Tumor Suppressor Lzap Suppresses Wnt/β-Catenin Signaling to Promote Zebrafish Embryonic Ventral Cell Fates via the Suppression of Inhibitory Phosphorylation of Glycogen Synthase Kinase 3

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Background: Lzap is a novel tumor suppressor.

Results: Elimination of Lzap in fish embryos or in a human tongue carcinoma cell line increases inhibitory phosphorylation of GSK3, which controls much cellular signaling, including Wnt/β-catenin signaling.

Conclusion: Lzap promotes GSK3 activity.

Significance: This introduces a new regulator of GSK3 and a potential target for cancer therapy.

Wnt/β-catenin signaling controls various cell fates in metazoan development, and its dysregulation is often associated with cancer formation. However, regulations of this signaling pathway are not completely understood. Here, we report that Lzap, a tumor suppressor, controls nuclear translocation of β-catenin. In zebrafish embryos disruption of lzap increases the expression of chordin (chd), which encodes a bone morphogenetic protein (BMP) antagonist that is localized in prospective dorsal cells and promotes dorsal fates. Consistently, lzap-deficient embryos with attenuated BMP signaling are dorsalized, which can be rescued by overexpression of zebrafish lzap or bmp2b or human LZAP. The expansion of chd expression in embryos lacking lzap is due to the accumulation of nuclear β-catenin in ventral cells, in which β-catenin is usually degraded. Furthermore, the activity of GSK3, a master regulator of β-catenin in ventral cells, is increased in lzap-deficient embryos via inhibitory phosphorylation. Finally, we also report that a similar regulatory axis is also likely to be present in a human tongue carcinoma cell line, SAS. Our results reveal that Lzap is a novel regulator of GSK3 for the maintenance of ventral cell properties and may prevent carcinogenesis via the regulation of β-catenin degradation.

Embryo body plan, including dorsal-ventral patterning, is established during early development. In frog and fish, certain maternal dorsal determinants (wnt5/11 mRNA in Xenopus (1, 2) and wnt8a mRNA in zebrafish (3)) are initially located at the vegetal pole and are subsequently transported to the future dorsal region to induce β-catenin nuclear localization. In zebrafish embryos, wnt3a, which is co-expressed with wnt8a at the gastrula margin (4), is also able to activate the maternal Wnt/β-catenin pathway (3). Downstream targets of β-catenin include bozozok/dharma and fibroblast growth factors (fgfs), which may act in parallel to activate transcripts involved in dorsal cell fate specification (5–7). For example, Chordin is a BMP antagonist involved in the establishment of the ventral to dorsal BMP signaling gradient (8). However, the mechanisms that restrict β-catenin nuclear localization to the future dorsal side have not been fully addressed.

Wnt/β-catenin signaling is evolutionarily conserved and controls many aspects of developmental processes (9). In the absence of Wnt ligands, GSK3 binds to the scaffold protein Axin and the adenomatous polyposis coli protein to form the β-catenin destruction complex. β-Catenin is phosphorylated by GSK3 and subjected to ubiquitin-dependent degradation. The binding of Wnt to its receptors (Frizzled and lipoprotein receptor-related proteins 5 or 6 (LRP5/6)) induces the phosphorylation of LRP5/6 PPPSPXS motif (a cytoplasmic tail), which consequently affects the catalytic pocket of GSK3 to directly block its activity (10). Thus, β-catenin is stabilized and allowed to translocate into the nucleus, where it modulates target transcription by interacting with members of the LEF1/TCF family of transcription factors. In addition to Wnt signaling,
GSK3 activity can also be suppressed by inhibitory phosphorylation mediated by insulin-like growth signaling or lithium (11–13). In vertebrates, two genes encode GSK3 (GSK3α and GSK3β) and Axin (Axin1 and Axin2). GSK3β is known to be involved in axis formation (14), whereas Axin2 is a direct target of Wnt/β-catenin signaling and acts as a feedback negative regulator for the same pathway (15). In zebrafish, two genes code for β-catenin. Only one, β-catenin2 (encoded by cateninb2, ctnnb2), is essential for dorsal axis formation (16).

LZAP (also called CDK5Rap3 or C53) is a putative tumor suppressor gene, which is not expressed in ~30% of neck and head squamous carcinoma (HNSCC) (17); in mice, loss of lzap accelerates tumor xenograft growth (17). The LZAP gene has been highly conserved during evolution, and it encodes two LXXLL motifs and a putative leucine zipper domain that are responsible for protein-protein interaction and DNA-binding, respectively (18). Recent studies have shown that LZAP suppresses cell cycle progression via the activation of the p53-mediated pathway (19), decreases cellular invasion and contact growth by directly inhibiting the NF-κB signaling pathway (17), and alters cell death and proliferation by suppressing mitogen-activated protein kinase p38 MAPK (20). A role for LZAP in embryogenesis has also been reported. lzap homozygous knock-out mice are embryonic lethal.6 In addition, lzap is maternally deposited and ubiquitously expressed in zebrafish embryos during early development, and knockdown of lzap results in slower cell division and delayed epibolic movement (the process by which blastomeres move from the animal pole to the vegetal pole to cover the yolk) (18). These results indicate that LZAP controls several cellular processes during development; nevertheless, it is less clear how its dysregulation causes developmental disorders and cancer formation.

In this study we further report that Lzap maintains the ventral cell fate of zebrafish embryos via GSK3. Disruption of lzap expression increases levels of GSK3 inhibitory phosphorylation, which in turn results in the accumulation of β-catenin nuclear localization; such accumulation causes expansion of dorsal-determining Wnt/β-catenin signaling and activation of dorsal-specifying genes in prospective ventral cells, which results in embryos becoming dorsalized. Furthermore, similar molecular expression patterns were observed in a human HNSCC line, SAS. These results are thus of relevance to many cancers in which Wnt/β-catenin signaling is elevated.

**Experimental Procedures**

**Fish Maintenance and Breeding**—The wild-type zebrafish AB strain was raised and maintained under standard laboratory conditions (22). Embryos were obtained by natural fertilization and then staged and fixed as previously described (23).

**Plasmid Constructs**—Total RNA was extracted from zebrafish embryos at 8 h post fertilization (hpf) or human nasal mucosal cells and converted to cDNA by reverse transcription (RT) (see details below). The coding regions of human LZAP and zebrafish lzap, bmp2b, bmp4, bmp7a, noggin1, chd, bozozok, and axin2 were amplified using gene-specific primers (sequences available upon request) and subcloned into the pCS2+ vector for capped mRNA synthesis or into the pGEMT-Easy vector (Promega) for generation of antisense probes.

**Morpholino and mRNA Injection**—Embryos were injected at the one-cell stage. The sequences of antisense morpholino (MO) oligonucleotides (Gene Tools) and siRNA (Life Technologies) used in this study were as follows: lzap 5’-UTR MO, 5’-AAGAATTACTAAAAAGCACCCTTGC-3’ (note that the label of lzap MO in the figure represents lzap 5’-UTR MO); lzap 5’-UTR 5-mismatch MO, 5’-AAACAATGATTAACCCCATCC-3’; lzap ATG MO, 5’-AGGGAGATTCTGGA-TGTTCCTCATT-3’; chd MO, 5’-ATCCACACAGCGCCCTTCCATCCATCCATC-3’ (24); gsk3α MO, 5’-CCGCTGGCGCTCATCATCCGGGTGGTCA-3’ (25); gsk3β MO, 5’-GGTCTGGGCGGACCCGCCGACATTTTTTC-3’ (25); lzap splice-blocking MO, 5’-AGTTATGTGTTTACCTcacCCATG3’-; ctnnb2 MO, 5’-CCTTTAGCTGAGCGACTTCCAAAC-3’ (16); control MO, 5’-CCGACTGTCGAAAGATTCGGTGCTCT-3’; control siRNA, 5’-UUCUCUCUCACCAGGGACUAAUU-3’; lzap siRNA1, 5’-UGGCAAGAGAUAGUGUCAGUGUAG-3’; lzap siRNA2, 5’-CAGCCUUAAGGAAGCAGGUGUCUA-3’. Capped mRNA from the coding region of lzap, bmp2b, gfp, or human LZAP with or without lzap 5’-UTR MO was injected into the cytoplasm of one-cell stage embryos.

**Whole Mount in Situ Hybridization and Immunocytochemistry**—Whole mount in situ hybridization was performed as described (26). Antisense probes labeled with digoxigenin-UTP (Roche Applied Science) were synthesized using cDNA encoding axin2 (5), bmp2b (27), bmp4 (28), bmp7a (29), chd (30), noggin1 (31), or bozozok (7) with AmpliCap™ SP6/T7 High Yield Message Maker kit (Epicenter). Digoxigenin-labeling probes were color-stained with NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) or BM purple (only used for bmp2b probes) (Roche Applied Science). Immunostaining for embryos was performed as previously described (32). The following primary antibodies were used: mouse anti-β-catenin antibody (Sigma catalog #C7207, 1:250), rabbit anti- phospho-Smad antibody (Cell Signaling, catalog #9511, 1:200), rabbit anti-phospho-GSK3 (Ser-21/-9) (Cell Signaling Technology, catalog #9331, 1:2000), and rabbit anti-phospho-ingenot-activated protein kinase antibodies (Sigma, catalog #M8159, 1:500). AlexaFluor 633-conjugated goat anti-mouse and -rabbit secondary antibodies (Sigma, catalog #C7207, 1:250), rabbit anti-phospho-GSK3 (Ser-21/-9) (Cell Signaling Technology, catalog #9331, 1:2000), and rabbit anti-phospho-ingenot-activated protein kinase antibodies (Sigma, catalog #M8159, 1:500). AlexaFluor 633-conjugated goat anti-mouse and -rabbit secondary antibodies (Molecular Probes, 1:400) and AP-labeled anti-rabbit secondary antibody (Cell Signaling, 1:2000) were used. Samples were stained with 0.5 μg/ml DAPI. Images were acquired using an upright fluorescence confocal microscope (Zeiss, LSM700).

**PKA/C Activity Assay**—PKA activities were examined by PepTag nonradioactive protein kinase assay (Promega). PKA activities are determined by the charge of phosphorylated fluorescent PepTag A1 and non-phosphorylated PepTag A1 peptides, and the charge ranges from +1 to +1 after phosphorylation. To perform the PKA activity assay, 2.5 μg of protein extracts of sphere-stage embryos were used. Bands were detected and photographed using UV light, and the fluorescence intensity of phosphorylated, compared with non-phosphorylated, peptides was quantified using an ELISA reader.

**PP2 Activity Assay**—Protein Phosphatase 2 activities were examined by the nonradioactive serine/threonine phosphatase.

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6 W. G. Yarbrough, unpublished results.
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assay system (Promega). In brief, 1 μg of protein extracts of stage-sphere embryos were used for each of protein phosphatases (PP) PP2A, PP2B, and PP2C activity assays. Embryos were homogenized in the buffers specific for PP2A, PP2B, and PP2C (33–35). After centrifugation, the supernatant was further passed through a Sephadex G-25 resin column to remove free phosphates. The eluants were used to detect the activity by the measurement of released phosphate amount from phosphopeptide (RRA(pT)/VA) according to the manufacturer’s instruction.

**Cell Culture and Plasmid Transfection**—Human embryonic kidney 293T, SAS, TW-01 (a nasopharyngeal cancer NPC cell line), and NNMs (normal nasal mucosal) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 1-glutamine (Life Technologies) and 10% fetal bovine serum (FBS; 15% FBS was for NNM) plus 1% penicillin-streptomycin in a 5% CO2 incubator. 50 sphere stage embryos were deyolked in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40) containing freshly diluted phosphatase and protease inhibitors (1 mM anti-CDK5RAP3 (BETHYL, catalog #A300-871A), mouse anti-phospho-ERK1/2 (Cell Signaling Technology, catalog #4695, 1:1000), mouse anti-GSK3 (Santa Cruz Biotechnology, catalog #sc-7291, 1:200), rabbit anti-phospho-Akt (Cell Signaling Technology, catalog #4060, 1:500), and rabbit anti-histone H3 (Abcam, catalog #ab1791, 1:1000). The following secondary antibodies were used: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 1:10,000) and HRP-conjugated goat-anti-mouse IgG (Merck Millipore, 1:10,000). Signals were detected by chemiluminescence assays using Western Lightning™ Plus-ECL kit (PerkinElmer Life Sciences). All blots are representative of at least two experiments.

**RT-PCR Analysis**—Total RNA of ~30 embryos of the desired stage or ~4 × 10^6 cells were extracted using TRIzol reagent (Invitrogen). One microgram of total RNA was used for first strand cDNA synthesis (Epicerent). For examining the transcript variants of **lzap** in embryos injected with **lzap** splice-blocking MO, we performed RT-PCR (see supplemental Fig. 2). For examining expression levels of transcripts, real time quantitative real-time PCR was performed with a LightCycler 480 II thermocycler (Roche Applied Science) together with the SYBR Green Master kit (Roche Applied Science) or a LightCycler 480 Probes master reagent kit (Roche Applied Science) equipped with specific hydrolysis TaqMan probes. Gene expression data for each individual group were normalized to the expression level of *actin* (for embryos) or GAPDH (for cells). Results were analyzed using a previously described formula (37). The sequence of primers (and probe number) used for RT-PCR, SYBR Green, or TaqMan Real Time PCR are available up request.

**Results**

**Lzap Controls Dorsal-Ventral Patterning**—Although Lzap was first identified as a novel tumor suppressor (17), it also plays critical roles during development (18). Knock-out of **lzap** in mice is embryonic lethal, and translational knockdown of **lzap** in zebrafish embryos using two independent **lzap** morpholinos causes epibolic movement delay (18). Interestingly, although ventral and dorsal genes were shown to be expressed in **lzap** morpholinos by Liu et al. (Ref. 18), a closer examination of these previously published figures reveals that the expression territories of ventral genes are reduced and dorsal gene expression areas are enhanced as compared with those of control embryos, suggesting a dorsal-ventral patterning defect in **lzap** morphants. Thus, to investigate the molecular mechanisms by which disruptions in tumor suppressor genes cause developmental defects and cancer growth, we carefully examined the role of Lzap during development using zebrafish embryos as a model; zebrafish are particularly advantageous on account of their rapid external growth and amenability to genetic manipulation (38). In agreement with a previous report (18), the application of one of the reported **lzap** MOs, 5’-UTR MO (4 ng), delayed epibolic movement in 78% ± 17% of embryos at 8 hpf (n = 1539). Furthermore, 63% ± 14 of the surviving embryos at this stage were dead at 24 hpf (supplemental Fig. S1 and Table S1). Strikingly, the remaining embryos exhibited dorsIALIZED phenotypes as compared with wild-type embryos or embryos injected with control MO (Fig. 1 and supplemental Table S1) when analyzed at 30 hpf. Based on the degrees of dorsализation
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(27, 39), the morphants could be categorized into five classes. Compared with those of wild-type embryos (Fig. 1A), the ventral tail fins of Class 1 and 2 embryos were partially lost (Class 1) or entirely absent with a smaller posterior trunk and shortened yolk extension (Class 2) (Fig. 1B). Class 3 and 4 embryos had a visible head structure but a shortened trunk and yolk extension; the arrow points to the twisted tail. G, percentage of surviving embryos displaying normal, mildly dorsalized (Class 1–2), moderately dorsalized (Class 3) or a trunk axis twisted around itself (Class 4) (Fig. 1, A–D). Class 5 embryos had unidentifiable tissue structures on top of the yolk ball (Fig. 1E). After injection with 4 ng of lzap 5'-UTR MO, 43 and 39% of surviving lzap morphants (n = 381) exhibited mild (Class 1–2) and severe (Class 3–5) dorsalized phenotypes (Fig. 1G), respectively. Consistently, application of another reported morpholino, the lzap ATG MO, also resulted in typically dorsalized embryos (supplemental Fig. S2 and Table S1) (note that embryos injected with lzap ATG MO caused less obvious epibolic delay). In addition, embryos injected with two different lzap siRNAs also showed dorsalized phenotype (supplemental Fig. S3).

We further examined the specificity of lzap 5'-UTR MO on dorsalized phenotypes by supplying the lzap-deficient embryos with exogenous lzap mRNA that only covers the coding region and thereby cannot be targeted by lzap 5'-UTR MO (Fig. 1, F and G, and supplemental Table S1). Our results showed that the ratio of lzap 5'-UTR MO-induced dorsalized embryos was markedly reduced by co-injection of lzap mRNA in a dosage-dependent manner as compared with embryos injected with lzap 5'-UTR MO alone or co-injected with lzap 5'-UTR MO and gfp mRNA. However, overexpression of lzap mRNA did not result in ventralized embryos (supplemental Fig. S4). Furthermore, the dorsalized phenotype could also be partially rescued by co-injection of human LZAP mRNA (supplemental Table S1). These results indicate that Lzap controls dorsal-ventral patterning and suggest a conserved function of Lzap during development among species.

To investigate whether maternal or zygotic Lzap contributes to dorsal-ventral patterning, we used a lzap splice-blocking MO, which targets the junction between exon 1 and intron 1 of lzap and thereby affects zygotic lzap RNA splicing (supplemental Fig. S5, A–C). However, altered lzap RNA splicing was only observed in embryos injected with lzap splice-blocking MO after 8 hpf (supplemental Fig. S5A), and <4% of embryos injected with lzap splice-blocking MO (n = 410) exhibited dorsalized phenotypes (supplemental Fig. S5D and Table S1).

These results indicate that maternal lzap has a dominant effect on early dorsal-ventral patterning.

BMP Signaling Activity Is Decreased in lzap Morphants—The BMP-Chordin axis is critical for dorsal-ventral patterning across species (2, 3, 40). BMPs are highly expressed in the ventral region and form a ventral to dorsal activity gradient, which arises mainly because of a dorsal to ventral gradient of their antagonists, such as Chordin. To determine whether lzap controls dorsal-ventral patterning via the BMP-Chordin axis, we first examined the expression of bmp2b, bmp7a, bmp4, chd, and noggin 1 (encoding another BMP antagonist [41] at the 75% epiboly stage (~8 hpf) (Fig. 2, A–H). In stage-matched lzap 5'-UTR MO-injected embryos, expression territories of bmp2b and bmp7a were decreased, whereas chd and noggin 1 expression regions were expanded as compared with wild-type embryos. Coincidently, quantitative PCR results revealed that expression levels of bmp2b, bmp7a, and bmp4 were decreased, but chd expression was increased in lzap 5'-UTR MO-injected embryos at the 75% epiboly stage as compared with wild-type embryos (Fig. 2I). To examine whether changes in bmps, chd, and noggin1 expression are associated with abnormal BMP sig-
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FIGURE 2. BMP signaling activity is decreased in lzap morphants. A–H, expression domains of bmp2b (A and B) and bmp7a (C and D) are reduced in lzap morphants, whereas those of chd (E and F) and noggin (G and H) are expanded in lzap morphants (MO) at the 75% epiboly stage. I, quantitative PCR analysis of the relative expression levels of the various mRNAs and chd mRNA in WT and stage-matched lzap morphants at the 75% epiboly stage. Error bars represent S.D. *p < 0.05; **p < 0.01. J and K, the phospho-Smad1/5/8 (p-Smad)-positive domain, which represents active BMP signaling, is decreased in lzap morphants at the 75% epiboly stage. A, C, E, G, and J are WT controls, and B, D, F, H, and K are stage-matched lzap morphants. Lateral views are seen in A–D, and animal views are seen in A′–H′, dorsal side to the right. Dorsal views are seen in E–H. Arrowheads in A′–H′ point to the limits of the signal-positive expression domain. The fraction of embryos displaying the corresponding phenotype is provided in each panel. All morphants were injected with 4 ng lzap 5′-UTR MO.

FIGURE 3. Overexpression of bmp2b rescues dorsalized phenotypes in lzap-diminished embryos. A–C, lateral views of embryos at 24 hpf. Uninjected (WT) embryos (A), embryos injected with lzap5'-UTR morpholino (MO) (B), and embryos overexpressing bmp2b (C) exhibit normal, C4 snailhouse dorsalization, and ventralization, respectively. D, percentage of embryos exhibiting dorsalized or ventralized phenotypes at 30 hpf. Numbers of analyzed embryos are shown above each bar. ***, p < 0.001 represents the significant difference between two groups as indicated, analyzed by the χ² test. Data for lzap5′-UTR MO-injected embryos are the same as shown in Fig. 1F.

signaling. Overexpression of bmp2b mRNA suppressed the dorsalization phenotype of lzap morphants, as compared with embryos injected with lzap5′-UTR MO alone (Fig. 3F), indicating that Lzap maintains BMP signaling to control dorsal-ventral patterning.
with controls (Fig. 4, c and d). Quantitative analysis of stage-embryo size also corroborated this result (Fig. 4e). Unexpectedly, we detected increased expression of bmp7a in lzap5′-UTR MO-injected embryos at the sphere stage (supplemental Fig. S6), suggesting a compensatory effect in response to the disruption of the BMP-Chordin gradient. Nevertheless, these results indicate that Lzap controls chd expression to maintain a proper gradient of BMP signaling. Furthermore, because the sphere stage occurs before the stage for epiboly initiation (dome stage, 4.3 hpf), we argue that lzap morphants exhibit a dorsal-ventral patterning defect in addition to the epiboly defect reported by an earlier study (18).

Wnt/β-Catenin-FGF Signaling Is Enhanced and Extended from Dorsal to Ventral in Lzap Morphants—The dorsal determinant Wnt/β-catenin signaling is known to activate transcription of fgfs and bozozok/dharma, which act in parallel to control chd expression in the prospective dorsal region during the mid-blastula transition stage (approximately 1 h before the sphere stage) (6, 7, 45). We then examined Wnt/β-catenin signaling activity in embryos injected with lzap5′-UTR MO by analyzing expression of its direct target, axin2 (which acts as a negative feedback regulator (15), at the sphere stage (Fig. 5, a, b, and g). Compared with control embryos, axin2 expression was increased ~2-fold in lzap morphants, indicating an enhancement of Wnt/β-catenin signaling. We proceeded to analyze Fgf signaling and expression of bozozok/dharma and fgfs in lzap morphants at the sphere stage. Expression of bozozok/dharma was slightly but significantly decreased in lzap morphants, as compared with controls (Fig. 5, c, d, and h). In contrast, phosphorylated p-ERK, downstream of active Fgf signaling (6, 46), was strongly detected in nearly every cell of lzap morphants, as compared with the dorsally restricted p-ERK signals of wild-type embryos (Fig. 5, e and f). In addition, quantitative PCR analysis revealed increased expression of fgf3 and fgf8a as well as up-regulation of FGF signaling downstream targets (47, 48), no tail (ntl) and sp5l, in lzap5′-UTR MO-injected embryos at the sphere stage (Fig. 5i). Although fgf17b has been reported to control dorsal-ventral patterning (49), no obvious changes in its expression were observed in lzap morphants (Fig. 5i). These results suggest that elevated Wnt/β-catenin-FGF signaling promotes chd expression in lzap morphants.

Lzap Suppresses Canonical Wnt/β-Catenin Signaling to Restrict chd Expression to the Dorsal Region—Upon the binding of Wnt ligands to their receptors, β-catenin translocates into the nucleus as a transcriptional co-activator to control several aspects of development, including determination of embryonic dorsal cell fate (9, 50). To determine if Lzap controls chd expression via Wnt/β-catenin signaling, we used quantitative PCR to examine Fgf signaling and chd expression in embryos co-injected with lzap5′-UTR MO and β-catenin2 MO (ctnnb2 MO) at the sphere to dome stage (4–4.3 hpf) (Fig. 6, A–F). Consistently, expression levels of axin2, fgf3, fgf8a, sp5l, ntl, and chd were increased in embryos injected with 4 ng lzap5′-UTR MO (see also Fig. 5), whereas such up-regulation was suppressed by the co-injection of ctnnb2 MO. In addition, expression levels of these genes were also decreased in embryos injected with ctnnb2 MO (23 ng) alone, supporting the known role of β-catenin2 in FGF signaling and chd expression. In situ hybridization results also revealed that chd expression level and territory were greater in lzap morphants, but such an increase was diminished in embryos co-injected with lzap5′-UTR and
CTNNB2 MO (Fig. 6, G–J), indicating that β-catenin2 acts downstream of Lzap to control chd expression.

To examine if up-regulation of Chordin expression caused dorsalization of Lzap 5′-UTR morphants, we co-injected embryos with Lzap 5′-UTR MO and chd MO (supplemental Fig. 7). Consistently, embryos injected with Lzap 5′-UTR MO exhibited dorsalization phenotypes, whereas chd morphants were ventralized in a dose-dependent manner, in agreement with previous reports (24). Embryos co-injected with Lzap 5′-UTR MO and chd MO are not dorsalized, but they exhibited compromised ventralization, as compared with chd MO morphants. This result suggests dorsalization caused by the loss of Lzap relied on the presence of Chordin.

Lzap Suppresses Nuclear Translocation of β-Catenin via Inhibitory Phosphorylation of GSK3—We further investigated how Lzap suppresses Wnt/β-catenin signaling (Fig. 7). Immunostaining results revealed that β-catenin accumulates in the nuclei of the dorsal and marginal cells of wild-type embryos at the sphere stage (Fig. 7A); in contrast, strong nuclear β-catenin signals were present in cells scattered throughout Lzap 5′-UTR-injected embryos (Fig. 7B). However, CTNNB2 mRNA levels in wild-type embryos and Lzap morphants were comparable (Fig. 7C), and expression levels of wnt3a and wnt8a (required for activating maternal β-catenin; Refs. 3 and 4) were decreased in Lzap morphants as compared with the wild-type control (Fig. 7D). These results indicate that elimination of Lzap may promote the stability of β-catenin independent of Wnts. Indeed, total β-catenin protein levels were increased in Lzap morphants at the sphere stage (Fig. 7E), and expression of p-ERK, downstream of FGF signaling, was also consistently and dramatically increased in Lzap morphants (supplemental Fig. 8; see also Fig. 5).

In addition to Wnt signaling (10), GSK activity can also be suppressed by inhibitory phosphorylation at serine 21 of GSK3α or serine 9 of GSK3β (p-GSK3α/β Ser-21/9–); this results in the accumulation of β-catenin and in turn causes the dorsalized phenotype (11–13). We, therefore, hypothesized that accumulation of β-catenin in Lzap morphants may result from the loss of GSK3β activity. To test this hypothesis, we examined GSK3 activity by subjecting embryos to Western blot with an anti-p-GSK3α/β Ser-21/9 antibody (Fig. 7E). Strikingly, inhibitory phosphorylation of GSK3 in Lzap morphants was greatly increased as compared with that in wild-type controls at the sphere stage (Fig. 7E); this finding was consistent with immunostaining data (Fig. 7F and G). Our results indicate that Lzap promotes GSK3 activity to promote the degradation of β-catenin in ventral cells via inhibitory phosphorylation.

Indeed, suppression of GSK3 expression in embryos by co-injecting gsk3α and gsk3β MO caused dorsalized phenotypes (supplemental Fig. S9), as previously described (25). The degree of dorsalization was enhanced when embryos injected with Lzap (5 ng), gsk3α (1 ng), and gsk3β (4 ng) MOs (supplemental Fig. S9), indicating a genetic interaction between LZAP and GSK3. However, knockdown of Lzap in embryos injected with higher concentrations of gsk3α MO (2 ng) and gsk3β (6 ng) did not increase dorsalization, and overexpression of lzap cannot rescue dorsalization of gsk3α and gsk3β double morphants, supporting our conclusion that GSK3 acts downstream of Lzap.

Human LZAP Controls Expression of β-Catenin and Inhibitory Phosphorylation of GSK3 in Oral Cancer SAS Cells—It has been reported that LZAP is absent from ~30% of HNSCCs (17). Interestingly, we found that SAS cells, a commonly used HNSCC cell line derived from tongue carcinoma (51), exhibited similar levels of LZAP expression, whereas a 40% reduction of LZAP and associated with higher levels of β-catenin and inhibitory phosphorylated GSK3 (Fig. 8, A and B, and supplemental Fig. S10) as compared with NNM epithelial cells (primary culture of NNM epithelia was performed during surgery on patients with nasal polyps) (52).

We further examined the effect of LZAP on GSK3 and β-catenin in human cancer cells by overexpressing LZAP in SAS cells. LZAP mRNA was increased ~1.6-fold after transfection (Fig. 8C), whereas LZAP protein expression was further decreased to 50% that of the original LZAP amount (Fig. 8D). Noticeably, similar phenomena were observed in 293T cells (supplemental Fig. S11). To determine the half-life of the LZAP protein, we first treated SAS cells with cycloheximide, an inhibitor for protein synthesis, in a time-dependent manner. Three hours after treating with cycloheximide (200 μg/ml), LZAP protein levels decreased to 30% that in SAS cells without treatment (supplemental Fig. S12). We further tested whether proteasome–dependent degradation was involved in the reduction of LZAP in Lzap-expressing SAS cells. To do this, SAS cells transfected with the empty control vector (as the control)
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FIGURE 7. β-Catenin nuclear localization is enhanced in lzap morphants via inhibitory phosphorylation of GSK3. A and B, animal views of sphere stage WT) (A) and 4 ng of lzap 5′-UTR MO-injected embryos (B) labeled with β-catenin (gray color). In WT embryos, the dorsal side is to the right, according to obvious β-catenin nuclear staining. The fraction of embryos displaying the corresponding phenotype is provided in each panel. C and D, quantitative PCR analysis of relative mRNA expression levels of ctnnb2 (C) and wnt3a and wnt8a (D) in sphere stage WT or 4 ng of lzap MO-injected embryos. *, p < 0.05 represents the significant difference between WT and morpholino-injected embryos, as analyzed by Student’s t test. E, representative Western blots for β-catenin (92–97 kDa), p-ERK (44/42 kDa), and p-GSK3-α/β levels (51/46 kDa) in WT or lzap 5′-UTR MO-injected embryos. Histone H3 (17 kDa) was used as an internal control. Molecular weight markers are indicated to the left of the blots. Membranes used for detecting expression of β-catenin, p-GSK3, and histone H3 and that of p-ERK were electroblotted from replicate lanes of a single gel. F and G, animal views of sphere stage WT (F) and 4 ng of lzap 5′-UTR MO-injected embryos (G) labeled with Ser-21/-9 p-GSK3α/β (green) and DAPI (blue). The fraction of embryos displaying the corresponding phenotype is provided in each panel.

or LZAP were treated with cycloheximide (200 μg/ml) alone or co-treated with cycloheximide (200 μg/ml) and MG132 (10 μM), an inhibitor for proteasome-dependent degradation. Three hours after cycloheximide treatment, a 30% reduction of LZAP protein levels was found in control cells, whereas a 50% reduction was found in the LZAP-overexpressing SAS cells, indicating a fast turnover rate of LZAP in the LZAP-overexpressing SAS cells. Co-treatment with cycloheximide and MG132 compromised the reduction of LZAP protein in LZAP-overexpressing SAS cells but not in controls. LZAP protein levels in LZAP-overexpressing SAS cells were returned to ~90% of the original amount (supplemental Fig. S12). These results suggest that too many LZAP proteins induce LZAP degradation via a proteasome degradation pathway and may be an explanation for the dorsalized embryos caused by lzap overexpression (see supplemental Fig. S4). The decrease of LZAP protein in LZAP-overexpressing SAS cells was further enhanced by inhibition of inhibitory phosphorylation of GSK3 and levels of β-catenin and cyclin D1 (a direct target of Wnt/β-catenin signaling (53) (Fig. 8D) as compared with levels in SAS cells transfected with the empty control vector. These results indicate that LZAP may also maintain tissue homeostasis via regulation of Wnt/β-catenin signaling, as a similar mechanism was observed in zebrafish embryos. Surprisingly, strengthened reduction of LZAP in SAS cells by shRNAi still increased levels of inhibitory phosphorylated GSK3, but β-catenin and cyclin D1 expressions were decreased (Fig. 8E). Knockdown LZAP in 293T by shRNAi also showed a similar result (supplemental Fig. S13). These results indicate a complex regulation of LZAP on Wnt/β-catenin signaling pathways. Nevertheless, our results strongly argue that LZAP is a novel dual regulator of GSK3 in fish and human cell lines.

Discussion

Wnt/β-catenin signaling promotes dorsal cell fates in frogs and fish, and it is also hyperactivated in several types of cancer (54, 55); however, its regulation remains incompletely understood. Here, we report that LZAP, a tumor suppressor, regulates Wnt/β-catenin signaling via the suppression of inhibitory phosphorylation of GSK3 in fish embryos and in cancer (Fig. 9). Within prospective dorsal cells of zebrfish embryos, maternal Wnt proteins activate their receptor, Frizzled, which directly inhibits GSK3 activity (54), allowing nuclear translocation of β-catenin. In contrast, within prospective ventral cells, GSK3 retains its activity, at least in part through Lzap, and this results in the degradation of β-catenin. Note that Lzap in dorsal cells may control GSK3 activity via a similar mechanism because chd expression is dramatically increased in the dorsal cells of lzap-
deficient embryos (Fig. 4B). This result may also reflect the ubiquitous expression of lzap mRNA during early embryogenesis (18).

**Lzap Controls Dorsal-Ventral Patterning in Parallel with Its Role in Epibolic Movement**—Zebrafish embryos lacking lzap exhibit defects in both epiboly initiation and movement (18) that are dependent on cell adhesion and radial intercalation of epithelial sheets (56). Here, we further demonstrate that Lzap controls dorsal-ventral patterning by restricting Wnt/β-catenin signaling to prospective dorsal cells. Epiboly is the first morphogenetic movement of the zebrafish embryo that drives cells toward the vegetal pole (57). The initiation of epiboly involves doming of the yolk cell up into the overlying blastoderm at the dome stage (~4.3 hpf), whereas maternal β-catenin starts to accumulate in the nuclei of dorsal blastomeres as early as the 128-cell stage (~2.25 hpf) (58, 59), indicating that dorsal-ventral patterning occurs earlier than epibolic movement. Given that mutations of several genes involved in dorsal-ventral patterning (e.g. bmp2b) did not exhibit severe epiboly defects (60), we speculate that Lzap controls pattern formation and morphogenetic movement in a parallel manner.

**Multiple Mechanisms Ensure Ventral Cell Formation**—Wnt/β-catenin signaling is activated in dorsal blastomeres of frog and zebrafish embryos during early development to induce genes that determine dorsal cell fate (61). This pathway is activated by maternal Wnts that are initially located at the vegetal pole and transported to cells that will become dorsal cells. Upon binding of Wnt proteins to their receptor, Frizzled, GSK3 activity is suppressed to facilitate the nuclear localization of β-catenin. However, β-catenin and wnt mRNAs, including wnt8a, are present throughout the blastoderm (at least in zebrafish) (3, 55), suggesting that other factors are required to ensure that β-catenin only accumulates in dorsal cells. Indeed, a recent study has shown that Sfrp1 and Frzb are two maternal Wnt inhibitors that limit the spatial context of Wnt proteins in dorsal cells (3). In addition, embryos treated with inhibitors that inactivate GSK3 (required for β-catenin degradation) from the 32-cell stage to the sphere stage also result in dorsalized phenotypes (14), indicating that GSK3 activity is promoted in ventral cells to ensure ventral cell fate. It is unclear, however, how GSK3 activity in ventral cells is controlled during early embryogenesis. In this study we propose that Lzap may
enhance GSK3 activity to ensure β-catenin degradation in ventral cells.

**LZAP-mediated Modulation of GSK3 Activity May Have Tumor Suppressive Effects—**LZAP has been shown to interact with several proteins in the context of its anti-tumor role. For example, LZAP binds ARF (alternative reading frame) for p53 activation to suppress tumor growth (19) and, together with RelA, RCAD (regulators of C53/LZAP and DDRGK1 (DDRGK domain-containing protein 1), or NLBP (novel LZAP-binding protein) inhibits NFκB to suppress tumor invasion (17, 63, 64). However, it is not clear if LZAP plays a role in cancer cells with aberrant Wnt/β-catenin signaling.

In this study we observed that diminished Lzap expression in fish embryos suppresses GSK3 activity and causes the accumulation of β-catenin, indicating that LZAP is able to modulate GSK3 activity. In addition, levels of LZAP were decreased and accompanied by increased β-catenin and phosphorylated GSK3 in human tongue cancer SAS cells, suggesting that LZAP may control GSK3 activity to suppress tumors. Interestingly, although another HNSCC cell line, human nasopharyngeal cancer TW-01 cells, also exhibited decreased LZAP expression, β-catenin and inhibitory phosphorylation of GSK3 are decreased in this cell line (supplemental Fig. S10), indicating that the regulation of Wnt/β-catenin signaling by LZAP occurs in a cell context-specific manner.

**Possible Mechanisms Underlying GSK3 Inactivation by Lzap—**GSK3 functions as a downstream regulatory switch for numerous signaling pathways, including cellular responses to Wnt and insulin-like growth factor and is involved in several cellular processes, ranging from glycogen metabolism to cell proliferation as well as cell movement, apoptosis, and dorsal-ventral patterning (21, 62). Therefore, GSK3 activity is tightly regulated (62). One critical regulatory mechanism is GSK3 phosphorylation. The most important phosphorylated residues are serine 21 for GSK3α and serine 9 for GSK3β, which inhibit GSK3 kinase activity, whereas phosphorylation of tyrosine residues (Tyr-216 for GSK3β and Tyr-279 for GSK3α) is required for activation. Several kinases, including Akt, protein kinases A (PKA) and PKC, suppress GSK3 activity via phosphorylation of serine 21/9. In contrast, protein phosphatases 1 (PP1) and 2A (PP2A) dephosphorylate the inhibitory site of GSK3, resulting in activation of GSK3. Furthermore, it has been shown that Lzap is able to interact with WIP1, a type 2C protein phosphatase (PP2C), to suppress p38 phosphorylation (20). To investigate how Lzap may control GSK3 activity, we further tested the involvement of the kinases and phosphatases described above (supplemental Fig. S14). We did not observe differences in PKA, PKB, PP2A, PP2B, or PP2C activities between wild-type and *lzap* 5′-UTR MO-injected embryos at the stage phase. In addition, we failed to detect PKC and PP1 activities in sphere-stage embryos. Further work will be needed to dissect the molecular mechanism by which LZAP promotes.

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