also found that low expression levels of BCL9L gene were a prognostic marker in ER-PR- patients (Supplementary Fig. 3B–D). In contrast, the high gene amplification and expression of BCL9 observed in this patient data set did not reveal a significant correlation with disease outcome in any of the breast cancer subtypes (Supplementary Fig. 3B–D).
These results show that Bc9/Bc9L proteins are expressed and potentially deregulated in invasive carcinoma in the MMTV-PyMT mouse model and in breast cancer patients.

**Complete loss of Bc9 and Bc9L functions affects tumor cell survival**

To investigate the functional relevance of Bc9 and Bc9L during tumor progression and invasion, we genetically deleted both Bc9 and Bc9L genes in mammary tumor cells of MMTV-PyMT mice. The simultaneous targeting of both genes was chosen to avoid any compensatory effect. MMTV-PyMT mice were crossed with Bc9\textsuperscript{fl/fl} and Bc9L\textsuperscript{fl/fl} mice [19] and with MMTV-Cre mice, expressing Cre-recombinaise exclusively in mammary epithelial cells [32]. Bc9\textsuperscript{fl/fl};Bc9L\textsuperscript{fl/fl};MMTV-PyMT mice and with or without MMTV-Cre expression were designated B9/B9L\textsuperscript{fl/fl} and B9/B9L\textsuperscript{fl/fl};MCre genotype mice, respectively (Fig. 3A). Surprisingly, at the age of 12 weeks, tumor growth (tumor mass) and the number of lung metastases were similar in B9/B9L\textsuperscript{fl/fl};MCre mice and B9/B9L\textsuperscript{fl/fl} control mice (Fig. 3B). There were also no significant differences in the percentage of hyperplasia, adenoma, and carcinoma areas and in the number and size of lung metastases between B9/B9L\textsuperscript{fl/fl} control and B9/B9L\textsuperscript{fl/fl};MCre mice (Fig. 3C–E). The reason seems to be a failure to delete both Bc9 and Bc9L genes in these tumors (Supplementary Fig. 3E). Immunohistochemical staining also revealed that Bc9\textsuperscript{fl} and Bc9L\textsuperscript{fl} proteins were both still expressed in cytosols and nuclei of tumor cells of MCre\textsuperscript{+} genotype (Fig. 3F), suggesting either a low recombination efficiency or a competitive selection against recombined tumor cells.

To confirm the functionality of Cre recombinase expressed by MMTV-Cre, B9/B9L\textsuperscript{wt/wt};Mpy/Mcre and B9/B9L\textsuperscript{fl/fl};Mpy/Mcre mice were crossed with GFP-reporter mice (R26-LSL-GFP). At 5 weeks of age, when Cre is first expressed in mammary epithelial cells, the amount of GFP was comparable between B9/B9L\textsuperscript{wt/wt};MCre mice and B9/B9L\textsuperscript{fl/fl};MCre mice (Supplementary Fig. 3F, G). Interestingly, at 12 weeks of age the percentage of GFP-positive cells was strongly reduced in B9/B9L\textsuperscript{fl/fl};Mpy/Mcre mice (Supplementary Fig. 3F, G). These results suggest there is a selection against tumor cells lacking both Bc9 and Bc9L.

To further validate whether there was a selection against tumor cells lacking both Bc9 and Bc9L, we examined whether the genetic deletion of Bc9/Bc9L affected tumor cell survival. We established cell lines from tumors of B9/B9L\textsuperscript{wt/wt}, B9/B9L\textsuperscript{fl/fl}, and B9/B9L\textsuperscript{fl/fl} genotypes and infected these cells with Adenoviruses expressing either only GFP (Ad-ires-GFP) or expressing Cre recombinase and GFP (Ad-Cre-ires-GFP) at a multiplicity of infection of 25 or 50. After 24 or 48 h, the percentage of Annexin V\textsuperscript{+} apoptotic cells was measured by flow cytometry. Consistent with the hypothesis counterselection, expression of Cre-recombinase in B9/B9L\textsuperscript{fl/fl} cells induced apoptosis, in particular after 48 h of viral infection (Fig. 3G).

Together these results indicate that the complete loss of Bc9 and Bc9L function in mammary tumor cells provokes their apoptotic death. As a consequence, there is a selection against Bc9/Bc9L-deficient cells. Such a lethal effect upon complete loss of Bc9 and Bc9L expression has also been reported during embryonic development [13].

**The interactions of Bc9/Bc9L with Pygopus moderately contribute to tumor progression**

As shown above, Bc9/Bc9L contribute to the initiation and maintenance of Tgfb\textsuperscript{-} induced EMT and cell migration in vitro, yet the complete loss of Bc9/Bc9L has led to tumor cell apoptosis in vitro and in vivo. We thus were motivated to investigate the specific contribution of Bc9 and Bc9L to β-catenin-mediated Wnt signaling and as a consequence to tumor progression in the MMTV-PyMT transgenic mouse model of breast cancer. Toward this end, we first used knock-in mouse lines carrying in-frame deletions of the conserved HD1 (Bc9/Bc9L\textsuperscript{ΔHD1/Δ}) [13]. This deletion ablates the interactions of Bc9 and Bc9L with Pygopus. Since B9/B9L double-homozygous mutants of HD1 were lethal [13], we crossed heterozygous Bc9\textsuperscript{fl};Bc9L\textsuperscript{fl} mutant mice with MMTV-PyMT transgenic mice to induce mammary tumor formation and with MMTV-Cre mice to delete the floxed allele in mammary tumors of Bc9\textsuperscript{fl};Bc9L\textsuperscript{ΔHD1/Δ} mutant mice. In Bc9\textsuperscript{fl};Bc9L\textsuperscript{ΔHD1/Δ};MMTV-PyMT;MMTV-Cre mice (B9/B9L\textsuperscript{ΔHD1/Δ};MCre), interaction of B9/B9L with Pygopus was disrupted (Fig. 4A). At 12 weeks of age of this mice, primary tumor growth was significantly reduced (Fig. 4B). Disruption of the B9/B9L-Pygopus interactions also caused a reduction of tumor progression and invasive phenotypes in primary tumors (Fig. 4C). Likewise we also observed a reduction in the numbers of lung metastasis and their nodular outgrowth (Fig. 4D, E). A comparable reduction in tumor progression was observed in B9/B9L\textsuperscript{ΔHD1/Δ} mice without MCre, yet not in B9/B9L\textsuperscript{fl/fl} mice with MCre. This suggests that the B9/B9L-
ΔHD1 mutant proteins exerted a dominant-negative effect over wild-type B9/B9L and that there was no haplosufficient effect caused by the ablation of single alleles of Bcl9 and Bcl9L.

To delineate the basis for the repressive effect of B9/B9L-ΔHD1 on primary tumor growth, we monitored potential changes in tumor cell proliferation and in tumor cell apoptosis by quantification of immunofluorescence staining for the mitosis marker phospho-histone 3 (pH3) and for the early apoptosis marker cleaved Caspase-3 (clCasp3), respectively (Supplementary Fig. 4A, B). These analyses did not show any significant differences in the rates of tumor cell proliferation or of apoptosis in B9/B9L-ΔHD1 tumors compared to wild-type controls (Fig. 4F, G). This might explain why the effect of the ΔHD1 mutations were relatively moderate on primary tumor growth and metastasis formation. In summary the interaction of Bcl9/Bcl9L with Pygopus appears to play only a limited role in the regulation of breast cancer tumor progression.

The interactions of Bcl9/Bcl9L with β-catenin substantially contribute to tumor progression

We next ablated the specific interaction of Bcl9 and Bcl9L with β-catenin by using knock-in mouse lines carrying an in-frame deletion of their conserved HD2 domains [13]. Since B9/B9L double-homozygous mutants of HD2 were lethal [13], we crossed heterozygous Bcl9/Bcl9LΔHD2/+ transgenic mice with MMTV-PyMT transgenic mice to induce mammary tumor formation and with MMTV-Cre mice to delete the floxed alleles in mammary tumors of Bcl9/Bcl9LΔHD2/+ mutant mice (Fig. 5A). We then assessed how disrupting the interaction of B9/B9L with β-catenin affected primary tumor growth, tumor progression, and metastasis...
Fig. 5 Disrupting the interaction of B9/B9L with β-catenin substantially reduces primary tumor growth and malignant tumor progression and metastasis. A Schematic representation of the knock-in strategy to express the ΔHD2 mutant forms of Bc9 and Bc9L and thus disrupting the interaction of Bc9 and Bc9L with β-catenin in mammary tumor cells of MMTV-PyMT transgenic mice. Mice carrying floxed alleles of Bc9 and Bc9L and carrying the knockin ΔHD2 mutant alleles of Bc9 and Bc9L were crossed with MMTV-PyMT and MMTV-Cre mice to generate composite transgenic mice expressing exclusively the ΔHD2 mutant forms of Bc9 and Bc9L in mammary tumor cells (B9/9LΔHD2/+;MMTV-PyMT;MMTV-Cre). B Tumor growth in heterozygous B9/9LΔHD2/+ transgenic mice with (n = 16) or without MCre (n = 14) compared to controls B9/9Lwt/+ mice with (n = 20) or without MCre (n = 25) expression. Tumor mass from 12-week-old females was calculated as total weight of thoracic, abdominal, and inguinal mammary glands with tumors lesions. Each dot represents one mouse. Data are presented as mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA multiple comparison test. **p < 0.01; ***p < 0.001; ****p < 0.0005. The bar graph represents the effects of tumor cell-specific expression of the ΔHD2 mutant forms of Bc9 and Bc9L on malignant tumor progression of primary tumors in Bc9/BCc9LΔHD2/+;MMTV-PyMT;MMTV-Cre transgenic mice. Quantification of tumor stages (hyperplasia, adenoma, and carcinoma) are shown for primary tumors based on histological analysis of stitched microscopy images of different tumor areas. Data are presented as mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA multiple comparison test. *p < 0.05; **p < 0.01. D Number of lung metastasis in heterozygous B9/9LΔHD2/+ transgenic mice with (n = 16) or without MCre (n = 14) compared to controls B9/9Lwt/+ mice with (n = 20) or without MCre (n = 25) expression. Lung metastasis nodules were counted in paraffin-embedded lung tissues, which were serially sectioned and stained with H&E. Each dot represents the total number of lung metastases per mouse. Data are presented as mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA multiple comparison test. *p < 0.05; **p < 0.01. E The bar graph represents the effects of tumor cell-specific expression of the ΔHD2 mutant forms of Bc9 and Bc9L on the outgrowth of lung metastases. The size of lung metastasis was determined in serially sectioned paraffin-embedded lung tissues isolated from the mice described in D. F Quantification of immunostaining for the mitosis marker phospho-histone 3 (pH3) on tumor sections of B9/9LΔHD2/+ transgenic mice with or without MCre compared to control B9/9Lwt/+ mice with or without MCre. Each data point represents one histological section. Data are presented as mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA multiple comparison test. ***p < 0.01; ****p < 0.0005. G Quantification of immunostaining for the apoptosis marker cleaved Caspase-3 (cCasp3) on tumor sections of B9/9LΔHD2/+ transgenic mice with or without MCre compared to control B9/9Lwt/+ mice with or without MCre. Each data point represents one histological section. Data are presented as mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA multiple comparison test. **p < 0.01; ***p < 0.001; ****p < 0.0005.

formation. At 12 weeks of age, primary tumor growth, tumor progression to invasive cancer and the numbers and sizes of lung metastases were significantly reduced in B9/9LΔHD2/+;MCre mice (Fig. 5B–E). Similar to the B9/9L−ΔHD1, B9/9L−ΔHD2 seemed be dominant over wild-type B9/9L tumor growth and progression were comparable between B9/9LΔHD2/+;MCre mice and B9/9LΔHD2/+;MCre mice, while B9/9LΔHD2/+;MCre mice did not show any difference to B9/9Lwt/+ mice (Fig. 5B–E), again excluding a haplosufficient effect. Immunofluorescence microscopy analysis of tumor sections stained with PH3 or cCasp3 revealed that the reduced tumor growth in B9/9L−ΔHD2-expressing mice was due to both diminished tumor cell proliferation and increased apoptosis (Fig. 5F, G and Supplementary Fig. 4A, B).

We next assessed whether a mutation of the N-terminal domain in β-catenin, which abrogated the interaction with both Bc9 and Bc9L on the side of β-catenin, recapitulated the effects of B9/9L−ΔHD2 in breast cancer progression. To this end, we employed knock-in mouse lines carrying a N-terminal point mutation abrogating the binding of Bc9 and Bc9L to β-catenin (D164A) [33]. Mice carrying one mutated allele and a conditional allele of β-catenin (designated as β-cateninΔD164A/+;MMTV-PyMT;MMTV-Cre mice = D164A/−) (Fig. 6A). At 12 weeks of age, the composite transgenic mice expressing only the mutant forms of
β-catenin showed a significantly lower tumor mass (Fig. 6B). The impairment of β-catenin binding to Bcl9/BcI9L also significantly reduced tumor progression and lung metastasis formation (Fig. 6C–E). Notably, composite transgenic mice harboring one allele of the mutant forms and still one allele of a conditional (fl) but fully functional allele of β-catenin also showed reduced tumor growth and metastasis formation, indicating a dominant-negative effect of β-catenin-D164A as observed with the mutant forms of Bcl9/BcI9L. Also here, we exclude a haploinsufficient effect of the loss of one allele of β-catenin, since tumor progression was not affected upon loss of only one allele of β-catenin in β-cateninΔHD2ΔHD1 mouse without Cre expression (fl/fl; n = 21), but significantly lower tumor mass (fl/fl; n = 19) and reduced tumor progression and lung metastasis formation (Fig. 6B, D, and Supplementary Fig. 5C). However, the expression of canonical Wnt signaling target genes c-Myc and Cyclin D1 known to regulate cell proliferation, like Axin 2 was not significantly affected by the abrogation of BcI9/BcI9L-β-catenin interactions in D164A cells (Supplementary Fig. 5D). Critically, the N-terminal mutation did not affect the interaction of β-catenin with E-cadherin: immunoprecipitation experiments (Supplementary Fig. 5E) and immunofluorescence microscopy analysis of co-localization at cell membranes (Supplementary Fig. 5F) demonstrated that the interaction with E-cadherin was retained by the D164A mutant form of β-catenin.

These data show that the interactions of BcI9 and BcI9L with β-catenin play a critical role in tumor cell proliferation, primary tumor outgrowth, malignant tumor progression, and metastasis formation.

BcI9/BcI9L-Pygopus-β-catenin interactions modulate gene expression

To examine the effects of ΔHD1, ΔHD2, and D164A knock-in mutations on Wnt/β-catenin and TGFβ-mediated transcriptional
Fig. 7 Comparative gene expression profiling of tumor-derived cell lines with defined B9/B9L and β-catenin mutations. A, B Primary epithelial tumor cells with B9/B9L wt/wt, wt/Δ, ΔHD1/Δ, ΔHD2/Δ, and Δβ-catenin 0/0 and D164A/Δ genotypes were isolated from mammary tumors of the various genotype mice. B9/B9L wt/wt, ΔHD1/Δ, ΔHD2/Δ, and Δβ-catenin D164A/Δ cell lines were generated by infection with Creexpressing Adenoviruses (Ad-Cre-IRES-GFP). Cells with and without Cre expressions were treated with no cytokine or Wnt3a (3 days, 100 ng/ml). Tumor-derived cell lines expressing exclusively wild-type B9/B9L (wt/wt, wt/Δ) or exclusively B9/B9L mutants (ΔHD1/- and ΔHD2/-) (A) or exclusively wild-type β-catenin (fl/fl-1 and fl/fl-2) or β-catenin-D164A (D164A/-) (B) were transfected with superTOPflash (TC/LEF-Fluciferase) or superFOPflash (mutated TCF-LEF-binding site) and treatment with or without Wnt3a for 3 days and canonical Wnt signaling output was determined by bioluminescence assays. Relative transcriptional output was calculated relative to wild-type cells in the absence of Wnt3a. Statistical analysis was performed using ordinary one-way ANOVA multiple comparison test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0005. C, D Cell lines derived from tumors of the various genotype MMT-PyMT transgenic mice were treated with recombinant Wnt3a (3 days; 100 ng/ml) or recombinant TGFβ (4 days; 2 ng/ml) and subjected to next-generation RNA-sequencing. Venn diagrams showing the overlap of differentially expressed genes between B9/B9L ΔHD1/-, B9/B9L ΔHD2/-, and Δβ-catenin D164A/- mutant cell lines compared to their wild-type control cell lines upon treatment with Wnt3a (C) and TGFβ (D). RNA changes with p ≤ 0.05 and a fold change ≥ 1.5 were considered differentially expressed. For the gene lists shared by the B9/B9L/Δβ-catenin interaction mutants ΔHD2/- and D164/- please see Supplementary Tables I and II.

output we established cell lines derived from tumors with the defined B9/B9L and β-catenin genotypes. Floxed alleles of Bcl9, Bcl9L, and β-catenin genes were recombined by infection with Adenovirus expressing Cre-recombinase and GFP (Ad-Cre-IRES-GFP). Adenovirus-transduced cells were sorted by flow cytometry for GFP expression, and their genotype was confirmed by PCR analysis. Wnt3a-induced β-catenin-mediated transcriptional activity was then determined using a Super TOPflash/FOPflash promoter-reporter assay (TC/LEF reporter). β-catenin/TCF-dependent transcriptional output was diminished in both ΔHD1/- and ΔHD2/- mutant cells (Fig. 7A), while β-catenin-D164A cells showed only a slight reduction in Wnt3a-induced transcriptional output as compared to two independent β-catenin-0/0 cell lines expressing exclusively wild-type β-catenin (Fig. 7B).

These cell lines were then used for whole transcriptome analysis by RNA-sequencing and comparative gene expression profiling under three conditions: no cytokine control or treatments with either Wnt3a (100 ng/ml) for 3 days or TGFβ (2 ng/ml) for 4 days. The reason for the rather long-term cytokine treatment was based on the observation that profound cell morphology changes were best detected after such time spans (Supplementary Fig. 6A). ΔHD1/-, ΔHD2/-, and D164A/- mutant cell lines expressed exclusively B9/B9L-ΔHD1, B9/B9L-ΔHD2, or β-catenin-D164A, while ΔHD1/-, ΔHD2/-, and D164A/- mutant cell lines expressed B9/B9L-ΔHD1, B9/B9L-ΔHD2, or β-catenin-D164A and the floxed (wild-type) alleles. Hemizygous control wt/Δ cells expressed only one wild-type allele of B9/B9L genes, while fl/fl cells expressed two floxed alleles which behaved as wild-type alleles. Unsupervised hierarchical clustering of the top 500 most variable genes between the various cell lines and the particular treatments revealed that the cell lines did not cluster according to their genotypes but rather to the treatments with Wnt3a or TGFβ.
DISCUSSION

Most breast cancer deaths are due to the growth of metastatic cells in vital organs. Both canonical Wnt and TGFβ-signaling are key pathways in the regulation of cellular state, cancer cell proliferation, and cancer cell invasion. In breast cancer the components of both pathways are rarely mutated, yet both pathways are frequently found activated in malignant and metastatic breast cancer [8, 34]. The functional interactions between these morphogenic signaling pathways are only poorly understood during the process of an EMT and the malignant progression and metastasis of breast cancer [4, 5, 29].

Here, we have dissected the roles of the interactions of Bc9 and Bc9L with Pygopus and with β-catenin during tumor progression and metastasis formation in the MMTV-PyMT mouse model of metastatic breast cancer. We have employed mouse lines carrying conditional alleles of Bc9/Bc9L and of Bcl9L with Pygopus and with β-catenin [13]. B9/B9L-ΔHD2 fail to bind to Bc9 and Bc9L may be critical for the expression of a number of genes, yet that these genes may not specify particular biological processes. Hence, the subtle changes in gene expression may underlie the modulatory role of Bc9 and Bc9L on β-catenin-mediated Wnt signaling and tumor progression (see “Discussion”).

( Supplementary Fig. 6B). This result suggests that the deficiencies in Bc9/Bc9L-Pygopus and β-catenin interactions were not dramatically affecting overall gene expression.

We thus analyzed which genes were differentially expressed upon treatment with Wnt3a or TGFβ and whether their expression was affected by the disruption of B9/B9L-β-catenin binding in the B9/B9L-ΔHD2 mutant and the β-catenin-D164A mutant cell lines. Only 38 Wnt3a-regulated genes were affected by the disruption of B9/B9L-β-catenin binding, while 229 TGFβ-regulated genes changed their expression upon loss of B9/B9L-β-catenin binding (Fig. 7C, D). Notably, these genes lists were not apparently enriched in any specific Wnt or TGFβ signaling target genes or any specific biological pathways (Supplementary Tables I and II). These results suggest that the interaction between Bc9 and Bc9L and β-catenin may be critical for the expression of a number of genes, yet that these genes may not specify particular biological processes.

Since the RNA-sequencing of cell lines expressing the mutant forms of B9/B9L and β-catenin did not reveal major changes, we postulate the interaction is critical for fine-tuning Wnt/β-catenin signaling during malignant breast cancer progression. In particular, we could not identify Wnt and TGFβ-specific gene signatures. It rather appeared that the Bc9/Bc9L-β-catenin binding deficiency caused a moderate decrease of the expression of genes of various pathways. Thus, Bc9 and Bc9L may promote mammary tumor growth and metastasis formation in MMTV-PyMT mice in a rather moderate manner and by fine-tuning the expression levels of direct and indirect Wnt target genes. These results are reminiscent of other studies where the disruption of Bc9 and Bc9L functions had no effect on normal tissue homeostasis, but moderately repressed carcinogenesis, an observation consistent with the “just-right” hypothesis of Wnt-driven tumor formation [39, 40]. Hence, the question remains whether Bc9 and Bc9L aid in the selection of specific β-catenin target genes or modulate the quantitative difference in β-catenin-dependent transcriptional output on specific target genes. Along these lines, loss of the specific B9/B9L-β-catenin/Pygopus interactions also affected the TGFβ-mediated signaling output. A functional interaction between Wnt and TGFβ signaling during EMT and other pathophysiological processes has been demonstrated before [42–44]. Our results underscore the importance of this crosstalk. However, a more complete picture is missing because of the challenges of identifying specific Wnt and TGFβ target genes in different cell types and in varying cell contexts.

Analysis of the expression of Bc9 and Bc9L revealed that both proteins are highly expressed during mammary tumor progression in MMTV-PyMT transgenic mice. Database mining indicated that Bc9L and only to a lesser extent Bc9L are found mutated in breast cancers of patients. Bc9L gene amplification appeared to be the most frequent genetic alteration in breast cancer with highest levels of amplifications in basal type and lowest in normal-type breast cancers. However, Bc9L gene alterations correlated with poor disease and progression-free survival of patients. Altogether, these results suggest the development of Bcl9L is critically players in breast carcinogenesis. These results are also consistent with previous work identifying nuclear BCL9 as a molecular driver of breast cancers. However, Bc9L and Bc9L may promote mammary tumor growth and metastasis formation in different cell types and in varying cell contexts.

The increased expression of Bc9L and Bc9L may be caused by downregulation of microRNAs, such as mir30a [45, 46], mir-30c-2

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 MATERIAL AND METHODS
See also Supplementary Material and Methods.

Mouse experiments
Mouse colonies were kept at the animal facility of the Department of Biomedicine, University of Basel, Switzerland. All animal experiments were carried out in accordance with the guidelines of the Swiss Federal Veterinary Office and the Cantonal Veterinary Office of Basel-Stadt. To examine the conditional ablation of Bcl9/Bcl9L in breast cancer cells, Bcl9fl/fl and Bcl9Lfl/fl mice [19] were crossed with MMTV-PyMT (a kind gift of N. Hynes, FMI, Basel, Switzerland) [24, 57] and MMTV-Cre (a kind gift of Lothar Henninghausen, NHI, Bethesda, USA) mice [32]. Bcl9/Bcl9Lfl/fl–MMTV-PyMT, and MMTV-Cre mice were then crossed with Bcl9/Bcl9L

Cell and tumor genotyping
To extract genomic DNA, cells from a confluent 10 cm petri dish were trypsinized, washed in PBS, and pelleted by centrifugation. DNA was extracted using GenElute™ Mammalian Genomic DNA Miniprep Kits (G1N70, Sigma-Aldrich) according to the manufacturer's protocol. Standard PCR was performed using the following primers: for the β-catenin floxed and mutant allele, sense primer RM41 (5′-AAG GTA GAG TGA AAG TGG TT-3′) and antisense primer RM42 (5′-CAC CAT GTC CTC TGT CTA TCT-3′) were used, generating 324 and 221 bp products from the floxed and mutant alleles, respectively. To detect the floxed allele, sense primer RM68 (5′-AAAT CAC AGG GAC TTC GTG AG-3′) and antisense primer RM69 (5′-GCC CAG CCT TAG CCC AAC T-3′) were used generating a 631 bp product from the deleted allele [58].

Genotyping Bcl9 loxP site (expected band: 350 bp for loxP and 600 bp WT)

Bcl9 forward primer: (5′-TGAGAAGACGGAGAGCTGTTC-3′).
Bcl9 reverse primer: (5′-ACACCCCGGAGGCTCTC-3′).

Genotyping Bcl9 WT and recombined (expected band: 254 bp for recombined: 162 bp for WT, and 400 bp for Lox)

Bcl9 D1 forward primer: 5′-CCACCCAGAATCCAGCAGTCTG-3′.
Bcl9 D2 reverse primer: 5′-CCCTGCTAGCTGTGGTTGC-3′.
Bcl9 D3 reverse primer: 5′-GGGTCCTGAAGTCTGTGTCTG-3′.

Cell line derivation
Cells were isolated from thoracic mammary glands with tumors lesions of 12-week-old MMTV-PyMT transgenic female mice (FVB/N background). Small tumor pieces were minced, and 10 ml of predigestion buffer (10 mM Hepes pH 7.4, 14 mM NaCl, 0.67 mM KCl, 1 mM EDTA supplemented with 50 g/ml gentamycin (Sigma, G1397) and 1X antibiotic-antimotic (15240062, Thermo Fisher Scientific) were added and incubated with 30 min at 37 °C on a shaker. After washing with PBS, the samples were digested using 6 ml digestion buffer (10 mM Hepes pH 7.4, 142 mM NaCl, 0.67 mM KCl, 0.67 mM CaCl2, 20 mM Glucose supplemented with 1 mg/ml Collagenase D (Roche, 11088858001), 50 g/ml gentamycin (Sigma-Aldrich, G1397) and 1X antibiotic-antimotic (15240062, Thermo Fisher Scientific) and incubated for 30 min at 37 °C on a shaker. Subsequently the samples were washed with PBS and then resuspended in growth medium and seeded in 10 cm plates. The medium was changed regularly and fibroblasts in culture were removed by differential trypsinization until only epithelial cells remained. The cell lines were further cultured in DMEM supplemented with glucose, penicillin, streptomycin, and 10% heat-inactivated FBS (F752, Sigma-Aldrich) and 10% horse serum (Amimed). To establish cell lines only expressing the mutant alleles, cells carrying one floxed allele and one mutant allele were seeded into 10 cm plates and on the next day infected either with Adeno-CRE-IRE5-GFP or with the Adeno-IRE5-GFP virus as control using FuGENE HD Transfection Reagent (E2311, Promega). Next day, the medium was changed, and after 3 days the cells were sorted for GFP-positive cells into 24-well plates using a BD FACSAria Flow Cytometer (BD Biosciences). Detachment of the cells was performed using trypsinization followed by two-times washing in 1× PBS and resuspension in 2% FBS, PBS and syringe filtering (40 μm mesh filter) immediately before FACs sorting into a polystyrene round bottom tube (302504, FALCON) filled with DMEM medium. After centrifugation, supernatants were discarded, and cells were resuspended and seeded into 24-well plates.

Adenovirus infection
Cells were plated onto 6 cm dishes in duplicates and transfected the next day with Adeno-CRE-IRE5-GFP or with Adeno-IRE5-GFP virus as a control (1710 and 1761, Vector Biolabs) using Fugene HD transfection reagent (E2311, Promega). The following day, medium was changed and cell culture continued.

Luciferase reporter assay
The firefly luciferase reporter constructs superTOPflash and superFOPflash (kindly provided by Konrad Basler, UZH Zürich) were used to quantify Wnt/β-catenin-mediated transcriptional output. Cells were plated in duplicates in 24-well plates and transfected the following day with 500 ng of the
superTOPflash or superFOPflash firefly luciferase reporter plasmid and 10 ng of a constitutive-active Renilla luciferase plasmid using Lipofectamine 3000 Reagent (Invitrogen) according to the manufacturer’s instructions. Luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (E1960, Promega). Firefly luciferase values were normalized to Renilla luciferase control values. For Smad and TCF reporter assays, we used the Smad reporter, TCF reporter and the Negative Control from the Cignal 45-Pathway Reporter Array (Qiagen, Lenti Reporter Assays). This assay is based on dual-luciferase technology and Negative control serves as a specificity control. The Smad reporter consists of Smad2/3/4 transcription factor-responsive firefly luciferase construct and a constitutively expressing Renilla luciferase construct. Cells were transduced with lentiviral particles for assessing canonical TGFβ activity, which is determined by comparing the normalized luciferase activities of the reporter in treated versus untreated cells.

siRNA transfections
NMuMG/E9, Py2T, and Py2T-LT cells (TGFβ > 20 days) were transfected with Silencer select siRNAs from Ambion (Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific). Three oligos against Bcl9 and Bcl9L were tested: sib9 (Oligo A, B, and C: s95054, s95055, and s95056) and sib9L (Oligo A, B, and C: s95949, s95950, and s95951). In addition, we tested siRNA pools against Bcl9 and Bcl9L and Smad4 from Dharmaco (SmartPool ON-Target Plus). For the study of EMT induction, cells were transfected with specific siRNAs 2 days before starting with TGFβ treatment (Py2T-LT is continuously under TGFβ). For EMT induction with 4 days of TGFβ treatment, cells were re-transfected at day 3 for NMuMG/E9 (20 nM each) and at days 3 and 5 for Py2T cells (40 nM each) to ensure proper siRNA-mediated gene downregulation until the end of the assay on day 6. Cells are then used for immunofluorescent staining, migration assay or for the isolation of RNA (Trizol) for quantitative RT-PCR.

Migration assays
Py2T-LT cells (TGFβ > 20 days) were transfected for 2 days with siRNA against Bcl9 and/or Bcl9L or control siRNA (scScr). In total, 2.5 × 10^4 cells were plated onto a 0.2–20% FBS/DMEM Gradient medium in a 24-well Boyden chamber plate with 8.0 μm Transparent PET Membrane (Corning, NY, USA) and incubated for 18 h at 37 °C. Cells on the membrane were fixed with 4% paraformaldehyde and counterstained with DAPI (1 μg/ml; Sigma D9542). Non-migrated cells from the upper surface of the transwell membrane were removed by scraping with a cotton swab. Migrated cells were imaged from the bottom of the Transwell membrane with a 10x objective on a Leica DMI microscope and quantified with ImageJ software.

RNA-sequencing and analysis
Established cell lines (B9/B9LWT/WT, B9/B9LWT/Bcl9/2Bcl9LD2/2, Bβ-catenin+/–, and B-β-cateninfl/fl/flwash) with and without floxed alleles were treated either with 100 ng/ml Wnt3a for 3 days or with 2 ng/ml TGFβ for 4 days. Untreated cells served as control. Biological duplicates were prepared, total RNA was isolated using the mirNeasy Mini Kit (Qiagen, 271004) with on-column DNAse digestion according to the manufacturer’s instructions. RNA quality control was performed using RNA ScreenTape on an Agilent 4200 TapeStation, and RNA concentration was measured using Quanti-IT RiboGreen RNA assay Kit (Life Technologies). RNA-sequencing libraries were prepared from total RNA using poly(A) enrichment using 200 ng input RNA with TruSeq stranded mRNA Sample prep (Illumina). QC was performed on a fragment analyzer using DNF-473-33-SS NGS Fragment 1–6000 bp kit. RNA sequence libraries were sequenced on a NextSeq 500 using 75 cycles kit High Output (Illumina). Single-end RNA-sequencing reads were mapped to the mouse genome assembly, version mm10, using RNA-STAR (PMID:23104866), with default parameters except for allowing only unique hits to genome (outFilterMultimapNmax = 1) and filtering reads without evidence in spliced junction table (outFilterType = “BySeqJout”). Using RefSeq mRNA coordinates from UCSC (genomic.ucsc.edu, downloaded in December 2015) and the cCount function from QuasR package (version 3.12.1) (PMID:25417205) we quantified gene expression as the number of reads that started within any annotated exon of a gene. The differentially expressed genes were identified using the edgeR package (version 1.10.1) (PMID:19910308). Genes with FDR ≤0.05 and minimum log2 fold change of ±1.0 were considered statistically significant and included in further analysis.

DATA AVAILABILITY
The RNA-sequencing data are deposited on GEO database under GSE148843 and GSE182404.

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AUTHOR CONTRIBUTIONS
VV designed and performed the experiments, analyzed the data, and wrote the manuscript. DB, NR, LB, and MS performed and analyzed experiments. RKRK performed bioinformatics analyses. TV, GH, CC, and KB provided mouse lines and reagents, designed the project, and proofread the manuscript. GC oversaw the project, designed experiments, analyzed data, and wrote the paper. All authors reviewed and approved the final manuscript.

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COMPETING INTERESTS
The authors declare no competing interests.

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