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Source: Zoological Science, 21(4) : 407-416

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.21.407

ZOOLOGICAL SCIENCE **21**: 407–416 (2004)

Inhibition of the Canonical Wnt Signaling Pathway in Cytoplasm: a Novel Property of the Carboxyl Terminal Domains of Two *Xenopus ELL* Genes

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ABSTRACT—The Wnt signaling pathways are important in many developmental events. The canonical Wnt pathway is one of the three major Wnt-mediated intracellular signaling pathways and is thought to activate Dvl followed by the stabilization of β-catenin. In *Xenopus*, this pathway is involved in dorsal determination, anterior-posterior patterning during gastrulation, and neural induction. Here we describe a role for the *Xenopus ELL* (*Eleven-nineteen Lysine-rich Leukemia*) gene product in canonical Wnt signaling. Translocation of *ELL* has been associated with acute myeloid leukemia and the protein possesses three functional domains. We identified *rELL-C* from a rat brain cDNA library as a binding factor for Dishevelled (Dvl); it represents a partial sequence of rat ELL lacking the pol II elongation domain and has been shown to suppress canonical Wnt signaling. Next, we isolated two *Xenopus* homologs of *ELL, xELL1* and *xELL2*. No obvious phenotypes were observed with microinjection of full-length xELL1 or xELL2 mRNA, however, microinjection with their occludin homology domain inhibited Wnt signaling at the level of Dvl and upstream of β-catenin. Intracellular localization of microinjected xELL1- and xELL2-GFP mRNAs showed localization of the full-length products in the nucleus and the occludin-homology domain products in cytoplasm. These results raise the possibility that ELL, which is thought to function as a transcription factor in nuclei, can serve other, novel roles to suppress canonical Wnt signaling in the cytoplasm.

Key words: ELL, *Xenopus*, Wnt, Dvl, localization

INTRODUCTION

The Wnt signaling pathways are important for the regulation of many biological events including axis formation, morphogenesis, and cell movement (Cadigan and Nusse, 1997; Peifer and Polakis, 2000; Taipale and Beachy, 2001; Moon *et al*., 2002). In *Xenopus* development, Wnt signaling is involved in dorsal determination, anterior-posterior patterning and gastrulation (Moon and Kimelman, 1998; Sokol, 1999; Niehrs, 1999; Wallingford *et al*., 2002). Wnt ligands transduce their signal via at least three independent pathways (Peifer and McEwen, 2002) with the canonical Wnt pathway being the most extensively investigated. Canonical Wnt signal transduction is currently thought to occur as follows. When Wnt signaling is off, Dishevelled (dsh/Dvl) protein is inactive and the Axin/GSK-3β/APC/β-catenin complex is stabilized (Behrens *et al*., 1998; Farr III *et al*., 2000; Ikeda *et al*., 1998; Kishida *et al*., 1998; Li *et al*., 1999; Rubinfeld *et al*., 1996). Consequently, β-catenin can be phosphorylated by GSK-3β on the complex and degraded via ubiquitin-dependent proteolysis (He *et al*., 1995; Hart *et al*., 1999; Kitagawa *et al*., 1999). It was recently shown that this phosphorylation requires a primary phosphorylation event mediated by casein kinase α (Liu *et al*., 2002; Amit *et al*., 2002). The Wnt ligand associates with Frizzled receptors, which activates Dvl and suppresses the phosphorylation of β-catenin. Consequently, when Wnt signaling is on, β-catenin can translocate into the nucleus, where it interacts with Tcf/Lef

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to stimulate target gene expression (Lemaire *et al*., 1995; Brannon *et al*., 1997; Laurent *et al*., 1997; McKendry *et al*., 1997). In addition to the factors described, many other proteins such as GBP, casein kinase ε, protein phosphatase 2A, Axam and Idax, are known to be involved with canonical Wnt signaling (Yost *et al*., 1998; Peters *et al*., 1999; Sakanaka *et al*., 1999; Seeling *et al*., 1999; Kadoya *et al*., 2000; Hino *et al*., 2001).

Dorso-ventral axis determination in *Xenopus* requires an accumulation of β-catenin at the dorsal half of the embryo and the subsequent activation of target gene expression (Larabell *et al*., 1997; Lamaire *et al*., 1995). Disruption of β-catenin at the dorsal side actually results in a complete loss of dorsal structure (Heasman *et al*., 1994; Heasman *et al*., 2000), and, during the cortical rotation, Dvl is enriched at the dorsal side of the embryo (Miller *et al*., 1999). Overexpression of a positive regulator of early canonical Wnt signaling, such as Dvl or β-catenin, at the ventral marginal zone (VMZ) of 4-cell stage embryos leads to the formation of secondary axis with a complete head (Sokol *et al*., 1995; Funayama *et al*., 1995). Conversely, negative regulators such as Axin and GSK-3β injected into the dorsal marginal zone (DMZ) causes a suppression of dorsalizing activity and head defects (Zeng *et al*., 1997; Fukui *et al*., 2000; He *et al*., 1995). Such an assay is therefore useful to determine whether a given protein is involved in the canonical Wnt pathway.

Recently, we screened a rat cDNA library using the yeast two-hybrid system to identify Dvl-binding proteins, using the PDZ domain of Dvl as a probe(Kikuchi, unpubished). One of the positive clones encoded the C-terminal half of the ELL (Eleven-nineteen Leucine-rich Leukemia) protein sequence (rELL: corresponding to a.a 402–602 in mouse ELL). This truncated protein was subsequently shown to interact with Dvl in an immunoprecipitation experiment (Kikuchi, unpubished). ELL was first isolated from myeloid leukemia cells (Thirman *et al*., 1994). It can induce acute myeloid leukemia (AML) by chimeric transcript generation when fused to the 5' region of *MLL* (*Myeloid Lymphoid Leukemia/Mixed Lineage Leukemia*) via chromosomal rearrangement (11;19)(q23; p13.1) (Thirman *et al*., 1994; Rubnitz *et al*., 1996). ELL was identified as an RNA polymerase II elongation factor (Shilatifard *et al*., 1996), and *ELL* genes have now been isolated from human (3), mouse (1), and fruit fly (1), although human ELLs are the only ones thus far shown to cause acute myeloid leukemia (Thirman *et al*., 1994; Thirman *et al*., 1997; Shilatifard *et al*., 1997; Khattak *et al*., 2002). It was recently found that ELL inhibits p53 transcriptional activity (Maki *et al*., 1999; Shinobu *et al*., 1999), and that this inhibition requires ELL to recruit p53 to the nucleus (Wiederschain *et al*., 2003). ELL protein contains three conserved functional domains: an RNA polymerase II elongation domain (Pol II-domain) spans about 350 residues from the N-terminus; a lysine-rich domain (K-domain) is located at the middle of the polypeptide; and a third conserved domain, the occludin homology domain (OH-domain) maps near the C-terminus. The OH-domain is necessary to the leukemia-inducing property of ELL (DiMartino *et al*., 2000; Luo *et al*., 2001).

In addition to its role in cell transformation, ELL also participates in normal early development. Mouse *ELL* is expressed in several tissues at embryonic day 16.5, such as liver and gastrointestinal tract (Thirman *et al*., 1997). In *Drosophila*, *dELL* is located within the *Suppressor Triplo-lethal* (*Su(Tpl)*) locus and deletion mutants of this gene cause abnormal embryonic segmentation and enhance the mutation phenotype of *Notch* and *cut* (Eissenberg *et al*., 2002), suggesting that *ELL* is important in early embryogenesis.

Here we report the role of ELL in Wnt signaling during *Xenopus* development. Preliminary experiments showed that expression of the C-terminal half of *rELL* caused loss of head structures. rELL interacts with Dvl *in vitro*, so we hypothesized that ELL was involved with canonical Wnt signaling. To test this suggestion, we first isolated the *Xenopus* homologue of ELL1 and ELL2 and examined the temporal and spatial patterns of their transcripts. Next, we found that the OH-domain of ELL can cause a loss-of-head phenotype that results from the inhibition of early canonical Wnt signaling. Furthermore, deletion of the ELL pol II domain re-directs the subcellular localization of xELL from nucleus to cytoplasm. Taken together, our studies implicate xELL as a cytoplasmic inhibitor of canonical Wnt signaling.

MATERIALS AND METHODS

Isolation of xELL1 and xELL2

Two *Xenopus* ESTs (AW642245 and AW640624) homologous to human ELL (hELL) were amplified by PCR from a stage-30 *Xenopus* embryo cDNA library using the following primers (xELL1 forward: 5'-GGCACGAGGGTTAAGATGGCGGC-3'; xELL1 reverse: 5'-CTGCAATCGCAAGATAAGTTCGGG-3'; xELL2 forward: 5'-GGGAGTCAGGAAGATGGCGGCGG-3'; xELL2 reverse: 5'-TCAGTGCCAGCAGATGAATCACCC-3'. Using these PCR fragments as probes, stage-10 and stage-28 *Xenopus* embryo cDNA libraries were screened by the plaque hybridization method. Positive clones were sequenced using a LIC-4200L(S) (Aloka, Japan).

Plasmid construction

For *in vitro* transcription, pxELL1/CS2 and pxELL2/CS2 were made by inserting full-length xELL1 or xELL2 ORF sequence, respectively, into the *Bam*HI site of pCS2. All deletion constructs of xELL1 and xELL2 (ΔOH, ΔPOL, ΔK, K and OH) were obtained by PCR amplification using *Pyrobest* DNA Polymerase (TAKARA, Japan) and the following primers: ΔOH forward: 5'-TAACAATTTTCCACTGGGTAGCAG-3'; reverse: 5'-GATAGAGTCCTGAAGAGTTCCACC- 3', ΔPol forward: 5'-GACTCTGTGAGTCATGAGACCACCG-3'; reverse: 5'-CATCTTAACCCTCGTGCCAATCACTAG-3'; ΔK forward: 5'-GATTTAAATGGTGCATGCAGTAAC-3'; reverse: 5'-TATGTGTTGGGCATCAGTTCTGGAG-3'. The primers were designed in the opposite direction to the deleted regions, and the fragments for the deletion constructs including plasmid vector sequence were amplified by PCR using pxELL1FL/CS2 or pxELL2FL/CS2 as the template. The 5-terminus of each PCR fragment was phosphorylated by T4 Polynucleotide Kinase (TAKARA, Japan).

Stage RT-PCR analysis

To examine the temporal pattern of ELL expression, we performed RT-PCR on *Xenopus* embryos at various stages of development. Total RNA was extracted from the embryos using ISOGEN (Nippon Gene, Japan). First-strand cDNAs were synthesized from 1 μg of total RNA with oligo(dT) primer using Superscript™II (Invitrogen, USA). Using the cDNA as a template, PCR was performed with following primers (xELL1: 5'-AAATGTTGGCAGAGACAGTCCCC-3' and 5'-ATCGCACCCCTACTCTTGTTCTCC-3'; xELL2: 5'-ACTGCATCCAGCAAACTGTCTCC-3'; and 5'-GGTCCCCCTGGTTTTATGACTTTAG-3'; siamois: 5'-AAGATAACTGGCATTCCTGAGC-3'; and 5'-GGTAGGGCTGTGTATTTGAAGG-3'; ODC: 5'-GTCAATGATGGAGTGTATGGATC-3' and 5'-TCCATTCCGCTCTCCTGAGCAC-3').

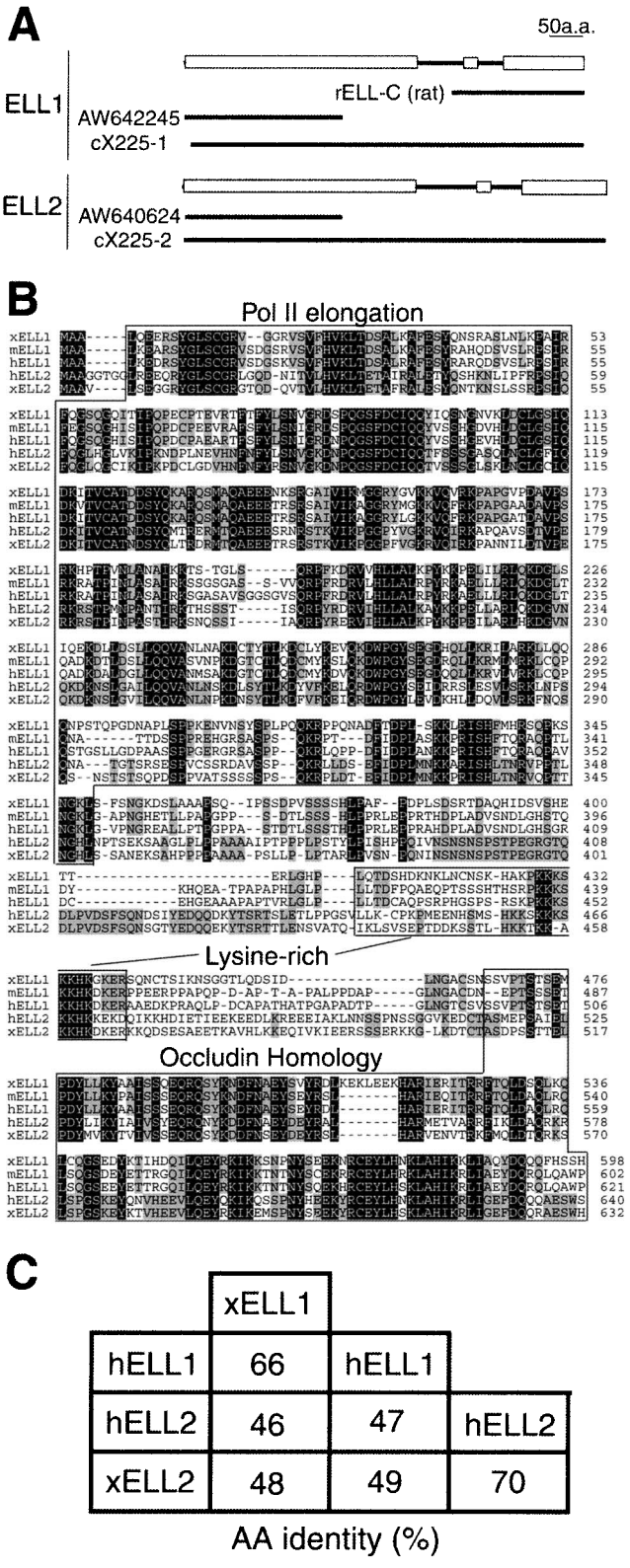


Fig. 1. (A) Diagrams of ELL1 and ELL2 structure. White boxes show the three conserved domains found in all ELL proteins. rELL-C was isolated from rat cDNA library, whereas cX225-1 and cX225-2 were isolated from *Xenopus* cDNA library. AW642245 and AW640624 are hypothetical fragments from the sequences of EST clones. (B) Deduced amino acid sequence of *Xenopus* ELL1 (xELL1) and ELL2 (xELL2), together with human ELL1 (hELL1), mouse ELL1 (mELL1) and human ELL2 (hELL2). Three highly conserved domains (Pol II elongation domain, Lysine-rich domain and Occludin homology domain) are indicated by the large open box. White characters on a black background indicate amino acid residues that are conserved across all ELL proteins, while black characters on gray indicate amino acids common to only ELL1 or ELL2 proteins. (C) Percentages of identity between the two ELL amino acid sequences are shown.

Microinjection with mRNAs

For microinjection into *Xenopus* embryos, we synthesized mRNA by *in vitro* transcription. Plasmid constructs were linearized with the appropriate restriction enzymes and these fragments were used as templates. Capped mRNAs were transcribed using mMessage mMachine kit (Ambion, USA). Fertilized eggs were dejellied by 100% Steinberg's solution containing 4.5% L-cysteine hydrochloride monohydrate. Embryos were injected with synthesized mRNAs in 100% Steinberg's solution containing 4% Ficoll and cultured. At stage 8, the Ficoll solution was substituted with 10% Steinberg's solution and the injected embryos were cultured for 3 days. Embryos were observed under a stereomicroscope (Olympus, JAPAN). For the expressing the degree of head defect, we used Dorso-anterior index (DAI) (Kao and Elinson, 1988).

Intracellular localization of ELL

EGFP-tagged xELL constructs were made by the insertion of full-length or truncated forms of xELL1 or xELL2 into EGFP-pCS2 plasmid in flame. EGFP-pCS2 was constructed by insertion of EGFP gene into pCS2 vector. mRNAs transcirbed *in vitro* with them were injected into the animal pole of 4-cell stage embryos. Injected embryos were cultured until stage 9, when animal caps were dissected out and observed under a fluorescence microscope (Olympus, JAPAN).

***In situ* hybridization**

DIG-labeled riboprobes were synthesized using T7, T3, or SP6 polymerase and a DIG NTP mix (Roche, Germany), and were digested into approximately 200-bp fragments using NaOH. The collected embryos were fixed with 1 × MEMFA for two hours and transferred to 1 × PTw (0.1% Tween-20/PBS). The embryos were then treated with Proteinase K for 3 minutes, and incubated in TEA for 20 minutes before a 20 minute post-fixation (4% paraformaldehyde/PBS). Pre-hybridization step was performed for two hours at 65°C prior to hybridization with the riboprobe for overnight. The embryos were washed three times with 2 × SSC, and twice with 0.2 × SSC. They were then incubated with anti-digoxygenin FAB fragment (Roche, Germany) overnight at 4°C. Washes with maleic acid buffer were followed by detection using the alkaline phosphatase substrate, BM-purple.

RESULTS

The C-terminal half of rELL has an inhibitory effect on canonical Wnt signaling

The C-terminal half of rat ELL (rELL-C) encodes a protein of approximately 200 residues corresponding to a.a. 402–602 of mouse ELL. This truncated protein interacts with Dvl, therefore it is thought to participate in the canonical Wnt signaling pathway. Indeed, dorsal injection with rELL-C mRNA (800pg) into *Xenopus* embryos caused head defects, with the occurrence of a cycloptic eye (data not shown). From this result, we thought that ELL might function negatively in the canonical Wnt signaling pathway. To address the hypothesis described above and to clarify the role of ELL in *Xenopus* early embryogenesis, we identified *Xenopus* homologues of the *ELL* gene. A search of the EST database revealed two highly homologous sequences, AW642245 and AW640624. AW642245 was 74% identical with the N-terminal 235-residue portion of human ELL1, while AW640624 was 77% identical with the N-terminal 232-residue portion of human ELL2 (Fig. 1A). Accordingly, we designed specific primers and amplified *xELL1* and *xELL2* fragments by RT-PCR. We then isolated positive clones by screening a lambda cDNA library. One positive clone for xELL1(cX225-1) was isolated from about 2.5×10^6 clones of the stage-28 cDNA library. The deduced amino acid sequence of cX225-1 showed that this clone contains almost the complete open reading frame, apart from 10 amino acid residues near the N-terminus. The overlapping region with AW642245 was 100% identical (Fig. 1A), therefore we adopted this EST sequence for the 10 truncated residues of xELL1. Nucleotide sequence analysis showed that *xELL1* contained the complete PCR fragment sequence. Thus, cX225-1 was identified as a Xenopus homologue of *ELL*. *xELL1* encodes a protein of 598 amino acids (Fig. 1B).

Another positive clone (cX225-2) was isolated by screening about 1×10^6 clones of a stage-10 cDNA library. CX225-2 included sequence consistent with that of AW640624 and encoded a protein of 632 amino acids (Fig. 1A, B). The deduced amino acid sequences showed that both xELL1 and xELL2 contain three conserved domains common to other ELL gene families: an RNA polymerase II elongation domain (Pol II-domain; xELL1: 4–349, xELL2: 4–349), lysine-rich domain (K-domain; xELL1: 409–440, xELL2: 436–466) and an occludin homology domain (OH-domain; xELL1: 467–598, xELL2: 508–632) (Fig. 1B). Comparison of the complete amino acid sequences of xELL1, xELL2, hELL and hELL2 indicated that xELL1 was 66% identical to hELL and that xELL2 was 70% identical to hELL2 (Fig. 1C). Of particular note was that xELL1 and xELL2 had high similarity with hELL1 and hELL2, respectively, in the three homologous regions (Fig. 1C). Another member of the ELL family, ELL3, was less homologous with both xELL1 and xELL2 (data not shown).

Spatial and temporal expression of xELL1 and xELL2

To determine the temporal expression patterns of *xELL*

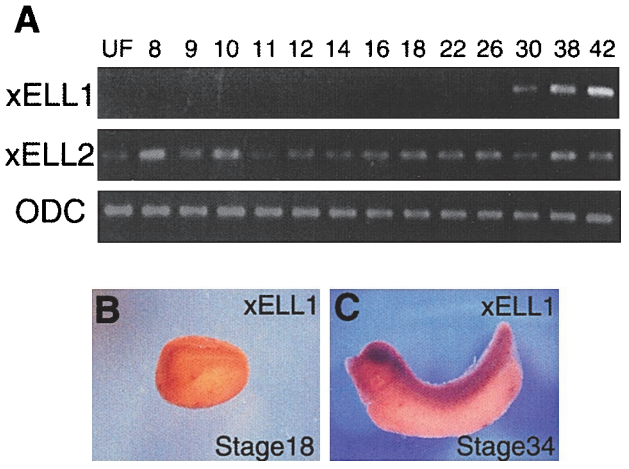


Fig. 2. Temporal and spatial patterns of xELL1 and xELL2 expression. (A) RT-PCR analysis of xELL1 (column 1) and xELL2 (column 2) expression with cDNAs synthesized from various stages of embryo development. ODC expression serves as the quantitative control. (B–C) *in situ* hybridization with xELL1 fragments as probes. Hybridization with the antisense probe on stage-18 embryo (B) or stage-34 embryo (C). Using the antisense probe, xELL1 expression can be detected in the neural crest region at stage 18 (B). At stage 34, xELL1 expression was localized at the dorsal region of the embryo, including eye and spinal cord (C).

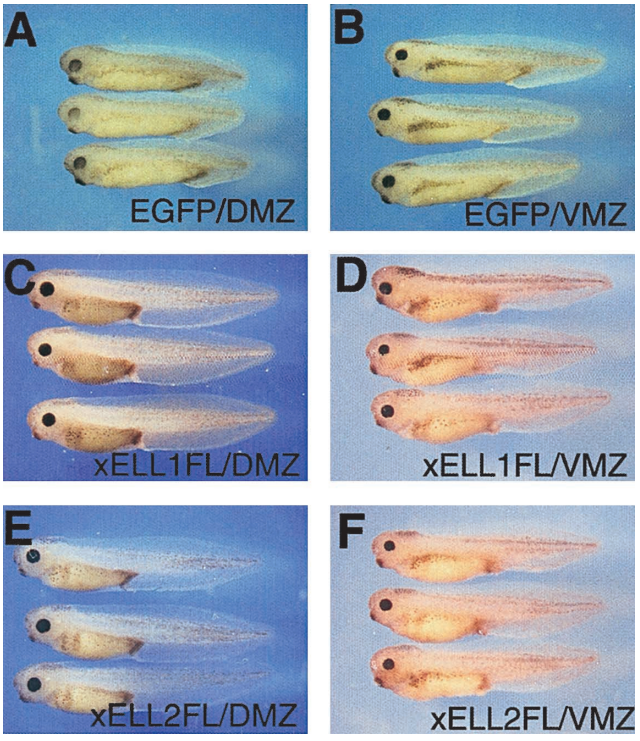
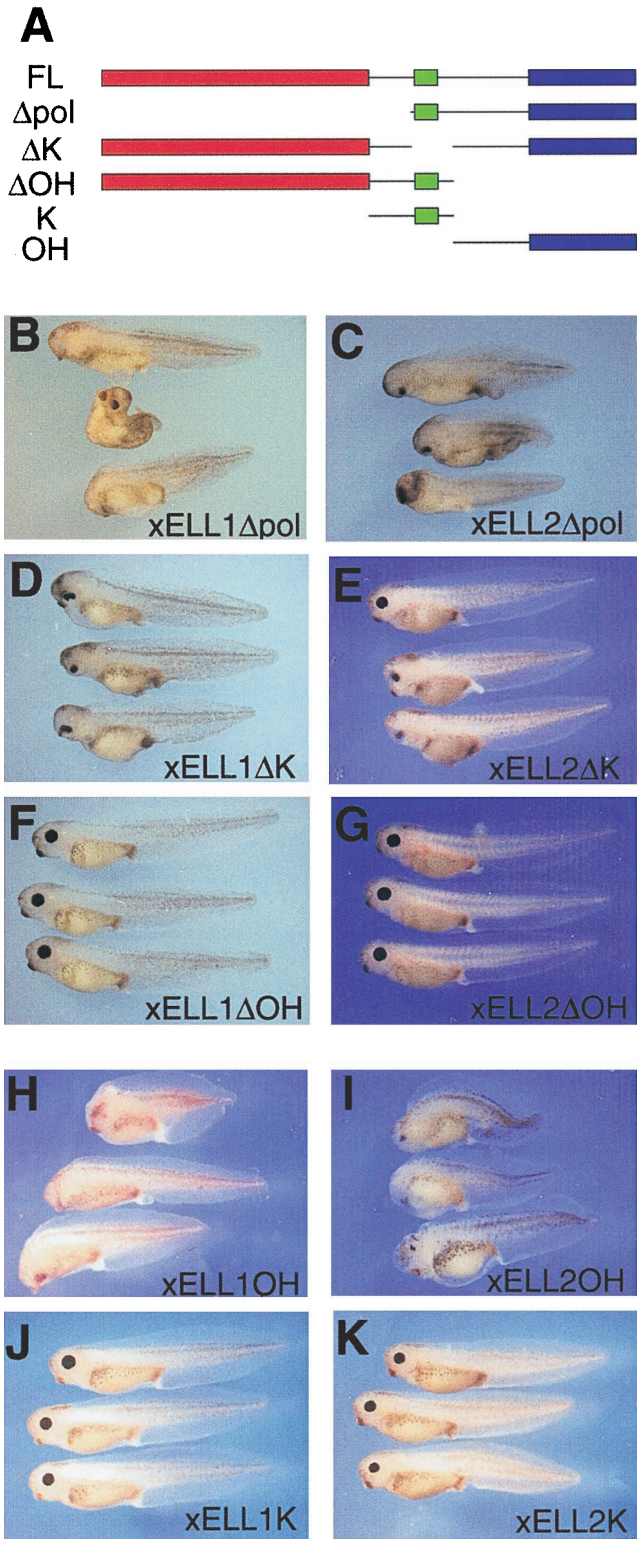


Fig. 3. Phenotypes derived by overexpression of full-length xELL1 and xELL2. EGFP (200 pg; A, B), xELL1FL (1 ng; C, D) and xELL2FL (1 ng; E, F) were injected into the dorsal marginal zone (DMZ; A, C, E) or the ventral marginal zone (VMZ; B, D, F). Injected embryos were observed at the 3-day tadpole stage. In each case, no obvious phenotypes were seen.

and *xELL2*, we synthesized cDNA using total RNA derived from various stages of Xenopus embryos and performed RT-PCR with specific primers for *xELL1* and *xELL2*. XELL1 mRNA was not seen before MBT or during gasturation and neurulation, but was increased at the 2-day tadpole stage (Fig. 2A, 1st column). Interestingly, *xELL2* showed a different pattern of expression: xELL1 mRNA was detected before MBT and constitutively expressed to the 4-day tadpole stage (Fig. 2A, 2nd column). Next, we carried out *in situ* hybridization to determine the spatial patterns of *xELL1* and *xELL2* expression. *XELL2* expression could not be detected, probably because of low levels of ubiquitous expression (data not shown). *XELL1* was not detected before neurulation. At the neurula stage, weak *xELL1* expression was detected in the neural plate (Fig. 2B). At the 2-day tadpole stage, *xELL1* transcripts were observed in the dorsal half of the embryo (Fig. 2D). In particular, *xELL1* was strongly expressed in the eye vesicle and the brain structure (Fig. 2D).



The truncated *xELL1* and *xELL2* genes inhibit dorsalization

To investigate the function of xELL1 and xELL2 in *Xenopus* development, we overexpressed these genes by microinjection. We first constructed xELL1FL/CS2 and xELL2FL/CS2, which contained the complete coding sequences of *xELL1* or *xELL2*, respectively, for in vitro transcription. The resultant mRNA was injected into *Xenopus* embryos. When 1 ng of xELL1FL mRNA was injected into the DMZ or VMZ at the 4-cell stage, no obvious phenotype was observed (Fig. 3A–D). Similarly, 1 ng of xELL2FL mRNA caused no defect (Fig. 3A–B, E–F). The pol II elongation domain-deficient rELL-C caused ventralization of the embryos, so we next examined deletion constructs of xELL1 and xELL2 lacking the Pol II-domain (xELL1(Δpol): Δ1–394, xELL2(Δpol): Δ1–418). When 500 pg of xELL1(Δpol) or xELL2(Δpol) were injected into the DMZ, formation of dorsoanterior structure was inhibited. In both cases, gastrulation was superficially normal (xELL1(Δpol): DAI=2.8, n=25; xELL2(Δpol): DAI=2.48 (n=23); Fig. 4B, C).

Next, we constructed other truncated forms of xELL1/2 lacking either the K-domain (xELL1(ΔK): Δ395–465, xELL2(ΔK): Δ419–526) or the OH-domain (xELL1(ΔOH): Δ466–598, xELL2(ΔOH): Δ527–632) and observed the phenotypes caused by overexpression of these mRNAs. When 1 ng of xELL1(ΔK) or xELL2(ΔK) was injected into DMZ or

Fig. 4. Observation of phenotypes caused by DMZ injection with truncated forms of xELL1 and xELL2. (A) Diagrams of several truncated forms of xELL1/2. Red box, green box, and blue box show pol II elongation domain, lysine-rich domain and occludin-homology domain, respectively. (B–K) Observed phenotypes caused by the ventral injection of the following mRNA; xELL1(Δpol) (1.0 ng; B), xELL2(Δpol) (1.0 ng; C), xELL1(ΔK) (1.0 ng; D), xELL2(ΔK) (1.0 ng; E), xELL1(ΔOH) (1.0 ng; F), xELL2(ΔOH) (1.0 ng; G), xELL1(OH) (1.0 ng; H), xELL2(OH) (1.0 ng; I), xELL1(K) (1.0 ng; J), xELL2(K) (1.0 ng; K). Injected embryos were observed at the 3-day tadpole stage. When xELL1Δpol or xELL2Δpol mRNA was injected into the DMZ, loss of head structure similar to that derived from rELL-C was observed (B, C). xELL1(OH) and xELL2(OH) also caused similar defects (H, I). Conversely, injection with xELL1(ΔK), xELL2(ΔK), xELL1(K) and xELL2(K) mRNA showed almost no phenotype (D, E, H, I). However, in the case of xELL1(ΔK) injection, a weak defect in the head region was detected (E).

VMZ, ventralization was not observed, although slight head defects were seen (Fig. 4D, E). Similarly, injection of 1 ng of xELL1(ΔOH) or xELL2(ΔOH) induced no effects (Fig. 4F, G). These results suggest that a deficiency in the Pol II-domain is required for the ventralization activity of xELL1/2. xELL1/2(Δpol) contained both the K-domain and OH-domains, so further experiments were done with xELL1(K) (xELL1(395–465)), xELL2(K) (xELL2(419–526)), xELL1(OH) (xELL1(466–598)) and xELL2(OH) (xELL2(527–632)) to delimit the domain necessary for the ventralization activity of the gene. When 200 pg of xELL1/2 (OH) mRNA was injected into DMZ at the 4-cell stage, loss of head structure was observed (xELL1(OH): DAI=3.56 (n=40); xELL2(OH): DAI=3.78 (n=42); Fig. 4H, I), while, as much as 500 pg of xELL1/2 (K) injection caused almost no effect on the developing embryo (Fig. 4J, K). Taken together, these results suggest that two conditions are essential for the ventralization activity of xELL1/2; that the genes encode for the OH-domain and not for the Pol II-domain.

The occludin homology domain of xELL1/2 inhibits canonical Wnt signaling

Our results showed that dorsal injection of xELL1(OH) and xELL2(OH) suppressed the formation of dorso-anterior structures (Fig. 4H, I). Thus, we suggested that this ventralization phenotype was due to an inhibition of the early canonical Wnt pathway by a truncated form of xELL. To prove this hypothesis, we next examined whether xELL1(OH) or xELL2(OH) inhibit canonical Wnt signaling. First, we carried out RT-PCR on xELL-injected embryos and checked the transcriptional levels of *siamois*, one of the direct target genes of early canonical Wnt signaling. Injec-

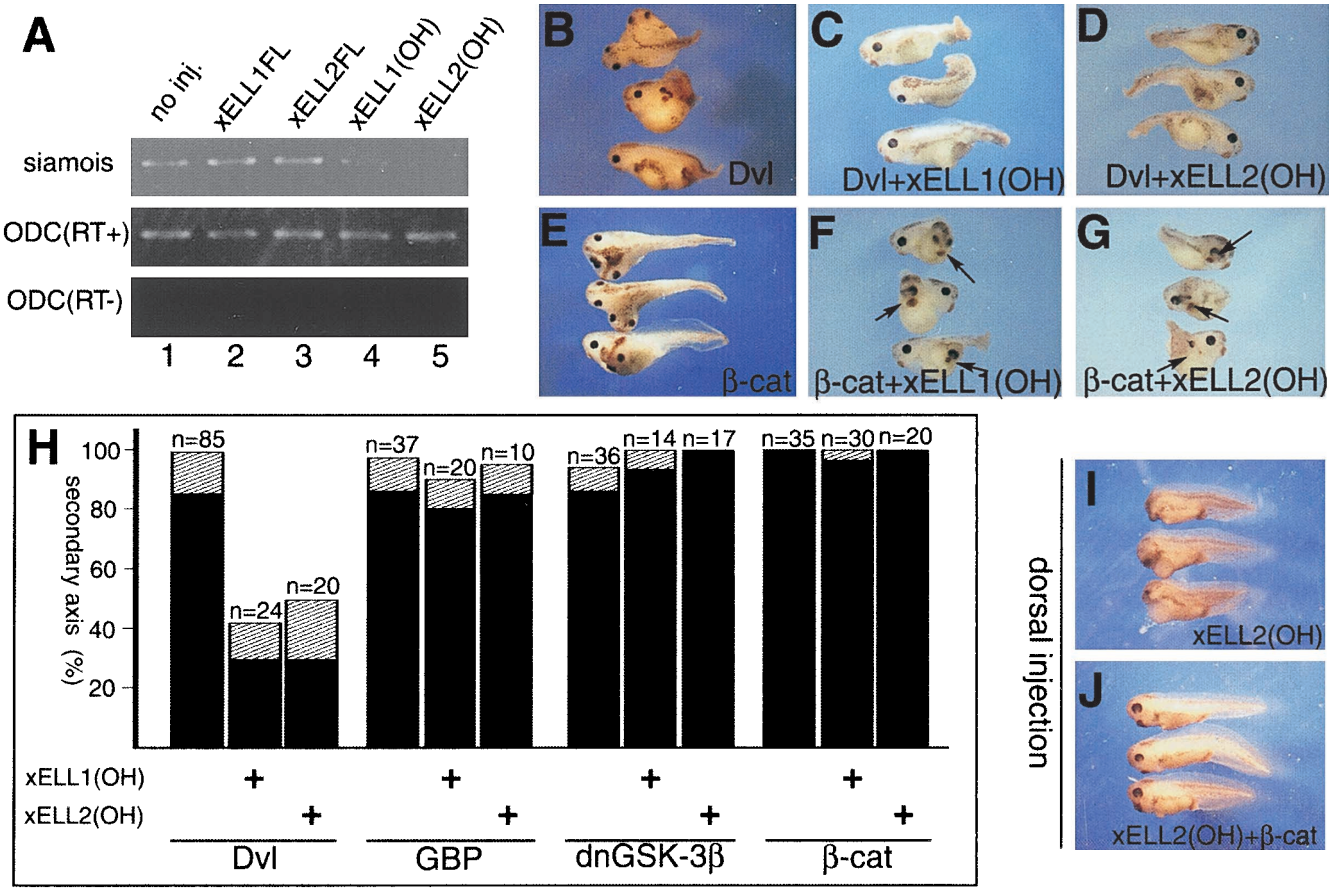
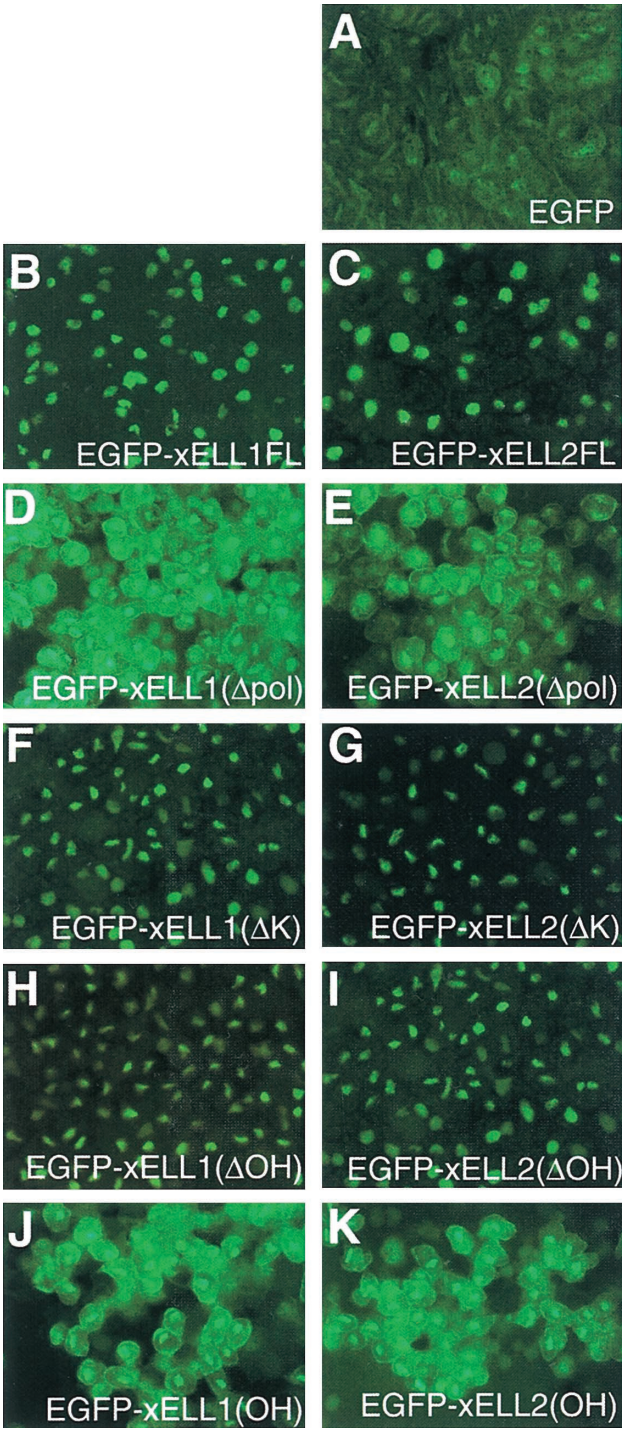


Fig. 5. Inhibition of the Wnt signaling pathway by ectopic expression of xELL1/2. (A) Effects on siamois expression from the overexpression of full-length or OH-domain of xELL1/2. Siamois expression was determined by RT-PCR using embryos injected with the indicated mRNA into the VMZ. cDNA samples were quantified using ornithine decarbozylase expression (ODC (RT+)). Experiments without RT were carried out to test whether genomic DNA was present in the cDNA samples (ODC (RT-)). (B-G) Phenotypes of embryos co-injected with xELL1/2(OH) and Wnt signaling regulators. Embryos were injected ventrally with the following mRNA; Dvl (500 pg; B), Dvl and xELL1(OH) (500 pg and 1.0 ng, respectively; C), Dvl and xELL2(OH) (500 pg and 1.0 ng; D), β-catenin (250 pg; E), β-catenin and xELL1 (OH) (250 pg and 1.0 ng; F), β-catenin and xELL2 (OH) (250 pg and 1.0 ng; G). (H) Frequency of secondary axis formation. In addition to Dvl and β-catenin, we examined the inhibition activity for secondary axis formation derived by GBP (500 pg) and dominant-negative form of GSK-3β (dnGSK-3β; 500 pg). The results shown in (B–G) are expressed as the percentage of embryos, which formed an ectopic secondary axis. Indicated mRNAs were injected ventrally with the volume shown in (B–G). The solid bars show complete axis duplication, which included eyes and cement grand. The open bars indicate incomplete axis duplication characterized by distinct branched axis without head structure. (I–J) Dorsal injection with xELL2(OH) (250pg; I) or xELL2(OH) and β-catenin (250pg and 500pg; J). By addition of β-catenin, head defect caused by xELL2(OH) was suppressed (J).

tion of 1 ng of xELL1FL or xELL2FL mRNA into the DMZ of 4-cell embryos did not alter the expression of *siamois* at the early gastrula stage, compared with normal, uninjected embryos (Fig. 5A, lane 2 and 3). Conversely, only 200 pg of xELL1(OH) or xELL2(OH) injection decreased *siamois* expression (Fig. 5A, lane 4 and 5). These results indicate that the OH-domain of xELL can act as a negative regulator of canonical Wnt signaling. To determine the part of the pathway affected by xELL(OH), we performed co-injection analysis with several positive regulators of canonical Wnt signaling. Injection of 500 pg of xDvl1 into the VMZ of 4- to 8-cell stage embryos induced secondary axis formation (Fig. 5B). Other factors such as GBP, the dominant-negative form of GSK-3β (dnGSK-3β), and β-catenin also induced an ectopic axis (Fig. 5E, H). When 1 ng of xELL1(OH) or xELL2(OH) was co-injected with Dvl into the DMZ, secondary axis formation was suppressed (Fig. 5C, D, H). Conversely, secondary axis induced by β-catenin was not suppressed by xELL1(OH) or xELL2(OH) (Fig. 5F, G, H). Secondary axes formations induced by GBP and dnGSK-3β were also not effectively suppressed, though a slight inhibition was seen in these embryos (Fig. 5H). Furthermore, β-catenin could suppressed the head defect derived from the dorsal injection with xELL2(OH) (Fig. 5I, J). These data suggest that the OH-domain of xELL1 inhibits canonical Wnt signaling at the level of Dvl and upstream of β-catenin.



Truncation of the N-terminus region of xELL1 and xELL2 alters their subcellular localization

Our results showed that truncated forms of xELL1 and xELL2, such as xELL(Δpol) or xELL(OH) inhibited canonical Wnt signaling (Fig. 4B, C, H, I). It is known that both ELL1 and ELL2 are localized within the nuclear body and function as RNA polymerase II elongation factors (Shilatifard, *et al*., 1996). In contrast, Dvl is thought to act in the cytoplasm to inhibit the kinase activity of GSK-3β. We hypothesized that full-length xELL resides in the nucleus and functions as a transcription factor, whereas a truncated form of xELL, containing only the OH-domain, acts in the cytoplasm to inhibit canonical Wnt signaling. To clarify this hypothesis, we examined the intracellular distribution of EGFP-xELL1 or EGFP-xELL2 fusion protein. Following injection of 200 pg of EGFP mRNA, the EGFP protein was localized to both nucleus and cytoplasm (Fig. 6A). When EGFP-xELL1FL and EGFP-xELL2FL were injected into animal pole cells of 2-cell stage embryos, staining was predominantly seen in the nuclear body (Fig. 6B, C). This result supported the role of ELL protein as a transcriptional factor.

We next injected the truncated forms of xELL1 and xELL2 fused with EGFP. EGFP-xELL1(ΔK), EGFP-xELL1(ΔOH), EGFP-xELL2(ΔK) and EGFP-xELL2(ΔOH) were all localized to the nucleus (Fig. 6F, G, H, I). In con-

Fig. 6. Intercellular localization of full-length or deletion constructs of xELL1/2. (A–K) mRNAs of EGFP and EGFP fusion full-length or deletion constructs of xELL1/2 were injected into the animal pole. Animal caps were removed from injected embryos at stage 10 and observed under the fluorescence microscope. The injected mRNAs were as follows; EGFP (A), EGFP fusion full-length of xELL1/2 (xELL1; B, xELL2; C), EGFP-xELL1/2(Δpol) (xELL1; D, xELL2; E), EGFP-xELL1/2(ΔK) (xELL1; F, xELL2; G), EGFP-xELL1/2(ΔOH) (xELL1; H, xELL2; I), EGFP-xELL1/2(OH) (xELL1; J, xELL2; K). 1.0 ng mRNA was injected in each case. In the case of EGFP-xELL1/2FL, nuclear localization was seen (B, C). Similar localization was observed in EGFP-xELL1/2(ΔK) and EGFP-xELL1/2(ΔOH) (F, G, H, I). Conversely, EGFP-xELL1/2(Δpol) and EGFP-xELL1/2(OH) were localized in the cytoplasm, as well as the nucleus (D, E, J, K).

trast, the proteins encoded by EGFP-xELL1(Δpol), EGFP-xELL2(Δpol), EGFP-xELL1(OH) and EGFP-xELL2(OH) were stained in the cytoplasm as well as in the nucleus (Fig. 6D, E, J, K). These observations are consistent with the results from the microinjection experiments: xELL located in cytoplasm caused a head defect (Fig. 4B, C, H, I), whereas several other forms of xELL located in nuclei caused no head defect (Fig. 3C, E; Fig. 4D, E, F, G). Actually, 0.5 ng of EGFP-xELL2(OH) injection into DMZ caused loss of head structure (DAI=2.1, n=16). Therefore, translocation of xELL protein from the nucleus to the cytoplasm may regulate its inhibition of the canonical Wnt signaling pathway.

DISCUSSION

This report demonstrates the involvement of xELL1 and xELL2 in the Wnt signaling pathway. We isolated *xELL1* and *xELL2* as two *Xenopus* homologues of the *ELL* gene. Sequence homology suggested that *xELL1* is the homologue of *hELL1* and that *xELL2* is the likely homologue of *hELL2*. Temporal expression of *xELL1* and *xELL2* in the developing embryo was shown to be quite different, and their 3'-prime UTR sequences do not show high homology (data not shown). We therefore concluded that *xELL1* and *xELL2* are not allelic variants of each other. The Pol II-domain, K-domain and OH-domain are all highly conserved among all five *ELL* genes, suggesting that these genes may serve similar biochemical functions. Previous reports showed that both hELL and hELL2 act as transcription factors and that they show little difference in function (Shilatifard *et al*., 1997). In contrast, RT-PCR revealed that *xELL1* expression is increased only after stage 26, while *xELL2* is expressed throughout development. This implies that xELL1 and xELL2 may play different roles in early embryogenesis, and that xELL2, at least, may play a role in the early canonical Wnt signaling. Amino acid comparison of the two genes showed that xELL2 but not xELL1 contains a proline-rich sequence between the Pol II-domain and K-domain. It is known that the PXXP motif can bind Src homology 3 (SH3) domain-containing proteins (Ren *et al*., 1993). In the proline-rich sequence of xELL2, several PXXP motifs are found, though it is remains unclear whether this domain ascribes a particular biochemical function to the protein.

We also showed that a loss of head phenotype is observed when the OH- domain of ELL is injected into the DMZ of 4-cell stage embryos. RT-PCR analysis indicated that overexpression of this domain inhibits target gene expression of canonical Wnt signaling target genes, such as siamois. These data implicate ELL in the inhibition of Wnt signaling via its OH-domain. Because the C-terminus half of rELL was isolated via an interaction with Dvl, it is thought that the OH-domain of xELL may interact with Dvl and inhibit Dvl activity. This claim is supported by the fact that the OH-domain of xELL1/2 is highly homologous with the ZO-1 binding domain of occludin, and Dvl contains a PDZ domain like ZO-1. Moreover, we also showed that the OH-domains of both xELL1 and xELL2 inhibited secondary axis formation induced by Dvl, but not by β-catenin. These results suggest that the OH-domain of xELL can down-regulate canonical Wnt signaling by acting at a point in the pathway between Dvl and GSK-3β. This is also consistent with the idea of an interaction between Dvl and ELL.

In contrast to the truncation mutants, full-length xELL1/2 caused virtually no phenotype and had no effect on siamois expression. In addition, it seems contradictory that ELL, which is known to function in the nucleus, can affect cytoplasmic factors such as Dvl and GSK-3β. However, we found that truncated forms of xELL1/2 proteins, possessing only the OH-domain were localized in the cytoplasm, whereas full-length xELL1/2 remained in the nucleus. These results raised the possibility that a nuclear localization signal is contained within the Pol II-domain of ELL. It is known that these signals ordinarily consist of bipartite basic clusters. However, our results also showed that the K-domain is dispensable for nuclear localization, which is consistent with previous studies (Kanda *et al*., 1998). The biochemical function of the K-domain remains unknown. It is interesting that although ELL is regarded as a transcription factor, no DNA-binding motifs such as zinc-finger, leucine zipper and helix-loop-helix sequences are present in the molecule. Clusters of basic residues are commonly found in the DNA-binding domains of several nuclear proteins, so the K-domain of xELL1/2 may possess DNA-binding properties.

Taken together, our results demonstrated that the truncated form of xELL1/2 acts to inhibit canonical Wnt signaling in the cytoplasm. The major problem for this argument is whether xELL actually exists in the cytoplasm and acts there during normal *Xenopus* development. Though direct evidence remains to be presented, there are several possible reasons for the existence of a truncated form of xELL1/2 in the cytoplasm. First, xELL1/2 may be post-translationally modified. To assess this working hypothesis, we made two plasmid constructs that encoded myc-xELL1-EGFP and myc-xELL2-EGFP fusion proteins and examined their products by Western blotting. In our preliminary experiments, the short forms of xELL1/2, especially those corresponding to the Δ(pol) type in molecular size, were not seen (data not shown). Moreover, both xELL1FL-EGFP and xELL2FL-EGFP were not localized in cytoplasm. Therefore, these results suggest that xELL1/2 protein is not effectively processed *in vivo*. The second possibility is that there are splicing variants of xELL1/2 and that these reside in the cytoplasm. In previous studies, several genes whose intracellular localization is different among their isoforms have been reported. For example, the erythrocyte protein 4.1R possesses seven different types of cDNAs, with four of the variants localized to the nucleus, and the other three in cytoplasm (Luque and Correas, 2000). Thus, we examined the transcription products of xELL1 and xELL2 by Northern blotting. A single positive band was detected in xELL1, and two major bands for the xELL2 transcript were seen (data not shown). This suggests the possibility that xELL2, at

least, has splice variant(s). Finally, the cytoplasmic localization may be due to several other events such as an interaction with other proteins and protein modification. It would be therefore informative to examine the subcellular localization of the truncated xELL protein in various stages of Xenopus embryos using an antibody against the OH-domain of xELL1/2.

It is clear that the role of full-length xELL should be examined by loss of function analysis using morpholino antisense oligonucleotides. In *Drosophila,* a deletion mutant of *dELL* causes abnormal embryonic segmentation (Eissenberg *et al*. 2002). However, ELL can inhibit the transcriptional activity of p53, so xELL may have a similar function. A recent study showed that Siah-1, a homolog of *Drosophila seven in absentia* gene, mediates a β-catenin degradation by interacting with APC (Liu *et al*., 2001), and it is known that Siah-1 is induced by p53 (Amson *et al*., 1996). So, xELL may normally inhibit Siah transcription via p53 and modulate Siah-mediated β-catenin degradation.

ACKNOWLEDGEMENTS

This work was supported in part by CREST and SORST of the Japan Science and Technology Agency (JST), and by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to M. A.

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(Received December 18, 2003 / Accepted February 7, 2004)