CASEIN KINASE I TRANSDUCES WNT SIGNALS

APPROVED BY SUPERVISORY COMMITTEE

Jonathan Graff, M.D., Ph.D._____

Jane Johnson, Ph.D. _____

Jin Jiang, Ph.D. _____

Melanie Cobb, Ph.D. _____

DEDICATION

I would like to dedicate this work to my family, and thank them for their continued patience and support throughout my education

CASEIN KINASE I TRANSDUCES WNT SIGNALS

by

JOHN MICHAEL PETERS

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

April, 2005

ACKNOWLEDGEMENTS

First I would like to thank my co-authors on this work for their invaluable contributions to this project. Additionally many members of the Graff Lab and Center for Developmental Biology who were not directly involved with this project, as well as my thesis committee, made helpful suggestions and provided valuable insight. My mentor Jon Graff provided unflagging support and encouragement throughout this project as well as technical expertise. Additionally I appreciate the collegiality of the scientific community and thank all of those researchers who contributed reagents and expertise to aid us in our studies. Finally I would like to thank UT Southwestern and the MSTP for providing the opportunity for myself and others to pursue research as part of a combined degree. Without the generosity of all of these people this work would not have been possible and I am grateful to have had the opportunity to study the role of CKI in the Wnt pathway.

CASEIN KINASE I TRANSDUCES WNT SIGNALS

Publication No.

John Michael Peters, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2005

Supervising Professor: Jonathan M. Graff M.D., Ph.D.

Wnt signaling controls a diverse array of processes including cell growth, oncogenesis, and development. Components of the Wnt cascade are altered in several human cancers including colon cancers and melanomas. We set out to identify novel components of this signal transduction pathway via expression cloning in *Xenopus* embryos. This assay identified a protein kinase, casein kinase I (CKI), as a novel component of the Wnt signaling pathway.

We first showed that expression of CKI produces Wnt phenotypes. First, CKI induced completely formed second axes, or Siamese twins, when injected into embryos. Second, embryos which had been ventralized by UV-irradiation were

completely rescued by CKI, producing normal embryos. In order to extend these observations, we tested CKI in three biochemical assays of Wnt signaling. First, CKI expression stabilized β-catenin protein levels, a hallmark of Wnt signaling. Second, CKI induced the expression of Siamois and Xnr3, direct transcriptional targets of Wnt signaling. Finally, CKI expression dorsalized the ventral marginal zone in *Xenopus*, inducing markers of the Spemann organizer which is a product of Wnt signaling.

We next investigated the ability of CKI inhibitors to block Wnt signaling *in vivo*. Two dominant negative forms of CKI and a specific pharmacological inhibitor of CKI all blocked the ability of Xwnt8 to induce dorsal axes and target genes in embryos. These data demonstrate that CKI function is required for transduction of Wnt signals. Additionally, we also demonstrated that CKI function is required for Wnt signaling in the nematode *C. elegans*, proving that CKI function in this pathway is conserved from invertebrates to vertebrates.

We then investigated the biochemical mechanism of CKI function. Epistasis experiments indicated that CKI acts between Dishevelled and GSK-3. Yeast two-hybrid assays showed that CKI strongly binds Dishevelled. Through *in vivo* phosphorylation experiments, we showed that CKI increases Dishevelled phosphorylation and *in vitro* experiments showed that CKI can directly phosphorylate Dishevelled. To extend our studies we performed further experiments using additional CKI isoforms and found that many other isoforms can also reproduce markers of Wnt signaling.

In summary, through a combination of gain-of-function and loss-of-function studies in invertebrates and vertebrates, we have shown that CKI is a novel and conserved component of the Wnt signaling pathway.

TABLE OF CONTENTS

PRIOR PUBLICATIONS RELATED TO CASEIN KINASE I

Peters JM*, McKay RM*, McKay JP, Graff JM (1999) Casein kinase I transduces Wnt signals. *Nature* 401: 345-350.

McKay RM*, Peters JM*, Graff JM (2001) The Casein Kinase I Family: roles in morphogenesis. *Developmental Biology* 235: 378-387.

McKay RM*, Peters JM*, Graff JM (2001) The Casein Kinase I Family in Wnt Signaling. *Developmental Biology* 235: 388-396.

* denotes authors share equal contribution

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF DEFINITIONS AND ABBREVIATIONS

C: Uninjected control
CKI: Casein kinase I
CKIα: *Bos taurus* CKI alpha
CKIβ: *Bos taurus* CKI beta
CKIγ: Human CKI gamma 3
CKIδ: Human CKI delta
DN: Dominant negative
D>N: CKI with inactivating aspartic acid to asparagine mutation
Dsh: Dishevelled
EF-1α: Elongation factor 1 alpha
KD: Kinase domain (CKI isoform modified to remove non-catalytic regions)
K>R: CKI with inactivating lysine to arginine mutation
MMR: Marc's Modified Ringer's solution
RT: Reverse Transcriptase
Xdsh: *Xenopus laevis* dishevelled
XCKIε: *Xenopus laevis* CKI epsilon
XGSK-3: *Xenopus laevis* glycogen synthase kinase 3
Xwnt-8: *Xenopus laevis* Wnt 8

CHAPTER ONE
Introduction

The highly conserved Wnt signal transduction pathway is essential for normal development in complex eukaryotes ranging from *C. elegans* to mammals (Cadigan and Nusse 1997, Moon *et al.* 1997). Additionally, mutations affecting Wnt signaling have been implicated in a broad range of pathological conditions including several types of cancer (Nusse and Varmus 1982, Kinzler and Vogelstein 1996, Rubinfeld *et al.* 1997, Morin *et al.* 1997, Chan *et al.* 1999, Koch *et al.* 1999). Despite the importance of this pathway, as yet not all elements of the signaling cascade have been described. Because of its widespread involvement in development and disease, understanding the Wnt pathway has broad implications. Therefore we undertook an expression cloning screen based on embryonic phenotypes and gene expression patterns in order to isolate components of the Wnt and other signaling pathways. This thesis describes a novel component of the Wnt signaling pathway identified in this manner, casein kinase I.

CANONICAL WNT PATHWAY

Through a combination of developmental, genetic, and biochemical studies, a preliminary outline of Wnt signaling has recently been espoused (Cadigan and Nusse 1997, Moon *et al.* 1997). These data were generated in a diverse array of organisms and have converged on a common set of molecules demonstrating that

the pathway has been conserved. The secreted Wnt ligands form a complex with the Frizzled family of receptor proteins (Bhanot *et al.* 1996, Xu *et al.* 1998). The signal is then transduced through Dishevelled to inhibit glycogen synthase kinase-3 (GSK-3), a negative regulator of the Wnt pathway. Dishevelled is a PDZ-domain containing protein with no known enzymatic activity which may act as a molecular scaffold (Li *et al.* 1999); details of its function and regulation are poorly understood. The mechanism by which the signal is propagated from Frizzled to GSK-3 is not currently understood, nor is it known how many steps are involved in these connections (Cadigan and Nusse 1997, Moon *et al.* 1997). In the absence of Wnt signaling, GSK-3 phosphorylates □-catenin targeting it for degradation (Yost *et al.* 1996). The Wnt-dependent inhibition of GSK-3 alleviates this negative regulation and increases □-catenin levels. Stabilized □-catenin then binds to the Lef/XTcf family of transcription factors and the complex translocates to the nucleus and alters gene expression by direct binding to the promoters of Wnt-target genes such as Xnr3 and Siamois (Molenaar *et al.* 1996, Behrens *et al.* 1996, McKendry *et al.* 1997, Brannon *et al.* 1997, Carnac *et al.* 1996). Several other genes have been implicated in the Wnt pathway. For example, Axin and APC are both negative regulators that enhance the degradation of □-catenin and are mutated in cancer (Groden *et al.* 1991, Kinzler *et al.* 1991, Rubinfeld *et al.* 1993, Satoh *et al.* 1993, Su *et al.* 1993, Zeng *et al.* 1997, Ahmed *et al.* 1998).

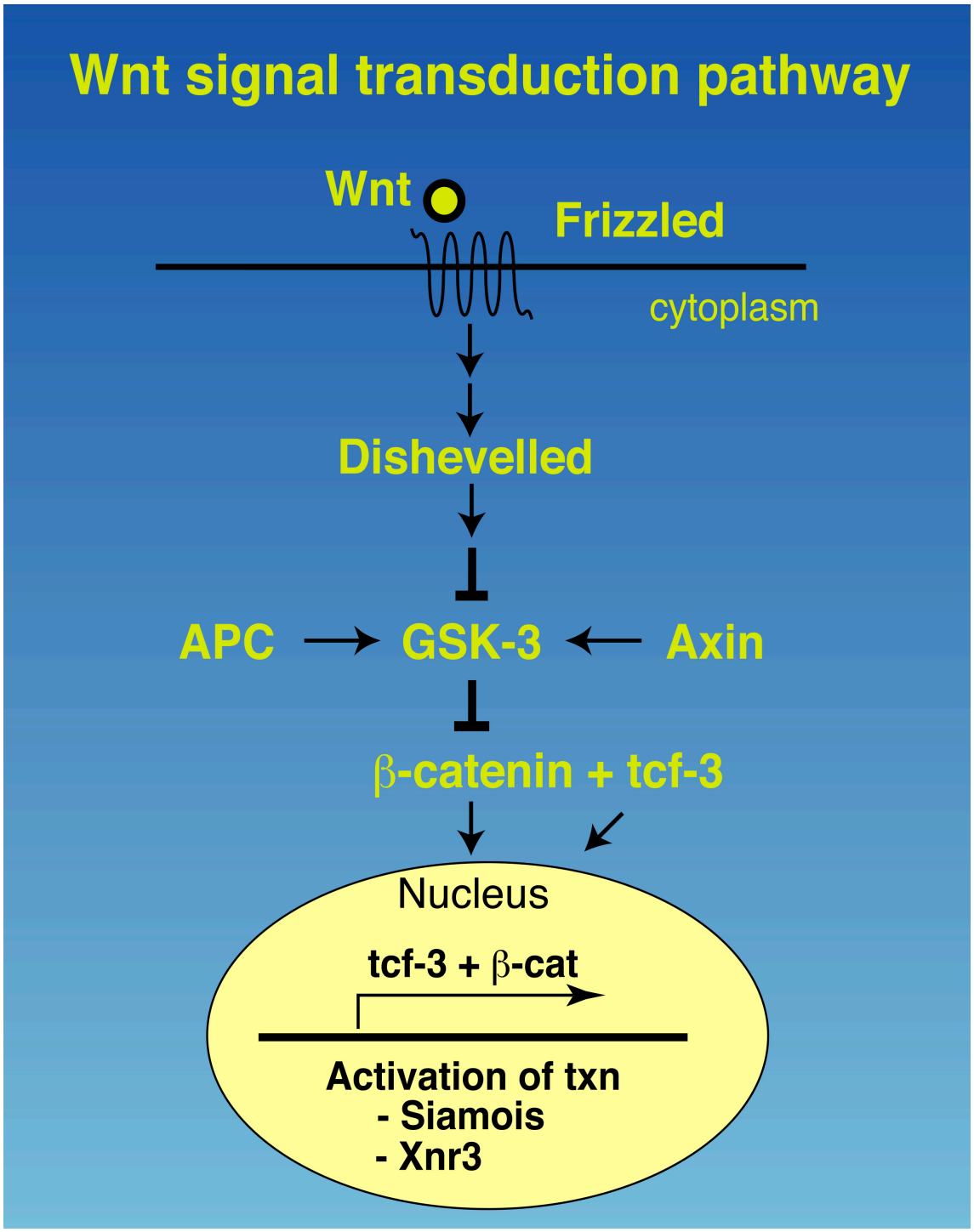


Figure 1. Overview of the Wnt signaling pathway. See text for discussion.

XENOPUS LAEVIS **AS A MODEL SYSTEM**

Xenopus laevis has been a model system for developmental biologists for decades. This species has many advantages including year-round reproduction, external fertilization and development, and large rapidly developing embryos. Additionally husbandry of the embryos requires only an incubator and simple buffered salt solutions. The embryos require no nutrition until the completion of their development and their external surface is freely observable after chemical removal of the protective jelly coating. As a result, it has been possible to map early cell cleavage patterns, tissue fates, and gene expression patterns extensively. This has allowed the generation of many highly informative embryo manipulation techniques as well as the ability to target treatments to specific tissues and examine gene expression patterns in specific tissues. However the great disadvantage of *Xenopus* is the difficulty of performing any classical genetic experiments. The generation time for *Xenopus* is over one year and this species is pseudotetraploid due to chromosome duplication. Therefore, genetic knockout studies are nearly impossible and even transgenics are time consuming to create and propagate. As a result, developmental studies in *Xenopus* often utilize the injection of *in vitro* synthesized mRNA to study gene function. Both oocytes and recently fertilized embryos are efficient at translating mRNA to protein. However, it is not simple to effectively express genes within the first few hours of development due to the handling time

required for embryo preparation and injection as well as a brief delay in the onset of translation. Loss-of-function studies may employ dominant negatives, chemical inhibitors, transcriptional or translational inhibitors, or with great difficulty mRNA depletion prior to fertilization (Sumanas *et al.* 2000, Heasman 2002).

Previous work has demonstrated that in *Xenopus laevis* Wnt signaling generates the Spemann organizer and thus controls formation of the dorsal axis (Heasman *et al.* 1994, Smith & Harland 1991, He *et al.* 1995, Pierce & Kimelman 1995, Molenaar *et al.* 1996, Behrens *et al.* 1996). This Spemann organizer acts to pattern the embryo and without its dorsalizing influence the embryos lack all dorsal and anterior tissues such as the head, nervous system, and skeletal muscle. Because an active Wnt signal is necessary and sufficient to produce a dorsalizing center in *Xenopus*, whole embryo experiments examining Wnt signaling are possible. For example, an ectopic Wnt signal introduced into the ventral side of a normal embryo can produce a second dorsalizing center. This results in the induction of a complete second body axis, mirroring the endogenous role of maternal Wnt signals in dorsal axis formation (Moon *et al.* 1997, Smith and Harland 1991, McMahon and Moon 1989).

When *Xenopus* embryos are irradiated with ultraviolet (UV)-light during the first cell cycle, the endogenous dorsalizing center is disrupted and all dorsal

structures are eliminated (Scharf and Gerhart 1983). This irradiation is limited to the vegetal pole of the one-cell embryo, opposite the nucleus, and does not cause any mutations or long-term injury. Rather it temporarily disrupts the microtubule network underlying the cell membrane and blocks formation of the endogenous dorsalizing center (Elinson and Rowning 1988). The embryo then becomes symmetrically ventral. As a result, only ventral tissues form and the embryos develop without any dorsal structures or tissues (Scharf and Gerhart 1983). Activation of Wnt signaling anywhere within the vegetal pole of the embryo after the UV-irradiation can restore all the dorsal structures to UV-embryos and produce apparently normal tadpoles (Smith and Harland 1991). Other endogenously expressed molecules such as activin or noggin also rescue some dorsal tissues in UV-embryos but rarely generate the full dorsal axis (Thomsen *et al.* 1990, Smith and Harland 1991); these other signals that can produce partial second axes or partially rescue UV-embryos do not induce a Spemann organizer (Lesueur and Graff 1999).

In addition to these developmental experiments in whole embryos, *Xenopus* can be used for gene expression and biochemical studies. Tissue fates and gene expression patterns have been extensively studied and are highly reproducible (Nieuwkoop and Faber 1967). Therefore it is possible to examine gene expression patterns in only specific tissues during development. For example, the animal cap portion of the embryo-which in isolation develops into skin-can be tested for the

expression of new markers after injection of exogenous mRNA. Early in development this tissue has limited plasticity and its gene expression pattern or even tissue fate can be altered through induction or expression of exogenous genes. For example, activation of signal transduction pathways such as Wnt can result in expression of their target genes. Additionally the consistent patterning of the embryo allows the ventral and dorsal sides to be examined separately for their gene expression patterns. This allows studies of patterning, such as altering ventral and dorsal fates by blocking endogenous or adding exogenous dorsalizing influences.

WNT SIGNALING IN *C. ELEGANS*

Because loss of function studies are complicated in *Xenopus*, we chose to pursue additional studies in *C. elegans* where RNA interference has been shown to reproduce the phenotypes of many genetic mutants (Fire *et al.* 1998). In *C. elegans*, one role of Wnt signaling is to control the fates produced by the EMS blastomere which normally generates two daughter cells, MS and E (Thorpe *et al.* 1997, Rocheleau *et al.* 1997). MS produces mesoderm and no endoderm and E generates only endoderm. Active Wnt signaling is essential for specification of the E blastomere, and in the absence of Wnt signals E is transformed into MS. The resultant phenotype, termed "*mom*" (more mesoderm), is a worm without endoderm and with excess muscle (Thorpe *et al.* 1997, Rocheleau *et al.* 1997). Several

components of the Wnt pathway were characterized by inhibiting their function through RNA interference (RNAi, Rocheleau *et al.* 1997). This approach results in a *mom* phenotype of variable penetrance, ranging from 2% with mom-5 (Frizzled) to 100% with wrm-1 (β-catenin, Rocheleau *et al.* 1997). Therefore *C. elegans* represents a useful model system for determining the necessity of putative Wnt components. Additionally, because of the great phylogenetic distance between *C. elegans* and *X. laevis*, consistency of results between the two systems suggests conservation of function across broad evolutionary lineages.

ALTERNATIVE FUNCTIONS FOR WNT PATHWAY COMPONENTS IN MORPHOGENESIS

In addition to the canonical Wnt β-catenin pathway, Dishevelled functions in another Wnt pathway that does not involve β-catenin stabilization (Boutros and Mlodzik 1999). In Drosophila, this β-catenin-independent cascade is termed the planar cell polarity pathway and it transduces its signal via some of the same molecules that function in the classical Wnt pathway including Frizzled, Dishevelled, and potentially others (Boutros and Mlodzik 1999). Downstream of Dishevelled, the planar cell polarity pathway diverges from the canonical Wnt/β-catenin pathway and acts through an unknown mechanism thought to involve the JNK cascade and rho/rac (Boutros *et al.* 1998, Eaton *et al.* 1996, Strutt *et al.* 1997). In cell culture,

Dishevelled can activate both the β-catenin and the JNK pathways (Boutros *et al.* 1998, Li *et al.* 1999). However, inhibitors of the canonical Wnt pathway including Axin, also activate the JNK cascade (Boutros *et al.* 1998, Zhang *et al.* 1999).

In *Xenopus* embryos, the β-catenin-independent pathway appears to control a set of cell movements termed convergence extension that occurs during gastrulation (Tada and Smith, 2000). Recent work in *Xenopus* demonstrated that cells blocked from undergoing convergence extension have a defect in cell polarity (Wallingford *et al.* 2000), which suggests that planar cell polarity in flies and convergence extension movements in frogs may be analogous processes. A role for Wnt signaling in convergence extension is indicated by embryonic expression of inhibitors or dominant negative forms of Wnts, Frizzleds, or Dishevelled (Sokol 2000, Djiane *et al.* 2000, Li *et al.* 1999, Tada and Smith 2000, Wallingford *et al.* 2000), which dramatically shorten the anterior-posterior axis, producing the "bent-back" phenotype (Deardorff *et al.* 1998, Djiane *et al.* 2000, Sokol 2000, Hoppler *et al.* 1996, Moon *et al.* 1997, Sokol 1996, Tada and Smith 2000). As these embryos contain all tissue types, the bent-back phenotype is thought to result from an inhibition of cell movements rather than a change in cell fate. These results contrast with those obtained upon injection of the negative Wnt regulators Axin and GSK-3 or a dominant negative form of Tcf-3; these embryos are ventralized and contain no dorsal tissues (He *et al.* 1995, Molenaar *et al.* 1996, Zeng *et al.* 1997). Ventralized

embryos are also observed when β-catenin is depleted from oocytes or embryos by anti-sense or morpholino technology (Heasman et al. 1994, Heasman *et al.* 2000). The molecular mechanism that underlies the distinct phenotypes observed when different components of the Wnt pathway are blocked has yet to be resolved.

CASEIN KINASE I

Through our expression cloning screen, we identified CKI as a gene capable of producing axis duplication in developing *Xenopus*. CKI was among the first serine/threonine kinases identified and studied biochemically (Tuazon and Traugh 1991, Fish *et al.* 1995). Based on sequence analysis, the CKI family includes the α, β, γ, δ, and ε isoforms, all of which contain highly conserved kinase domains (Gross and Anderson 1998) which are believed to have similar substrate specificity (Tuazon and Traugh 1991, Fish *et al.* 1995). Although the biochemistry of these enzymes has been studied in detail, until recently their biological roles remained poorly defined. The different isoforms are distinguished by amino and carboxyl extensions that flank the kinase domain (Figure 22). The α and δ isoforms have short amino and carboxyl extensions. The β and γ isoforms have short amino extensions and relatively long carboxyl-terminal tails that are of similar length and primary structure. Three highly related (>90% amino acid identity in the kinase domain) γ isoforms are known, γ1, γ2, and γ3. The ε isoforms are distinguished from the rest of the CKI

family by the presence of both carboxyl and amino extensions as well as the most sequence divergent catalytic domains. It has been postulated that for the CKI family the amino- and carboxyl-terminal extensions are important for conferring differential function and regulation to the various isoforms (Graves and Roach 1995, Gross and Anderson 1998, Santos *et al.* 1996). The CKI family has been recently found to be involved in a variety of processes including DNA repair, cell-cycle control, and circadian rhythm (Gross *et al.* 1997, Kloss *et al.* 1998, Lowrey *et al.* 2000, Santos *et al.* 1996). Until this study however, no developmental role had been described for CKI.

OBJECTIVES OF DISSERTATION

This dissertation is intended to describe a novel component of the Wnt signaling pathway, casein kinase I. The focus will be on the evidence generated to indicate that CKI is both sufficient and required for Wnt signaling in biological studies. This will be accomplished by describing a series of gain-of-function, loss-of-function, and biochemical experiments which compare the characteristics of CKI to those of known Wnt components.

CHAPTER TWO
Materials and methods

LIBRARY SCREENING AND IDENTIFICATION OF CASEIN KINASE I

Poly (A)+ RNA from 2,000 two-cell stage embryos was synthesized into complementary DNA with the Superscript Plasmid System (GibcoBRL), ligated into SalI-NotI digested pCS105, transformed and plated. Pools of 100 clones were then generated and plasmid DNA purified using Promega Wizard miniprep kits. Pooled plasmid DNA was linearized with AscI, purified by solvent extraction and ethanol precipitation, and mRNA was synthesized as described using the Ambion SP6 megascript kit (Smith and Harland 1991). Single clones from pool 450 were isolated by sib-selection roughly as described (Smith and Harland 1991). The active clone was sequenced and found to have over 97 percent amino-acid identity to the human sequence for casein kinase I epsilon. The isolated open reading frame of *Xenopus laevis* CKI epsilon (XCKI□) was then subcloned into pCS2+ for use in all subsequent experiments. Plasmid DNA was linearized with Not I and transcribed using the Ambion SP6 Megascript kit. After purification, mRNA was injected into appropriate oocytes or embryos as described (Lesueur and Graff 1999).

CLONING OF ADDITIONAL ISOFORMS

All CKI isoforms used were subcloned into pCS2+ for consistency using standard methods. After subcloning, all clones used for further study were completely sequenced to verify their identities and rule out cloning artifacts and

mutations. *Bos taurus* CKI alpha (CKIα) and *Bos taurus* CKI beta (CKIβ) were the generous gift of Dr. Melanie Cobb. Jun Kusuda provided human CKI gamma 3 (CKIγ) and human CKI delta (CKIδ). Catalytically inactive forms of all CKI isoforms were generated via SOEing PCR then sequenced for accuracy (Ho *et al.* 1989). CKIα (K>R) has lysine 38 mutated to arginine. For all CKI isoforms, the D>N mutants have the first aspartic acid mutated to asparagine within the conserved sequence RDI/VKPD/ENFL of the conserved protein kinase domain VIb (amino acid 128 of XCKIα).

XENOPUS **EMBRYO EXPERIMENTAL METHODS**

Female frogs (*Xenopus laevis*) were primed by injection of hCG eighteen hours prior to use. Expressed eggs were fertilized *in vitro* using macerated testis from male *Xenopus* in 0.05XMMR then allowed fifteen minutes to determine the success of fertilization. Embryos were de-jellied by 2x3 minute treatments in three percent cysteine then thoroughly rinsed with 0.1X MMR. Prior to injection, embryos were transferred to 0.1XMMR with 5% Ficoll. After injection, embryos were serially diluted to 0.5XMMR and cultured at eighteen to twenty-four degrees. For long-term culture, gentamycin was used.

For the animal cap/RT-PCR assays, fertilized embryos were injected at the one cell stage. At stage eight to nine explants of animal cap tissue were made by removing the membrane with forceps and cutting the embryo using a hair knife. Caps were then cultured in 0.5XMMR until harvest for RT-PCR. MRNA was harvested from 2 embryos or 5 animal caps by digesting in a solution of 50mM Tris (pH 7.5), 50mM NaCL, 5mM EDTA, 0.5% SDS, and 250ug/mL Proteinase K at 45 degrees for 35 minutes. After solvent extraction and precipitation with ethanol and ammonium acetate, genomic DNA was digested using RQ-a DNAse. Following a second extraction/precipitation, cDNA was synthesized using MMLV reverse transcriptase and an oligo-dT primer. This cDNA was then used in PCR reactions to determine gene expression patterns. For all experiments a control reaction without reverse transcriptase was used to detect any contamination by genomic DNA. Marginal zone experiments were performed identically except that explants were cut from the dorsal or ventral marginal zone areas at the onset of gastrulation.

For the UV-Rescue experiments, embryos were prepared as described above. Precisely 30 minutes post fertilization the embryos were irradiated for 82.5 seconds using a hand-held UV source at 260nm wavelength. The embryos were then allowed to develop undisturbed until the eight-cell stage when they were transferred to 5% Ficoll and injected into one vegetal cell with the appropriate mRNA. Second-axis experiments were performed similarly except that 1) no UV

was used and 2) ventral vegetal cells were chosen for injection. Embryos were scored for dorso-anterior index (DAI) after reaching stage 21 or later (Kao and Elinson 1988) for UV-rescue or second axis index (SAI) for the second axis assay.

For β-catenin stabilization studies, embryos were injected with myc-tagged β-catenin mRNA at the one cell stage along with experimental mRNA. 4.5 hours post-fertilization, before the onset of zygotic transcription, embryos were harvested by lysing 15 embryos in 150uL of: 10mM HEPES pH7.5, 150mM NaCl, 2mM EDTA, 2mM EGTA, 1% NP-40/IGEPAL, .002mg/mL PMSF, .001mg/mL Leupeptin, .001mg/mL SBT1. Lysates were immediately spun at 10,000g for 10 minutes at 4°C. The isolated supernatants were then prepared by SDS-PAGE followed by Western blotting directed against the myc-tag using Amersham's ECL system.

***XENOPUS* OOCYTE EXPERIMENTS**

Oocyte phosphorylation experiments were performed by first surgically harvesting oocytes from *X. laevis* anesthetized in a solution of ice water followed by 0.2% MS222 (tricane-methane sulfonate). The oocytes were then defolliculated by incubation in collagenase at room temperature with constant gentle mixing. After recovery by incubating for eight to twenty-four hours at 16°C, healthy stage six oocytes were injected with mRNA encoding the gene of interest. The oocytes were

then cultured for 24 h at 18°C with 0.6 □Ci $^{32}P_i$/□L. Samples were lysed, centrifuged at 10,000g for 5 min, and the supernatants immunoprecipitated and subjected to SDS-PAGE and autoradiography or Western blotting (anti-Myc, 9E10; anti-CKI antibodies C40520, Transduction Laboratories).

For the oocyte phosphorylation experiments with multiple CKI isoforms, oocytes were injected with 30 ng myc-Dishevelled mRNA and incubated for 24 hours at 17^{0}C. The same oocytes were then injected with 10 ng mRNA encoding CKI□, CKI□, CKI□3, CKI□3KD, CKI□, CKI□, or CKI□KD and transferred to medium containing 0.3 mCi/ml $^{32}P_i$ for an additional 24 hours at 17^{0}C. Then, the oocytes were lysed in 50 mM Tris-HCl, 190 mM NaCl, 6 mM EDTA, 1% Triton X-100, and protease inhibitors. myc-Dishevelled was immunoprecipitated with 9E10, and the immunoprecipitates were subjected to SDS-PAGE and autoradiography. For the Dishevelled mobility shift assay, oocytes were co-injected with 20 ng myc-Dishevelled and 5 ng CKI mRNA, incubated for 20 hours at 15.5°C, lysed, immunoprecipitated, transferred, and probed with 9E10 antibody.

C. ELEGANS **METHODS**

Standard methods were used to culture *C. elegans* wild-type strain Bristol N2. Antibody staining with pharyngeal muscle-specific monoclonal antibody 3NB12

(Rocheleau *et al.* 1997) and gut specific monoclonal antibody ICB4 (Bowerman *et al.* 1992) was done as described (Bowerman *et al.* 1993), except that monoclonal cell supernatants were diluted 1:20. Templates for RNA were full-length cDNAs from C03C10.1 (kin-19) and F46F2.2 (kin-20) generated by RT-PCR. Partial cDNAs for mom-2 and apr-1 were generated in a similar fashion. RNA was generated with Megascript (Ambion). The sense and antisense RNAs were annealed for 15 min at 68°C in TE and then for 1 h at 37°C. The annealed RNA (1 mg/ml) was injected into both gonad arms of young adult hermaphrodites. Embryos for analysis were collected 3-24 h after injection. Laser ablations were as described (Thorpe *et al.* 1997, Epstein and Shakes 1995).

IN VITRO **PHOSPHORYLATION**

Xenopus Dishevelled fragment 2 (AAs 231–531) was cloned into the bacterial expression vector pET15b, produced in E. coli and purified with nickel beads. Purified fragment 2 was incubated with 1000 units of purified CKI (New England Biolabs) in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 5mM DTT, 200 mM ATP, 2 □Ci g^{32}P-ATP at 18°C for the indicated periods of time, and phosphorylation evaluated by SDS-PAGE and autoradiography.

CHAPTER THREE
CKI expression reproduces Wnt signaling

CKI GENERATES SECONDARY DORSAL AXES IN NORMAL EMBRYOS AND RESTORES UV-VENTRALIZED EMBRYOS

During the process of screening cDNA pools for the induction of molecular markers of changes in cell fate, we found that in pool 450 several embryos showed variable duplication of the dorsal-ventral body axis. Through a process of sib-selection of the clones within this pool we identified the responsible gene as the *X. laevis* orthologue of casein kinase I epsilon (CKI). We then proceeded to further test this gene for its ability to reproduce markers and phenotypes of Wnt signaling.

Ectopic Wnt signals induce a complete second axis, mirroring the endogenous role of maternal Wnt signaling in dorsal axis formation (Moon *et al.* 1997, Smith and Harland 1991, McMahon and Moon 1989, Figure 2). CKI was isolated from a maternal source and therefore is expressed at the appropriate time to be involved in generation of the dorsal axis. While other molecules not in the Wnt pathway such as chordin or activin can induce second axes, typically these axes are incomplete and do not include the most anterior structures such as eyes (Thomsen *et al.* 1990, Sasai *et al.* 1994). To characterize the axis-inducing activity of CKI, we injected: CKI, a kinase inactive CKI (CKI (K>R)) (Fish *et al.* 1995, DeMaggio *et al.*

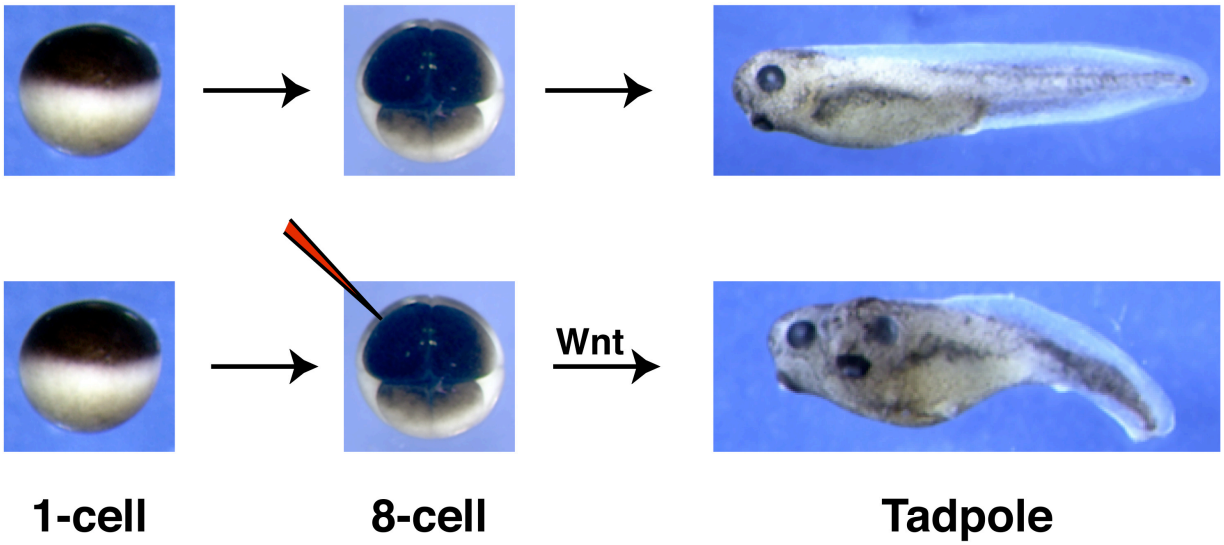


Figure 2. Wnt expression produces complete secondary axes. At the eight cell stage, after the first dorsal/ventral cell division, expression of active Wnt signaling molecules by mRNA microinjection on the ventral side of developing *Xenopus* embryos causes the formation of a second dorsalizing center. As a result, the embryos develop with two dorsal body axes and appear as twins attached at the tail.

1992), and as a comparison a Wnt ligand (Xwnt-8). Approximately 80% of CKI (234/298) or Xwnt-8 (58/73) injected embryos formed second dorsal axes, while the K>R mutant did not, confirming the specificity of the effect (Figure 3). Of note, CKI and Xwnt-8 produced, at roughly the same frequency (11% v 16%, respectively), embryos in which the second axis was indistinguishable from the primary axis.

When *Xenopus* embryos are irradiated with ultraviolet (UV)-light during the first cell cycle, dorsal structures are eliminated and only ventral tissues form (Scharf and Gerhart 1983, Figure 4). Wnt signaling molecules can restore all the dorsal structures to UV-embryos and produce apparently normal tadpoles (Smith and Harland 1991). To determine whether CKI, like Wnt signaling molecules, could restore the dorsal axis to UV-irradiated embryos, we microinjected UV-embryos with CKI, Xwnt-8 as a comparison, and the inactive CKI (K>R) as a specificity control. Both CKI (111/114) and Xwnt-8 (60/61), but not the CKI (K>R) mutant, produced dorsal axes in UV embryos with high frequency. Notably, CKI, like known Wnt signaling components, completely rescued the dorsal axis, producing embryos that were identical to unirradiated, sibling controls (Figure 5).

CKI STABILIZES β-CATENIN AND ACTIVATES WNT-SPECIFIC TARGET GENES

Wnt transduces its signal by inhibiting GSK-3 which leads to a post-

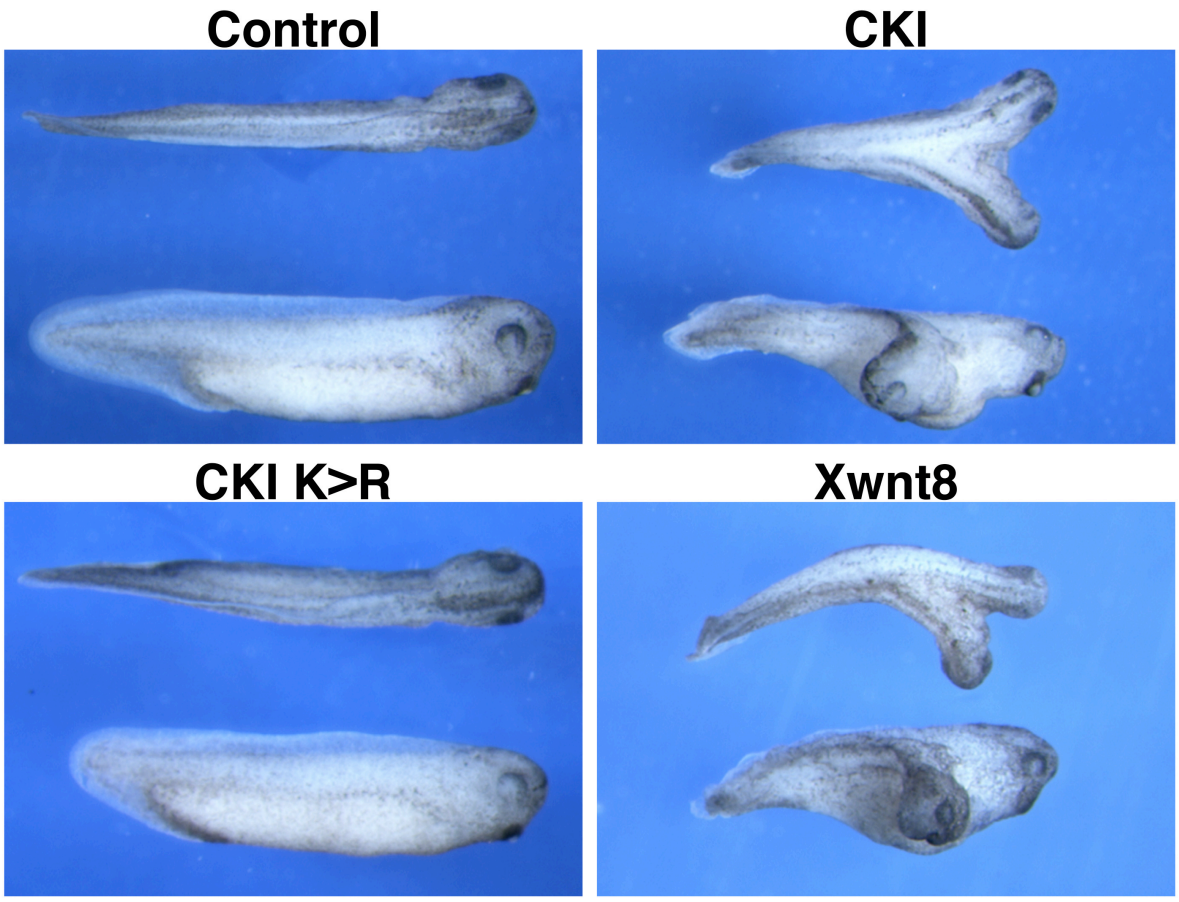


Figure 3. CKI produces complete secondary dorsal axes. Synthetic mRNA encoding XCKI (350 pg), mutant XCKI (K>R) (350 pg), or Xwnt-8 (25 pg) was injected at the 8-cell stage into one ventral vegetal blastomere. XCKI or Xwnt-8, but not inactive XCKI (K>R), injected embryos developed complete secondary dorsal axes.

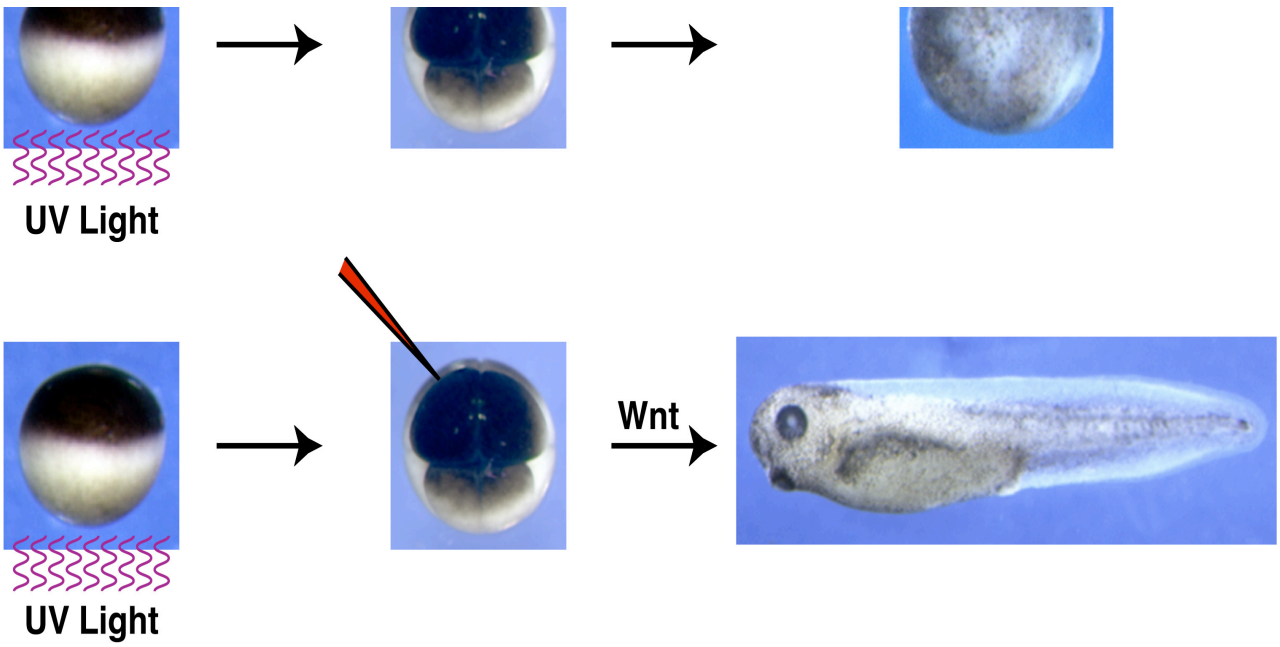


Figure 4. Wnt expression rescues UV-ventralized embryos. Exposure to UV-light disrupts the microtubule array in the vegetal pole of the embryo. As a result, the dorsalizing center does not form and the embryo develops as a completely ventralized ball of tissue. Because it has no dorsalizing influence, the embryo never elongates and never patterns or forms any dorsal structures such as a head or nervous system.

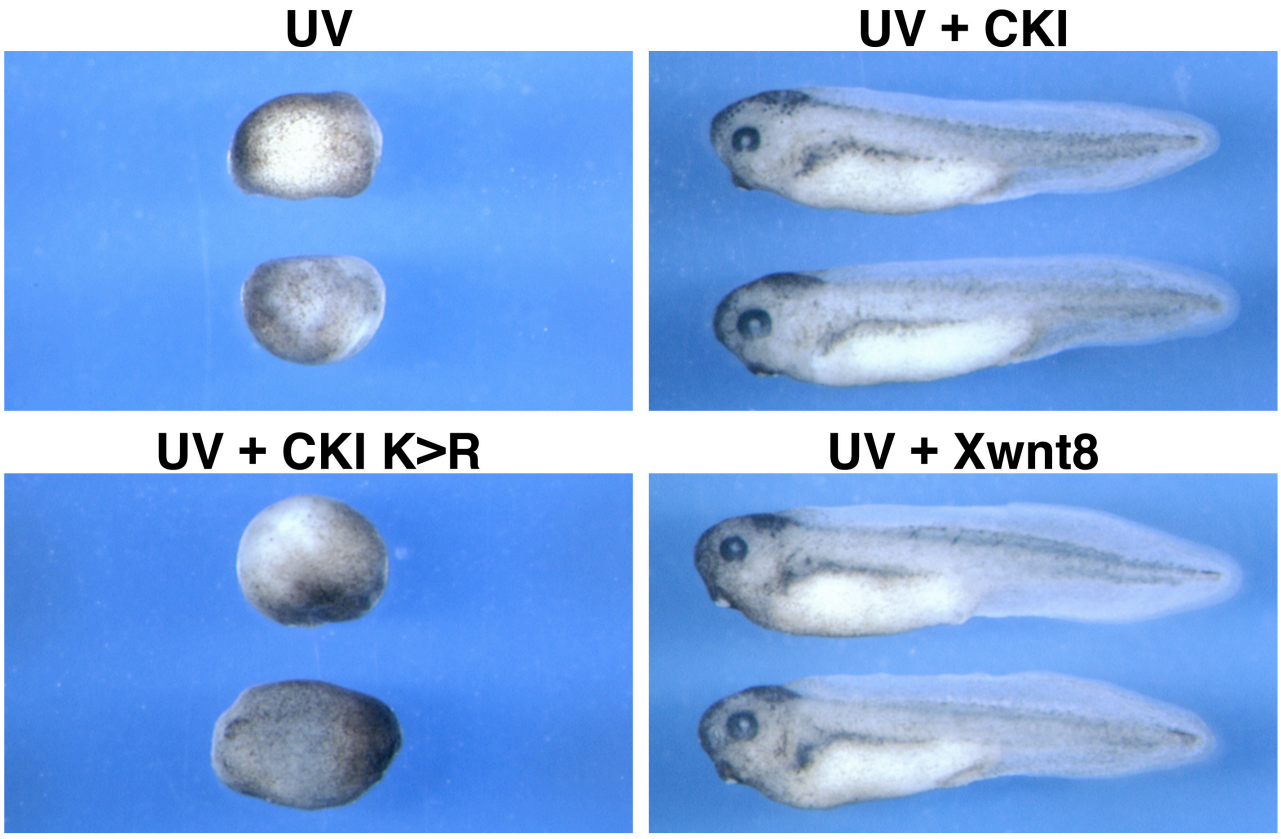


Figure 5. CKI rescues UV-Ventralized embryos. The embryos' vegetal poles were UV-irradiated for 82.5 seconds using a wavelength of 260nm exactly 30 minutes post-fertilization. At the eight-cell stage, one vegetal cell was injected with synthetic mRNA encoding XCKI (350 pg), mutant XCKI (K>R) (350 pg), or Xwnt-8 (25 pg). As in the second-axis assay, only catalytically active XCKI was effective. All embryos shown are at the same developmental age.

translational increase in β-catenin protein levels (Yost *et al.* 1996). Therefore, stabilization of β-catenin protein is a direct and specific readout of Wnt signaling. To determine if CKI could increase the levels of β-catenin, we expressed a myc-tagged version of β-catenin alone, with CKI (K>R), or with CKI, and then analyzed the levels of β-catenin with a Western blot probed against the myc-epitope. To ensure that we were assessing a direct effect, β-catenin protein levels were analyzed prior to initiation of transcription (Newport and Kirschner 1982). We found that CKI increased β-catenin levels and the inactive K>R mutant did not (Figure 6). Therefore, CKI, like Wnt signals, directly stabilizes β-catenin.

Wnt-stabilized β-catenin binds to the Lef/XTcf family of transcription factors and alters gene expression. As CKI also increased β-catenin levels, it seemed plausible that if CKI is in the Wnt pathway then it should also induce Wnt specific-markers. Wnt signaling components activate the expression of dorsal, Spemann organizer-specific markers in ventral tissues (Smith and Harland 1991). In contrast, other signals that can produce second axes or rescue UV-embryos do not induce organizer markers (Lesueur and Graff 1999). To test whether CKI could also induce dorsal markers in ventral tissue, 2-cell embryos were injected with CKI, CKI (K>R), or Xwnt-8 and ventral marginal zones were excised and analyzed for expression of organizer-specific genes as described (Lesueur and Graff 1999, Graff *et al.* 1994). CKI and Xwnt-8, but not CKI (K>R), induced all the organizer markers (Figure 8). Of note, this included the expression of Siamois and Xnr3, direct targets of Wnt signaling (McKendry *et al.* 1997, Brannon *et al.* 1997).

To extend the molecular comparison between CKI and Wnt signaling, we

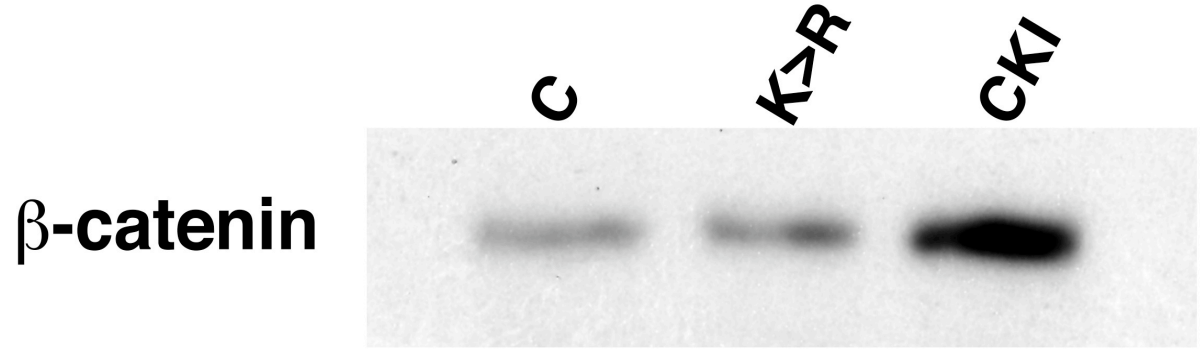


Figure 6. CKI stabilizes ß-catenin. One-cell embryos were injected with myc-tagged ß-catenin (100 pg) alone or with either XCKI (K>R) (350 pg) or XCKI (350 pg). 3-4 hours after fertilization, embryo lysates were analyzed by Westerns with an antibody to the myc-tag. XCKI but not the inactive mutant increased ß-catenin protein levels.

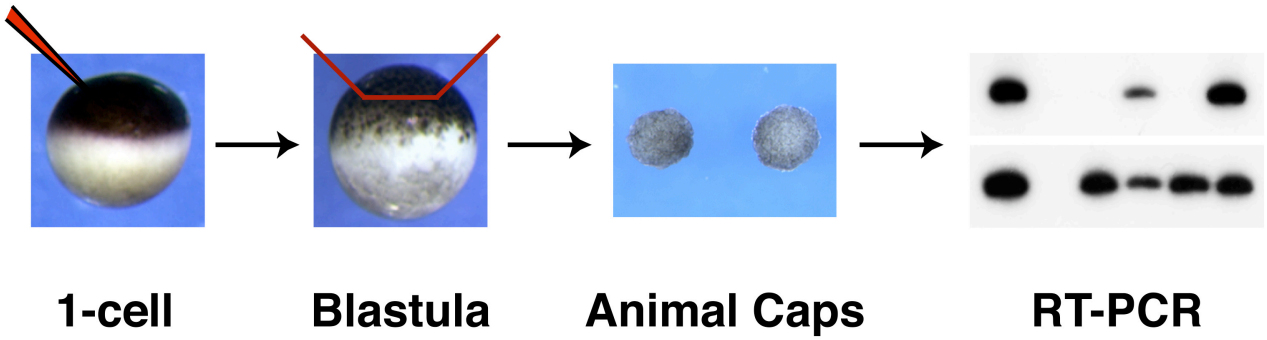


Figure 7. Overview of embryo gene expression studies. Early embryos are microinjected with mRNA encoding a gene of interest. Later at the blastula stage (stage 8-9) explants of specific regions are made. These explants are then cultured until the appropriate developmental age then RNA is extracted for RT-PCR analysis. For marginal zone experiments the explants are made at the onset of gastrulation .

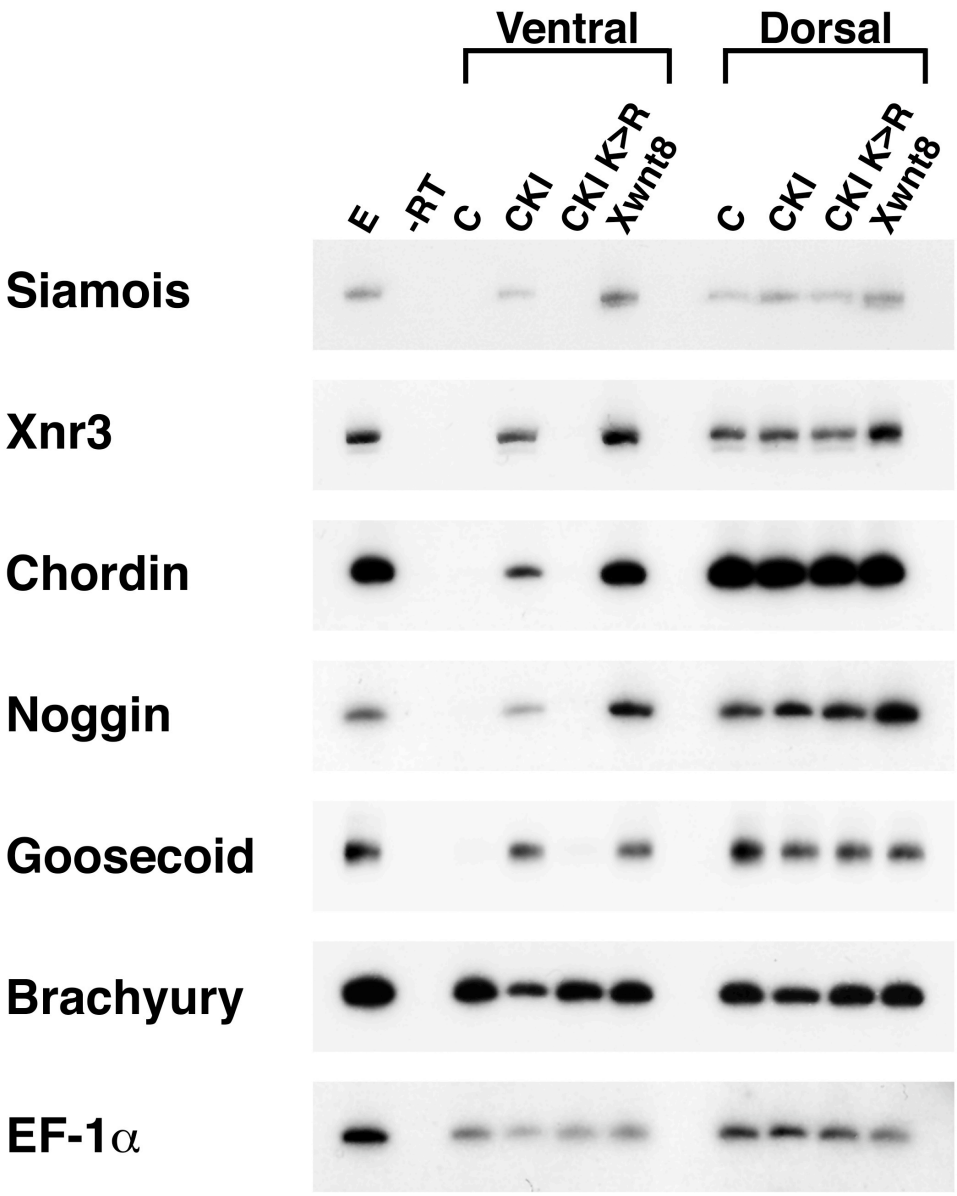


Figure 8. CKI and Wnt dorsalize ventral marginal zones (VMZ). VMZs expressing XCKI (350 pg), XCKI (K>R) (350 pg), or Xwnt-8 (25 pg) were analyzed by RT-PCR. The lane marked E contains total RNA harvested from whole embryos. The lane marked -RT is identical to E except that reverse transcriptase (RT) was omitted. EF-1, a ubiquitously expressed message, is a loading control. The lane C corresponds to control. XCKI and Xwnt-8 but not XCKI (K>R) induced the ectopic expression of all the organizer markers in the VMZ. Dorsal marginal zones from the same embryos are shown for reference.

turned to the animal cap assay. In animal caps, Wnt signals induce the expression of a unique subset of organizer genes, including chordin, Siamois and Xnr3, in the absence of other organizer markers such as goosecoid or of the general mesodermal marker brachyury (Lesueur and Graff 1999, Figures 7 and 9). These latter two genes are normally expressed in the organizer but are not themselves Wnt targets. To test whether CKI could activate the expression of the Wnt-specific subset of genes, we microinjected mRNA encoding CKI, CKI (K>R), or Xwnt-8 into 1-cell embryos, explanted animal caps, and analyzed gene expression. CKI and Xwnt-8 induced the expression of the same subset of genes including the direct targets of Wnt signals, Siamois and Xnr3, while the CKI (K>R) mutant did not (Figure 9).

If CKI and Wnt function in a common pathway, then they might interact synergistically. To test for synergy, we expressed a relatively low dose of CKI or Xwnt-8 alone and also co-expressed them and then assessed the induction of molecular markers. In animal caps, limiting amounts of CKI did not induce expression of Xnr3 and Siamois, while Xwnt-8 induced a relatively low level of expression (Figure 10). When CKI and Xwnt-8 were co-injected, Siamois and Xnr3 expression were synergistically increased (Figure 10). CKI and Xwnt-8 also synergized in rescuing UV-embryos (not shown).

To summarize, in four distinct assays, CKI specifically mimics expression of known Wnt signaling components. CKI is able to produce secondary dorsal axes in normal embryos and restore organizer function to UV-ventralized embryos. Biochemically, CKI stabilizes β-catenin and increases the expression of direct Wnt-

target genes. In all cases the expression of CKI precisely reproduced the phenotypes or markers produced by known Wnt signals.

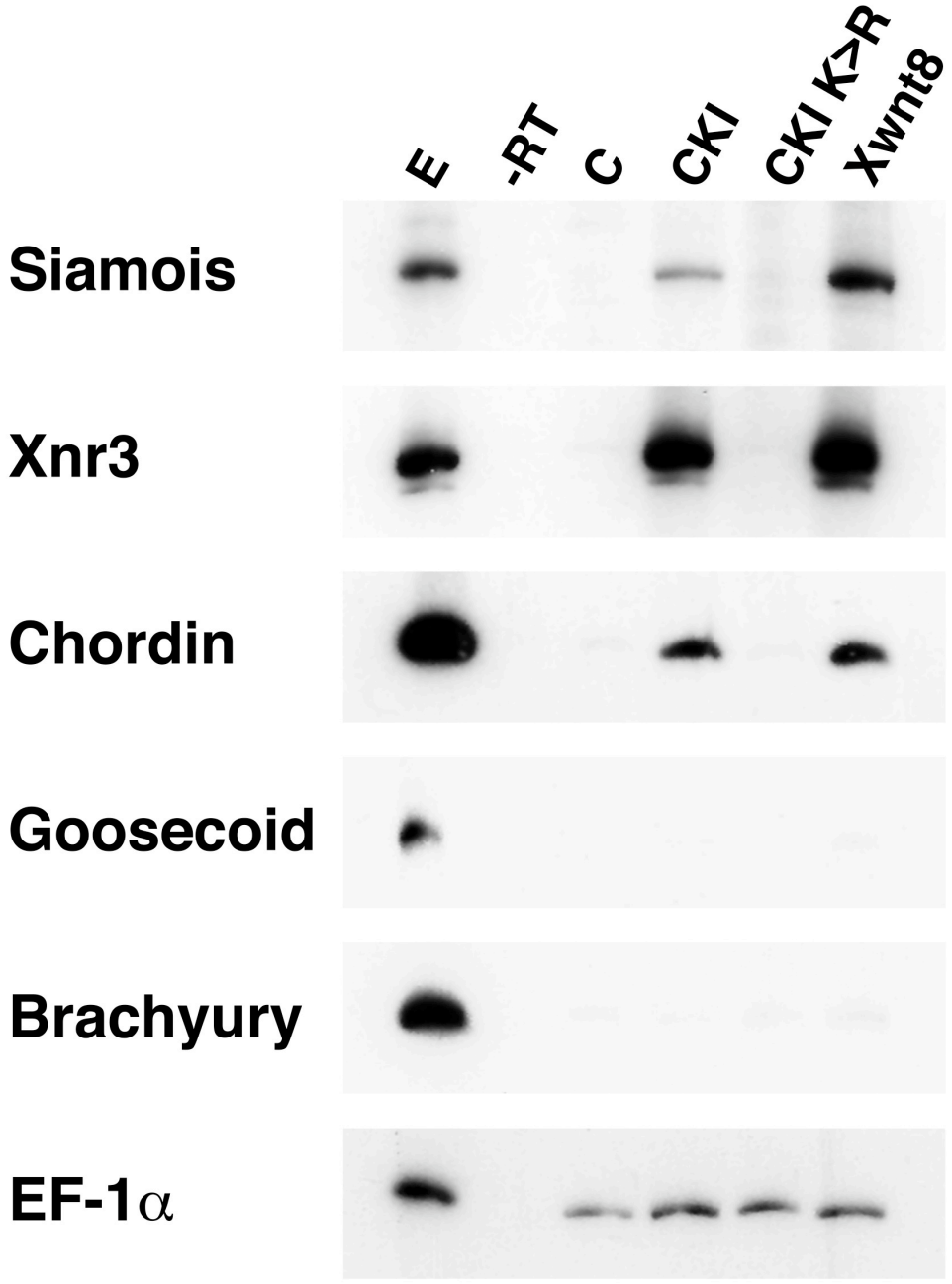


Figure 9. CKI induces Wnt-specific markers in the animal cap. Animal caps expressing XCKI (350 pg), XCKI (K>R) (350 pg), or Xwnt-8 (25 pg) were analyzed by RT-PCR. XCKI and Xwnt-8 induced the expression of the same markers.

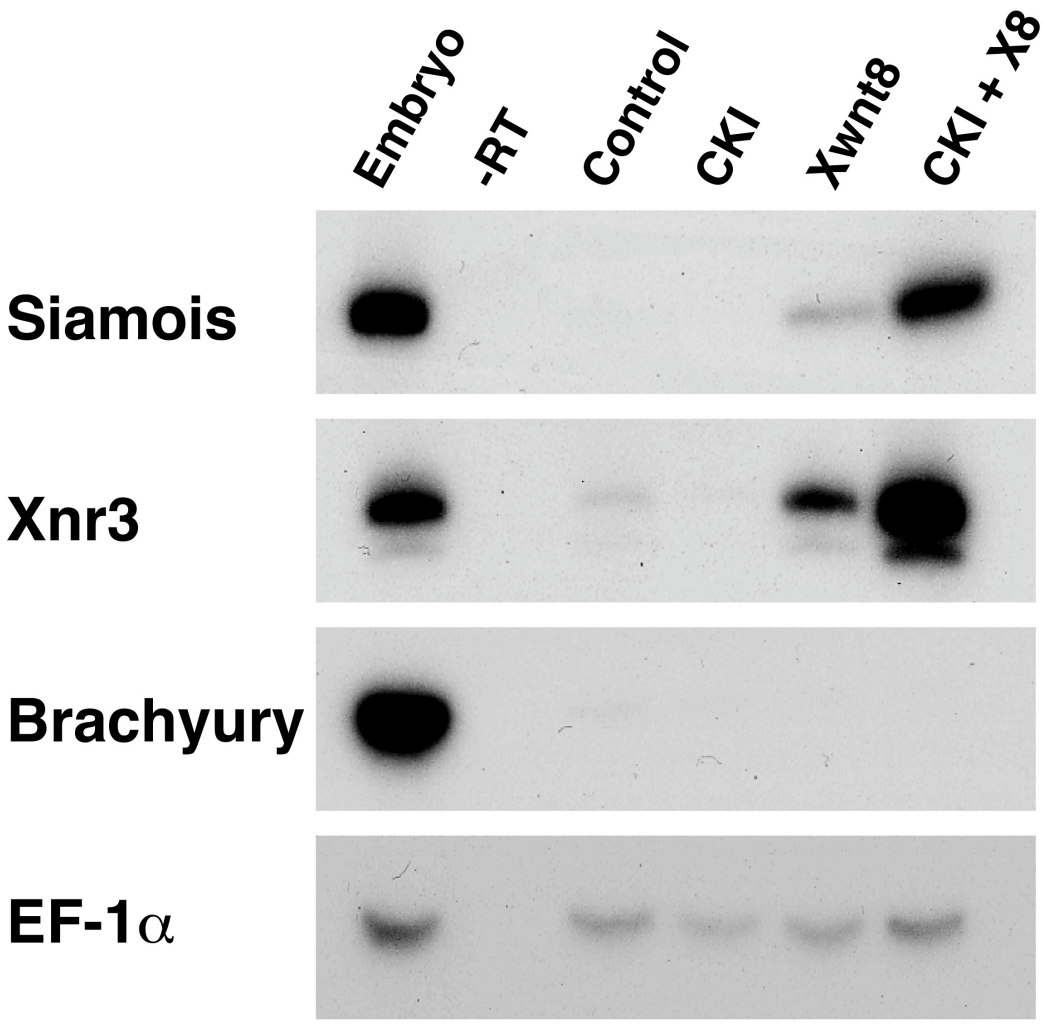


Figure 10. CKI and Wnt synergize to induce the expression of markers in the animal cap. XCKI (25 pg) and Xwnt-8 (X8, 0.5 pg) were injected alone or together and animal caps were analyzed as described in Figure 7. C denotes uninjected control. Experimental data in this figure by RM McKay.

CHAPTER FOUR
CKI is required for Wnt signaling

BLOCKING CKI INHIBITS WNT SIGNALING IN *XENOPUS*

Loss-of-function studies are key to concluding that CKI is in the Wnt pathway and we provide such evidence in two systems, frog and worm (see below). We attempted to derive two dominant negative forms of CKI that could block Wnt signaling. Kinases that are catalytically inactive due to K>R mutations are often dominant negatives (He *et al.* 1995). A D>N mutation in CKI at amino acid 131 was proposed to be a dominant negative (Zhu *et al.* 1998). Both K>R and D>N decreased CKI activity (not shown). Next, we determined whether these two mutants could also block Wnt function in either the double axis or animal cap assay using an approach previously described for Wnt inhibitors (Hoppler *et al.* 1996). Expressing β-galactosidase at the same doses as the mutants did not block Wnt signaling while expression of GSK-3, a negative regulator of Wnt signaling, did in the second axis assay (Table 1). CKI (K>R), in a dose-dependent manner, inhibited Wnt-dependent second axis formation (Table 1). CKI (K>R) also inhibited Wnt-induced gene expression in the animal cap assay (not shown). CKI (D>N) inhibited Wnt in the double axis assay (Table 1); however, CKI (D>N) produced substantial toxicity to embryos decreasing the reliability of this assay. In the animal cap assay, CKI (D>N) was not toxic and blocked Wnt signaling in 100% of experiments (5/5, Figure 12).

Sample	n	mean
Uninjected	21	0.0
Xwnt8 + β-gal (1 ng)	73	1.5
Xwnt8 + GSK-3 (0.5 ng)	73	1.1
Xwnt8 + K>R (1 ng)	110	0.9
Xwnt8 + K>R (2 ng)	57	0.6
Uninjected	38	0.0
Xwnt8 (8 pg)	56	1.5
Xwnt8 + β-gal (1 ng)	85	1.5
Xwnt8 + D>N (0.5 ng)	56	0.9
Xwnt8 + D>N (1 ng)	43	0.4
Uninjected	73	0.0
CKI (350 pg)	44	1.4
CKI + CKI-7 (2.9 ng)	48	0.5
Xwnt8 (10 pg)	31	1.5
Xwnt8 + CKI-7	61	0.3
Dsh (500pg)	23	1.4
Dsh + CKI-7	18	0.4
β-cat (1 ng)	36	1.8
β-cat + CKI-7	36	1.8

Table 1. Blocking CKI inhibits Wnt signaling in Xenopus. To evaluate blockade, embryos were scored as no duplication (0), partial duplication (1), and second axis with a head and cement gland (2). For the dominant negative studies, ß-galactosidase, XGSK-3, XCKI (K>R) or XCKI (D>N) were injected at the one-cell stage and then Xwnt-8 was injected at the 16-cell stage into 1 ventral vegetal blastomere. For the CKI-7 studies, XCKI, Xwnt-8, and -catenin with or without CKI-7, were injected into 1 ventral vegetal blastomere at the 8-cell stage. Both dominant negative forms of XCKI and the pharmacological inhibitor, CKI-7, inhibited Wnt.

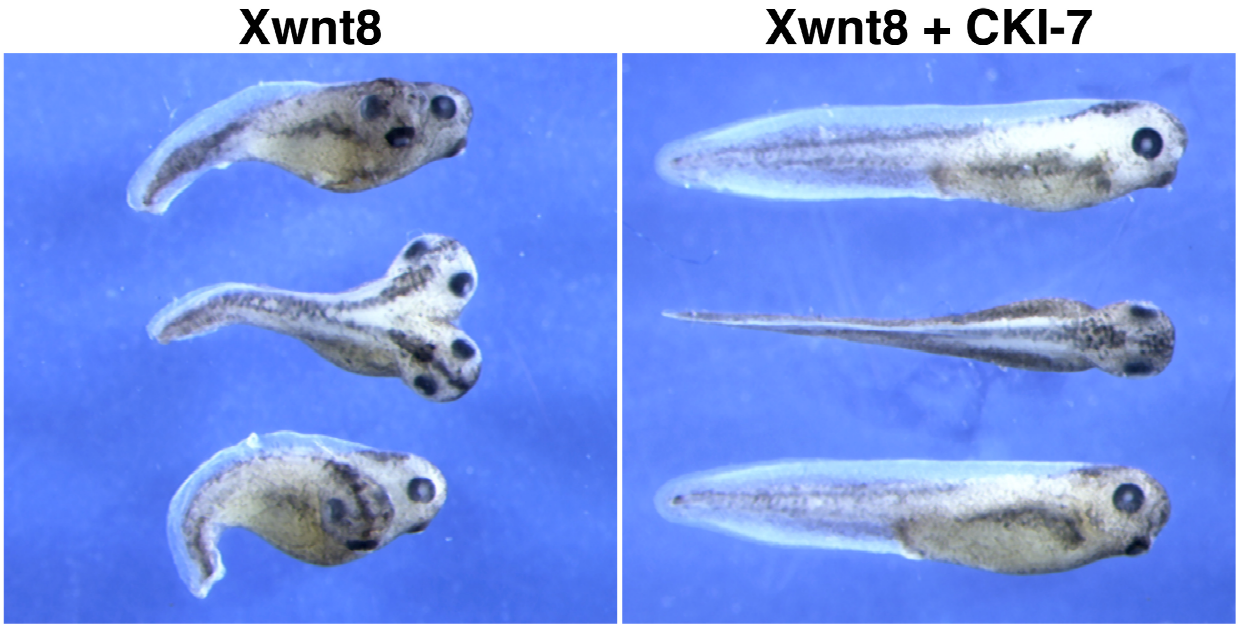


Figure 11. CKI inhibitors block Wnt signaling. Dominant negative forms of CKI or the chemical inhibitor CKI-7 (shown) were injected into embryos at the one cell stage. At the eight-cell stage Xwnt-8 (8pg) was injected into one ventral vegetal cell to produce secondary axes. As shown, blocking CKI prevented Wnt mediated axis duplication. See Table 1 for details and controls.

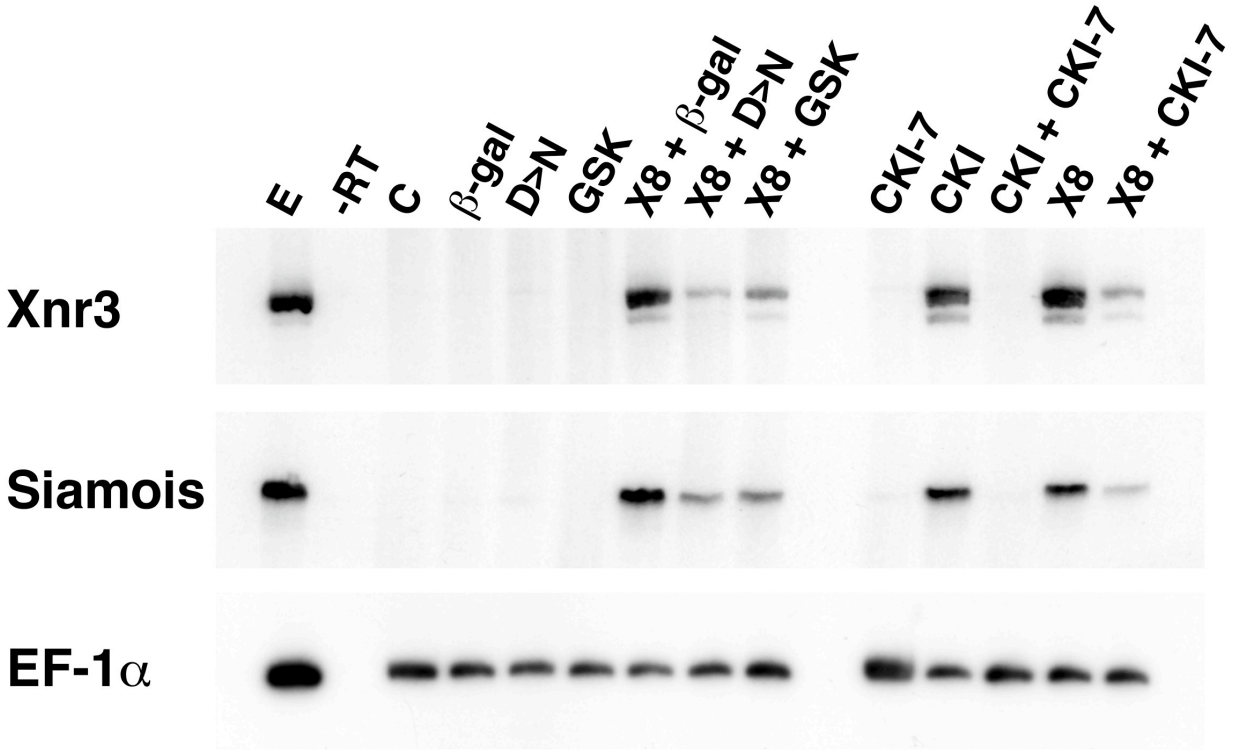


Figure 12. CKI inhibitors block the expression of Wnt target genes. Animal caps expressing □-gal (4 ng), XCKI□ (D>N) (4 ng), XGSK-3 (1 ng), Xwnt-8 (X8, 10 pg) with □-gal, Xwnt-8 with XCKI□ (D>N), Xwnt-8 with XGSK-3, CKI-7 (2.9 ng), XCKI□ (350 pg), XCKI□ with CKI-7, or Xwnt-8 with CKI-7 were analyzed by RT-PCR. The dominant negative XCKI□ (D>N) and the pharmacological inhibitor of CKI, CKI-7, inhibited Wnt.

Pharmacologic inhibitors provide a distinct and alternative approach to blocking CKI activity. CKI-7 is a specific and selective inhibitor of CKI (Chijiwa *et al.* 1989) and, in two assays, was a more potent blocker than either of the dominant negative forms. In both the second axis and the animal cap assay, CKI-7 inhibited both CKI and Wnt in a dose-dependent fashion, at doses that approximate the k_i for inhibition of CKI (Chijiwa *et al.* 1989) (Figure 11 and Table 1). However, CKI-7 did not block β-catenin demonstrating the specificity of the blockade (Table 1). Taken together, these data support the idea that CKI is in the Wnt pathway.

DOMINANT NEGATIVE CKI'S BLOCK CONVERGENCE EXTENSION MOVEMENTS

In *Xenopus*, inhibiting Wnt signals produces two distinguishable phenotypes: ventralized embryos or bent-back embryos. In embryos, ventralization only occurs when Wnt signaling is blocked below the level at which GSK-3 functions. Blocking at upper levels, such as Frizzled, does not produce a ventralized embryo unless the block is executed prior to fertilization (Sumanas *et al.* 2000). Zygotic block of upstream Wnt components instead produces embryos with relatively normal tissue patterning but altered morphogenesis, the bent-back phenotype. The dominant negative forms of CKIδ produced the bent-back phenotype and did not block primary axis formation (Figure 13, bottom right panel). However, CKI is a large family of enzymes and another CKI isoform might play a role in primary axis formation. We

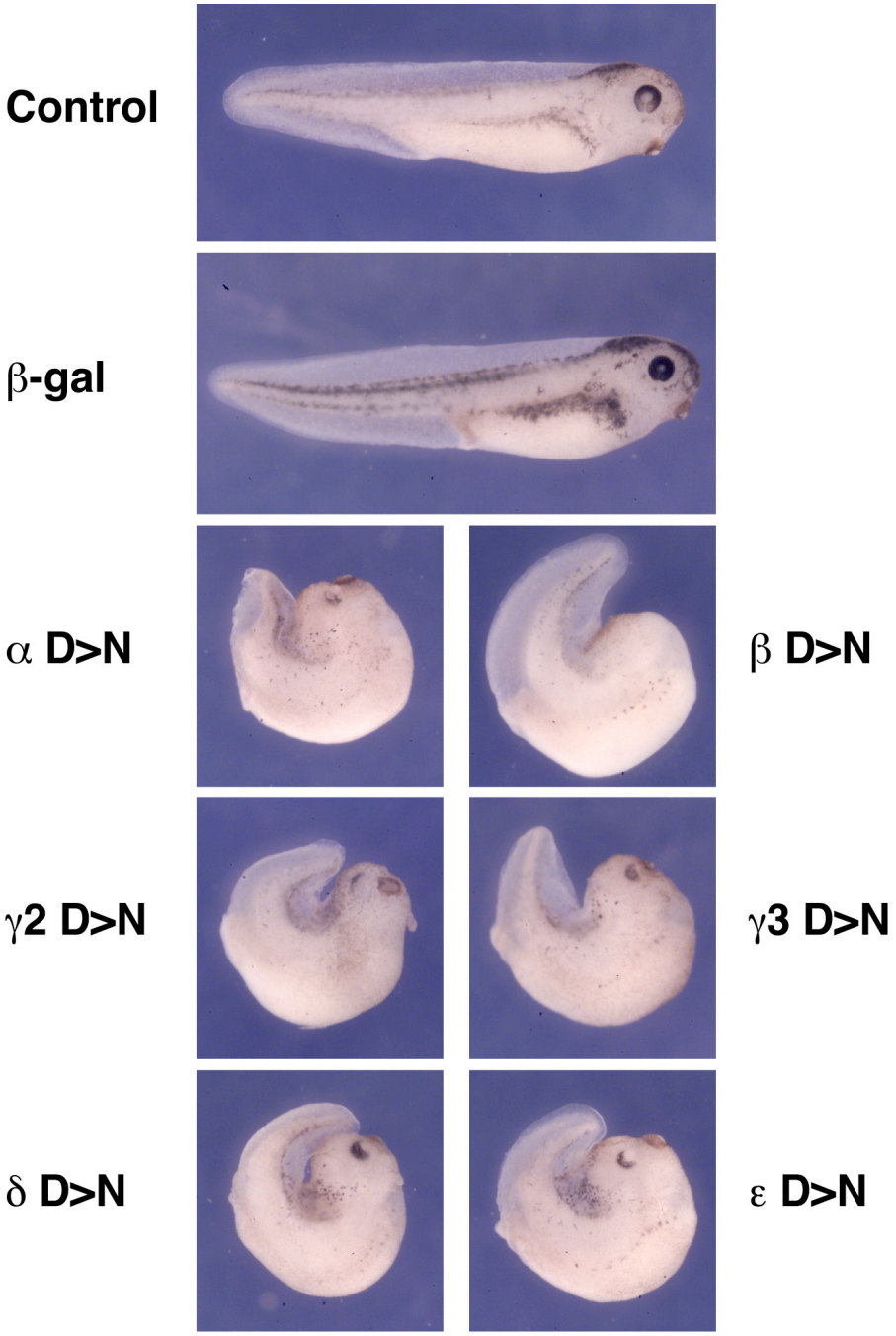


Figure 13. Inhibiting CKI generates truncated axes. mRNAs encoding β-galactosidase (2 ng) or D>N forms of CKIα (1 ng, n = 50), CKIβ (1.5 ng, n = 43), CKIγ2 (1 ng, n = 48), CKIγ3 (1 ng, n = 58), CKIδ (1 ng, n = 58), or CKIε (1 ng, n = 47) were injected into 1-cell embryos. Tadpoles were then photographed and scored for the percentage displaying the bent-back phenotype (see Table 2 below).

therefore extended the loss-of-function experiments and expressed dominant negative forms (D>N) of the CKI α, δ, ε2, ε3, and γ isoforms in embryos. All of the CKI (D>N) isoforms, with varying penetrance, produced the same bent-back phenotype, while β-galactosidase did not (Figure 13 and Table 2). Histological analysis of the bent-back, CKIε (D>N) tadpoles confirmed the presence of normal tissues as well (Figure 14). Therefore, the dominant negative forms of CKIε do not alter cell fate but rather inhibit morphogenesis.

Although none of the dominant negative isoforms of CKI altered primary axis formation, there were many possible explanations for the lack of a ventralized phenotype: the dominant negatives may not be sufficiently potent, the timing of the blockade may be too late, or redundancy among the isoforms could confound the dominant negative studies. To attempt to address these issues, we blocked CKI function with CKI-7, which potently inhibits several CKI isoforms, by microinjecting either 1-cell or 2-cell stage embryos with a range of CKI-7 doses (6-60 ng). Although 3 ng of CKI-7 blocked Wnt and Dishevelled function in *Xenopus* embryos, doses up to 60 ng did not alter primary axis formation (Table 3). Taken together, these data suggest that if CKI is required for axis formation, it is necessary prior to the time at which we inject embryos.

Sample	n	%Bent Back
Uninjected	50	0%
βgal (2ng)	54	0%
Alpha D>N (1ng)	50	78%
Beta D>N (1.5ng)	43	51%
Gamma2 D>N (1ng)	48	46%
Gamma3 D>N (1ng)	58	83%
Delta D>N (1ng)	58	69%
Epsilon D>N (1ng)	47	94%

Table 2. Summary of bent-back results. Dominant negative forms of all CKI isoforms produced bent-back embryos with varying penetrance. Methods are as described in Figure 13.

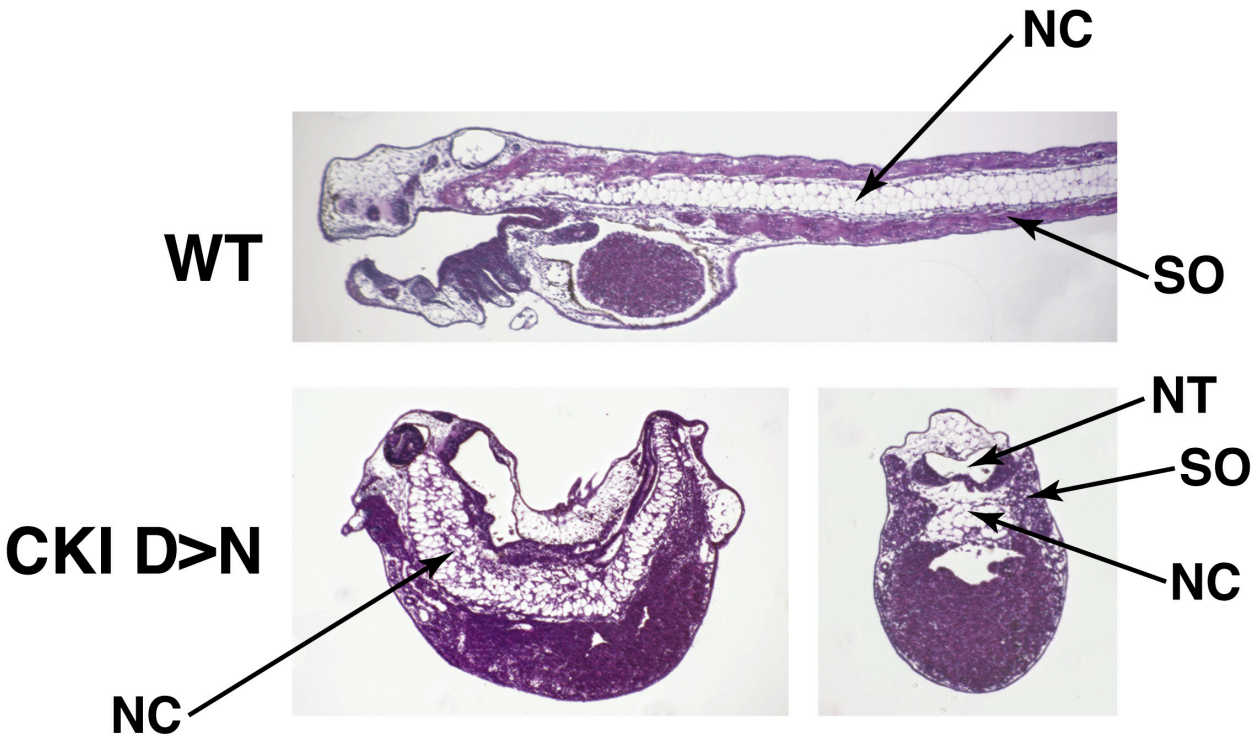


Figure 14. Bent-back embryos retain normal tissue patterning. Sagittal and transverse sections of CKI (D>N)-injected embryos (stage 35) show the presence of somites (SO), notochord (NC), and neural tissue (NT), tissues which form only in embryos with normal dorsal-ventral patterning. Emrbyos were fixed using MEMFA then stained via hematoxilin and eosin, paraffin embedded, and sectioned.

1-cell

Treatment	n	Ventralized
Control	87	0%
12ng CKI-7	66	0%
30ng CKI-7	35	0%
60ng CKI-7	35	0%
1ng Axin	40	53%

2-cell

Treatment	n	Ventralized
Control	43	0%
6ng CKI-7	29	0%
12ng CKI-7	12	0%
2ng Axin	22	87%

Table 3. CKI-7 does not block primary axis formation. Embryos were injected at the one-cell or two-cell stage with the chemical inhibitor CKI-7 then scored for ventralization. Axin, a negative regulator of the Wnt pathway, served as a positive control. The k_i for CKI-7 is approximated with a dose of 3ng.

BLOCKING CKI INHIBITS WNT SIGNALING IN *C. ELEGANS*

As Wnt signaling is conserved from nematodes to mammals, the function of CKI in the Wnt pathway might also be conserved. In *C. elegans*, Wnt signaling controls the fates of the two daughter cells, MS and E, produced by the EMS blastomere (Thorpe *et al.* 1997, Rocheleau *et al.* 1997). MS produces mesoderm, and E produces only endoderm. Active Wnt signaling is essential for specification of the E blastomere, and in the absence of Wnt signals E is transformed into MS (Figure 15). The resultant phenotype, *mom*, is a worm without endoderm and with excess muscle (Thorpe *et al.* 1997, Rocheleau *et al.* 1997).

Database analysis identified two *C. elegans* genes, CO3C10.1 (kin-19) and F46F2.2 (kin-20), with high sequence identity to *Xenopus* CKI. In *C. elegans*, several components of the Wnt pathway were characterized by inhibiting their function through RNA interference (RNAi) (Rocheleau et al 1997, Tabara *et al.* 1998, Sharp 1999, Fire *et al.* 1998). This approach resulted in a *mom* phenotype of variable penetrance, ranging from 2% (mom-5, Frizzled) to 100% (wrm-1, □-catenin). RNAi with kin-20 produced a sterile phenotype, precluding the ability to score embryonic phenotypes (not shown). When RNAi was performed with kin-19, 8% of the resulting worm embryos had the *mom* phenotype as judged by a variety of criteria (Figure 16). First, differential interference contrast (DIC) microscopy

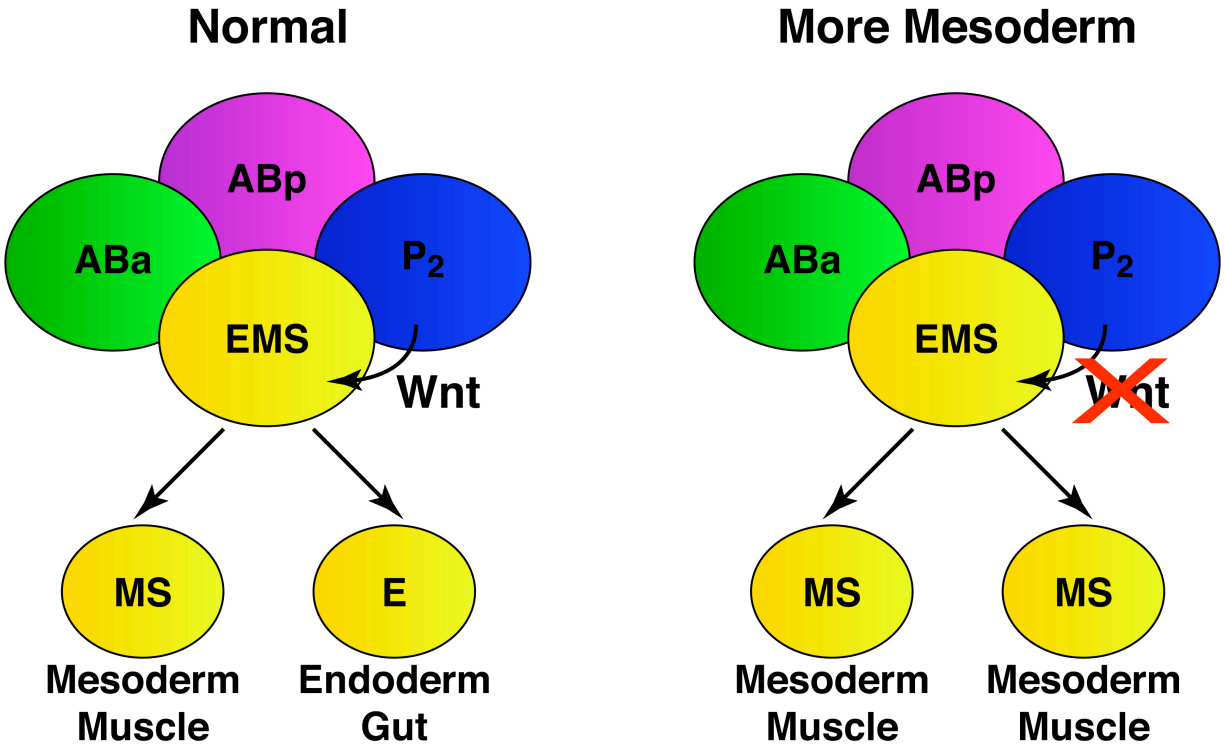


Figure 15. Overview of EMS patterning in *C. elegans*. A Wnt-dependent signal from the P_2 blastomere polarizes EMS, causing it to give rise to two dissimilar daughter cells. MS gives rise to mesodermal tissues such as the pharyngeal muscle and E yields endodermal tissues such as the gut. In the absence of the Wnt signal, EMS is not polarized and both daughter cells develop in a MS like fashion, resulting in the absence of endodermal derivatives and an excess of mesoderm.

demonstrated loss of both morphogenesis and endodermal differentiation with excess mesoderm formation (Figure 16 a, c, d). Second, polarized light microscopy revealed that the embryos contained no gut granules, consistent with a loss of endodermal fates (Figure 16b). Third, the absence of endoderm was confirmed by tissue-specific antibody staining (Figure 16c). Fourth, tissue-specific antibody staining showed that these embryos had excess pharyngeal muscle, an MS derivative (Figure 16d). These features are all hallmarks of the *mom* phenotype, which results from loss of Wnt signals.

To determine whether this phenotype was due to an E-to-MS transformation, we used RNAi followed by laser ablation of all blastomeres except E and analyzed the descendants of E (Thorpe *et al.* 1997, Rocheleau *et al.* 1997). When wild-type embryos were ablated and analyzed, E always produced endoderm (18/18). In roughly 11% of kin-19 RNAi-treated embryos, E did not form endoderm (3/28). Instead, these partial embryos generated pharyngeal muscle (not shown). In addition, we undertook RNAi with just the C-terminal domain of kin-19, a region outside the kinase domain which does not share homology with other protein kinases. This determined whether the *mom* phenotype might result from promiscuous inhibition of another kinase; if so, then RNAi with the tail alone would not produce the *mom* phenotype. As with the full-length clone, however, RNAi with the tail of kin-19 produced embryos that lacked endoderm and contained excess

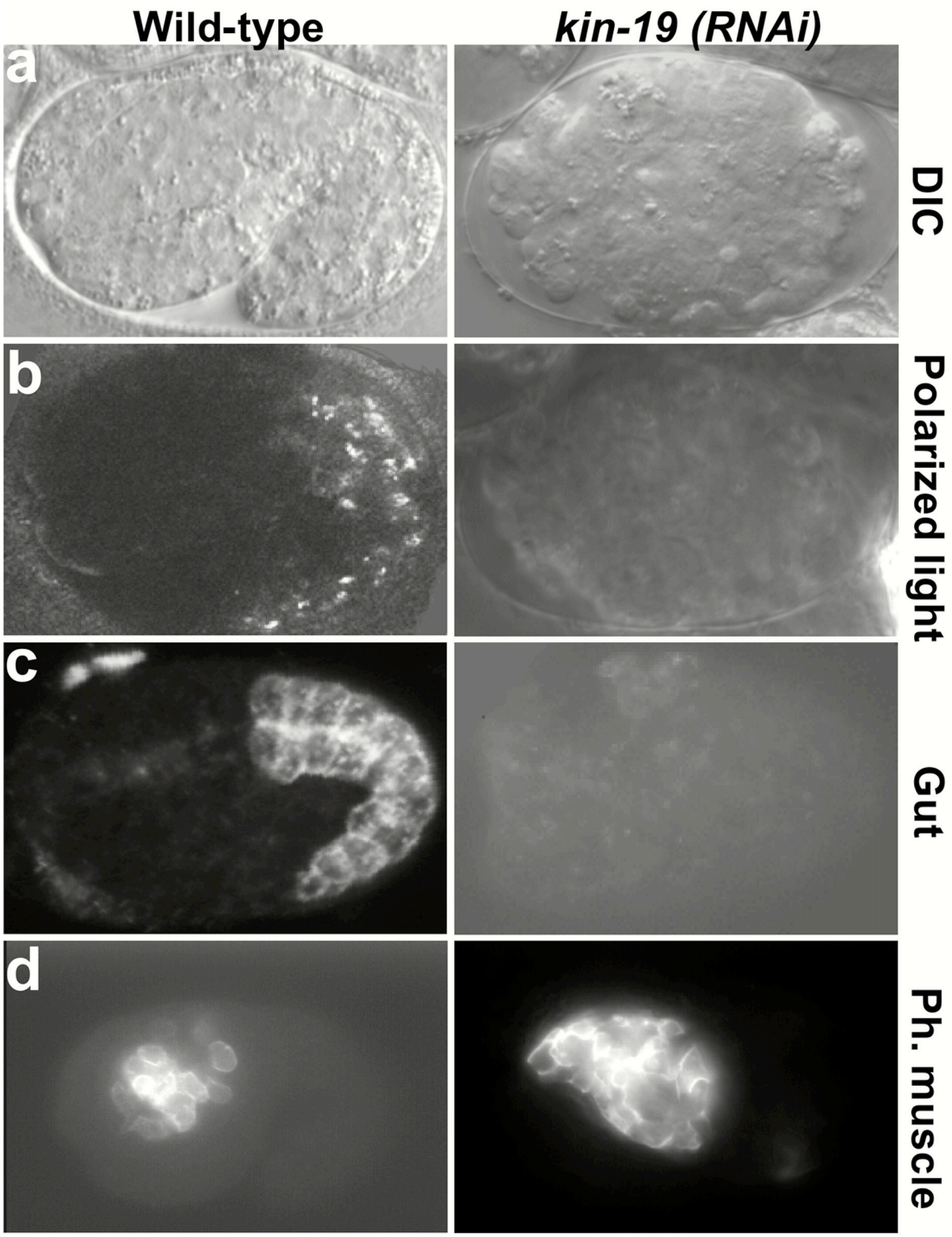

Wild-type
kin-19 (RNAi)
a
b
c
d
DIC
Polarized light
Gut
Ph. muscle

Figure 16. CKI RNA interference produces the *mom* phenotype in *C. elegans*. a, DIC micrographs of living embryos. In contrast to a wild-type embryo, kin-19 RNAi blocked morphogenesis and altered differentiation. b, Polarized light microscopy revealed birefringent gut granules (white) in a wild-type embryo but no gut granules in kin-19 (RNAi)-embryos. c, Gut-specific antibody (ICB4) staining showed that, in contrast to wildtype embryos, kin-19 (RNAi)-embryos lack endoderm. d, Staining with a pharyngeal (Ph.) muscle-specific antibody (3NB12) demonstrated that, compared with wild-type embryos, kin-19 (RNAi)-embryos had an excess of the tissues that can be derived from MS. Experimental data in this figure by RM McKay and JP McKay.

pharyngeal muscle (not shown). As another specificity control, we inhibited casein kinase II (CKII), another serine kinase that binds to Dishevelled (Willert et al. 1997); the resultant CKII RNAi embryos did not have the *mom* phenotype (not shown). Thus, RNAi of CKI in the worm specifically produced the same phenotype, *mom*, as loss of Wnt signaling.

Some components of the Wnt cascade, including the receptor, GSK-3 and β-catenin, have a linear relationship downstream of Wnt and are directly modulated by the ligand (Rocheleau *et al.* 1997). Other components, such as adenomatous polyposis coli (APC) modify the cascade but are not directly downstream of Wnt (Rocheleau *et al.* 1997). When two components of the linear pathway are simultaneously eliminated, no synergy of the *mom* phenotype is observed (Rocheleau *et al.* 1997). In contrast, when a linear component and APC are eliminated together, there is a marked increase in the percentage of *mom* mutant embryos (Rocheleau *et al.* 1997). To determine any synergy with CKI and other components of the Wnt cascade, we simultaneously decreased the function of CKI and either mom-2 (Wnt) or apr-1 (APC) by RNAi and analyzed the resultant embryos. RNAi combining kin-19 and mom-2 had no effect on the penetrance of the phenotype (Table 4). In contrast, we observed a synergistic enhancement of the mutant phenotype by inhibiting both kin-19 and apr-1 (Table 4). This was observed with other linear components of the Wnt pathway and is consistent with CKI functioning directly in the cascade (Rocheleau *et al.* 1997).

Embryo Type	% gutless (n)
WT	0 (100)
kin-19	8 (247)
mom-2	20 (190)
apr-1	18 (168)
kin-19; mom-2	16 (132)
kin-19; apr-1	80 (157)
mom-2; apr-1	84 (210)

Table 4. kin-19 (RNAi) synergizes with apr-1 (RNAi) but not with mom-2 (RNAi). Methods are described in figure 16. Experimental data in this table by RM McKay and JP McKay.

CHAPTER FIVE

Epistatic and biochemical results place CKI within the Wnt pathway

CKI FUNCTIONS BETWEEN DISHEVELLED AND GSK-3

We used epistasis tests to determine where CKI functions in the Wnt pathway. Initially, we analyzed Wnt, Dishevelled or β-catenin function using the second axis assay while blocking CKI and found that CKI functions downstream of Wnt and Dishevelled and upstream of β-catenin (Table 1, experiments replicated in Table 5). We then extended this assay with dominant negative forms of GSK-3 and Axin, two negative regulators of the Wnt pathway (Yost *et al.* 1996, Zeng *et al.* 1997). The dominant negative forms of these two molecules activate the Wnt pathway. CKI-7 did not block either DN-GSK-3 or DN-Axin, again suggesting that CKI acts relatively upstream in the Wnt pathway. As a complementary approach, we assessed CKI activity in the presence of Wnt pathway inhibitors. In the animal cap assay, Nfz (Xu *et al.* 1998, Deardorff *et al.* 1998), a dominant-negative Frizzled, and Xdd1 (Sokol 1996), a dominant-negative Dishevelled, both blocked Wnt, but neither blocked CKI (Figure 17). GSK-3 functions downstream of Frizzled and Dishevelled and is a negative regulator of the Wnt pathway (Cadigan and Nusse 1997). GSK-3 inhibited CKI in the animal cap assay (Figure 18). GSK-3 and Axin (not shown) also blocked CKI function in the ultraviolet rescue assay. Thus, CKI functions downstream of Dishevelled and upstream of GSK-3 based upon this assay.

Sample	n	mean
Uninjected	148	0.00
Xwnt-8	82	1.49
Xwnt-8 + CKI-7	93	0.22
Xdsh	38	1.16
Xdsh + CKI-7	34	0.26
DN-GSK3	39	1.34
DN-GSK3 + CKI-7	23	1.25
DN-Axin	29	1.07
DN-Axin + CKI-7	33	1.13
β-cat	68	1.77
β-cat + CKI-7	62	1.75

Table 5. CKI acts between Dishevelled and GSK-3. Embryos were microinjected into one ventral vegetal cell at the eight-cell stage with 10pg Xwnt-8, 500pg Xenopus dishevelled (Xdsh), 1ng DN-GSK-3, 1ng DN-Axin (□RGS), or 1ng □-catenin with or without 2.9ng of the CKI inhibitor CKI-7. After development the embryos were scored for the presence of ectopic axes (2=complete, 1=partial, 0=normal).

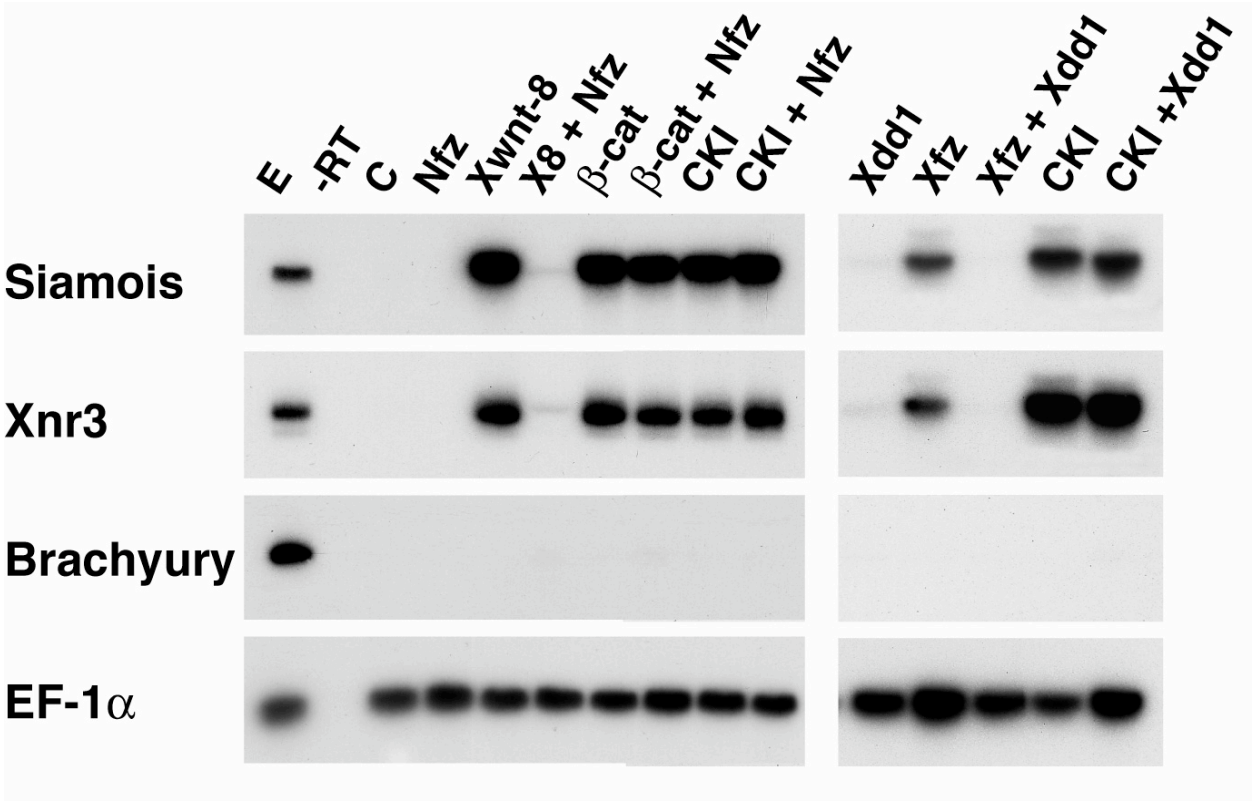


Figure 17. CKI functions below Frizzled and Dishevelled. CKI activity is not blocked by dominant-negative Frizzled (Nfz) or dominant-negative Dishevelled (Xdd1). Nfz (1 ng), Xwnt-8 (25 pg), Xwnt-8 (X8) mixed with Nfz, □-catenin (□-cat, 1 ng), □-catenin mixed with Nfz, XCKI□ (350 pg), or XCKI□ mixed with Xdd1 were injected and animal caps analysed for the expression of markers. Experimental data in this figure by RM McKay.

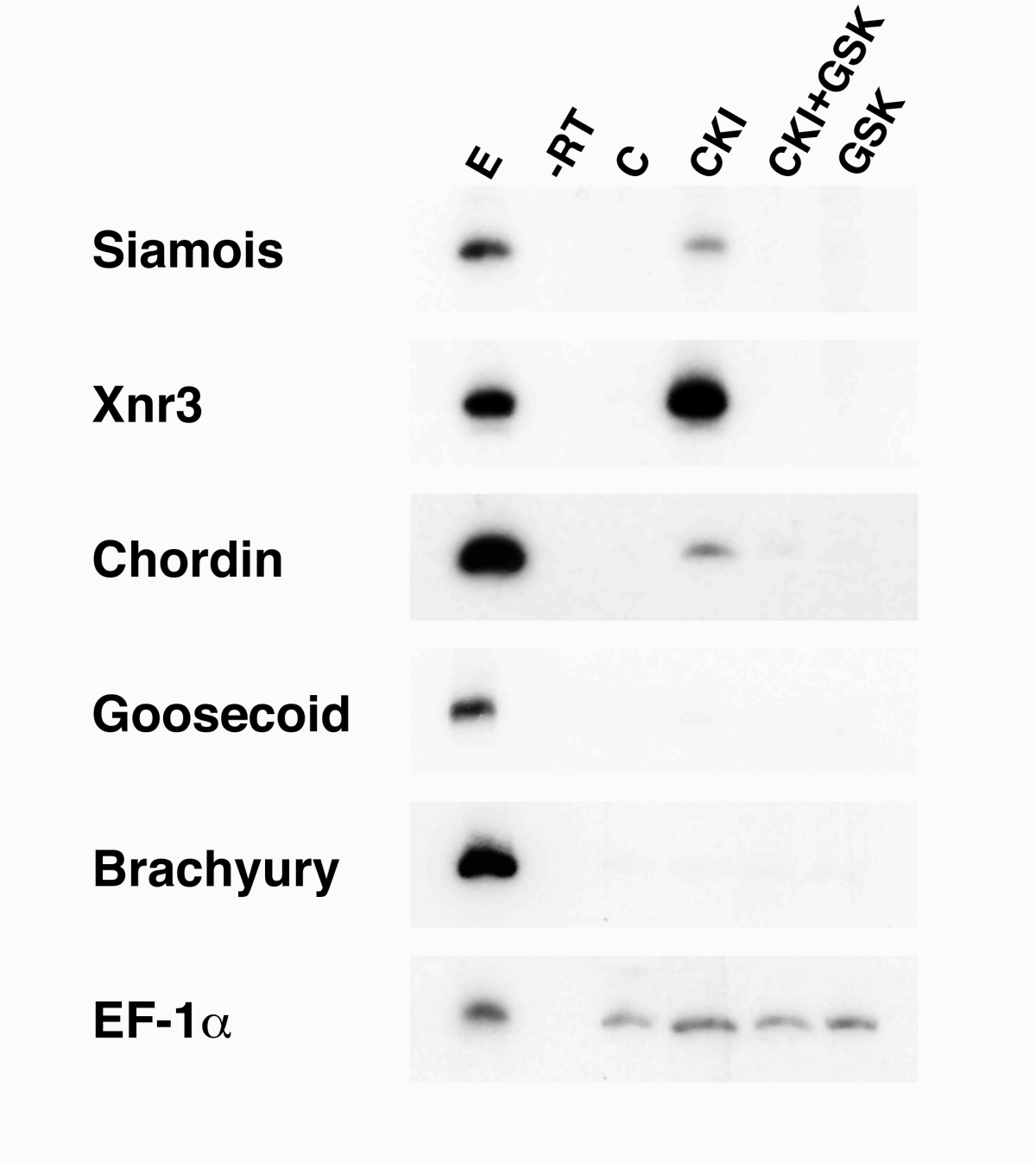
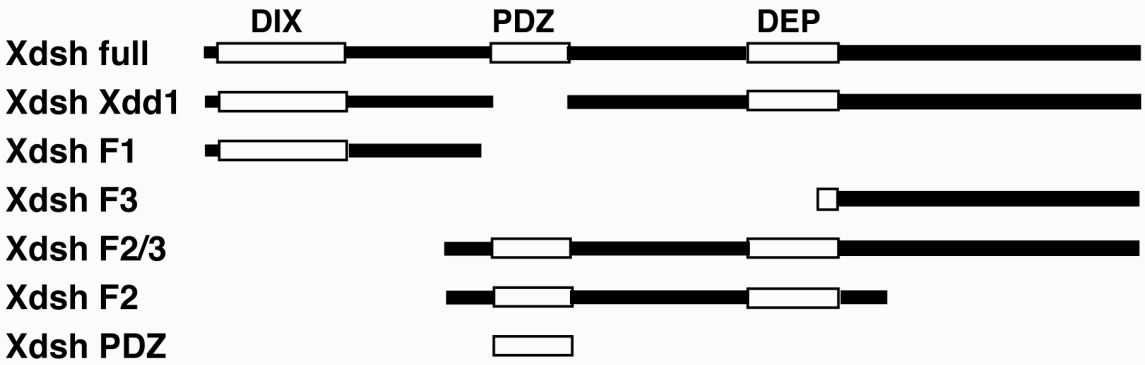


Figure 18. CKI is blocked by GSK-3. Emrbyos were injected at the one-cell stage with 350ng of XCKIe, XCKIe plus 500pg of XGSK-3, or 500pg of XGSK-3. Animal cap explants were then analyzed for gene expression.

CKI BINDS AND INCREASES THE PHOSPHORYLATION OF DISHEVELLED

Presumably, CKI regulates Wnt signaling by phosphorylating an element of the cascade. Thus we expressed, in *Xenopus* oocytes, Myc-tagged components of the Wnt pathway with or without CKI or CKI (K>R) and incubated the oocytes in $^{32}P_i$. Next, we immunoprecipitated the epitope-tagged constructs and evaluated phosphorylation levels. CKI did not increase the phosphorylation of GSK-3, Axin, or □-catenin in this assay (not shown); however, it did increase the phosphorylation of Dishevelled, whereas CKI (K>R) did not, confirming specificity (Figure 20). To define the region where CKI binds, we divided Dishevelled into three roughly equal fragments and tested these fragments for interaction in yeast (Figure 19). Unlike the first and third fragments, the middle fragment of Dishevelled bound to CKI (Figure 19). Consistent with the two-hybrid data, CKI increased the phosphorylation of the middle fragment (Figure 20). This middle region of Dishevelled contains an 80-amino-acid PDZ protein-interaction domain that is critical for wild-type function (Sokol 1996). Notably, CKI directly bound this domain (Figure 19). Deleting these 80 amino acids converts Dishevelled into a dominant-negative form, Xdd1 (Sokol 1996), which did not interact with CKI (Figure 19) and was not phosphorylated by CKI in oocytes (not shown). In order to determine whether CKI might directly phosphorylate Dishevelled, we performed an *in vitro* phosphorylation assay, testing the ability of bacterially expressed and purified CKI to phosphorylate a bacterially

a



b

pGAD-series	Binding	pACT-series	Binding
CKI + XDsh	+++++	CKI + XDsh	+++++
K>R + XDsh	++++	K>R + XDsh	++++
CKI + GSK	-	CKI + XDshF1	-
CKI + GBP	-	CKI + XDshF3	-
Lam + XDsh	-	CKI + XDshF2/3	+++
CKI + XDshF2	++	CKI + XDshF2	++
CKI + Xdd1	-	CKI + XDshPDZ	++

Figure 19. CKI binds to Dishevelled. a, The Dishevelled protein (Xdsh) and fragments used in the yeast two-hybrid and phosphorylation assays (Figures 20, 21, and 25). b, CKI bound Dishevelled in a yeast two-hybrid assay. XCKI and XCKI (K>R) baits interacted strongly with Xdsh. XCKI also interacted with Xdsh fragments 2/3 (F2/3; amino acids 231-737), fragment 2 (231-531), and the PDZ domain (301-381). XCKI did not interact with GSK-3, GBP, Xdsh fragment 1 (1-289), Xdsh fragment 3 (485-737) or a dominant negative form of Xdsh, Xdd1, that lacks the PDZ domain. Relative strength of binding was determined by a liquid -galactosidase assay. To establish reproducibility, two different reporter vectors, pGAD and pACT (Clonetech), were used. None of the clones activated in the absence of the interacting partner. Experimental data in this figure by RM McKay.

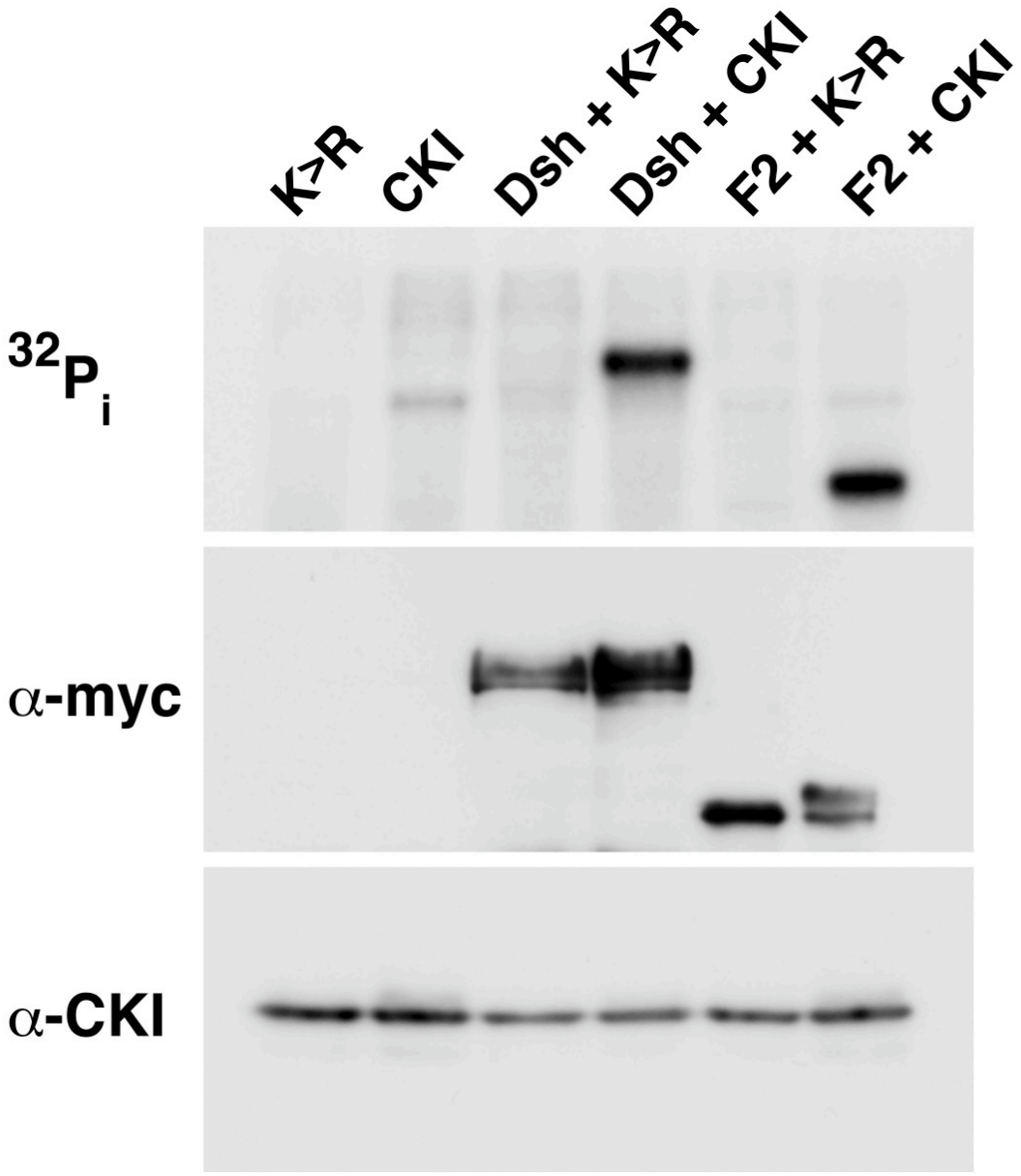


Figure 20. CKI increases Dishevelled phosphorylation in Xenopus oocytes. Xenopus oocytes were injected with mRNA encoding either XCKI or XCKI (K>R) (15 ng) in the presence of either Myc-tagged Dishevelled (Dsh, 30 ng) or Myc-tagged Dishevelled fragment 2 (F2, 30 ng), incubated with $^{32}P_i$, lysed and immunoprecipitated via the Myc-tag. Protein levels were analysed by western blots against the Myc-epitope or CKI. Dishevelled fragments 1 and 3 were not phosphorylated by XCKI (not shown).

expressed and purified Dishevelled fragment 2. Control lysates, from bacteria that did not express Dishevelled fragment 2, were not phosphorylated by purified CKI (Figure 21). In contrast, CKI directly phosphorylated the middle fragment of Dishevelled in a time-dependent fashion (Figure 21). In addition, the mobility of Dishevelled fragment 2 decreased upon phosphorylation, mirroring the Dishevelled upshifts observed in *Xenopus* oocytes and cell lines (Figure 20, 21). This is consistent with the notion that the CKI-dependent phosphorylation of Dishevelled detected *in vivo* is direct.

At what position in the Wnt pathway does CKI function? CKI and APR synergize in producing the *mom* phenotype which suggests that CKI has a linear relationship with Wnt, the Wnt receptor (Frizzled), GSK-3 and □-catenin in the cascade (Rocheleau *et al.* 1997). Furthermore, epistasis studies in the frog indicate that CKI is downstream of Dishevelled and upstream of GSK-3, which is consistent with CKI increasing □-catenin protein levels. In addition, CKI directly binds Dishevelled, a component of the Wnt pathway that is upstream of GSK-3 (Moon *et al.* 1997)). Together, these data indicate that CKI may function between Dishevelled and GSK-3. Dishevelled phosphorylation increases in response to Wnt signaling yet this has not been shown crucial to Wnt signaling (Yanagawa *et al.* 1995). Possibly,

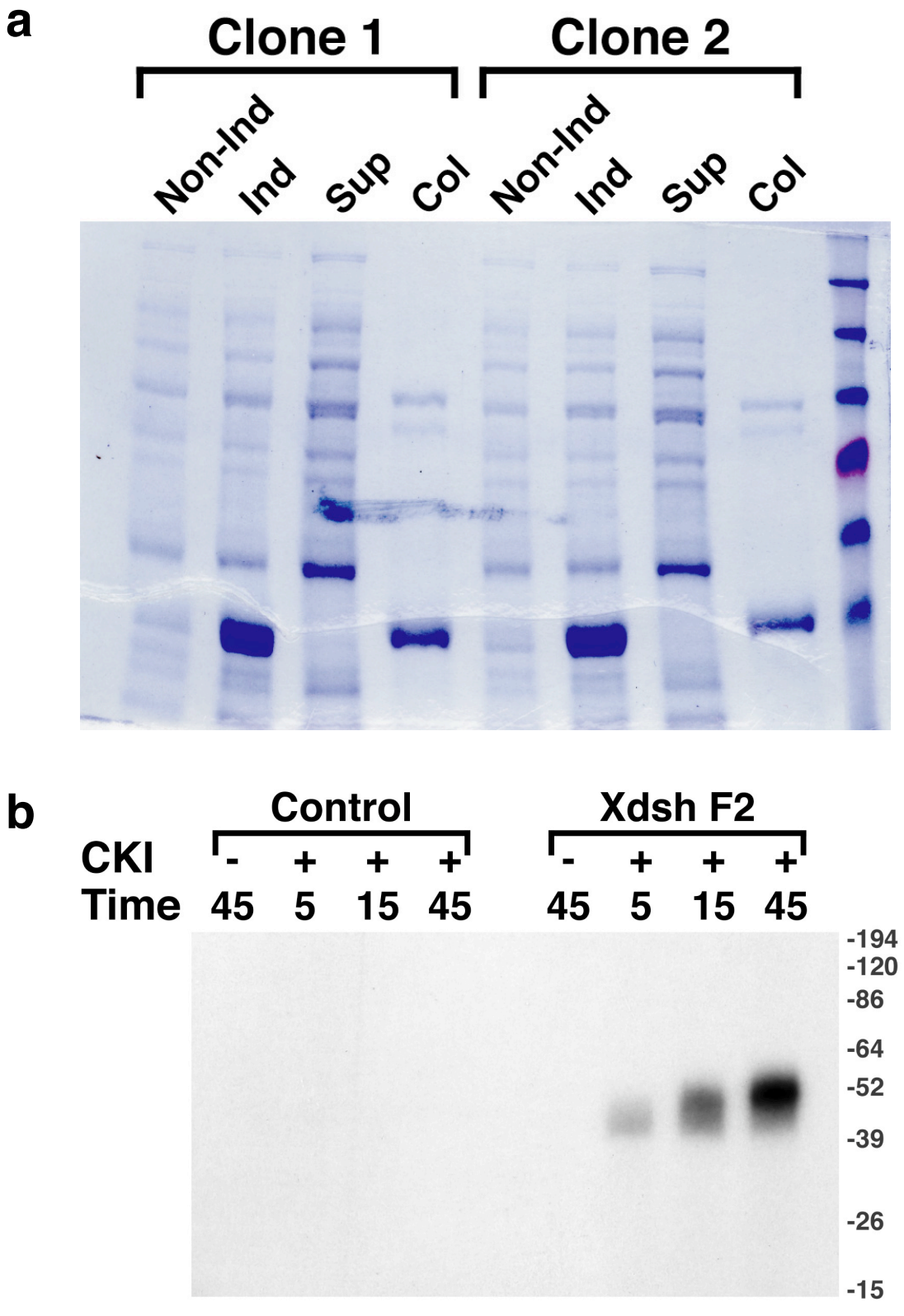

a
Clone 1
Clone 2
Non-Ind
Ind
Sup
Col
Non-Ind
Ind
Sup
Col
b
Control
Xdsh F2
CKI
- + + + - + + +
Time
45 5 15 45 45 5 15 45
-194
-120
-86
-64
-52
-39
-26
-15

Figure 21. CKI directly phosphorylates Dishevelled fragment 2 *in vitro*. a, purification of Xdsh fragment 2. Histidine tagged Dishevelled was purified from bacterial lysates after cloning into the inducible vector pET 15b. Two independent clones are shown. Samples are uninduced bacterial lysates, bacterial lysates after induction, flow-through from a nickel purification column, and the column-purified eluate. b, Purified recombinant CKI KD (New England Biolabs) was incubated with purified recombinant Xdsh fragment 2 at 18 degrees C for the specified times in minutes with [-^{32}P]-dATP. Control lanes replace the purified Xdsh fragment 2 with another purified histidine-tagged protein.

CKI is the responsible kinase, as it binds to and increases the phosphorylation of Dishevelled. Xdd1, a form of Dishevelled with an 80-amino-acid deletion, functions as a dominant negative (Sokol 1996). These 80 amino acids, when expressed in isolation, bind to CKI whereas Xdd1 does not; thus, these 80 amino acids may be critical for the CKI-Dishevelled interaction. The abrogation of CKI binding may account for the dominant-negative action of Xdd1. The epistasis studies suggest that CKI is downstream of Dishevelled, yet CKI phosphorylates Dishevelled. Perhaps the CKI-dependent phosphorylation of Dishevelled is not responsible for the biological role that CKI plays in the Wnt pathway. For example, the phosphorylation of Dishevelled may just be a marker of CKI activation. Equally plausible, however, is that the CKI-dependent phosphorylation of Dishevelled modulates or modifies downstream signaling.

CHAPTER SIX
Multiple CKI isoforms reproduce Wnt signaling

While gain-of-function studies in *Xenopus* embryos and mammalian cell lines have shown that CKI□ and □ activate the canonical Wnt pathway, the ability of the other CKI isoforms to activate Wnt signaling is unknown (Peters *et al.* 1999, Sakanaka *et al.* 1999). Although it has been reported that the □ isoform does not activate Wnt signaling in frogs or mammalian cell lines (Sakanaka *et al.* 1999), loss-of-function studies in worms suggest that the □ isoform is required for Wnt signaling (Peters *et al.* 1999). This incongruity emphasizes the need for further studies to clarify which isoforms activate the Wnt cascade.

MULTIPLE CKI ISOFORMS ACTIVATE WNT SIGNALING

We wanted to determine whether the other CKI isoforms (Figure 22) could also activate the canonical Wnt signaling pathway. When *Xenopus* embryos are irradiated with ultraviolet (UV)-light during the first cell cycle, dorsal axis formation is blocked and the embryos develop as ventralized balls of tissue (Scharf and Gerhart 1983, Figure 4). Positive regulators of the Wnt pathway, including CKI□, rescue this ventralized phenotype (Peters *et al.* 1999, Smith and Harland 1991, Figure 5). To determine whether the other CKI isoforms could also rescue the UV-phenotype, we irradiated embryos and microinjected the various CKI isoforms into one vegetal blastomere at the 8-cell stage. Doses for □, □, □ and □KD were empirically chosen

for optimal rescue; for □, □3, and □3KD the maximum non-toxic dose was used. As a negative control, we expressed the inactive CKI□ (K>R) mutant and it did not generate any dorsal axes (Figure 23). In contrast, all of the wild-type CKI isoforms rescued UV-irradiated embryos (Figure 23). In this assay, one can quantitate the degree of rescue with the dorso-anterior index (DAI), in which normal embryos are scored as 5 and completely ventralized embryos are scored as 0 (Kao *et al.*, 1986). Based upon DAI results, all the isoforms rescued UV-irradiated embryos. Of note, the □3 isoform, which is the most divergent in primary structure (Gross and Anderson 1998), produced the lowest degree of rescue (lowest DAI, Table 6). Similar results were obtained with the □2 isoform (data not shown). Elimination of the carboxyl terminal tail is thought to increase the catalytic activity of CKI family members (Cegielska *et al.*, 1998; Graves and Roach, 1995). So, we attempted to increase the activity of the □3 isoform by generating a form that contained only the kinase domain (KD). We generated a similar form of CKI□ as a control. In both cases, the isolated kinase domains produced slightly better rescues than the wild-type versions (Figure 23, Table 6).

As another test of the ability of the CKI isoforms to activate the Wnt pathway, we turned to the second axis assay (Figure 2). Positively acting components of the Wnt pathway, including Wnt, Frizzled, Dishevelled, CKI□, □-catenin, and XTcf-3/LEF-1 induce formation of a second dorsal axis (Behrens *et al.*, 1996; Deardorff *et al.*,

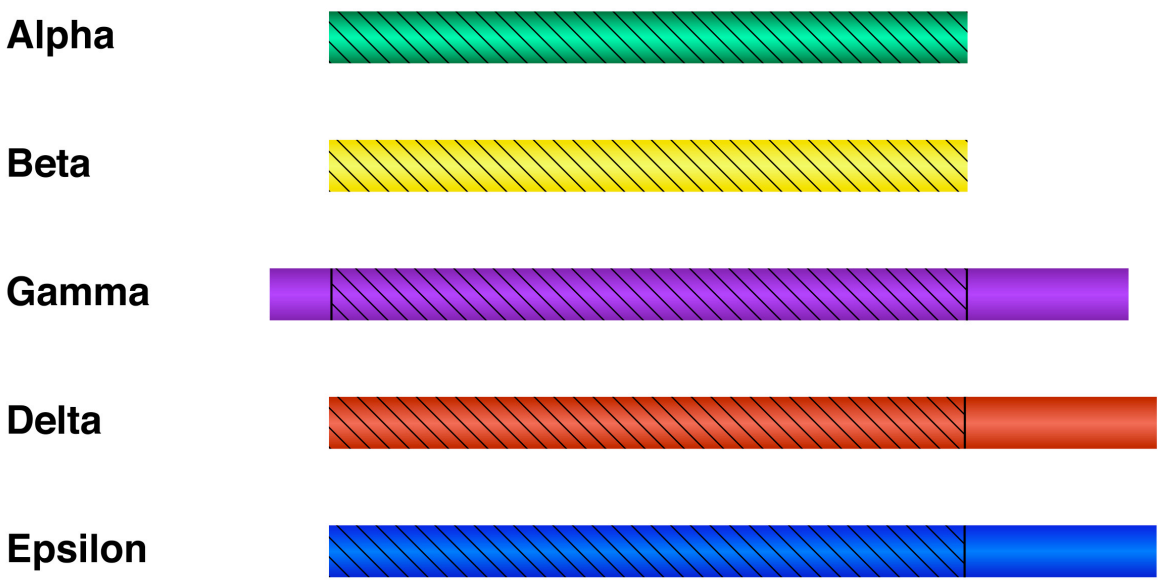


Figure 22. Multiple CKI isoforms exist in all eukaryotes. All CKI isoforms show homology within the kinase domain of fifty percent or greater (stippled area). The delta and epsilon isoforms are highly related including their carboxy-terminal tails. At least three gamma isoforms exist in mammals. Although the gamma isoforms are closely related to each other, they are the most distantly related to all other isoforms.

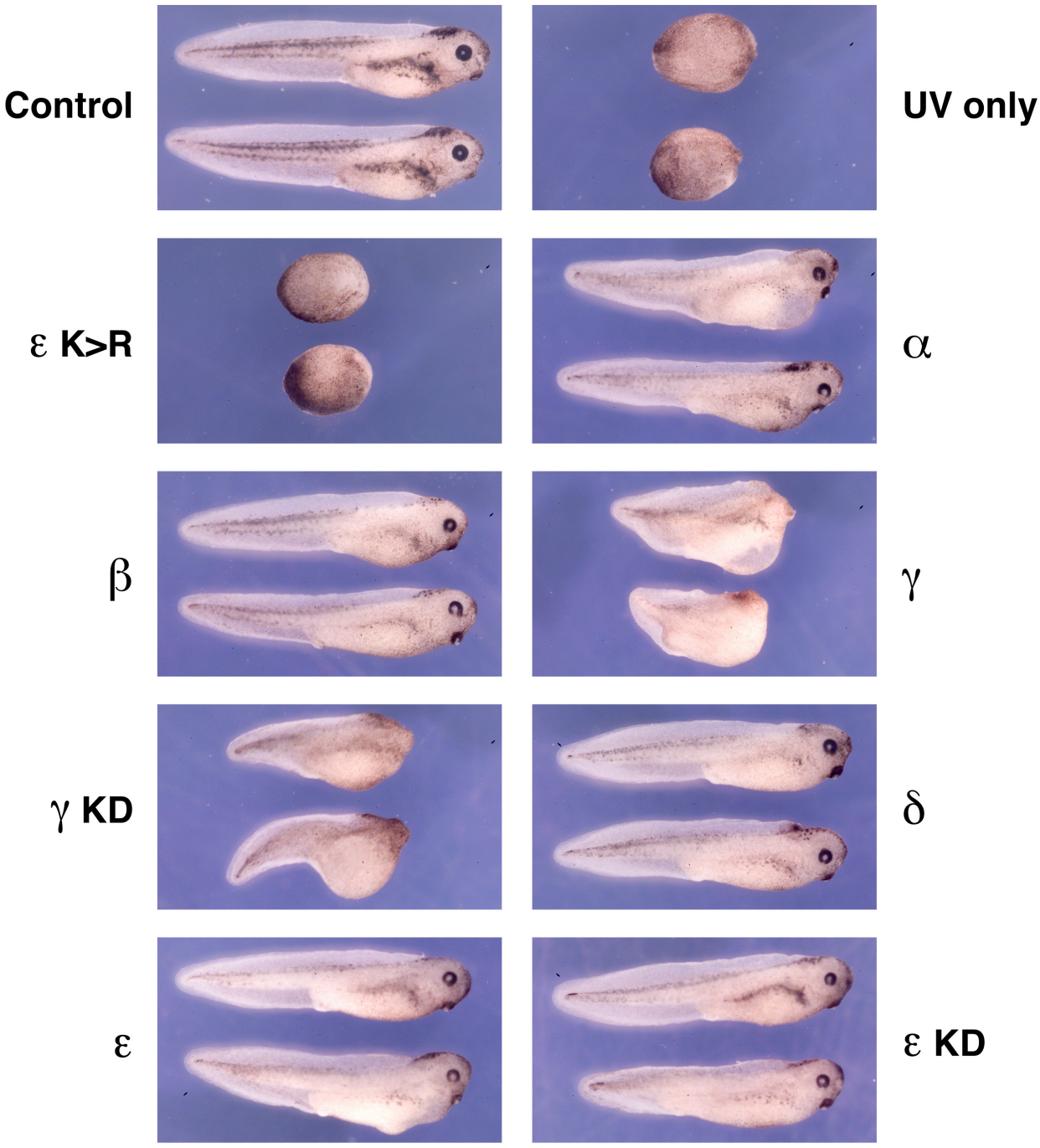

Control
UV only
ε K>R
α
β
γ
γ KD
δ
ε
ε KD

Figure 23. All CKI isoforms rescue UV-irradiated embryos. Emrbyos were irradiated as standard and then mRNA encoding various CKI isoforms was injected into one vegetal cell at the eight cell stage. Only the gamma isoforms were unable to effect a complete or near complete rescue. Doses were titrated for optimal rescue except for gamma, for which the highest non-lethal dose was used. Doses were: epsilon K>R-350pg, alpha-175pg, beta-25pg, gamma and gamma KD-1400pg, delta-350pg, epsilon-350pg, and epsilon KD-175pg.

Sample	n	mean
Uninjected	295	5.00
UV only	311	0.05
Epsilon K>R (700pg)	79	0.01
Alpha (175pg)	130	3.71
Beta (25pg)	56	3.20
Gamma-3 (1400pg)	55	0.43
Gamma-3 KD (1400 pg)	92	1.16
Delta (350pg)	109	3.80
Epsilon (350pg)	205	3.79
Epsilon KD (175pg)	110	3.99

Table 6. All CKI isoforms rescue UV-ventralized embryos. For both the gamma and epsilon isoforms, eliminating regions outside the kinase domain (KD) either increases the effectivness of rescue (gamma) or decreases the dosage required for rescue (epsilon), suggesting that the carboxyl-terminal tails may be partially inhibitory in this assay. Embryos were scored from 0 (completely ventralized) to 5 (normal). Doses were: epsilon K>R-350pg, alpha-175pg, beta-25pg, gamma and gamma KD-1400pg, delta-350pg, epsilon-350pg, and epsilon KD-175pg.

1998; Funayama *et al.*, 1995; Molenaar *et al.*, 1996; Peters *et al.*, 1999; Sakanaka *et al.*, 1999; Smith and Harland, 1991; Sokol *et al.*, 1995). mRNA encoding the various isoforms was microinjected into one ventral vegetal blastomere at the 8-cell stage and the embryos were then scored for formation of second axes using the second axis index (SAI) (Peters *et al.* 1999). In this scale, complete second axes are scored as a 2, partial axes are a 1, and no second axes are a 0. Uninjected embryos and embryos injected with the inactive CKI□ (K>R) mutant had an SAI of 0 (Table 7). In contrast, all the CKI isoforms generated second axes and, with the exception of gamma, were similarly effective (Table 7). Again, □3 and □2 (data not shown) produced the least degree of axis formation and were slightly activated when the isolated kinase domains were expressed (Table 7).

Wnt transduces its signal by generating a complex between □-catenin and the transcription factor XTcf/LEF-1, which binds directly to the promoter of certain genes, such as Xnr-3 and Siamois, and activates their transcription (Brannon *et al.* 1997, McKendry *et al.* 1997, Figure 7). If the CKI isoforms activate the Wnt pathway, then they should induce the expression of these Wnt-specific markers. To evaluate this, we microinjected mRNA encoding the various isoforms and the inactive CKI□(K>R) into embryos, explanted animal caps, and analyzed Siamois and Xnr-3 expression. Control animal caps and caps expressing the CKI□(K>R) mutant did not induce Xnr-3 or Siamois (Figure 24). In contrast, all isoforms tested induced

Sample	n	mean
Uninjected	125	0.00
Epsilon K>R (700pg)	62	0.00
Alpha (175pg)	61	1.55
Beta (25pg)	71	1.13
Gamma-3 (1400pg)	83	0.14
Gamma-3 KD (1400 pg)	79	0.21
Delta (350pg)	62	1.49
Epsilon (350pg)	61	1.41
Epsilon KD (175pg)	59	1.48

Table 7. All CKI isoforms induce second axes. As in the UV-rescue experiments, all isoforms rescue with the gamma isoforms being the least effective. Normal embryos were injected into one ventral vegetal cell at the eight cell stage with the indicated doses of mRNA. Embryos were then scored as normal (0), partial duplication (1), or complete duplication (2).

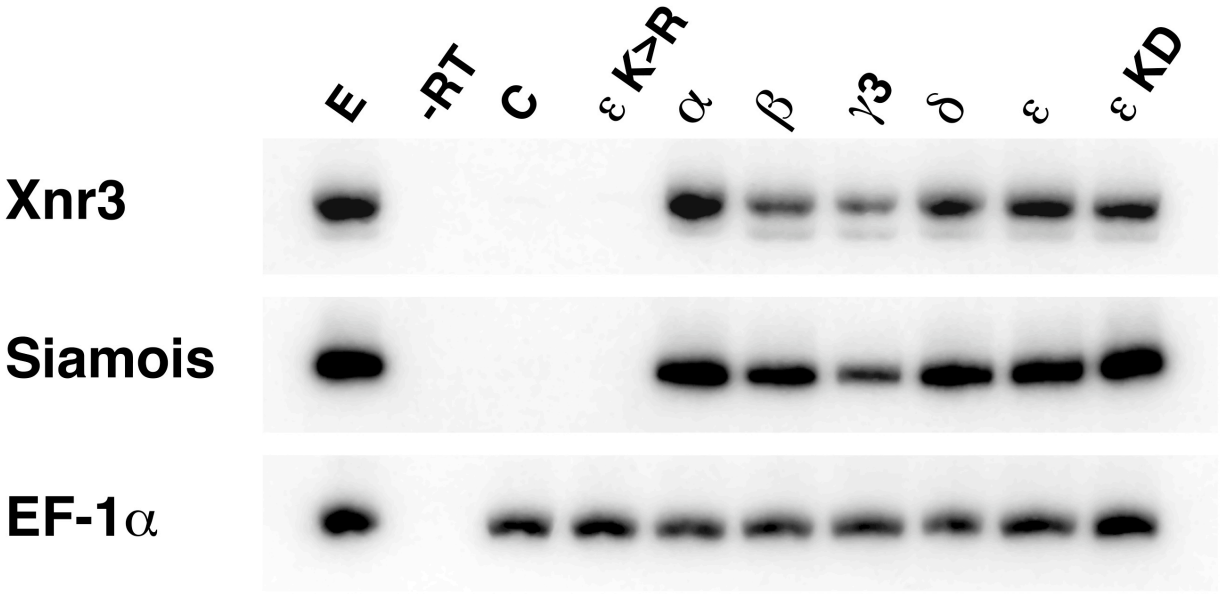


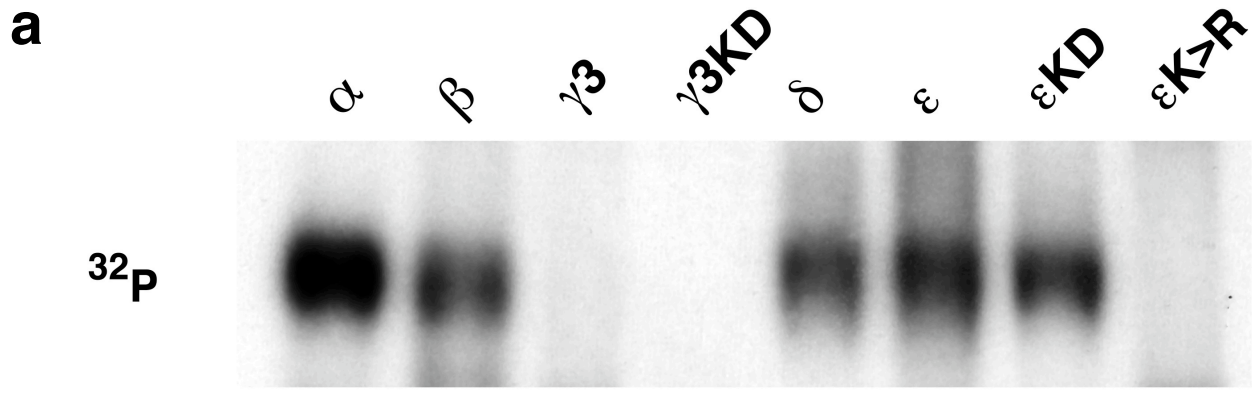
Figure 24. All CKI isoforms induce Wnt target genes. Animal cap explants were examined at stage 10.5 for expression of the Wnt-target genes Siamois and Xnr-3. EF-1 serves as a loading control. Doses were: epsilon K>R-350pg, alpha-175pg, beta-10pg, gamma 700pg, delta-350pg, epsilon-350pg, and epsilon KD-175pg. C denotes uninjected control caps.

the expression of both Wnt-specific markers (Figure 24). This figure represents the highest level of activation of Xnr-3 and Siamois by the δ3 isoform, which, in general, produces the lowest level of gene induction in this assay, similar to what we observed in the two whole embryo assays. Taken together, these data demonstrate that all the CKI isoforms can activate the Wnt pathway in *Xenopus* embryos. Although the gamma isoforms were injected at a relatively high mRNA concentration and produced the least robust phenotype, it is plausible that this is due to low levels of protein expression.

MOST CKI ISOFORMS PHOSPHORYLATE DISHEVELLED

Wnt signaling induces the phosphorylation of Dishevelled in intact cells and CKIε increases the phosphorylation of Dishevelled in *Xenopus* oocytes (Lee *et al.* 1999, Peters *et al.* 1999, Yanagawa *et al.* 1995). To extend our analysis of the CKI isoforms, we tested whether all the isoforms could induce this post-translational modification. To that end, we co-injected *Xenopus* oocytes with mRNA encoding the various CKI isoforms, the isolated catalytic domains of δ3 and ε, or the catalytically inactive CKIε (K>R) as a negative control, along with myc-tagged Dishevelled. We then incubated the oocytes in $^{32}P_i$, lysed them, immunoprecipitated Dishevelled with a monoclonal antibody directed against the myc-tag, and subjected the precipitates to SDS-PAGE. Autoradiography demonstrated that alpha, beta, delta, epsilon, and

the epsilon catalytic domain increased Dishevelled phosphorylation (Figure 25). The exception was the gamma3 isoform, which, as in the functional assays, produced the lowest response. In order to further assess the extent of phosphorylation, we performed a Western blot against the myc-tag on Dishevelled. All isoforms other than gamma appeared to phosphorylate Dishevelled with high efficiency based upon a decrease in its electrophoretic mobility (Figure 25b).


a
α
β
γ3
γ3KD
δ
ε
εKD
εK>R
32P

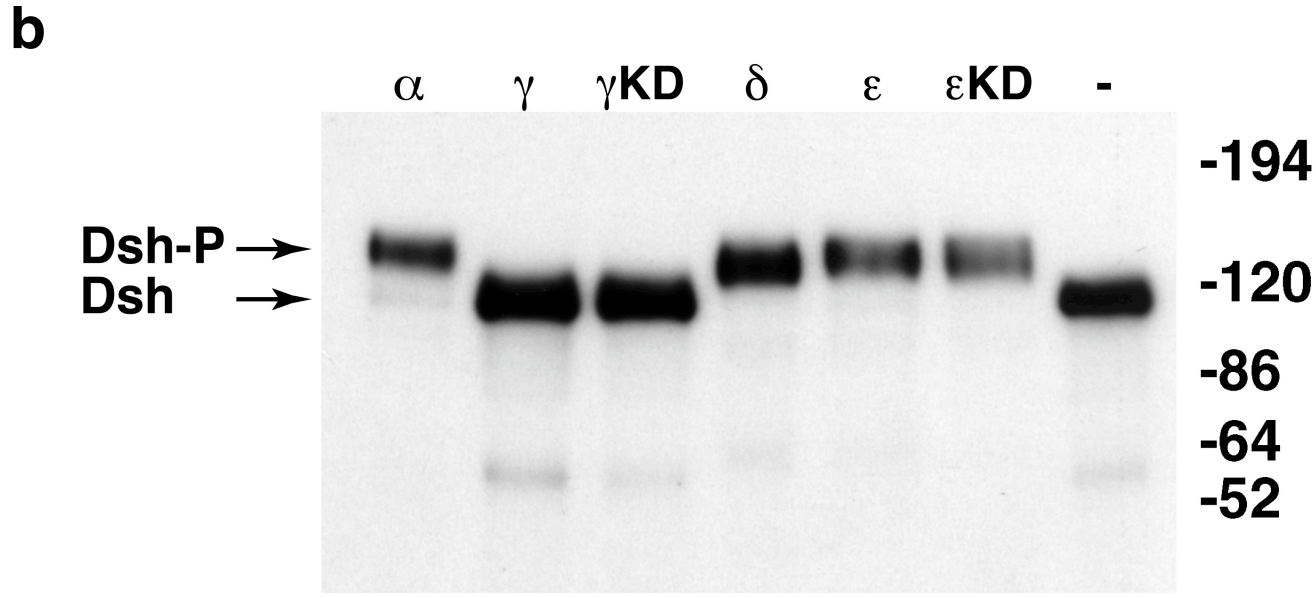

b
α
γ
γKD
δ
ε
εKD
-
Dsh-P
Dsh
-194
-120
-86
-64
-52

Figure 25. Multiple CKI isoforms phosphorylate Dishevelled and decrease its electrophoretic mobility. a, CKI isoforms □, □, □, □, or □KD increase Dishevelled phosphorylation. *Xenopus* oocytes were injected with the indicated CKI isoforms and myc-tagged dishevelled, incubated in medium containing $^{32}P_i$, lysed, and immunoprecipitated with an antibody directed against the myc epitope. The immunoprecipitates were subjected to SDS-PAGE and autoradiography. b, Expression of the CKI isoforms □, □, □, or □KD in oocytes decreased the electrophoretic mobility of dishevelled. Here the oocyte lysates are examined by Western blotting for the myc-tag present on dishevelled. Upshift results correlate with phosphorylation studies based on autoradiography.

CHAPTER SEVEN
Perspectives and conclusions

ROLES OF THE VARIOUS CKI ISOFORMS

CKI is a recently identified component of the -catenin-dependent Wnt cascade (Peters *et al.* 1999, Sakanaka *et al.* 1999). In *Xenopus*, CKI induces second axes, rescues UV-treated embryos, and induces expression of Wnt-signaling markers (Peters *et al.* 1999, Sakanaka *et al.* 1999). In cell lines, CKI activates transcription of a LEF-1 reporter gene and depletion of CKI abolishes Wnt-1 induced LEF-1 reporter activity (Sakanaka *et al.* 1999). These data demonstrate a role for CKI as a positive component of the Wnt pathway. In addition to CKI, other CKI isoforms exist including , , , and . While these isoforms differ due to variable amino- and carboxy-terminal extensions, all of the isoforms contain a highly conserved kinase domain, suggesting a similar substrate specificity (Gross and Anderson 1998). Previous studies have shown various roles for these isoforms including DNA repair, circadian rhythm in flies and rats, and cell budding in yeast (Gross *et al.* 1997, Kloss *et al.* 1998, Santos *et al.* 1996).

In this study, we have begun to characterize the role and the mechanism of action of CKI in Wnt signaling. We find that all of the CKI isoforms activated the canonical Wnt pathway in *Xenopus* embryos. In addition, expression of each of the isoforms, with the exception of gamma, led to Dishevelled phosphorylation in *Xenopus* oocytes. The lack of Dishevelled phosphorylation by gamma coupled with

its ability to activate the Wnt pathway could be interpreted to suggest that Dishevelled phosphorylation is not required for Wnt signaling. However, several other explanations are also possible. For example, measuring Dishevelled phosphorylation in oocytes and Wnt signaling in embryos may not reflect the same events. Consistent with that, preliminary studies indicate the Wnt pathway cannot be activated in oocytes as measured by the lack of □-catenin stabilization and activation of transcriptional reporters (not shown). Epistasis tests placed CKI downstream of Dishevelled and phosphorylation of Dishevelled may only be maximal with upstream activation. Formation of second axes, UV-rescue, and transcriptional activation, however, may only require coupling to the downstream components. Another possible explanation is that the dose of gamma required to activate Wnt in embryos is four times greater than any other isoform, yet this increased dose only produces a weak effect. For technical reasons, we only injected equal amounts of mRNA encoding all the isoforms for the oocyte phosphorylations. This dose of gamma may therefore have been too low to observe Dishevelled phosphorylation in this assay. It is not known whether the relative differences in levels required for the various isoforms to activate Wnt signaling are due to differences in the isoforms' protein translation efficiency, stability, catalytic activity, or substrate preferences. However, all CKI isoforms used in this study are of similar length and have identical non-coding elements, suggesting a high likelihood of similar expression levels. Additionally for the less active isoforms, such

as gamma, multiple independent clones were generated and tested and found to have similar levels of activity. Epitope tagging at either the carboxy or amino terminal of CKI largely inactivated the enzymes in all functional assays, and therefore could not be used to compare protein levels of functioning enzymes.

How many isoforms of CKI participate in Wnt signaling endogenously? Previous reports demonstrate that CKI is a required component of the vertebrate Wnt pathway (Peters *et al.* 1999). The sufficiency tests presented here demonstrate that many isoforms of the CKI family can activate the Wnt pathway. *C. elegans* also contains several isoforms of CKI. However, loss-of-function of one isoform, kin-19, which is most similar to the vertebrate alpha, produced a Wnt mutant phenotype (Peters *et al.* 1999). This suggests that the alpha isoforms also transduce Wnt signals. Loss-of-function of another worm CKI isoform, kin-20, which is most similar to mammalian epsilon, generates a distinct phenotype that is also produced by loss-of Wnt signals (J. Kimble, personal communication). Of note, when KIN-20 is expressed in frogs, it activates the Wnt pathway (data not shown). This is consistent with the notion that distinct CKI isoforms function in the Wnt pathway, but may do so in a temporally and spatially regulated manner. Consistent with that notion, frog embryos contain at least four CKI isoforms and, although not yet studied in depth, they appear to have distinct expression patterns.

POSSIBLE MECHANISMS OF CKI FUNCTION

When Wnt binds to its receptor, Dishevelled becomes phosphorylated (Lee *et al.* 1999, Yanagawa *et al.* 1995). CKI binds to the region of Dishevelled that is required for this phosphorylation and for the subsequent activation of downstream components of the Wnt cascade (Peters *et al.* 1999, Sokol 1996, Yanagawa *et al.* 1995). This suggests that CKI may be involved in both of these processes. In support of that, we have demonstrated that CKI is required for Wnt signaling and that CKI increases Dishevelled phosphorylation in *Xenopus* oocytes (Peters *et al.* 1999). Additionally, CKI directly phosphorylates Dishevelled *in vitro* and is required for the Wnt-dependent phosphorylation of Dishevelled in intact cells. Taken together, these data are consistent with a model that Wnt leads to the activation of CKI, which, in turn, phosphorylates Dishevelled. One observation that does not seem to fit with this model is that epistasis studies place CKI downstream of Dishevelled (Figure 26). These epistasis studies could be misleading as they were not done with true loss-of-function mutants. In addition, CKI, Dishevelled, and several other molecules in the Wnt pathway function in a large multi-protein complex, which could complicate epistatic analyses (Li *et al.* 1999, Sakanaka *et al.* 1999). However, biochemical studies support the placement of CKI downstream of Dishevelled; CKI directly binds to Axin, which functions downstream of Dishevelled in the Wnt cascade (Sakanaka *et al.* 1999). CKI could function downstream of

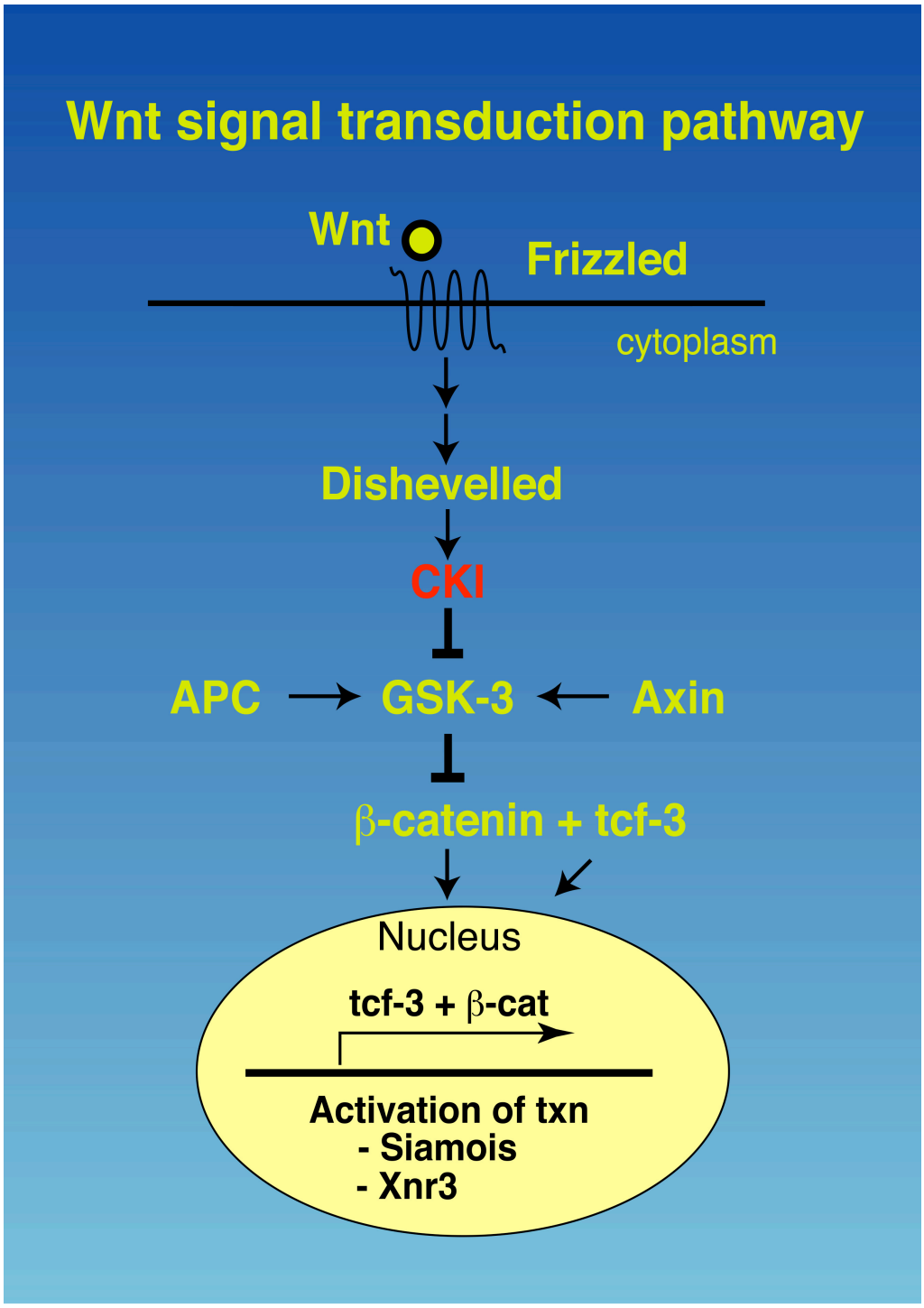


Figure 26. Overview of the Wnt signaling pathway. See text for discussion.

Dishevelled yet still phosphorylate Dishevelled. For example, CKI and Dishevelled might associate in the absence of Wnt signaling. Upon ligand binding, Dishevelled could activate CKI, and the activated form of CKI could in turn phosphorylate Dishevelled. This phosphorylation might then disrupt the interaction of Dishevelled and CKI, releasing CKI to activate downstream components of the pathway. Alternatively, the phosphorylation of Dishevelled might simply be a marker of activation of CKI and not a requirement for Wnt pathway function. Indeed, while Rothbacher *et al.* have shown that Dishevelled phosphorylation is significantly higher on the dorsal side of the embryo during the onset of dorsalizing events, they also conclude that phosphorylation of Dishevelled is not required for second-axis formation (Rothbacher *et al.* 2000). This conclusion is based upon deletion analysis involving large deletions of Dishevelled. Mapping and mutagenesis of specific Dishevelled phosphorylation sites coupled with functional analysis may be required to clarify the role of the Wnt/CKI-dependent phosphorylation of Dishevelled.

It was previously reported that CKI□ and a form of CKI□ lacking the carboxy-terminal tail did not generate second axes (Sakanaka *et al.* 1999). In addition, forms of CKI that lacked the C-terminal tail did not interact with Axin in co-immunoprecipitations. Based upon these data, it was concluded that the C-terminal tail was critical for activation of the Wnt pathway and that the interaction with Axin was key to that signaling process (Sakanaka *et al.* 1999). However, biochemical

studies demonstrated that forms of CKI without the tail are activated (Cegielska *et al.* 1998, Graves and Roach 1995). Consistent with that, our data demonstrate a strong activation of the Wnt pathway with forms of CKI (□, □, and □KD) that lack the C-terminal tail. These data are reproducible in several distinct assays including second axis formation, UV-rescue, and induction of Wnt-specific target genes. Furthermore, forms of CKI□ and CKI□ that lack the tail (CKI□KD, CKI□ KD) are slightly more active in the Wnt pathway than the wild-type enzymes. We also found that the tail of CKI□ is neither required nor sufficient for binding to Axin in yeast and that the isolated CKI□ kinase domain interacted strongly with Axin (McKay *et al.* 2001b). We cannot reconcile our data with the previous report. However, in support of our data, *Xenopus* CKI□ was recently isolated in an expression cloning screen based upon its ability to induce dorsal axes (Grammer *et al.* 2000). In addition, necessity tests suggest that CKI□ (kin-19) is required in worms to transduce Wnt signals at least for an early cell polarity decision (Peters *et al.* 1999). Therefore, we conclude that the C-terminal tail of CKI is neither required for binding to Axin nor for activation of the Wnt pathway.

Recently this controversy over the role of CKI in the Wnt pathway has continued. Since our original report, multiple groups have shown positive effects of CKI on the Wnt pathway including biological/functional assays and biochemical effects such as □-catenin stabilization (Sakanaka *et al.* 1999, Lee *et al.* 2001,

Kishida *et al.* 2001, Gao *et al.* 2002, McKay *et al.* 2001a, McKay *et al.* 2001b, Zhang *et al.* 2002). However, in 2002 several papers demonstrated an apparent negative effect of CKI on β-catenin stability (Liu et al 2002, Yanagawa *et al.* 2002, Amit et al 2002, Sakanaka 2002). Specifically, these authors report that phosphorylation of β-catenin by CKI primes β-catenin for subsequent phosphorylation by GSK-3 and degradation. Currently this issue remains unresolved (Polakis 2002). However, one likely explanation is that CKI is involved in regulating the assembly and function of the β-catenin degradation complex (Polakis 2002). In fact, previous reports have shown that CKI can phosphorylate and destabilize this degradation complex resulting in β-catenin accumulation (Gao *et al.* 2002). Additionally, Lee at al. (2001) have shown that CKI increases the binding of the transcription factor TCF-3 to β-catenin, leading to β-catenin stabilization. Perhaps the most likely explanation is that CKI can have both positive and negative effects on Wnt signaling due to its regulation of complex assembly. The specific effect of CKI in any situation may be determined by which other Wnt pathway components are present, the baseline state of Wnt signaling and complex assembly, the transcriptional cofactors present, and other ongoing signaling events. A further complication is the existence of multiple splice variants of individual isoforms (Rowles *et al.* 1991, Zhang *et al.* 1996, Green and Bennett 1998, Yong *et al.* 2000, Fu *et al.* 2001). Although much work remains to be done on CKI splice variants, it is already known that they have differential expression patterns (Green and Bennett 1998), subcellular localizations (Fu *et al.*

2001, Burzio *et al.* 2002), and biochemical profiles including k_m and sensitivity to chemical inhibitors such as CKI-7 (Burzio *et al.* 2002).

CKI IN MORPHOGENESIS AND ENDOGENOUS PATTERNING

A β-catenin-independent Wnt pathway also exists and, in *Xenopus*, this pathway is important for the convergence extension movements that are the main driving force for axis elongation (Keller 1991, Tada and Smith 2000, Wallingford *et al.* 2000). To examine the role that CKI plays during vertebrate development, we inhibited CKI function in *Xenopus* embryos with dominant negative forms of CKI, with a morpholino that blocks CKIε mRNA translation, and with CKI-7, a pharmacological inhibitor of CKI. Our results suggest that CKI plays a role in convergence extension movements; dominant negative forms of CKI generated embryos with shortened axes and a bent-back phenotype. This phenotype is similar to that observed with dominant negative forms of Wnt, Frizzled, and Dishevelled (Deardorff *et al.* 1998, Hoppler *et al.* 1996, Sokol, 1996).

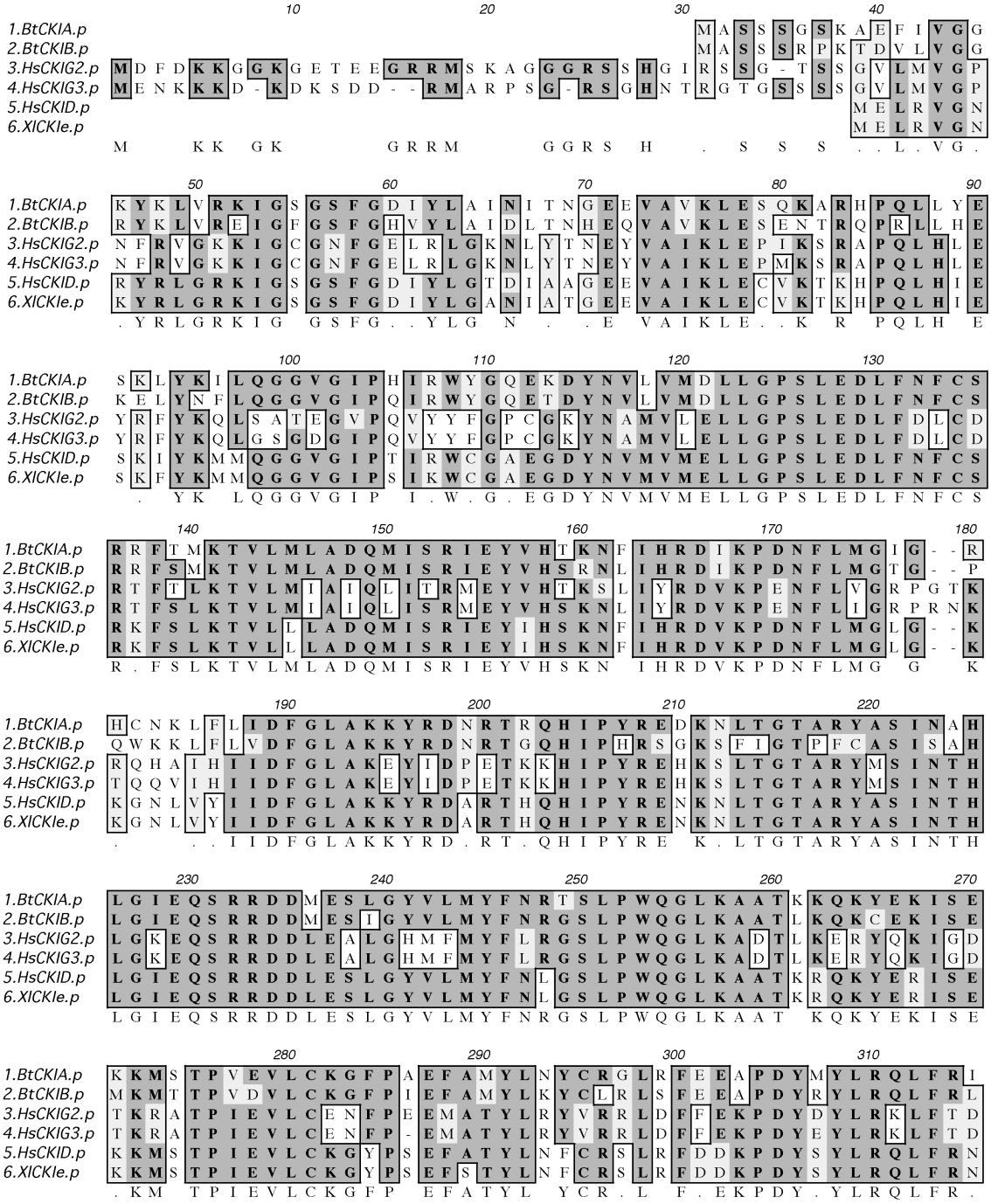
In *Xenopus* embryos, CKIε generates complete dorsal axes in both the second axis assay and the UV-rescue assay, which suggests a possible role for CKI in dorsal axis specification. Yet, blocking CKI function with dominant negatives, CKI-7, or the CKIε morpholino (McKay et al 2001a) did not alter dorsal axis formation.

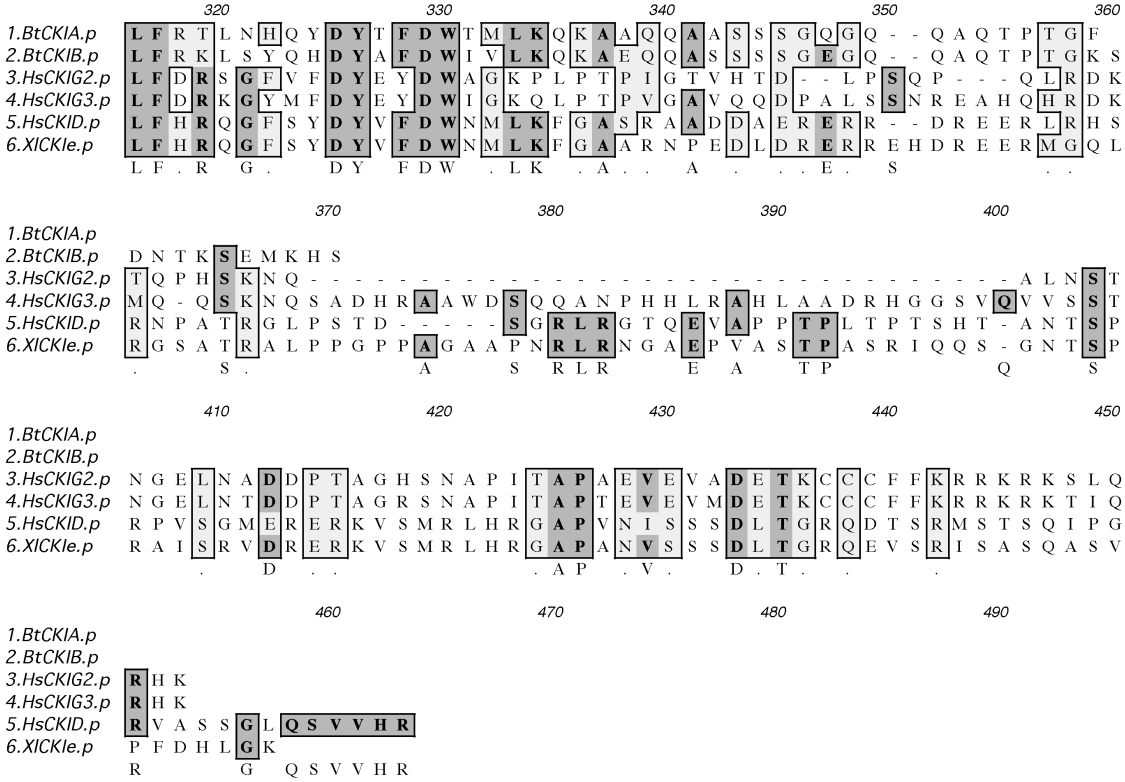
These data are consistent with other reports in which components of the Wnt pathway that act upstream of GSK-3 were blocked in embryos. For example, embryos microinjected with inhibitors of Wnts, Frizzleds, or Dishevelled all contained dorsal axes and had the bent-back phenotype (Deardorff *et al.* 1998, Hoppler *et al.* 1996, Hsieh *et al.* 1999, Sokol 1996). Of note, embryonic expression of dominant negative forms of Frizzled-7 blocked morphogenetic movements but not axis formation (Djiane *et al.* 2000). However, when Frizzled-7 was blocked by antisense-depletion in oocytes, primary axis formation was inhibited (Sumanas *et al.* 2000). This suggests that many if not all components of the canonical □-catenin pathway might be important in axial determination.

One possible explanation for the lack of effect of CKI inhibition on primary axis formation is that the dominant negative forms of CKI, Wnt, Dsh, and Frizzled are not potent enough to completely inhibit the endogenous gene products. Another possibility is that dorsal axis specification occurs very early and the dominant negatives are not expressed soon enough to alter this process. When might the endogenous process occur? In the dominant negative experiments, mRNA is microinjected and must then be translated. In an attempt to circumvent this delay, we microinjected the drug CKI-7, which should rapidly inhibit CKI function (Chijiwa et al. 1989). However, no dose of CKI-7 nor any time of microinjection into embryos affected axis formation. This suggests that the upstream components of the

pathway are required prior to the time in which we are able to microinject the drug. So, the key event(s) for axis specification might occur soon after fertilization. To resolve this issue and to determine the role that the CKI family plays in dorsal axis formation may require depletion of maternal stores of CKI mRNA in oocytes (Heasman et al. 1994). To date, we have tested twenty-five different CKIε anti-sense oligonucleotides and none have depleted the CKIε message to a substantial degree (not shown). Dominant negative forms (D>N) of the other CKI isoforms also generated the same bent-back phenotype as observed with the dominant negative forms of CKIε. Although it is possible that all of the different CKI isoforms play a role in convergence extension movements, it seems unlikely. An alternative explanation is that all of the dominant negatives interact or interfere with a common target. Preliminary data support this idea as dominant negatives of one isoform can inhibit the activity of a different CKI isoform.

APPENDIX A
Alignment of CKI Isoforms




320 330 340 350 360
1.BtCKIA.p
2.BtCKIB.p
3.HsCKIG2.p
4.HsCKIG3.p
5.HsCKID.p
6.XlCKIe.p
370 380 390 400
410 420 430 440 450
460 470 480 490

APPENDIX B
Oligos Used for Cloning

Template	S/A	Linker	Sequence
	Purpose		
XCKIe	S	ClaI	CG ATCGAT ATG GAG CTG AGA GTG GGG
	Forward primer for 450.6.1 (XCKIepsilon) ORF with Cla I site		
XCKIe	A	XbaI	CG TCTAGA TCA TTT CCC AAG GTG GTC
	Reverse primer for 450.6.1 (XCKIepsilon) ORF with Xba I site		
XCKIe	S	None	AG GAG GTT GCC ATT CGG CTG GAA TGT GTC
	Sense primer to make the K -> R mutation in CKI		
XCKIe	A	None	TC CTC CAA CGG TAA GCC GAC CTT ACA CAG
	Antisense primer to make the K -> R mutation in CKI		
XCKIe	A	XbaI	CG TCTAGA TCA CTG TCC CAT TCT TTC TTC
	Reverse primer for truncated 450.6.1 (XCKIepsilon) ORF (AA 1-319) with Xba I site		
XCKIe	S	ClaI	CG ATCGAT ATG TTT GGT GCT GCT AGG AAT
	Forward primer to amplify 450.6.1 (XCKIepsilon) COOH tail only (AA 295-416) with ClaI site		
XCKIe	S	ClaI	CG ATCGAT ATG GAG CAA AAG CTC ATT TCT GAA GAG GAC TTG GAG CTG AGA GTG GGG AAC
	Forward primer to amplify 450.6.1 (XCKIepsilon) ORF and add 5’ myc 9E10 tag		

XCKIe	A	ClaI	CG ATC GAT TCA CAA GTC CTC TTC AGA AAT GAG CTT TTG CTC TTT CCC AAG GTG GTC AAA

Reverse primer to amplify 450.6.1 (XCKIepsilon) ORF and add 3' myc 9e10 site

XCKIe	S	None	TTC ATA CAC CGA AAT GTA AAA CCA GAT AAC TTC

Forward primer to soe Asp 128 to Asn in 450.6.1 (XCKI-epsilon)

XCKIe	A	None	ATC TGG TTT TAC ATT TCG GTG TAT GAA GTT CTT

Reverse primer to soe Asp 128 to Asn in 450.6.1 (XCKI-epsilon)

XCKIe	S	KpnI	CG GGTACC CAGGGTAAAC ATG GAG CTG

Forward oligo to amplify XCKIe beginning 10bp before ATG and adding a 5' Kpn I site for cloning into pUA

XCKIe	A	XbaI	CG TCTAGA TCA AGC GTA GTC TGG GAC GTC GTA TGG GTA TTT CCC AAG GTG GTC AAA

Reverse oligo to amplify XCKIepsilon adding a 3' Xba I site and 3' HA tag

Y106G6	S	None	CCAGGATTCTGCAGGTAA

5' tail primer to clone just the tail for RNAi

apc-related	S	None	ATTGACGTGGTTTCCTCA

5' primer to clone the C. elegans apr-1 cDNA

apc-related	A	None	GGCTAATTGTCCGGCGGCGGCCAGGGCGGCCACCAAGCTCTT

3' primer to clone the C. elegans apr-1 cDNA

XBcat	S	EcoRI	CG GAATTC A ATG GCA ACT CAA GCA GAT

Forward oligo to clone XL B-catenin into pCS2+MT in frame with EcoRI site

XBcat **A** **SacI** CG GAGCTC TTA CAA GTC AGT GTC AAA
Reverse oligo to amplify XL B-catenin with SacI site for cloning into pCS2+MT (don't cut)

Xfz8 **S** **EcoRV** CG GATATC ATG GAG AGT CTG TCG CTG
Forward oligo to amplify Xfz8 into pCS2+MT adding a EcoRV site (don't cut)

Xfz8 **A** **ClaI** CG ATCGAT C GAC CTG AGA TAA GGG CAT
Reverse oligo to clone Xfz8 into pCS2+MT in frame with ClaI site

Xdsh **S** **EcoRI** CG GAATTC A ATG GCG GAG ACT AAA GTG
Forward oligo to clone Xdsh into pCS2+MT in frame with EcoRI site

Xdsh **A** **SpeI** CG ACTAGT TCA CAT GAC ATC CAC AAA
Reverse oligo to clone Xdsh into pCS2+MT with SpeI site (don't cut)

Xgsk3b **S** **BglII** CG AGATCT TA ATG TCG GGA AGG CCG AGA
Forward oligo to clone Xgsk3b into pCS3+MT with BglII site

Xgsk3b **A** **SacI** CG GAGCTC TCA GGA GGA GTT GGA GGC
Reverse oligo to clone Xgsk3b into pCS3+MT with SacI site (don't cut)

CKI **S** **SalI** GGGATCCGTCGACCT GGT GCT GCT AGG AAT CCA GAG
Forw. primer to clone CKI tail into pBTM116

GBP **S** **EcoRI** AACCCCGAATTCCC ATG CCG TGT CGC AAG GAG AG
Forw. primer to clone into yeast pGAD vector

GBP **A** **XhoI** TTTCCCCTCGAGG TCA TTG CAC GGT TGT CTC AG
Rev primer to clone into yeast pGAD vevctor

Xdsh **S** **EcoRI** AACCCCGAATTCCC ATG GCG GAG ACT AAA GTG
Forw. primer to clone into yeast pGAD vector

Xdsh **A** **SalI** TTTCCCGTCGAC TCA CAT GAC ATC CAC AAA G
Rev primer to clone into yeast pGAD vevctor

BCKIb **S** **EcoRI** CG GAATTC A ATG GCG AGC AGC AGC AGA
Forward oligo to clone Bovine CKIB into pCS2+ or pCS2+MT with EcoRI linker

BCKIb **A** **XhoI** CG CTCGAG TTA AGA ATG TTT CAT TTC
Reverse oligo to clone Bovine CKIB into pCS2+ or pCS2+MT with XhoI linker

BCKIa **S** **ClaI/BglII** CG ATCGAT AGATCT AG ATG GCG AGC AGC AGC GGC
Forward oligo to clone Bovine CKIA into pCS2+ (ClaI) or pCS3+MT (BglII)

BCKIa **A** **XhoI** CG CTCGAG TTA GAA ACC TGT GGG GGT
Reverse oligo to clone Bovine CKIA into pCS2+ or pCS3+MT with XhoI linker

HCKId **S** **ClaI/BglII** CG ATCGAT AGATCT AG atg gag ctg aga gtc ggg
Forward oligo to clone Human CKID into pCS2+ (ClaI) or pCS3+MT (BglII)

HCKId **A** **XhoI** CG CTCGAG tca tcg gtg cac gac aga
Reverse oligo to clone human CKID into pCS2+ or pCS3+MT with XhoI linker

XCKIe S SalI CG GAATTC A TCA CGC CTT CTC AAA CGG
5' ORF primer for cloning into pPRO-EX-HTb in frame

XCKIe A SalI CG GAATTC TCA TAG AGG CCA TGG GGA AGC
5' ORF primer for cloning into pGEX-4T3 in frame

Xdsh S EcoRI CGGAATTCAATGGCGGAGACTAAAGTG
Forward oligo Xdsh AA's 1-6 to clone into pCS2+MT

Xdsh A EcoRI CGGAATTCTCAGCCCTTCATTATAGAGCC
Reverse oligo Xdsh AA's 289-284 plus STOP to clone into pCS2+MT

Xdsh S EcoRI CGGAATTCATCACGCCTTCTCAAACGG
Forward oligo Xdsh AA's 216-221 to clone into pCS2+MT

Xdsh A EcoRI CGGAATTCTCATAGAGGCCATGGGGAAGC
Reverse oligo Xdsh AA's 543-538 plus STOP to clone into pCS2+MT

Xdsh S EcoRI CGGAATTCAACAGTGAACAAGATCACA
Forward oligo Xdsh AA's 485-490 to clone into pCS2+MT

Xdsh A EcoRI CGGAATTCTCACATGACATCCACAAA
Reverse oligo Xdsh AA's 737-732 plus STOP to clone into pCS2+MT

Xdsh S EcoRI CG GAATTC A CGCCTGGAACGGACGTCC
Forward oligo Xdsh AA's 232-238 to clone into pCS2+MT

Xdsh A EcoRI CG GAATTC TCA GGCCAGAGTGTCCTGGTC
Reverse oligo Xdsh AA's 531-525 plus STOP to clone into pCS2+MT

Xdsh A EcoRI CG GAATTC TCA CCG TTC CAG GCG CGG GGG
Reverse oligo Xdsh AA's 236-231 plus STOP to clone into pCS2+MT

Xdsh S EcoRI CG GAATTC A CGC CTT CTC AAA CGG CAC
Forward oligo Xdsh AA's 217-222 to clone into pCS2+MT

Xdsh A EcoRI CG GAATTC TCA CAT ATT CTC AAA GTT AAT
Reverse oligo Xdsh AA's 315-310 plus STOP to clone into pCS2+MT

Xdsh S EcoRI CG GAATTC A ATA ATG AAG GGC GGA GCT
Forward oligo Xdsh AA's 285-290 to clone into pCS2+MT

Xdsh A EcoRI CG GAATTC TCA GAC CCA GGC AGC CGG ATC
Reverse oligo Xdsh AA's 367-362 plus STOP to clone into pCS2+MT

Xdsh S EcoRI CG GAATTC A CTC CCC CGC AAT GAG CCC
Forward oligo Xdsh AA's 351-356 to clone into pCS2+MT

Xdsh A EcoRI CG GAATTC TCA GAT TTT GAG CCA CAT GCG
Reverse oligo Xdsh AA's 442-437 plus STOP to clone into pCS2+MT

Xdsh S EcoRI CG GAATTC A GGC CTC GAG GTC CGA GAT
Forward oligo Xdsh AA's 430-435 to clone into pCS2+MT

pCS2+	**S**	**None**	**GATCAGATCTATGCACCACCACCACCACCACCACCACCACAGATCTGGATCCCAAAAT**
	Make pCS2+9His		
pCS2+	**A**	**None**	**CGATTTTGGGATCCAGATCTGTGGTGGTGGTGGTGGTGGTGGTGCATAGATCT**
	Make pCS2+9His		
XCKIe	**S**	**XhoI**	**CG CTCGAG ATG GAG CTG AGA GTG GGG**
	Clone XCKIe into pET15b for bacterial expression with His tag		
XCKIe	**A**	**XhoI**	**CG CTCGAG TCA TTT CCC AAG GTG GTC**
	Clone XCKIe into pET15b for bacterial expression with His tag		
XCKIe	**A**	**XhoI**	**CG CTCGAG TCA CTG TCC CAT TCT TTC TTC**
	Clone XCKIe KD only into pET15b for bacterial expression with His tag		
XCKIe	**S**	**None**	**TAC ATT ATT GcT TTC GGA CTT GCC AAG**
	Forward primer to soe Asp 148 to Ala in 450.6.1 (DFG->AFG)		
XCKIe	**A**	**None**	**AAG TCC GAA AgG AAT AAT GTA GAC TAG**
	Reverse primer to soe Asp 148 to Ala in 450.6.1 (DFG->AFG)		
XCKIe	**S**	**None**	**ATA CAC CGA GcT GTA AAA CCA GAT ggC TTC CTA ATG GGG CTT**
	Forward primer to soe Asp 128 to Ala and Asn 133 to Gly in 450.6.1 (DVKPDN->AVKPDG)		
XCKIe	**A**	**None**	**CAT TAG GAA Gcc ATC TGG TTT TAC AgC TCG GTG TAT GAA GTT**
	Reverse primer to soe Asp 128 to Ala and Asn 133 to Gly in 450.6.1 (DVKPDN->AVKPDG)		

XCKIe	**S**	**None**	**CGG CGT GAT AaC TTG GAG gcT CTG GcA TAT GTC acT ATG TAC TTT AAT CTG**

Forward primer to soe DDLESLGYVL TO DNLEALAYVT in 450.6.1 (XCKI-epsilon)

XCKIe	**A**	**None**	**AAA GTA CAT Agt GAC ATA TgC CAG Tgc CTC CAA GTt ATC ACG CCG ACT TTG**

Reverse primer to soe DDLESLGYVL TO DNLEALAYVT in 450.6.1 (XCKI-epsilon)

XCKIe	**S**	**ClaI**	**CG ATCGAT CC ATG GAG CTG AGA GTG GGG**

Forward oligo to clone XCKIe into ClaI of pCS2+9His

XCKIe	**A**	**ClaI**	**CG ATCGAT TCA TTT CCC AAG GTG GTC**

Reverse oligo to clone XCKIe into ClaI of pCS2+9His

Xdsh	**A**	**SalI**	**CG GTCGAC TCA CAT GAC ATC CAC AAA**

Reverse oligo to clone Xdsh into XhoI of pET15b

HCKIg2	**S**	**EcoRI**	**CG GAATTC A ATGGATTTTGACAAGAAAGG**

Forward oligo to clone HCKIg2 into pCS2+ or pCS2+MT with EcoRI linker

HCKIg2	**A**	**EcoRI**	**CG GAATTC TCACTTGTGTCGCTGCAG**

Reverse oligo to clone HCKIg2 into pCS2+ or pCS2+MT with EcoRI linker

BCKIa	**S**	**None**	**TTTATACACAGAAATATTAAACCAGATAACTTC**

Forward oligo to soe '128' D>N in BCKIa

BCKIa	**A**	**None**	**ATCTGGTTTAATATTTCTGTGTATAAAATTCTT**

Reverse oligo to soe '128' D>N in BCKIa

BCKIb	**S**	**None**	**CTTATACACAGGAATATTAAACCAGATAACTTC**
Forward oligo to soe '128' D>N in BCKIa			
BCKIb	**A**	**None**	**ATCTGGTTTAATATTCCTGTGTATAAGATTGCG**
Reverse oligo to soe '128' D>N in BCKIa			
HCKIg2	**S**	**None**	**CTAATCTACCGGAATGTGAAGCCCGAGAACTTC**
Forward oligo to soe '128' D>N in BCKIa			
HCKIg2	**A**	**None**	**CTCGGGCTTCACATTCCGGTAGATTAGGCTCTT**
Reverse oligo to soe '128' D>N in BCKIa			
HCKIg3	**S**	**None**	**TTGATATACAGAAATGTAAAACCTGAGAACTTC**
Forward oligo to soe '128' D>N in BCKIa			
HCKIg3	**A**	**None**	**CTCAGGTTTTACATTTCTGTATATCAAGTTCTT**
Reverse oligo to soe '128' D>N in BCKIa			
HCKId	**S**	**None**	**TTCATCCACCGGAATGTGAAGCCAGACAACTTC**
Forward oligo to soe '128' D>N in BCKIa			
HCKId	**A**	**None**	**GTCTGGCTTCACATTCCGGTGGATGAAGTTCTT**
Reverse oligo to soe '128' D>N in BCKIa			
HCKIg2	**S**	**EcoRI**	**CG GAATTC A ATG GTC CTG ATG GTG GGC CC**
Forward HCKIg2 KD oligo for pCS2+ or pCS2+MT			

HCKIg2	**A**	**EcoRI**	**CG GAATTC TCA GTC CCG GAG CTG AGG CTG**

Reverse HCKIg2 KD oligo for pCS2+ or pCS2+MT

HCKIg3	**S**	**EcoRI**	**CG GAATTC A ATG GTT TTA ATG GTT GGA CCT**

Forward HCKIg3 KD oligo for pCS2+ or pCS2+MT

HCKIg3	**A**	**EcoRI**	**CG GAATTC TCA ATC TCT GTG TTG ATG TGC**

Reverse HCKIg3 KD oligo for pCS2+ or pCS2+MT

Xdsh	**S**	**BglII**	**GA AGATCT G CGCCTGGAACGGACGTCC**

Forward oligo to clone Xdsh Fragment 2 into pET15b BamHI site

Xdsh	**A**	**BglII**	**GA AGATCT TCATAGAGGCCATGGGGAAGC**

Reverse oligo to clone Xdsh Fragment 2 into pET15b BamHI site

BIBLIOGRAPHY

Amit, S., Hatzubai, A., Birman, Y. andersen, J.S., Ben-Shushan, E., Mann, M., Ben-Neriah, Y. and Alkalay, I. (2002). Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes and Development 16, 1066-1076.

Behrens, J., von Kries, J., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of □-catenin with the transcription factor LEF-1. Nature 382, 638-642.

Bhanot, P., Fish, M., Jemison, J., Nusse, R., Nathans, J., and Cadigan, K. (1999). Frizzled and DFrizzled-2 function as redundant receptors for Wingless during Drosophila embryonic development. Development 126, 4175-4186.

Boutros, M., and Mlodzik, M. (1999). Dishevelled: at the crossroads of divergent intracellular signaling pathways. Mechanisms of Development 83, 27-37.

Boutros, M., Paricio, N., Strutt, D., and Mlodzik, M. (1998). Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell 94, 109-118.

Bowerman, B., Tax, F., Thomas, J. & Priess, J. (1992). Cell interactions involved in development of the bilaterally symmetrical intestinal valve cells during embryogenesis in *Caenorhabditis elegans*. Development 116, 1113-1122.

Bowerman, B., Draper, B., Mello, C. & Priess, J. (1993). The material gene skn-1 encodes a protein that is distributed unequally in early *C. elegans* embryos. Cell 74, 443-452.

Brannon, M., Gomperts, M., Sumoy, L., Moon, R., and Kimelman, D. (1997). A □-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in *Xenopus*. Genes & Development 11, 2359-2370.

Burzio, V., Antonelli, M., Allende, CC., Allende, JE. (2002). Biochemical and cellular characteristics of the four splice variants of protein kinase CK1 alpha from zebrafish (Danio rerio). Journal of Cellular Biochemistry 86, 805-814.

Cadigan, K., and Nusse, R. (1997). Wnt signaling: a common theme in animal development. Genes & Development 11, 3286-3305.

Carnac, G., Kodjabachian, L., Gurdon, J. & Lemaire, P. (1996). The homeobox gene Siamois is a target of the Wnt dorsalization pathway and triggers organizer activity in the absence of mesoderm. Development 122, 3055-3065.

Cegielska, A., Gietzen, K., Rivers, A., and Virshup, D. (1998). Autoinhibition of Casein Kinase I is relieved by protein phosphatases and limited proteolysis. Journal of Biological Chemistry 273, 1357-1364.

Chijiwa, T., Hagiwara, M., and Hidaka, H. (1989). A newly synthesized selective Casein Kinase I inhibitor, N-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide, and affinity puriification of Casein Kinase I from bovine testis. Journal of Biological Chemistry 264, 4924-4927.

Deardorff, M., Tan, C., Conrad, L., and Klein, P. (1998). Frizzled-8 is expressed in the Spemann organizer and plays a role in early morphogenesis. Development 125, 2687-2700.

DeMaggio, A., Lindberg, R., Hunter, T. & Hoekstra, M. (1992). The budding yeast HRR25 gene product is a casein kinase I isoform. Proc. Natl. Acad. Sci. 89, 7008-7012.

Djiane, A., Riou, J., Umbhauer, M., Boucat, J., and Shi, D. (2000). Role of frizzled 7 in the regulation of convergent extension movements during gastrulation in *Xenopus laevis*. Development 127, 3091-3100.

Eaton, S., Wepf, R., and Simons, K. (1996). Roles for Rac1 and cdc42 in planar polarization and hair outgrowth in the wing of Drosophila. Journal of Cell. Biology 135, 1277-1289.

Elinson, R., Rowning, B. (1988). A transient array of parallel microtubules in frog eggs: potential tracks for a cytoplasmic rotation that specifies the dorso-ventral axis. Developmental Biology 128, 185-197.

Fire, A., Xu, S., Montgomery, M., Kostas, S., Driver, S., Mello, C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-811.

Fish, K., Cegielska, A., Getman, M., Landes, G. & Virshup, D. (1995). Isolation and characterization of human casein kinase I epsilon (CKI), a novel

member of the CKI gene family. Journal of Biological Chemistry 270, 14875-14833.

Fu, Z., Chakraborti, T., Morse, S., Bennett, GS., Shaw, G. (2001). Four casein kinase I isoforms are differentially partitioned between nucleus and cytoplasm. Experimental Cell Research 269, 275-286.

Funayama, N., Fagotto, F., McCrea, P., and Gumbiner, B. M. (1995). Embryonic axis induction by the armadillo repeat domain of beta-catenin: evidence for intracellular signaling. J Cell Biol 128, 959-68.

Gao, Z.H., Seeling, J.M., Hill, V., Yochum, A., and Virshup, D.M. (2002). Casein kinase I phosphorylates and destabilizes the beta-catenin degradation complex. Proc. Natl. Acad. Sci. U.S.A. 99, 1182-1187.

Graff, J., Thies, R., Song, J., Celeste, A., and Melton, D. (1994). Studies with a *Xenopus* BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals *in vivo*. Cell 79, 169-179.

Grammer, T., Liu, K., Mariani, F., and Harland, R. (2000). Use of large-scale expression cloning screens in the *Xenopus laevis* tadpole to identify gene function. Developmental Biology 228, 197-210.

Graves, P., and Roach, P. (1995). Role of COOH-terminal phosphorylation in the regulation of casein kinase I. Journal of Biological Chemistry 270, 21689-21694.

Green, CL., Bennett, GS. (1998). Identification of four alternatively spliced isoforms of chicken casein kinase I alpha that are all expressed in diverse cell types. Gene 216, 189-195.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., and *et al.* (1991). Identification and characterization of the familial adenomatous polyposis coli gene. Cell 66, 589-600.

Gross, S., and Anderson, R. (1998). Casein Kinase I: spatial organization and positioning of a multifunctional protein kinase family. Cell. Signal 10, 699-711.

Gross, S., Simerly, C., Schatten, G., and Anderson, R. (1997). A casein kinase I isoform is required for proper cell cycle progression in the fertilized mouse oocyte. Journal of Cell Science 110, 3083-3090.

He, X., Saint-Jeannet, J., Woodgett, J., Varmus, H., and Dawid, I. (1995). Glycogen synthase kinase-3 and dorsoventral patterning in *Xenopus* embryos. Nature 374, 617-622.

Heasman, J. (2002). Morpholino oligos: Making sense of antisense?. Developmental Biology 243, 209-214.

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C., and Wylie, C. (1994). Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. Cell 79, 791-803.

Heasman, J., Kofron, M., and Wylie, C. (2000). Beta-catenin signaling activity dissected in the early *Xenopus* embryo: a novel antisense approach. Dev Biol 222, 124-34.

Hino, S., Michiue, T., Asashima, M., Kikuchi, A. (2003). Casein kinase I epsilon enhances the binding of Dvl-1 to Frat-1 and is essential for Wnt-3a-induced accumulation of beta-catenin. Journal of Biological Chemistry 278, 14066-14073, 2003.

Hoppler, S., Brown, J., and Moon, R. (1996). Expression of a dominant-negative Wnt blocks induction of MyoD in *Xenopus* embryos. Genes and Development 10, 2805-2817.

Hsieh, J. C., Kodjabachian, L., Rebbert, M. L., Rattner, A., Smallwood, P. M., Samos, C. H., Nusse, R., Dawid, I. B., and Nathans, J. (1999). A new secreted protein that binds to Wnt proteins and inhibits their activities. Nature 398, 431-6.

Kao, K. R., and Elinson, R. P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. Developmental Biology 127, 64-77.

Kao, K. R., Masui, Y., and Elinson, R. P. (1986). Lithium-induced respecification of pattern in *Xenopus laevis* embryos. Nature, 371-373.

Keller, R. (1991). Early embryonic development of *Xenopus laevis*. Methods Cell. Biology 36, 61-113.

Kinzler, K., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. Cell 87, 159-170.

Kishida, M., Hino, S., Michiue, T., Yamamoto, H., Kishida, S., Fukui, A., Asashima, M. and Kikuchi, A. (2001). Synergistic activation of the Wnt signaling pathway by Dvl and casein kinase I epsilon. Journal of Biological Chemistry 276, 33147-33155.

Kloss, B., Price, J., Saez, L., Blau, J., Rothenfluh, A., Wesley, C., and Young, M. (1998). The Drosophila clock gene double-time encodes a protein closely related to human casein kinase Ie. Cell 94, 97-107.

Lee, E., Salic, A. and Kirschner, M.W. (2001). Physiological regulation of beta-catenin stability by Tcf3 and CK1epsilon. Journal of Cell Biology 154, 983-993.

Lee, J., Ishimoto, A., and Yanagawa, S. (1999). Characterization of mouse Dishevelled (Dvl) proteins in Wnt/Wingless signaling pathway. Journal of Biological Chemistry 274, 21464-21470.

LeSueur, J., and Graff, J. (1999). Spemann organizer activity of Smad10. Development 126, 137-146.

Li, L., Yuan, H., Xie, W., Mao, J., Caruso, A., McMahon, A., Sussman, D., and Wu, D. (1999). Dishevelled proteins lead to two signaling pathways. The Journal of Biological Chemistry 274, 129-134.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G.H., Tan, Y., Zhang, Z., Lin, X. and He, X. (2002). Control of beta-catenin phosphorylation/ degradation by a dual-kinase mechanism. Cell 108, 837-847.

Lowrey, P., Shimomura, K., Antoch, M., Yamazaki, S., Zemenides, P., Ralph, M., Menaker, M., and Takahashi, J. (2000). Positional cloning and functional characterization of the mammalian circadian mutation tau. Science 28, 483-491.

McKay, R.M., Peters, J.M. and Graff, J.M. (2001a). The Casein Kinase I Family: Roles in Morphogenesis. Developmental Biology 235, 378-387.

McKay, R.M., Peters, J.M. and Graff, J.M. (2001b). The Casein Kinase I Family in Wnt Signaling. Developmental Biology 235, 388-396.

McKendry, R., Hsu, S., Harland, R., and Grosschedl, R. (1997). LEF-1/TCF proteins mediate wnt-inducible transcription from the *Xenopus* nodal-related 3 promoter. Developmental Biology 192, 420-431.

McMahon, A., and Moon, R. (1989). Ectopic expression of the proto-oncogene int-1 in *Xenopus* embryos leads to duplication of the embryonic axis. Cell 58, 1075-1084.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates β-catenin-induced axis formation in *Xenopus* embryos. Cell 86, 391-399.

Moon, R., Brown, J., and Torres, M. (1997). WNTs modulate cell fate and behavior during vertebrate development. Trends In Genetics 13.

Newport, J., and Kirschner, M. (1982). A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. Cell 30, 687-696.

Nieuwkoop, P., Faber, J. (1967). "Normal Table of *Xenopus laevis*." North-Holland, Amsterdam.

Nusse, R. & Varmus, H. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31, 99-109.

Peters, J. M., McKay, R. M., McKay, J. P., and Graff, J. M. (1999). Casein kinase I transduces Wnt signals. Nature 401, 345-50.

Polakis, P. (2002). Casein kinase 1: A Wnt'er of disconnect. Current Biology. 12, R499-R501.

Rocheleau, C., Downs, W., Lin, R., Wittmann, C., Bei, Y., Cha, Y., Ali, M., Priess, J., and Mello, C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. Cell 90, 707-716.

Rothbacher, U., Laurent, M., Deardorff, M., Klein, P., Cho, K., and Fraser, S. (2000). Dishevelled phosphorylation, subcellular localization and

multimerization regulate its role in embryogenesis. The EMBO Journal 19, 1010-1022.

Rowles, J., Slaughter, C., Moomaw, C., Hsu, J., Cobb, M. (1991). Purification of casein kinase I and isolation of cDNAs encoding multiple casein kinase I-like enzymes. Proc. Natl. Acad. Sci. USA 88, 9548-9552.

Rubinfeld, B., *et al.* (1997). Stabilization of β-catenin by genetic defects in melanoma cell lines. Science 275, 1790-1792.

Sakanaka, C. (2002). Phosphorylation and Regulation of b-catenin by Casein Kinase I. Journal of Biochemistry 132, 697-703.

Sakanaka, C., Leong, P., Xu, L., Harrison, S., and Williams, L. (1999). Casein kinase Ie in the Wnt pathway: regulation of β-catenin function. PNAS 96, 12548-12552.

Santos, J., Logarinho, E., Tapia, C., Allende, C., Allende, J., and Sunkel, C. (1996). The casein kinase I a gene of Drosophila melanogaster is developmentally regulated and the kinase activity of the protein induced by DNA damage. Journal of Cell Science 109, 1847-1856.

Sasai, Y., Lu, B., Steinbesser, H., Geissert, D., Gont, L., De Robertis, E. (1994). *Xenopus* chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell 79, 779-790.

Satoh, S., Diago, Y., Furukawa, Y., Kato, T., Miwa, N., Nishiwaki, T., Kawasoe, T., Ishiguro, H., Fujita, M., Tokino, T., and al., e. (2000). AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. Nature Genetics 24, 245-250.

Scharf, S. & Gerhart, J. (1983). Axis determination in eggs of *Xenopus laevis*: A critical period before first cleavage, identified by the common effects of cold, pressure, and ultraviolet irradiation. Developmental Biology 99, 75-87.

Sharp, P. (1999). RNAi and double-strand RNA. Genes and Development 13, 139-141.

Smith, W., and Harland, R. (1991). Injected Xwnt-8 RNA acts early in *Xenopus* embryos to promote formation of a vegetal dorsalizing center. Cell 67, 753-765.

Sokol, S. (1996). Analysis of Dishevelled signaling pathways during *Xenopus* development. Current Biology 6, 1456-1467.

Sokol, S. (2000). A role for Wnts in morphogenesis and tissue polarity. Nature Cell Biology 2, 1-2.

Sokol, S., Klingensmith, J., Perrimon, N., and Itoh, K. (1995). Dorsalizing and neuralizing properties of XDishevelled, a maternally expressed *Xenopus* homolog of dishevelled. Development 121, 1637-1647.

Strutt, D., Weber, U., and Mlodzik, M. (1997). The role of RhoA in tissue polarity and Frizzled signalling. Nature 387, 292-295.

Sumanas, S., Strege, P., Heasman, J., and Ekker, S. C. (2000). The putative wnt receptor *Xenopus* frizzled-7 functions upstream of beta-catenin in vertebrate dorsoventral mesoderm patterning. Development 127, 1981-90.

Tabara, H., Grishok, A. & Mello, C. (1998). RNAi in *C. elegans*: soaking in the genome sequence. Science 282, 430-431.

Tada, M., and Smith, J. (2000). Xwnt11 is a target of *Xenopus* Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. Development 127, 2227-2238.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W., Melton, D. (1990). Activins are expressed early in *Xenopus* embryogenesis and can induce axial mesoderm and anterior structures. Cell 63, 485-493.

Thorpe, C., Schlesinger, A., Carter, J. & Bowerman, B. (1997). Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. Cell 90, 695-705.

Tuazon, P. & Traugh, J. (1991). Casein kinase I and II - multipotential serine protein kinases: structure, function, and regulation. In Advances in Second Messenger and Phosphoprotein Research Vol 123: 123-164 (Raven Press, Ltd., New York).

Wallingford, J., Rowning, B., Vogeli, K., Rothbacher, U., Fraser, S., and Harland, R. (2000). Dishevelled controls cell polarity during *Xenopus* gastrulation. Nature 405, 81-85.

Willert, K., Brink, M., Wodarz, A., Varmus, H. & Nusse, R. (1997). Casein kinase 2 associates with and phosphorylates Dishevelled. EMBO Journal 16, 3089-3096.

Xu, Q., D'Amore, P., and Sokol, S. (1998). Functional and biochemical interactions of Wnts with FrzA, a secreted Wnt antagonist. Development 125, 4767-4776.

Yanagawa, S., van Leeuwen, F., Wodarz, A., Klingensmith, J., and Nusse, R. (1995). The dishevelled protein is modified by Wingless signaling in Drosophila. Genes & Development 9, 1087-1097.

Yanagawa, S., Matsuda, Y., Lee, J., Matsubayashi, H., Sese, S., Kadowaki, T., Ishimoto, A. (2002). Casein kinase I phosphorylates the Armadillo protein and induces its degradation in Drosophila. EMBO Journal 21, 1733-1742.

Yong, TJK., Gan, YY., Toh, BH., Sentry, JW. (2000). Human CKI alpha(L) and CKI alpha(S) are encoded by both 2.4-and 4.2-kb transcripts, the longer containing multiple RNA-destablising elements. Biochimica et Biophysica Acta - Gene Structure & Expression 1492, 425-433.

Yost, C., Torres, M., Miller, J., Huang, E., Kimelman, D., and Moon, R. (1996). The axis-inducing activity, stability, and subcellular distribution of □-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. Genes & Development 10, 1443-1454.

Yost, C., Farr, G., Pierce, S., Ferkey, D., Chen, M., and Kimelman, D. (1998). GBP, an inhibitor of GSK-3, is implicated in *Xenopus* development and oncogenesis. Cell 93, 1031-1041.

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T., Perry, W., Lee, J., Tilghman, S., Gumbiner, B., and Costantini, F. (1997). The mouse fused locus encodes axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell 90, 181-192.

Zhang, J., Gross, S., Schroeder, M., Andersons, R. (1996). Casein kinase I alpha and alpha L: alternative splicing-generated kinases exhibit different catalytic properties. Biochemistry 35, 16319-16327.

Zhang, Y., Neo, S., Wang, X., Han, J., and Li, S. (1999). Axin forms a complex with MEKK1 and activates c-Jun NH2-terminal kinase/stress-activated

protein kinase through domains distinct from Wnt signaling. The Journal of Biological Chemistry 274, 35247-35254.

Zhang, Y., Qiu, W.J., Chan, S.C., Han, J., He, X. and Lin, S.C. (2002). Casein kinase I and casein kinase II differentially regulate Axin function in Wnt and JNK pathways. Journal of Biological Chemistry 277, 17706-17712.

Zhu, J., Shibasaki, F., Price, R., Guillemot, J., Yano, T., Dotsch, V., Wagner, G., Ferrara, P., and McKeon, F. (1998). Intramolecular masking of nuclear import signal on NF-AT4 by casein kinase I and MEKK1. Cell 93, 851-861.

CURRICULUM VITAE

John Michael Peters was born February 11, 1972 in Fort Worth, Texas to John and Frances Peters. He grew up in Fort Worth attending J.T. Stevens elementary school, McMaster middle school, and Trinity Valley for high school. In 1990 he enrolled at Rice University in Houston, Texas. There he majored in Biology, graduating with a B.A. in 1994. For the next three years he continued his studies at Cornell University in Ithaca, NY where he studied social behavior in insects with Dr. H. Kern Reeve. In 1997 he returned to Dallas, Texas to pursue and M.D./Ph.D. at Southwestern Medical School. There he performed his thesis research in the laboratory or Dr. Jonathan M. Graff before returning to medical school to complete the M.D. portion of his degree.

PREVIOUS PUBLICATIONS UNRELATED TO CKI:

1. **Peters JM**, Queller DC, Strassmann JE, Solís CR (1995) Maternity assignment and queen replacement in a social wasp. *Proceedings of the Royal Society of London Series B* 260:7-12.

2. Strassmann JE, Solís CR, **Peters JM**, Queller DC (1996) Strategies for finding and using highly polymorphic DNA microsatellite loci for studies of genetic relatedness and pedigrees. In: *Molecular methods in zoology and evolution* (eds Ferraris J, Palumbi S), Wiley, New York, NY pp. 163-178, 528-549.

3. Queller DC, **Peters JM**, Solís CR, Strassmann JE (1997) Control of reproduction in social insect colonies: individual and collective relatedness preferences in the paper wasp, *Polistes annularis*. *Behavioral Ecology and Sociobiology* 40:3-16.

4. **Peters JM** (1997) Microsatellite loci for *Pseudomyrmex pallidus* (Hymenoptera: Formicidae). *Molecular Ecology* 6:887-888.

5. Strassmann JE, **Peters JM**, Barefield K, Solís CR, Hughes CR, Queller DC (1997) Trinucleotide microsatellite loci and increased heterozygosity in cross-species applications in the social wasp, *Polistes*. *Biochemical Genetics* 35:273-279.

6. **Peters JM**, Queller DC, Imperatriz Fonseca VL, Strassmann JE (1998) Microsatellite loci for stingless bees. *Molecular Ecology* 7:784-787.

7. **Peters JM**, Barnes B, Bennett B, Gitomer WM, Bowcock AM, Garg A (1998) Localization of the gene for familial partial lipodystrophy (Dunnigan Variety) to

chromosome 1q21-22. *Nature Genetics* 18:292-295.

8. Ezenwa VO, **Peters JM**, Zhu Y, Arévalo E, Hastings MD, Seppa P, Pederson JS, Zacchi F, Queller DC, Strassmann JE (1998) Ancient conservation of trinucleotide microsatellite loci in polistine wasps. *Molecular Phylogenetics and Evolution* 10:168-177.

9. Reeve HK, **Peters JM**, Nonacs P, Starks PT (1998) Dispersal of first workers in social wasps-causes and implications of an alternative reproductive strategy. *Proc. Natl. Acad. Sci. USA* 95:13737-13742.

10. **Peters JM**, Queller DC, Imperatriz-Fonseca VL, Roubik DW and Strassmann JE (1999) Mate number, kin selection, and social conflicts in stingless bees and honey bees. *Proceedings of the Royal Society of London Series B* 266: 379-384.

11. Reeve HK, Starks PT, **Peters JM**, Nonacs P (2000) Genetic support for the evolutionary theory of reproductive transactions in social wasps. *Proceedings of the Royal Society of London Series B* 267: 75-79.