SGK196 CONTROLS STEM CELL FATES BY PROMOTING THE DEGRADATION OF TGFβ FAMILY RECEPTORS

BY

STEPHANIE TSANG MUI CHUNG

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Cell and Developmental Biology in the Graduate College of the University of Illinois at Urbana-Champaign, 2014

Urbana, Illinois

Doctoral Committee:

Professor Andrew Belmont, Chair
Professor Jie Chen, Director of Research
Assistant Professor Eric Bolton
Professor Jonathan Henry
ABSTRACT

Much remains to be understood regarding the molecular mechanisms controlling stem cell fate. Through studies in human pluripotent stem cells (hPSCs), I have identified SGK196, an atypical kinase of relatively unknown function, as a novel regulator of cell fate and the TGFβ family signaling pathway. SGK196 depletion in human embryonic stem cells (hESCs) inhibits neural induction and enhances mesoderm induction, while SGK196 overexpression represses mesoderm and endoderm induction. SGK196 negatively regulates TGFβ family signaling by interacting with and promoting the degradation of TGFβ family receptors. Interactions were observed to occur within the SGK196 transmembrane (TM) domain. Mutation of specific Asn, Asp, and His residues in the SGK196 TM domain resulted in the diminished degradation of TGFβ family receptors and reduced interactions with the type I BMP receptor (BMPR1B), suggesting that these polar residues may play a key role in arbitrating SGK196 activity. The functional effects of Sgk196 in *Xenopus laevis* animal caps and embryos are in agreement with the results in hESCs. Defects in Sgk196 depleted *Xenopus* embryos include impaired development of neural tissues and increased development of mesodermal tissues. Overall, I demonstrate that SGK196 is an essential regulator of stem cell fate determination and early development. Through uncovering the regulatory mechanism of SGK196 I unveil valuable insight into receptor-level regulation of the TGFβ family pathway.
To my Father, Mother, and Ryan
I would not have endured this experience without the support of key individuals throughout these years. First, thank you to the amazing committee members that truly believed in me. To Dr. Andrew Belmont: Words cannot express my gratitude for all the guidance and help you have given me. I would not be where I am without your support. Thank you for believing in my scientific potential. Most of all, thank you for supporting me from the beginning to the end. I am blessed to have such an amazing mentor. To Dr. Jie Chen: Thank you for taking on the role as my advisor, for the great support you have given me, and for your immense efforts to help make my graduation a reality. I especially want to also express my deepest appreciation to both Dr. Andrew Belmont and Dr. Jie Chen for their unwavering encouragement during the completion of my manuscript. To Dr. Philip Newmark: Thank you for your scientific guidance and support. Seeing the way you carry and think about science has always inspired me. To Dr. Eric Bolton: Thank you for kindly stepping in to be on my committee, for your inspirational advice, and continued support during these past years.

I am grateful to Dr. Jonathan Henry and Ms. Kimberly Perry for their many years of collaboration with me on all the *Xenopus* work. I want to acknowledge Kim for her enormous efforts in performing numerous morpholino injections, in situ hybridizations, and animal caps experiments. And to both Jon and Kim for their significant involvement and invaluable contributions in preparing and editing the early drafts of my manuscript.
I thank Dr. Fei Wang for providing funding to support this work, and for the suggestion of performing a screen of human kinases.

I would like to express my deepest gratitude to my parents and my best friend Ryan for their unwavering love and incessant support. Thank you for always listening to me, constantly pointing me towards the light during my difficult times, and for reminding me that you were my biggest fans. And, thank you to all my friends who have believed in me with compassion and support.

Last but not least, thank you to the University of Illinois at Urbana-Champaign for the generous financial support through the Illinois Distinguished Fellowship and to the Department of Cell and Developmental Biology for partial support of my work.
CHAPTER 1

INTRODUCTION

Human embryonic stem cells (hESCs) are pluripotent stem cells (PSCs) derived from the inner cell mass of blastocyst-stage human embryos, possessing the remarkable ability to self-renew almost indefinitely in culture and give rise to derivatives of all three embryonic germ layers. In the interest of advancing methods to generate homogenous cell types from human PSCs (hPSCs) for therapeutic applications and the study of normal and abnormal human developmental processes, the mechanisms that control pluripotency, direct differentiation and early embryogenesis are areas of active investigation (Rajamohan et al., 2013). Mouse ESCs (mESCs) also serve as an in vitro model for mammalian embryos and share some conserved properties with hESCs, including expression of pluripotency transcription factors SOX2, OCT4, and NANOG. However mouse and human ESCs exhibit differences in morphology and cell cycle, and utilize distinct signaling networks involved in maintaining pluripotency (Ginis et al., 2004, Yeo and Ng, 2013). mESCs require leukemia inhibitory factor acting through the Janus Kinase-signal transducer and activator (JAK/STAT) pathway and Bone morphogenic protein 4 (BMP4) to maintain pluripotency. Activation of the JAK/STAT pathway was shown to be insufficient to prevent differentiation in hESCs (Humphrey et al., 2004, Daheron et al., 2004). BMP4 is an inducer of mesoderm and trophoblast lineages in hESCs (Zhang et al., 2008, Xu et al., 2002). In hESCs, signaling is induced by the basic fibroblast growth factor (FGF) and Activin/Nodal ligands to support pluripotency (Yeo and Ng, 2013). Considering these observed species specific differences, hESCs remain the ideal in vitro tool to investigate the molecular and cellular processes of human development.
Thus far, studies in hPSCs have indicated the signaling intensity and crosstalk of the fibroblast growth factor, insulin growth factor (IGF), Wnt, and Transforming Growth Factor (TGF) β family pathways play important roles in the cellular decisions between self-renewal and distinct differentiation states (Dalton, 2013). The intricacy of the signals that mediate fate decisions of hPSCs are reflected by the concerted actions of a large number of extracellular factors and complex cytoplasmic and nuclear networks from multiple pathways (Vazin and Freed, 2010, Hodonsky et al., 2013, Boyer et al., 2005). Many questions remain on the mechanistic details surrounding these signaling events, as well as their effects in vivo.

Of the pathways investigated in hESCs, the role of the TGFβ family signaling pathway in hESC pluripotency and lineage specification has been extensively documented. Present throughout all metazoans, the TGFβ family pathway controls diverse biological processes including differentiation, apoptosis, migration, and proliferation at both the cellular and developmental levels (Moustakas and Heldin, 2009). Unsurprisingly, the aberrant signaling of the TGFβ family pathway is linked to numerous disease states (Gordon and Blobe, 2008, Waite and Eng, 2003). The TGFβ family pathway is comprised of two branches: TGFβ/Activin/Nodal and the bone morphogenetic protein (BMP) signaling. Signaling of the TGFβ family pathway is mediated through more than 30 ligands, including TGF-βs, BMPs, activins, nodal, and seven type I and five type II serine/threonine kinase receptors (Moustakas and Heldin, 2009). The binding of ligands to a specific type II receptor dimer leads to the recruitment of a type I receptor dimer and subsequent formation of a hetero-tetrameric complex. Within this complex, the constitutively active type II receptor phosphorylates the type I receptor, leading to the activation of its kinase
activity and the subsequent phosphorylation of receptor-regulated (R) Smads (R-Smads; 1,5,8 for BMP and 2,3 for TGFβ/Activin/Nodal signaling). Phosphorylated R-Smads then complex with a common-mediator (Co) Smad, Smad4, allowing for nuclear translocation and interaction with other co-factors to mediate transcriptional regulation of target genes (Shi and Massagué, 2003, Moustakas and Heldin, 2009). Signaling may be further attenuated by inhibitory (I) Smads 6 and 7 (Moustakas and Heldin, 2009).

The frog *Xenopus* has contributed greatly to our understanding of the role of various signaling pathways in controlling embryonic development. For instance, it was work done in the *Xenopus* system that first demonstrated an instrumental role for TGF-β signaling in the formation of the mesoderm (Smith, 1987, Slack et al., 1987, Kimelman and C. Bjornson, 2004, Harland and Grainger, 2011). This early work was soon followed by the discovery of key antagonistic ligands: noggin, chordin and cerberus (Bouwmeester et al., 1996, Zimmerman et al., 1996, Piccolo et al., 1996, Piccolo et al., 1999), which led to our fundamental understanding that the BMP pathway inhibition is required for induction of the nervous system.

Both the TGFβ/Activin/Nodal and BMP signaling branches have also been shown to profoundly impact the fate decisions of hESCs. The TGFβ/Activin/Nodal pathway supports pluripotency, as demonstrated by the positive regulation of the pluripotency-maintenance transcription factor NANOG, exhibited by the inhibitory effects of NANOG on BMP mediated differentiation (Suzuki et al., 2006). In contrast, the BMP pathway alters the regulatory activities of OCT4 and thus suppresses pluripotency (Wang et al., 2012). In addition to regulating pluripotency, TGFβ/Activin/Nodal signaling can mediate endoderm differentiation (D'Amour et al., 2005, Teo
et al., 2012), while BMP signaling has been shown to induce mesoderm, trophoblast, and extraembryonic lineages (Xu et al., 2002, Zhang et al., 2008). Conversely, inhibition of the BMP pathway alone or in combination with the TGFβ/Activin/Nodal pathway, leads to the induction of neural differentiation of hPSCs (Zhou et al., 2010, Chambers et al., 2009, Kim et al., 2010).

The capacity of the TGFβ family pathway to elicit a plethora of biological outcomes requires tightly controlled spatial and temporal regulation of the signaling components at multiple levels. Extracellular regulation by ligand antagonists, cytosolic regulation by inhibitory Smads (I-Smads) and modulation of R-Smad activity, and nuclear transcriptional regulation by Smad interaction with co-activators/co-repressors have been reported (Miyazono, 2000, Massagué et al., 2005, Bruce and Sapkota, 2012, Miyazono et al., 2006). Regulation at the receptor level is particularly important due to the impact changes in receptor levels and activities have on downstream signaling. Such regulation has been demonstrated by the functional interactions of the receptors with other regulators, including co-receptor betaglycan (Bilandzic and Stenvers, 2011, Blobe et al., 2001, Lewis et al., 2000), pseudoreceptor BAMBI (Onichtchouk et al., 1999), FKBP12 (Chen et al., 1997, Okadome et al., 1996, Huse et al., 1999), DRAGON (Samad et al., 2005), RGMa (Babitt et al., 2005), and more recently Jiriaya (Aramaki et al., 2010). Given the broad implications of the TGFβ family pathway in the regulation of pluripotency and early lineage specification of hPSCs, a better delineation of the repertoire of TGFβ family regulators and associated mechanisms, especially at the receptor level, is essential for a deeper understanding of the molecular programs that govern fate determination of hPSCs.

To identify novel signaling regulators of cell fate specification, I performed a shRNA screen directed against human kinases in NTERA-2 cells, a human embryonal carcinoma cell line
derived from the stem cells of a teratocarcinoma risen from transformed germ cells. These cells are often referred to as the malignant counterpart of hESCs, as they possess limited capacity to differentiate into other cell types and share similarities with hESCs, including morphology of which growth as tight compact colonies with defined borders and the expression of pluripotency transcription factors OCT4, SOX2, and NANOG, are associated with the state of pluripotency (Pal and Ravindran, 2006, Przyborski et al., 2004). Upon loss of pluripotency and differentiation, colonies flatten and spread out and there is a reduction in pluripotency transcription factors. Relative to hESCs, NTERA-2 cell culture requires less time and resources, and thus serves as an ideal platform for initial high volume screening. Primarily based upon my observations on morphological changes, in my screen I identified SGK196, and went on to discover this molecule to be a key regulator of hESC multi-lineage specification.

SGK196 is single-pass transmembrane receptor containing a serine/threonine kinase domain. Suggestive of its functional significance, SGK196 has homologs present throughout the genomes of a variety of species, including mouse, zebrafish, *Xenopus*, and green anole (NP_083313.1, NP_001007415.1, NM_001095489.1, XP_003222408.1). SGK196 belongs to an interesting class of kinases, termed pseudokinases, predicted to be inactive based on the lack of one or more of the three key residues each within conserved motifs known to be essential for kinase activity (Boudeau et al., 2006). The kinase domain of SGK196 deviates from all three conserved residues (Boudeau et al., 2006). Pseudokinases are distributed throughout the different kinase subfamilies, representing roughly 10% of the kinome. Despite being predicted to be inactive, some pseudokinases have been found to possess kinase activity, while others have been shown to regulate active kinases, or act as signaling scaffolds (Zeqiraj and van Aalten, 2010). Much
remains to be characterized of this understudied class of proteins. Interestingly, it has been reported SGK196 exhibited substrate specific kinase activity on O-mannose but the mechanism by which this occurs is not clear (Boudeau et al., 2006, Yoshida-Moriguchi et al., 2013). SGK196 knockout mice were reported to develop hydrocephalus (Vogel et al., 2012), however mechanistic studies clearly linked to the biological role of SGK196 remains to be addressed.

SGK196 depletion was found to enhance mesoderm differentiation but inhibit neural differentiation of hESCs. Conversely, overexpression of SGK196 resulted in the inhibition of both mesoderm and endoderm differentiation. I demonstrated that SGK196 is a negative regulator of TGFβ family signaling and showed that the degradation of, the TGFβ family receptors and interaction with the type I BMP receptor (BMPR1B), was reduced upon the mutation of the Asn, Asp, and His amino acid residues within the SGK196 transmembrane domain. Thereby, these results suggest interactions occurring within the SGK196 transmembrane domain are linked to the ability of SGK196 to promote TGFβ family receptor degradation. I extended the functional study of SGK196 to the in vivo Xenopus model and showed that SGK196 depletion resulted in developmental abnormalities in neural and mesoderm tissues and axis formation. Together, I reveal SGK196 as a key regulator of stem cell fate and embryonic development, uncover a mechanism underlying SGK196 functional activity, and present novel insights into the receptor-level regulation of the TGFβ family signaling pathway.

In this work, I present the first clear demonstration of the functional significance of SGK196 in hESC multi-lineage specification and present the first mechanistic characterization accounting
for its biological role by revealing SGK196 to be a negative regulator of the TGFβ family signaling pathway.

My dissertation research has focused on the characterization of the role of SGK196 in the specification of mesoderm, endoderm, and neural lineages through knockdown and overexpression studies in hESCs and the effects of Sgk196 depletion on early development in the *Xenopus* model. The other focus of my work was on the signaling aspect of SGK196 attributed to its functional regulation, by dissection of the regulatory mechanism by which SGK196 negatively regulates the TGFβ family signaling pathway.
CHAPTER 2

MATERIALS AND METHODS

Antibodies, Growth Factors, and Inhibitors

Primary Antibodies: SGK196 (ab57908) and α-tubulin (AB11304) were purchased from Abcam (Cambridge, MA), P-Smad1/5/8 (9511), P-Smad2 (3108), and Smad2/3 (3102) were purchased from Cell Signaling (Beverly, MA), Smad 1/5/8 (SC-6031-R) was purchased from Santa Cruz Biotechnology (Dallas, Texas) M2 (anti-FLAG, F1804) was purchased from Sigma-Aldrich, anti-HA (MMS-101R-500) was purchased from Covance (Princeton, NJ) BMPR1B (AP2005b) was purchased from Abgent (San Diego, CA). Activin A was purchased from R&D Systems (Minneapolis, MN), BMP4 and TGF-β1 was purchased from Invitrogen (Grand Island, NY). Cycloheximide was purchased from Fisher Scientific (Pittsburg, PA).

Cell Culture

hESCs H1 and H9 (WiCell, Madison, WI) were maintained in mTeSR medium (STEMCELL Technologies, Vancouver, BC, Canada) with daily medium changes, on 6-well plates coated with Matrigel (BD Biosciences, Franklin Lakes, NJ). hESCs were passaged at ratios of 1:4 to 1:6 every 4-6 days using 1mg/mL dispase (Invitrogen, Grand Island, NY). Endoderm (D'Amour et al., 2005) and mesoderm (Zhang et al., 2008) differentiation were conducted as previously described. Retinoic acid mediated neural differentiation was carried out in DMEM/F12 supplemented with 1x N2 supplement (Invitrogen) and 10uM retinoic acid (Sigma-Aldrich, St Louis, MO). Compound C (EMD Millipore, Billerica, MA) mediated neural differentiation was conducted as previously described (Zhou et al., 2010). HEK293T and NTERA2 cells were
maintained in DMEM supplemented with 10% FBS, 2mM L-glutamine, 0.1mM non-essential amino acids and 1% penicillin/streptomycin.

**Plasmid Construction for Overexpression, Knockdown, and Mutant Constructs**

The pSin vector backbone containing the EF1α promoter and IRES-puromycin selection marker was used to generate all overexpression constructs, including mKate and mCherry control. Wild-type and mutant forms of SGK196 and TGFβ family receptor genes were tagged with HA or FLAG epitopes and subcloned into the pSin vectors by replacing SOX2 or OCT4 (Addgene, 16577, 16579, Cambridge, MA). SGK196 deletion mutants were generated by PCR-directed mutagenesis. Generation of the SGK196 44-81AA deletion mutant was generated as previously described (Science Gateway, 2013a). cDNAs were obtained from Open Biosystems (SGK196, 8069054; BMPR1B, 6202951; TGFBR1, 30344890; ACVR2B, 40005760, Thermo Scientific, Waltham, MA) and Addgene (BMPR2, 23669; UBQLN1, 8663; TGFBR2, 19147; FGFRL1, 23600). The pLKO.1-TRC cloning vector (Addgene, 10878) was used in the generation of shRNA constructs

shSGK196-1 (5'GTCTTTGGGATACACTTAGAttctcgagaaTCTAAGTGTATCCAAGACC 3’)

and shSGK196-2 (5'AGTTACAGCATTCTACTCTtctcgagaaAGAGTAGAATGCTGTAACT 3’). Hairpin sequences were designed using [http://sirna.wi.mit.edu/](http://sirna.wi.mit.edu/). pLKO.1 scramble and SGK196-A1 shRNA was purchased from Sigma-Aldrich (NM_032237.x-1338s1c1).

Overexpression in HEK293T cells was conducted by transient calcium phosphate transfection as previously described (Kingston et al., 2001) or stable lentivirus infection (Cohen, 2008).

Overexpression and knockdown in hESCs were conducted by stable lentivirus infection.

Lentivirus was generated by co-transfection of pLKO.1 or pSin lentiviral vector of interest with
pCMV-dR8.91 and pCMV-VSV-G in HEK293T cells, as described previously (Science Gateway, 2013b).

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was extracted with TRIzol reagent (Invitrogen) according to manufacturer’s instructions. cDNA was synthesized using the Improm II Reverse Transcription System (Promega, Madison, WI) per manufacturer’s instructions. Quantitative real-time PCR (QPCR) was conducted using the SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) on a StepOne Plus Real Time PCR machine (ABI). Primer sequences are provided in Table 1. QPCR values represent the average of at least three independent trials and error bars represent the standard error of the mean. Asterisks indicate a statistical significance of \( p \leq 0.05 \) compared to the control within each experimental group, as determined by the Student’s t-test.

**Immunoprecipitation and Western Blot Analysis**

Immunoprecipitation of FLAG and HA tagged proteins was carried out using M2 and EZview Red Anti-HA Affinity Gel antibody-agarose conjugates (Sigma-Aldrich), respectively, in lysis buffer containing 20mM Tris HCl pH 8, 0.1-1% NP-40, 150mM NaCl, 2mM EDTA, supplemented with protease inhibitor cocktail (Sigma-Aldrich). After binding and extensive washing, bound proteins were eluted by boiling in 2X SDS sample buffer (Biorad, Hercules, CA), separated by SDS-PAGE and transferred onto nitrocellulose membranes. Signals were detected with ECL reagents (Pierce Thermo Scientific, Rockford, IL). HEK293T cells subjected to Western blot analysis were directly lysed in 2X SDS sample buffer. Western blot quantifications were done using Image J (Schneider et al., 2012).
Immunostaining

Cells on coverslips were washed in cold PBS, fixed in 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and blocked in 5% bovine serum albumin in PBS. Primary antibodies were incubated with samples overnight at 4°C, and detection was carried out using Alexa-conjugated secondary antibodies (Invitrogen). For live staining, cells on coverslips were cooled to 4°C, rinsed in cold PBS, and incubated with primary antibody for 30 minutes at 4°C. After PBS washes, cells were incubated with Alexa-conjugated secondary antibodies (Invitrogen) for one hour at 4°C. Following PBS washes, cells were fixed in 3.7% paraformaldehyde for 10 minutes at room temperature. All coverslip samples were mounted with Vectashield Mounting Medium with DAPI (Invitrogen).

Plasma Membrane Staining

Cells grown on coverslips were incubated with 0.5X concentration of CellMask green plasma membrane stain (Invitrogen) in PBS at 37°C for 10-15 minutes. Cells were then fixed with prewarmed 3.7% paraformaldehyde at 37°C for 10 minutes then mounted with Vectashield Mounting Medium with DAPI (Invitrogen).

Determination of Transmembrane Protein Orientation

HEK293T cells transfected with N or C terminal HA tagged SGK196 were singularized with 0.05% Trypsin-EDTA (Mediatech, Manassas, VA) for 40 minutes at 37°C. After trypsin treatment, cells were neutralized in 10% FBS containing media and pelleted by centrifugation. Cells were lysed in 2X SDS sample buffer and subject to Western blot analysis.
**Luciferase Reporter Assay**

The pID-120-luc reporter construct (kindly provided by Dr. Renhe Xu, University of Connecticut, (Lopez-Rovira et al., 2002)) was used to measure BMP signaling and the pARE-GL3 luciferase reporter (kindly provided by Dr. Yisrael Sidis, Partners Healthcare, (Sidis et al., 2002)) and FAST1 constructs were used to measure Activin signaling. Renilla-luciferase reporter (Promega, Madison, WI) was used for the internal control. Reporters were cotransfected with different quantities of HA-SGK196 in HEK293T cells using Lipofectamine 2000 (Invitrogen, Grand Island, NY). 24 hours later cells were serum starved overnight then treated with ligand for 12 hours. Samples were prepared according to the manufacturer's protocol using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured with the LUMIstar plate reader (BMG Labtech, Cary, NC).

**Animals**

Adult *Xenopus laevis* were acquired from Nasco (Fort Atkinson, WI). Fertilized eggs were obtained according to methods previously published in (Henry and Grainger, 1987), and subsequent embryos and larvae were reared according to methods described in (Henry and Mittleman, 1995). Developmental staging was based on the descriptions of (Faber and Nieuwkoop, 1956).

**Sgk196 and In situ Hybridization**

The *Xenopus Sgk196* full-length clone was obtained from Open Biosystems (Clone ID: 6868253, Thermo Scientific, Waltham, MA) and used to generate digoxygenin (DIG)-labeled RNA sense (T3) and antisense (T7) probes. *In situ* hybridization reactions contained of a range of embryonic
stages (st8-41) and were conducted according to the published protocol (Harland, 1991) with a few modifications (described in (Malloch et al., 2009).

**Morpholino Design and Rescue RNA**

Lissamine-tagged MOs were designed and obtained from Gene Tools (Philmath, OR). The SGK196MO targets the translational start site of SGK196, including the downstream region adjacent to the start site (5’–TGCAGACACACTAGGTTTTCTCTCCAT; translational start site is underlined). A commercially available CONMO was used as a standard control and has been used in previous studies (Perry et al., 2010, Wolfe and Henry, 2006, Elkins and Henry, 2006). To demonstrate the specificity of SGK196MO, an altered SGK196 transcript was generated. Primers for the altered SGK196 transcript were designed to include third-base substitutions to target the nucleotide binding sequence of SGK196MO in a manner to preserve the original protein coding sequence (Forward 5’-BamHI:ATGGAAAGgAAgCCcAGcGTtTGtA and Reverse 5’-EcoRI: TTACAACATCTCTCGAGTTTCTGGCC). Underlined bases refer to the translational start site and lowercase bases represent those altered from the original nucleotide sequence. The altered Sgk196 cDNA was digested with BamHI and EcoRI, directionally cloned into pCS2+ (Clontech, Mountain View, CA) and sequence verified (Carver Biotechnology Center, Urbana, IL). Capped altered sgk196 mRNA noggin mRNA (plasmid provided by Dr. Richard Harland) were synthesized from PCR template using the SP6 mMessage mMACHINE kit (Life Technologies).
Microinjection Techniques

Dejellied two-cell *Xenopus* embryos were placed in a solution of 5% ficoll in clay dishes containing small recesses to hold each individual embryo (Elkins and Henry, 2006, Wolfe and Henry, 2006) and injected with MOs, capped mRNAs or combinations of both. Morpholinos were diluted to a concentration of 2 mM in sterile water and each injection was performed either one-cell stage embryos or two-cell stage embryos using glass microinjection needles and a Narishige micromanipulator (Narishige USA, East Meadow, NY). A pressure injection apparatus (Harvard Apparatus, Holliston, MA) was used to deliver specific quantities of morpholino, mRNA only and morpholino combined with capped mRNA. Capped mRNA only injections required combining the non-toxic dye Fast Green or fluorescent dextran (green) with the mRNA sample for visualization. Following injection, embryos were allowed to recover in 1/20X normal amphibian media (NAM) through st41 with daily solution changes.

Phenotypes were characterized by changes in morphology. Mild phenotypes were close in size to wild-type specimens, but had minor disruptions in axis formation with a clearly developed tail. Mild cases also had slightly reduced eye sizes in comparison to wild-type control eyes. Severe phenotypes displayed at least two of the following defects. Changes included a dramatically reduced body size in comparison with wild-type specimens. There was a significant disruption in axis formation that included tails that were greatly reduced or even absent and ectopic tissue growth was common. Many of these animals displayed grossly reduced eye sizes (at least 50% smaller than the contralateral control eye) or in the most severe cases some animals displayed cyclopic phenotypes (statistical analysis described in Elkins and Henry, 2006)
Histology
Specimens from a range of stages (8-41) were fixed in MEMFA (3.7% formaldehyde, 100mM MOPS, 2mM EGTA, 1 mM MgSO₄), and stored in methanol at –20°C. For histological preparations, specimens were washed in graded ethanol up to 100%, xylene, and embedded in Paraplast Plus (Fisher Scientific, Pittsburg, PA). Specimens were sectioned to a thickness of 8µm, dewaxed in xylene and stained in Harris hematoxylin/Eosin (Fisher Scientific) according to published protocols (Humason, 1972).

Animal Cap Assay
_Xenopus_ embryos at st8 were collected and animal caps were excised according to (Ariizumi et al., 2009). Embryos at st8 were placed in 3/4X NAM solution in clay dishes containing depressions to hold each individual embryo. The vitelline membrane was removed from each st8 specimen and animal caps were removed using a finely pulled glass needle, making sure to excise only the ectodermal cells of the animal cap and not the adjacent marginal zone which might include mesodermal cells. All caps were placed in a clean petri containing 3/4X NAM solution overnight in an incubator at 16°C. The next morning, caps were washed in 1/20X NAM and monitored for staging in comparison with sibling control embryos until st26-28. Animal caps were pooled in groups of 5 caps per tube and processed with sibling control embryos for total RNA extraction using Trizol (Invitrogen, Grand Island, NY).

Total RNA from pooled caps (5 caps/tube) was used for RT-PCR and the presence of the following transcripts were examined by PCR: positive control _elongation factor 1-alpha (eef1a1;_
(Krieg et al., 1989)), epidermal marker *epidermal cytokeratin (sxk81a1;* (Jonas et al., 1989)) and pan-neural marker *ncam1* (Kintner and Melton, 1987). For primer sequences see Table 2.
CHAPTER 3

RESULTS

SGK196 is a key regulator of hESC lineage specification

In search of novel regulators of fate determination of hPSCs, we examined the involvement of a subset of human kinases by RNAi. Kinases were depleted by the use of shRNAs in NTERA2 cells and the disruption of pluripotency was identified based on cell morphology. NTERA2 cells are a human pluripotent embryonal carcinoma line that exhibits high-level expression of pluripotency markers and possesses the capacity for limited multilineage differentiation in vitro and in vivo (Pal and Ravindran, 2006, Przyborski et al., 2004). SGK196 emerged as a potential regulator based on the loss of distinct colony borders and cell spreading upon SGK196 depletion (Figure 1A). These screening experiments led to the identification of SGK196 (Figure 1B), an atypical transmembrane kinase, as a potential regulator of hPSC fate determination.

I next investigated the role of SGK196 in hESCs. In agreement with the results in NTERA2 cells, shRNA mediated knockdown of SGK196 in hESCs cultured under feeder-free conditions led to noticeable morphological changes including the flattening and spreading of cells (Figure 1C). These changes are in contrast to control cells, which grew as tight compact cell colonies (Figure 1C). Interestingly, SGK196 overexpression did not appear to affect hESC morphology (Figure 1D). In my preliminary studies using hESCs cultured under mouse embryonic fibroblast feeder conditions, I found that SGK196 depletion resulted in the spontaneous up-regulation of endoderm (SOX17, GATA4, GATA6) and mesoderm markers (T, MIXL1) without significantly altering the level of neural markers (PAX6, SOX1) or pluripotency markers (OCT4, NANOG, 17
SOX2; data not shown). SGK196 knockdown and overexpression under feeder-free conditions also had minimal effects on the expression of pluripotency markers (Figure 2A and B), suggesting that SGK196 may not directly regulate pluripotency.

The observed selective up-regulation of endoderm and mesoderm markers upon SGK196 depletion indicated an involvement in lineage specification. I therefore induced hESCs to differentiate into neural, mesoderm, and endoderm lineages and assessed whether SGK196 depletion and overexpression affected these differentiation activities. Following treatment with Compound C (also known as Dorsomorphin), which has previously been used for neural induction of hPSCs (Zhou et al., 2010, Yu et al., 2008), the neural progenitor markers PAX6, SOX1, and OTX2 were up-regulated, but were markedly attenuated upon SGK196 depletion (Figure 3A). Compound C mediated up-regulation of PAX6, SOX1, and OTX2 was also attenuated upon SGK196 overexpression (Figure 3B). I also treated hESCs with retinoic acid (RA), a known inducer of neural differentiation in pluripotent stem cells (Tonge and Andrews, 2010), and found that PAX6 up-regulation was dramatically impaired upon SGK196 depletion (Figure 3C). SGK196 overexpression exerted minimal effects on RA-induced PAX6 up-regulation (Figure 3D).

SGK196 depletion and overexpression in hESCs also influenced endoderm and mesoderm differentiation. Upon BMP4-mediated mesoderm differentiation, SGK196 depletion further elevated the level of mesoderm markers (T and MIXL1; Figure 4A). Induction of these mesoderm markers was inhibited in the presence of overexpressed SGK196 (Figure 4B). Upon Activin A-mediated endoderm induction, SGK196 depletion had minimal effects on the up-
regulation of endoderm markers (*SOX17, GSC, FOXA1*; Figure 4C). Induction of these markers was inhibited by SGK196 overexpression (Figure 4D). *SGK196* levels appeared largely unaffected during hESC differentiation into the three embryonic lineages. Thus, SGK196 is involved in lineage specification of hESCs during early differentiation.

**SGK196 inhibits BMP and TGFβ/Activin signaling**

I next sought to investigate the mechanism by which SGK196 regulates early differentiation in hESCs. Previously, Barrios-Rodiles *et al.* (2005) developed a high-throughput screen to map protein-protein interactions within signaling pathways. When applied to the TGFβ family signaling pathway, it was found that mouse Sgk196 can interact with type I receptors TGFBR1 and BMPR1B, but the function of this interaction was not elucidated. The observed differential regulation of SGK196 on mesoderm/endoderm and neural induction (Figures 3 and 4) is reminiscent of the positive and negative roles of TGFβ family signaling on mesoderm/endoderm (Zhang *et al.*, 2008, D'Amour *et al.*, 2005) and neural specification (Zhou *et al.*, 2010, Kim *et al.*, 2010, Chambers *et al.*, 2009), respectfully. Taken together, these observations led us to hypothesize that SGK196 may be involved in the regulation of TGFβ family signaling.

SGK196-mcherry fusion proteins expressed in HEK293T cells (Figure 5A), showed colocalization with the general plasma membrane stain CellMask (Figure 5B), indicating that SGK196 is localized to the plasma membrane. This pattern of membrane localization was previously observed for ectopically expressed mouse Sgk196 in COS-7 cells, a monkey kidney fibroblast-like cell line (Barrios-Rodiles *et al.*, 2005).
Given that SGK196 is localized to the plasma membrane, I assessed the effect of SGK196 depletion and overexpression on R-Smad phosphorylation. Treatment of hESCs with BMP4 and Activin A induced R-Smad1/5/8 and 2/3 phosphorylation, respectively, and upon SGK196 depletion both ligand-induced R-Smad1/5/8 and 2/3 phosphorylation were elevated (Figures 5C and 5D). In contrast, SGK196 overexpression repressed ligand-induced R-Smad1/5/8 and 2/3 phosphorylation (Figures 5E and 5F). These results suggest that SGK196 acts as a negative regulator of BMP and Activin signaling in hESCs.

To ask whether SGK196 played a similar regulatory role in other cell types, I tested the effect of SGK196 ectopic expression in HEK293T cells. Consistent with the findings in hESCs, SGK196 overexpression inhibited BMP4-induced Smad1/5/8 phosphorylation (Figure 6A) and Activin /TGFβ-1-induced Smad2/3 phosphorylation (Figure 6B). In experiments with BMP and Activin luciferase reporters carried out in HEK293T cells, ectopic expression of SGK196 reduced the reporter activity in a dose-dependent manner (Figures 6C and 6D). Thus, the SGK196 regulatory function within the TGFβ family pathway is consistent in HEK293T cells.

Together, I present the first functional characterization of SGK196 as a potential key regulator of the TGFβ family signaling pathway. The inhibitory role of SGK196 in the regulation of TGFβ family signaling provides a mechanistic explanation for the effects of SGK196 depletion and overexpression on endoderm, mesoderm and neuroectoderm differentiation of hESCs.
SGK196 promotes the degradation of TGFβ family receptors

The localization of SGK196 to the plasma membrane (Figure 5A and 5B) and effects of SGK196 depletion/overexpression on ligand-induced R-Smad phosphorylation (Figure 5C-F; Figure 6A and 6B), indicated that SGK196 regulation of TGFβ family signaling may occur at the receptor level. To study the potential SGK196 regulation on the receptor proteins, I ectopically co-expressed SGK196 with FLAG-tagged TGFBR1 (a type I TGFβ receptor, also referred to as ALK5), BMPR1B (a type I BMP receptor, also referred to as ALK6), and BMPR2 (a type II BMP receptor) in HEK293T cells. Upon SGK196 overexpression, the levels of TGFBR1, BMPR1B, and BMPR2 (Figures 7A and 3B) were markedly decreased. SGK196 overexpression did not affect the protein levels of UBQLN1, a non-TGFβ family pathway molecule known to interact with proteasome and ubiquitin ligases (Lee and Brown, 2012) (Figures 7A and 3B). SGK196 overexpression also promoted the degradation of the type II Activin receptor ACVR2B protein (Figure 7C).

I next examined the half-life of FLAG-tagged TGFBR1 and BMPR1B in HEK293T cells by administering cycloheximide (CHX), an inhibitor of protein synthesis. After a 5-h time course, CHX treatment reduced TGFBR1 and BMPR1B levels by 39% and 37%, respectively (Figures 7D and 7E, respectively). In the presence of HA-SGK196, enhanced degradation of TGFBR1 and BMPR1B was observed, with protein levels reduced by 92% and 68%, respectively (Figures 7D and 7E, respectively). I also assessed whether SGK196 may affect receptor expression by examining the mRNA levels of TGFBR1, BMPR1B, TGFBR2 and BMPR2 in hESCs depleted of or overexpressing SGK196 (Figure 8A and 8B). No distinct regulatory patterns were observed.
Thus, SGK196 inhibits BMP and TGFβ/Activin signaling by promoting the protein degradation of receptors.

**The SGK196 transmembrane domain is key for SGK196 function**

The negative regulatory role of SGK196 on the TGFβ/Activin and BMP signaling at the receptor level, along with the previously reported potential interaction between mouse Sgk196 with TGFβR1 and BMPR1B (Barrios-Rodiles et al., 2005), led us to test whether SGK196 could complex with the TGFβ family receptors and ask if such interactions are essential for the protein degradatory function of SGK196.

I performed co-immunoprecipitation (co-IP) experiments to look for possible interactions between SGK196 and TGFβR1, BMPR1B, BMPR2, and ACVR2B, all of which were shown to be degraded in the presence of ectopic SGK196 (Figure 7A-C). HEK293T cells were transfected with HA-SGK196 and FLAG-tagged TGFβ family receptors and IP was carried out against either the HA or FLAG epitope. Tagged proteins were used due to lack of commercially available antibodies able to readily detect specific endogenous receptors. To ensure sufficient expression of TGFβ family receptors, the TGFβ family receptors were expressed in excess relative to the lower levels of SGK196. Interestingly, the co-IP studies revealed that all the TGFβ family receptors examined could complex with SGK196 (Figure 9A-D).

**SGK196 is a type II transmembrane receptor**

To investigate the nature of the interaction between SGK196 and the receptors, I first assessed if SGK196 was a type I or type II transmembrane protein. Type I and II proteins are single pass
transmembrane proteins that are classified by the N-terminus occurring at either the extracellular or cytosolic side, respectfully. SGK196 tagged with the HA epitope at the N (HA-SGK196) or C (SGK196-HA) terminus were expressed in HEK293T cells (Figure 10A). Subsequent IF studies revealed clear detection of the HA epitope for SGK196-HA under both live as well as fixed permeabilized staining conditions (Figure 10C and 10D). IF detection of the HA epitope for HA-SGK196 occurred only under fixed permeabilized conditions (Figure 10C and 10D). In addition, western blot analysis of HEK293T cells expressing SGK196-HA showed trypsin treatment dramatically reduced the amount of detectable HA tag of full length SGK196-HA, in contrast to the effects observed for HA-SGK196 (Figure 11A and 11B). A commercial antibody raised against the C-terminal region of SGK196 (amino acids 251-350) also detected a reduction of HA-SGK196 and SGK196-HA protein levels following trypsin treatment (Figure 11A and 11B). These findings indicate SGK196 is a type II transmembrane protein. This is in agreement with a study reporting that SGK196 is a protein O-mannose kinase, as illustrated by experiments that showed SGK196 was required for the successive phosphorylation of the 6-position on specific O-mannosyl trisaccharide substrates (Yoshida-Moriguchi et al., 2013). While it was not confirmed, the nature of this reported kinase activity places the SGK196 kinase domain in the luminal side of the endoplasmic reticulum/Golgi.

The SGK196 transmembrane domain is important for SGK196-receptor interactions and SGK196 activity

To investigate whether the interaction between SGK196 and the receptors is linked to its protein-degradatory function, I examined the relationship between SGK196 and BMPR1B to identify the region within SGK196 that mediates the interaction. As a type II transmembrane protein,
SGK196 is comprised of a cytoplasmic domain (1-20 amino acid; AA), a transmembrane domain (TM) (21-43 AA) and an extracellular domain (ECD) (44-350 AA; Figure 1B). HA-tagged SGK196 mutants were generated with the following deletions: 1-81 AA mutant missing the majority of the ECD, Δ1-21 AA mutant missing the cytoplasmic region, Δ44-81 AA mutant missing a short region between the TM and kinase domain, and the Δ1-43 AA mutant missing both the cytoplasmic and TM domains (Figure 12A). All HA-SGK196 mutants containing the TM domain retained the ability to co-IP with BMPR1B, independent of the presence of the intact ECD or cytoplasmic domain (Δ1-21 AA), with the exception of the Δ1-43 AA mutant that contains only the ECD (Figure 12B). This suggests that the ECD, thereby SGK196 kinase activity, is dispensable for the SGK196-BMPR1B interaction. Taking into account the 1-81 AA SGK196 fragment, which lacks a majority of the ECD, was sufficient to co-IP with BMPR1B and with a BMPR1B mutant lacking the cytoplasmic domain (Figure 12B-D), I inferred the SGK196 TM domain (21-43 AA) is likely a major site that mediates the interaction with BMPR1B. While these findings clearly support the SGK196 TM domain to be a necessary region for receptor-interactions, it does not rule out the possibility for additional points of interactions to exist in the other regions of SGK196.

The HA-SGK196 mutants shown to interact with BMPR1B were all found to be capable of promoting BMPR1B and BMPR2 protein degradation (Figure 12E and 12F). IF against the N-terminal HA tag of the SGK196 1-81AA, 1-100AA, Δ1-20 AA and the Δ44-81 AA mutants in HEK293T cells showed localization patterns similar to the full length SGK196 (Figure 13).
To examine the specificity of the interaction between BMPR1B and the SGK196 TM domain, I created a chimeric protein by replacing the SGK196 TM domain with the TM domain of fibroblast growth factor receptor-like 1 (FGFRL1), a type I topology single-pass transmembrane receptor of the FGFR family that lacks an intracellular tyrosine kinase domain (Figure 14A). The SGK196 chimera exhibited a reduced capacity to promote the degradation of BMPR1B (Figure 14B). BMPR2 and TGFBR1 was also observed to not be degraded by the SGK196 chimera (Figure 14C and 14D). The affinity between the SGK196 chimera and BMPR1B was reduced, as shown by the co-IP studies (Figure 14E and 14F). IF against the N-terminal HA tag of the SGK196 chimera displayed distribution patterns similar to wild type SGK196 (Figure 14G). Although the FGFRL1 TM domain is of a type I orientation in contrast to the type II orientation of the SGK196 TM domain, these results indicate that the interactions occurring in the SGK196 TM domain and the effects on receptor protein degradation are not nonspecific.

**Asn31, Asp39 and His40 in the SGK196 transmembrane domain are critical for mediating SGK196 activity and receptor interactions**

Polar residues in the TM domain have been shown to serve as signals to influence endocytic trafficking (Reggiori et al., 2000, Popa et al., 2012, Sato et al., 2003). It has been demonstrated that the transplantation of three polar residues into the transferrin receptor TM domain can cause the down-regulation of recycling transferrin receptors (Zaliauskiene et al., 2000). The SGK196 TM domain contains Asn, Asp, and His, all of which are polar residues rarely observed in the TM domains of single pass proteins (Landolt-Marticorena et al., 1993). With the exception of ACVR2A (Activin receptor Type IIA), which contains an Asn, these three polar residues are not present in the TM domains of the other TGFβ family receptors. To examine whether these polar
amino acids may be involved with SGK196 activity, Asn31, Asp39 and His40 residues in the SGK196 TM domain were mutated to Ala (Figure 15A). Interestingly, major loss of SGK196 degradatory activity for BMPR1B (Figure 15B) was observed upon mutation of all three polar residues. The Asn31Ala and the Asp39Ala / His40Ala SGK196 mutants retained the general ability to promote BMPR1B degradation (Figure 15B). The Asp39Ala and His40Ala SGK196 mutants similarly retained protein degradatory function (data not shown). These mutants exhibited the same effects on TGFBR1, BMPR2, and ACVR2B receptors (Figure 15C-E).

Polar residues in the TM domain have also been shown to influence TM domain interactions (Nordholm et al., 2013, Zhou et al., 2001). The SGK196 Asn31Ala / Asp39Ala / His40Ala (triple mutant) exhibited decreased co-IP with BMPR1B (Figure 15F and 15G). This reduced interaction indicates the Asn, Asp, and His polar residues may play a role in mediating the interactions with receptors that occur within the SGK196 TM domain. IF against the N-terminal HA tag of the SGK196 Asn31Ala, Asp39Ala / His40Ala , and Asn31Ala / Asp39Ala / His40Ala mutants in HEK293T cells showed localization patterns similar to the wild type SGK196 (Figure 16).

The reduced degradation of and interaction with BMPR1B resulting from the mutation of the polar residues in the SGK196 TM domain, further suggests that SGK196 function is contingent upon SGK196-receptor interactions occurring at the SGK196 TM domain. The SGK196 deletion and chimera mutants were observed to exert similar effects on the degradation of other TGFβ family receptors (Figure 12F and 15C-E). Overall, the findings presented here imply there may be a common mechanism of SGK196 to regulate other TGFβ family receptors.
SGK196 is essential for Xenopus embryonic development

The morpholino injections described in this section were performed in collaboration with Kim Perry in Dr. Jonathan Henry’s lab. All *insitu* were performed by Kim Perry. The TGFβ family pathway plays key roles in a wide array of developmental processes including gastrulation, germ layer formation, axis polarization, and the differentiation and patterning of specific organ systems (Kitsin et al., 2007, Herpin and Cunningham, 2007). An understanding of the developmental and tissue-specific functions of SGK196 requires evaluation in the context of an *in vivo* whole animal system. The frog *Xenopus* represents a model system for studies of vertebrate molecular, cell and developmental biology (Kay BK, 1991, Henry et al., 2008, Sive et al., 2000), having played a major role in deciphering the activities of various signaling pathways, including the TGFβ/Activin/Nodal and BMP pathways. This is due to the relative ease with which one can procure and rear embryonic material, and a powerful array of functional tools that have been developed to examine gene function. Here the function of SGK196 was examined during embryogenesis in *Xenopus*.

*In situ* hybridization were first carried out in embryos over a range of stages (10-36; gastrula through early larval stages) to pinpoint the expression of *sgk196* during *Xenopus* early development. It was found that *sgk196* was expressed throughout all stages of embryonic development examined (Figures 17A-5L). Initially, *sgk196* mRNA was expressed broadly in embryos at st10-12, (Figures 17A-5D), but eventually became more restricted to the developing central nervous system including brain, eyes and spinal cord (Figures 17E-L). RT-PCR analyses also verified that *sgk196* was expressed during all stages examined (Figures 17M). *sgk196* transcripts are present maternally as the original *Xenopus sgk196* clone was isolated from eggs.
Next, loss-of-function analyses were carried out to determine the effect of Sgk196 depletion. Injecting a morpholino (MO) to block the translation of *Xenopus* Sgk196 (SGK196MO) led to a range of developmental defects. Many defects were associated with the central nervous system, including truncated anterior-posterior axes and neural defects (Figures 18A-H). Severe phenotypes observed following Sgk196 depletion included embryos with an overall reduction of body size, major axis defects, tail malformation, and reduced head and eye sizes (Figures 18A and 18B). Some embryos exhibited cyclopia and spina bifida (data not shown). Sections of a 40ng SGK196MO injected embryo show disrupted brain development, including reduced size and thickness of the neural tube (Figure 18C and 18D). In contrast, there was increased development of axial mesodermal tissues including the notochord and somatic mesoderm (Figure 18C and 18D). Similar defects were observed to a lesser extent in mild cases injected with 20ng SGK196MO (Figure 18E-H). Sgk196 depletion did not alter development of the otic vesicles, indicating that the identity of hindbrain tissue, which is involved in otic vesicle induction, was still preserved (Gallagher et al., 1996). Impaired development of neuronal tissues and increased mesoderm development are reminiscent of the changes detected in differentiating hESCs following SGK196 depletion. Injections of SGK196MO also resulted in cloacal defects and defective digestive tracts, which appeared to be smaller in diameter and comprised of disorganized thickened tissue (Figure 18A and 18E). The severity of defects was dependent on the amount of MO injected (Figure 18A-H, 18CC). In contrast, injections of a control morpholino (CONMO) had no deleterious effects on development (Figure 18I-L, 18CC). To further verify the specificity of the MO injection and in the absence of a reliable antibody for *Xenopus* Sgk196, it was observed that the defects observed following injections of SGK196MO
could be rescued by co-injection of a synthetic RNA encoding an altered form of Sgk196, which was not targeted by the SGK196MO. While no significant rescue was seen with a lower dose of co-injected RNA (454pg), a dose dependent improvement in development was detected using higher concentrations of RNA (745pg and 1000pg, respectively Figure 18CC). Sections of an embryo co-injected with SGK196MO and 1000pg sgk196 RNA showed relatively normal organization of internal tissues (Figure 18Q-T). Injections of the sgk196 RNA at the higher 1000pg concentration did not result in apparent developmental defects (Figure 18U-X, 18CC).

The increase in mesoderm formation observed following Sgk196 depletion led to the pursuit whether this phenotype could be rescued by co-injection of a truncated Activin receptor. A previous study (Hemmati-Brivanlou and Melton, 1992) found that the activity of the endogenous type II Activin receptor Xar1 could be inhibited by injecting embryos with a dominant negative form, ΔXAR1, generated by the truncation of nearly the entire cytoplasmic region which includes the serine/threonine kinase domain. It was found that injection of ΔXAR1 mRNA blocked induction of the mesodermal marker brachyury in the marginal zone cells of st11 Xenopus embryos, and animal cap explants injected with ΔXAR1 expressed higher levels of the neural-specific marker ncam. Also, these ΔXAR1 injected Xenopus embryos did not form notochord (Hemmati-Brivanlou and Melton, 1992). Our experiments used the same concentration of ΔXAR1 mRNA (labeled XAR1DN; 4ng) co-injected with 30ng SGK196MO, which resulted in a partial rescue of Xenopus embryos (Figure 18Y-BB; 18CC). There was a reduction in the number of severe phenotypes and an increase in mild and normal phenotypes following co-injection of XAR1DN mRNA and SGK196MO (Figure 18CC).
same dose of XAR1DN mRNA alone resulted in death for majority of the embryos by st30 (data not shown).

**SGK196 overexpression stimulates neural differentiation in Xenopus animal cap**

The animal cap assays described in this section were performed by Kim Perry and Dr. Jonathan Henry. The *Xenopus* animal cap assay has been used as a standard to examine the role of various inductive signaling pathways. During normal *Xenopus* early development (at blastula stages) the animal-most cap of ectoderm that lies above the hollow blastocoel represents a pluripotent tissue capable of generating a variety of cell fates that ultimately contributes to the formation of the epidermis as well as neural tissues (Okabayashi and Asashima, 2003, Ariizumi et al., 2009). When isolated alone, animal cap tissue obtained from st8-9 embryos forms “atypical” epidermis. This is due to the fact that the animal cap, specifically those cells of the ventral region, expresses BMP4, which antagonizes the development of neuronal cell fates. Treatment or injecting animal caps with agents that antagonize the activity of BMP4, such as noggin, leads to the formation of neural fates (Lamb et al., 1993).

The potential activity of Sgk196 to antagonize BMP4 signaling and to stimulate neuronal development of the animal cap was tested. Since *sgk196* mRNA is present in animal cap ectoderm at st8 of development (Figure 17M), st8 animal caps were isolated from untreated control embryos or embryos that had either been injected with synthetic *sgk196* mRNA or the SGK196MO (Figure 18DD). Overexpression of *sgk196* results in the expression of the pan-neural marker *ncam* (Figure 18DD). In contrast, no *ncam* expression was detected in control untreated animal caps or those isolated from the embryos injected with SGK196MO. As a
further control, it was shown that injection of *noggin* mRNA led to the expression of *ncam* in animal caps as expected (Figure 18DD).
CHAPTER 4

DISCUSSION AND CONCLUSION

In this work I identified SGK196 as a key regulator of hESC lineage specification and found SGK196 to be a negative regulator of BMP and Activin/TGFβ/Nodal signaling. SGK196 functions by promoting the degradation of TGFβ family receptors, which is contingent upon SGK196-receptor interactions occurring at the SGK196 TM domain.

Despite the lack of conserved catalytic residues within the kinase domain (Figure 1B), SGK196 has been reported to exhibit \textit{in vitro} kinase activity on O-mannose, as part of a series of post-translational modifications leading to the proper post-translational modification of dystroglycan (DG) (Jae et al., 2013, Yoshida-Moriguchi et al., 2013). The molecular basis that gives rise to the kinase activity was not defined. Defects in the genes responsible for the DG glycosylation are associated with congenital muscular dystrophy (CMD), of which mutations to \textit{SGK196} have been identified in CMD patients (von Renesse et al., 2014, Yoshida-Moriguchi et al., 2013). In a separate study, SGK196 knockout mice were found to develop congenital hydrocephalus and exhibited improper neuronal migration in the cerebellum, hippocampus, and cerebral cortex tissues (Vogel et al., 2012). The expression of endogenous SGK196 transcript and protein was not examined in the mutant mice, and the pathogenic mechanism for the observed defects was unclear. Overall, these reports indicate the significance of SGK196 and the diverse roles that this molecule may play in other biological functions.
In this study I show that SGK196 negatively regulates TGFβ family signaling and accordingly influences hESC fate determination. SGK196 depletion results in elevated R-Smad phosphorylation in response to BMP4 and Activin A ligand treatment (Figure 2C and 2D). Under SGK196 depletion, mesoderm induction is augmented, and endoderm induction appears unaffected (Figure 4A and 4C). In the case of SGK196 overexpression, R-Smad ligand-induced phosphorylation is repressed (Figure 2E and 2F), accompanied by inhibition of both mesoderm and endoderm specification (Figure 4B and 4D). These findings are in agreement with established reports showing Activin and BMP signaling play an active role in the induction of definitive endoderm (D'Amour et al., 2005) and mesoderm (Zhang et al., 2008) differentiation, respectively, in hESCs. Inhibition of BMP and Activin/TGFβ signaling, alone or in combination, results in neural induction (Chambers et al., 2009, Zhou et al., 2010, Kim et al., 2010, Smith et al., 2008). In line with these studies, upon SGK196 depletion, RA and compound C mediated neural induction is repressed (Figure 3A and 3C). Inhibition of TGFβ family signaling upon SGK196 overexpression is also observed in HEK293T cells (Figure 6A and 6B).

Intriguingly, RA-mediated neural induction is not affected by elevated SGK196 levels (Figure 3D); while Compound C-mediated neural induction appears to be repressed (Figure 3B). It is known that RA and Compound C act through distinct signaling mechanisms to elicit neural induction. In vitro, RA signaling instructively promotes neural differentiation of pluripotent cells (Bain et al., 1995, Bibel et al., 2004, Fraichard et al., 1995). Compound C causes neural induction by blocking BMP signaling through the inhibition of type I BMP receptors, ALK2, ALK3 and ALK6 (Zhou et al., 2010, Yu et al., 2008, Kim et al., 2010). Emerging evidence suggests neural induction is not a simplistic ‘default’ phenomenon occurring in the absence of
repressive BMP signaling (Stern, 2006, Stern, 2005), but instead a complex process involving instructive signals from FGF, IGF, RA, Wnt pathways and their cross talk with the BMP pathway (Sheng et al., 2010, Pera et al., 2003, Pera et al., 2001, Baker et al., 1999, Delaune et al., 2005, Sasai et al., 1996, Launay et al., 1996, Stern, 2005). It has been also been found that the TGFβ family pathway can transduce signals via non-canonical Smad independent pathways, including the mitogen-activated protein kinase and RhoA cascades (Zhang, 2009, Derynck and Zhang, 2003). Smad independent TGFβ signaling has been shown to be highly relevant in pathological states (Iwata et al., 2013, Holm et al., 2011). The effects from the steady state levels of TGFβ family receptors influenced by SGK196 is not necessarily analogous to the effects of signaling inhibition by BMP ligand antagonists. Degradation of TGFβ family receptor proteins would result in the overall reduction of any associated receptor-dependent signaling. Thereby, I speculate the attenuated neural induction in the presence of SGK196 overexpression with repressed BMP signaling, may be due to impacted Smad-independent pathways downstream of TGFβ family signaling. Another speculation is SGK196 could interact with other effectors which may consequently affect other pathways. In both cases the instructive cues required for neural induction could be altered. Further work is needed to address these remaining questions.

The TGFβ family pathway controls an extensive array of homeostatic and developmental processes. Diverse spatial and temporal regulation is achieved through functional interactions with outside effectors, including proteins that modulate the stability and availability of TGFβ family receptors (Satow et al., 2006, Aramaki et al., 2010, Onichtchouk et al., 1999, Zhao et al., 2012, Zhang et al., 2012, Chen, 2009, Zhang and Laiho, 2003, Eichhorn et al., 2012). I have
expanded the mechanistic understanding of the TGFβ family pathway regulation by finding that SGK196 can promote the degradation of TGFβ family receptors (Figure 7). In my studies I show that degradation of TGFβ family receptors is likely mediated through specific receptor-SGK196 interactions occurring at the SGK196 TM domain (Figures 9, 12, and 14). The SGK196 deletion mutant studies demonstrate that the ECD is dispensable for SGK196 activity and for BMPR1B interaction, suggesting that SGK196’s ability to promote receptor degradation may be kinase independent (Figure 12B, 12E-F). Mutation of all three Asn31, Asp39, and His40 polar amino acids residues in the SGK196 TM domain resulted in the decreased interaction with BMPR1B, concomitant with the loss of the ability to degrade BMPR1B (Figure 14). Polar residues in the TM domain have been shown to influence receptor trafficking and TM domain interactions (Zhou et al., 2001, Nordholm et al., 2013, Cosson et al., 2013, Reggiori et al., 2000, Zaliauskiene et al., 2000). Altogether these findings accentuate the link between the SGK196-receptor interaction and the ability for SGK196 to promote receptor degradation. Further studies are needed to understand the nature by which these polar amino acids mediate the SGK196 TM domain interactions and the means by which SGK196 promotes the degradation of the TGFβ family receptors. Given the differences in the structure and oligomerization between and amongst TGFβ family receptor subtypes, the exact nature of the interaction with SGK196 may vary among individual receptors (Hinck, 2012, Rechtman et al., 2009, Groppe et al., 2008).

One speculation is SGK196 could function as an adapter, recruiting components linked to these degradatory pathways in proximity to SGK196-bound receptors to promote receptor degradation. A number of adapters, including I-Smads, Axin and TGIF have been reported to promote the degradation of TGFβ family signaling members (Murakami et al., 2003, Liu et al., 2006, Kavsak
et al., 2000, Seo et al., 2004). Another speculation is the polar amino acids in the SGK196 TM domain may play a role in affecting the trafficking and subsequent degradation of the SGK196-bound TGFβ family receptors.

To place SGK196 in a developmental context, the expression and function in the model vertebrate *Xenopus* was examined. *sgk196* is expressed broadly during early development, including in the animal cap ectoderm (Figure 17A-M). At later stages *sgk196* is more highly expressed in the CNS and the eye (Figure 17I-L). Consistent with an increased level of BMP and Activin/TGFβ signaling, MO-mediated Sgk196 knockdown results in a reduction in the development of brain tissues and expanded development of axial mesodermal tissues (e.g., the notochord, see Figure 18C-D). These defects could be rescued by co-expression of *sgk196* mRNA (Figure 19Q-T), confirming the specificity of the knockdown. Isolated animal cap ectoderm, which normally does not exhibit signs of neural differentiation in culture, expressed the pan-neural marker *ncam* when caps were obtained from embryos injected with *sgk196* mRNA, but not those injected with either SGK196MO or CONMO (Figure 6DD). Interestingly, no apparent developmental defects resulted from the expression of *sgk196* mRNA alone. Correspondingly, it was also observed SGK196 overexpression had little effect on pluripotency in hESCs (Figure 1C and Figure 1 - figure supplement 1C). Expression of a dominant negative type II activin receptor, ΔXAR1, (called XAR1DN; Figure 18Y-6CC), which blocks many forms of TGFβ family signaling (Hemmati-Brivanlou and Melton, 1992; Schulte-Merker et al., 1994) leads to severe axial defects in *Xenopus* embryos. Co-injection of SGK196MO along with XAR1DN mRNA resulted in partial rescue of those phenotypes, indicating that the loss of Sgk196 activity counteracted some of the repressed TGFβ signaling resulting from expression of
the XAR1DN (Figure 18CC). Together, these findings suggest that Sgk196 plays a key role in negatively modulating the levels of TGFβ family signaling during Xenopus embryonic development.

In this first mechanistic and functional characterization of SGK196, I present novel insight into SGK196’s role as an essential regulator for stem cell fate and embryonic development, and as a negative regulator of the TGFβ family pathway. I provide evidence suggesting that SGK196 acts by promoting the degradation of BMP and TGFβ/Activin receptors to attenuate signaling. The indispensable role of SGK196 in hESCs and Xenopus embryos suggests a conserved function for this protein, thereby underscoring the significance of SGK196 activity. As a regulator of TGFβ family signaling, SGK196 may play important roles in other biological processes, which requires further investigation.
CHAPTER 5
TABLES AND FIGURES

Table 1. QPCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 1</th>
<th>Reverse 1</th>
<th>Forward 2</th>
<th>Reverse 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5' CTGGTAAAGTGGATATTGTTGCCAT 3'</td>
<td>5' TGGAATCATATTGGAAACATGTAAACC 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGK196</td>
<td>5' CCACCTCTTCATCGCTCCCT 3'</td>
<td>5' ACAGCCAAGGTGAGCAGTTT 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOX17</td>
<td>5' GCATGACTCCGGGTGTAATCT 3'</td>
<td>5' TCACACGTCAAGGATGTGAGT 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXA2</td>
<td>5' CTGAAGCCGGAACACACACTAC 3'</td>
<td>5' CGAGGACATGAGGTTGTTGAGT 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSC</td>
<td>5' ACCTCCGCAGGAGAAAGTG 3'</td>
<td>5' CTTCCTCGCGTCTCCGACT 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAX6</td>
<td>5' GCTTCACCATGGCAATAACC 3'</td>
<td>5' GGCGACATGAGGAGTATGA 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOX1</td>
<td>5' AAATGTAGTAAGGCAAGGTCTCA 3'</td>
<td>5' ACCCAGATAATATAACTCCGCC 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTX2</td>
<td>5' CAACAGCAGAATGGGAGATCA 3'</td>
<td>5' ATGGGCCACTTGGTCCACTC 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>5' CAGCAAAAGTCAAGCTCACCA 3'</td>
<td>5' CCCCAACTCTCCTACTATGTTGATT 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCT4</td>
<td>5' GGAGGAAGCGTGAACAAATGAAA 3'</td>
<td>5' GGCCCTGCACGAGGGTTT 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOX2</td>
<td>5' TCGAGGCAGCTGCACAT 3'</td>
<td>5' CATGAGCGTCTTGGTCCACT 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NANOGE</td>
<td>5' CCAACATCCTGAACCTACCTAC 3'</td>
<td>5' GCCTTCCGGTCACACCATT 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIXL1</td>
<td>5' GCAGTTCCAGAGTTGGGAATCCC 3'</td>
<td>5' GCAGTTACATCTACCTCCAGAG 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMPR1B</td>
<td>5' CCCCTCATCCTCCAAACC 3'</td>
<td>5' TTAAACCGCAGGGCTGTC 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMPR2</td>
<td>5' CTTTACTGAGAATTTCCACCTTCTC 3'</td>
<td>5' GCCAAAAGCAATGATTATTGTTCTACATC 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFB1</td>
<td>5' AAGTCACTCACTCGGCCTTGG 3'</td>
<td>5' TCGAATGGAATGACAGTGTC 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFB2</td>
<td>5' GTTGCGAACAACACTCAACC 3'</td>
<td>5' AGTGATTGCCTCGCTCTCG 3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. *Xenopus* primers used in RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 3’</th>
<th>Reverse Primer 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ef1a1</td>
<td>CAGATTGGTGCTGGATATGC</td>
<td>ACTGCCTTGATGACTCCTAG</td>
</tr>
<tr>
<td>sxk81a1</td>
<td>CACCAGAACACAGAGTAC</td>
<td>CAACCTTCCCATCAACCA</td>
</tr>
<tr>
<td>ncam1</td>
<td>CACAGTTCCACCAAATGC</td>
<td>GGAATCAAGCGGTACAGA</td>
</tr>
</tbody>
</table>
Figure 1. SGK196 knockdown and overexpression in hPSCs

(A) Morphology of NTERA2 cells (top) shScramble control and (bottom) shSGK196-A1 mediated knockdown (bar=200µm). (B) Deduced protein structure of human SGK196, showing the location of the transmembrane (TM) and kinase domains. Key residues within conserved motifs essential for catalytic kinase activity are indicated in red boldface type and corresponding substituted residues (S108, V201, N220) found in SGK196 are denoted by blue boldface type. (C) Morphology of stable SGK196 knockdown and control H1 hESCs (bar=200µm), right: expanded view. (D) Morphology of H1 hESCs stably expressing mKate (control) or HA-SGK196 (bar=100µm).
Figure 2. Effects of SGK196 levels on hESC pluripotency markers

qPCR analysis of pluripotency genes in hESCs upon (A) SGK196 knockdown and (B) SGK196 overexpression. SGK196 knockdown data represents pooled results from shSGK196-1, shSGK196-2, and shSGK196-A1. Data represents mean ±SEM. Asterisks indicate a statistical significance level of p≤0.05 compared to control mKate or shScramble lines.
Figure 3. Effects of SGK196 knockdown and overexpression on neural differentiation in hESCs

QPCR analysis of gene expression levels in H1 hESCs upon SGK196 depletion and overexpression during (A and B, respectively) 1μM compound C (ComC) mediated neural differentiation, (C and D, respectively) 10μM retinoic acid (RA) mediated neural differentiation. Asterisks indicate a statistical significance level of p≤0.05 compared to control mKate or shScramble lines. Data are represented as mean ± standard error of the mean (SEM).
Figure 4. Effects of SGK196 knockdown and overexpression on mesoderm and endoderm differentiation in hESCs

QPCR analysis of gene expression levels in H1 hESCs upon SGK196 depletion and overexpression during (A and B, respectively) BMP4-mediated mesoderm differentiation and (C and D, respectively) Activin A-mediated endoderm differentiation. Asterisks indicate a statistical significance level of $p \leq 0.05$ compared to control mKate or shScramble lines. Data are represented as mean ± standard error of the mean (SEM).
Figure 5. SGK196 negatively regulates ligand-induced Smad1/5/8 and Smad2 phosphorylation

(A) Fixed HEK293T cells showing the localization of (top) overexpressed mCherry and (bottom) fusion SGK196-mCherry with a magnified image to the left (bar=20μm). (B) Live staining of HEK293T cells overexpressing SGK196-mCherry fusion proteins with CellMask green plasma membrane stain (bar=10μm). Under mTeSR culture conditions, stable H9 hESC SGK196 knockdown and H1 hESC HA-SGK196 overexpression lines (C and E, respectively) were treated with BMP4 (10ng/mL) at the indicated time points or with (D and F, respectively) Activin A (20-80ng/mL) for 30 minutes prior to lysis and Western blot analysis to assess R-Smad phosphorylation levels.
Figure 6. SGK196 negatively regulates BMP and Activin signaling at the receptor and transcriptional level in HEK293T cells

Overnight serum starved HEK293T cells stably expressing HA-SGK196 or mKate were treated with (A) BMP4 (5ng/mL) or (B) Activin A (30ng/mL) and TGFβ1 (1.8ng/mL) for 30 minutes prior to Western blot analysis. Arrow indicates protein of interest. Western blot bands were normalized to respective alpha-tubulin levels and quantifications were presented as intensities relative to the non-treated control group. Luciferase assays assessing the effect of ectopic HA-SGK196 on the transcriptional activity of ligand induced (C) BMP and (D) Activin signaling using the ID-120-luc and ARE-GL3 reporters, respectively, in HEK293T cells. 24-48 hours post transfection, cells were serum starved overnight then treated with BMP4 (5ng/mL) or Activin A (30ng/mL) for 12 hours prior to detection.
Figure 7. SGK196 promotes the protein degradation of BMP and Activin/TGFβ receptors

Western blot analysis with anti-FLAG M2 antibody (F denotes FLAG peptide) to detect (A) FLAG-TGFBRI and FLAG-BMPR1B and (B) BMPR2-FLAG protein levels upon co-expression with HA-SGK196 or control FLAG-Ubiquitin1 in HEK293T cells. Effect of HA-SGK196 overexpression on (C) ACVR2B-FLAG protein levels. Assessment of (C) FLAG-TGFBRI and (D) FLAG-BMPR1B receptor half-life in the presence of HA-SGK196, by inhibition of protein synthesis via time course treatment with cycloheximide (CHX, 100μg/mL) in HEK293T cells. Western blot quantification of protein levels represents proportion of remaining protein relative to starting amount at t=0 within each experimental group.
Figure 8. Effects of SGK196 levels on the expression of TGFβ family receptors

QPCR analysis of TGFβ family receptor mRNA levels in H1 hESCs upon SGK196 (A) knockdown and (B) overexpression under pluripotency (mTeSR) conditions. Data are represented as mean ± SEM. Asterisks indicate a statistical significance level of p≤0.05 compared to control mKate or shScramble lines.
Figure 9. SGK196 is a type II transmembrane protein that interacts with TGFβ family receptors

Interactions between ectopic HA-SGK196 and FLAG-tagged TGFβ family receptors in HEK293T cells assessed by immunoprecipitation (IP) of the (A, C) HA or (B, D) FLAG epitopes followed by Western blotting to detect co-IP proteins.
Figure 10. Live immunofluorescence staining indicates SGK196 is a type II transmembrane protein

(A) Diagram describing the positioning of the N and C terminal HA tags on SGK196 to discern a type I from type II transmembrane protein orientation. (B) Immunofluorescence detection of the HA epitope by live staining of ectopic (top) N-terminal tagged HA-SGK196 and (bottom) C-terminal tagged SGK196-HA in HEK293T cells (bar=100µm). (C) Immunofluorescence detection of the HA epitope in fixed permeabilized HEK293T cells expressing (top) HA-SGK196 and (middle) SGK196-HA (bar=100µm), and (bottom) a magnified view of SGK196-HA (bar=20µm).
Figure 11. Trypsin digestion indicates SGK196 is a type II transmembrane protein

Western blot analysis showing the effect of trypsin treatment on the detection of the HA tag and SGK196 protein of (A) N-terminal and (B) C-terminal HA tagged SGK196 expressed in HEK293T cells. Western blot bands were normalized to respective alpha-tubulin levels and quantifications were presented as intensities relative to the first non-treated control group.
Figure 12. SGK196-mediated receptor degradation is linked to the interaction with TGFβ family receptors

(A) Schematic diagram of the HA-SGK196 deletion mutants describing the deleted regions containing the cytoplasmic, extracellular (ECD), and transmembrane (TM) domains. (B) IP of FLAG-BMPR1B to detect interactions with the I-81 AA, Δ44-81 AA, ΔECD, and Δ1-43AA (extracellular domain only) HA-SGK196 deletion mutants. (C) Schematic diagram of the FLAG-BMPR1B mutants showing the deleted regions containing the kinase domain, or the ECD and TM domains, as indicated. (D) IP of the FLAG-BMPR1B mutants to detect interactions with HA-SGK196. Asterisk indicates protein of interest. Effect of the HA-SGK196 deletion mutants on (E) FLAG-BMPR1B and (F) BMPR2-FLAG protein levels.
Figure 13. Cellular localization of the SGK196 deletion mutants

Immunofluorescence staining of the HA epitope of HA-SGK196 deletion mutants expressed in HEK293T cells (bar=50μm). The bottom panel is a higher magnification showing staining of the full length HA-SGK196 (bar=10μm).
Figure 14. Effects of replacing the SGK196 transmembrane domain with the FGFR1L transmembrane domain

(A) Schematic diagram of the HA-SGK196 chimera, describing the replacement of the SGK196 TM domain with the FGFR1 TM domain. Effect of the HA-SGK196 chimera on (B) FLAG-BMPR1B (C) BMPR2-FLAG and (D) FLAG-TGFB1 protein levels relative to wild-type HA-SGK196. IP against the (E) FLAG and (F) HA epitopes to assess the interaction affinities of the chimeric and wild-type HA-SGK196 forms with FLAG-BMPR1B. (G) Immunofluorescence detection against the N terminal HA epitope of the chimeric HA-SGK196 in HEK293T cells (bar=20μm).
Figure 15. The Asn31, Asp39, and His40 polar residues in the SGK196 transmembrane domain are important for the interaction with and degradation of TGFβ.

(A) Schematic diagram showing the location of the Asn31Ala, Asp39Ala, and His40Ala mutations in the SGK196 TM domain. HEK293T cells. Western blot analysis showing the effects of the Asn31Ala, Asp39Ala, and His40Ala mutations on the (B) FLAG-BMPR1B, (C) FLAG-TGFBR1, (D) BMPR2-FLAG, and (E) ACVR2B-FLAG protein levels. Assessment of the interaction between FLAG-BMPR1B and the HA-S GK196 mutant containing the Asn31Ala, Asp39Ala and His40Ala mutations relative to wild-type HA-S GK196 by IP against the (F) HA and (G) M2 epitopes.
Figure 16. Cellular localization of the Asn31Ala, Asp39Ala, and His40Ala SGK196 mutants

Immunofluorescence staining of the HA epitope of the full length HA-SGK196 containing the Asn31Ala, Asp39Ala, and His40Ala mutations in HEK293T cells (bar=25μm).
Figure 17. *In situ* hybridization and RT-PCR analysis of *Xenopus sgk196*

St10 embryo (A-B) displaying broad expression across animal cap region in the (A) animal view and (B) corresponding vegetal view. St12 embryo (C-D) with broad expression shown in the (C) dorsal view and (D) ventral view with the yolk plug to the left side (posterior) of the embryo and developing neural plate extending to the right. Lateral view (E) of st19 embryo with expression bordering the neural fold region. Broad anterior expression and regional localization is shown in the spinal cord region of st22 embryo (F). An oblique lateral view of st25 embryo (G) showing expression in the primary eye vesicles, forebrain, midbrain and hindbrain region, also shown in the corresponding lateral view (H). Localization of *sgk196* becomes restricted to the central nervous system in the dorsal view of a st28 embryo (I). The lateral view of a st33 embryo (J) and corresponding dorsal view (K) show discrete labeling in the forebrain, hindbrain and along the animal axis. Brain and spinal cord localization (L) are present at st36, along with restricted lens localization. RT-PCR results (M) showing *sgk196* transcripts present in all *Xenopus* st2-36 and in isolated st8 animal caps (AC). (+) represents the positive control lane where *sgk196* containing plasmid was used as template. (-) denotes the negative control lane (no template DNA added). Asterisk (*) marks the 100bp band on the DNA ladder, which is close to the expected size of the *sgk196* amplification product. Scale bar in L=400μm.
Figure 18. Effects of MO and mRNA injections on *Xenopus* development

Lateral bright-field images of representative injection cases shown at st41 (A, E, I, M) and st36 (Q, U, Y), and corresponding fluorescence images showing uniform distribution of lissamine-tagged MO or dextran at st41 (B, F, J, N), and st36 (R, V, Z). Hematoxylin/eosin stained transverse sections are shown at the level of the otic vesicles (C, G, K, O, S, W, AA) and the more posterior location (D, H, L, P, T, X, BB). The severe 40ng SGK196MO phenotype (A-D) displays major axis and size defects, reduced head and eye sizes and tail malformation. Disrupted brain development and disrupted mesoderm tissue are seen in C-D. The mild 20ng SGK196MO phenotype (E-H) shows a moderate reduction of eye and head size (compare to control in I-L), with axis deformities (kinks) and sections (G-H) show reduced brain tissues with enlarged notochord (compare to K-L). CONMO injected (40ng; I-L) specimen exhibits normal morphology, including normal brain and notochord tissue (K-L). Injection with 745pg *sgk196* RNA (M) co-injected with fluorescent dextran for visualization (N) displays a normal phenotype (O-P). Representative rescue cases (Q-T) co-injected with 30ng SGK196MO/1000pg altered *sgk196* RNA display an overall normal morphology and relatively normal organization of internal tissues (S-T). Maximum injection of *sgk196* RNA (1000pg; U-X) displays a relatively normal morphology with good organization of internal tissues (W-X). This specimen was co-injected with non-fluorescent tracer fast green, which shows no fluorescence (V). Rescue cases (Y-BB; 4ng XARIDN RNA + 30ng SGK196MO) display mostly normal external morphology and internally the neural tube (AA-BB) is somewhat reduced.
Figure 18. (cont.)

The graphical representation of injection data is shown in CC, where specimen morphology is noted as no defect, mild defect and severe defect. The number and severity of defects increased in a dose-dependent manner as the concentration of SGK196MO increased. Error bars indicate standard error. RT-PCR data for the animal cap assay (DD) is shown in tabular form with the corresponding gel bands. All sets of caps expressed the positive control elongation factor l-alpha (efl1a) and an epidermal marker (epidermal cytokeratin, ssk81a1). sgk196 RNA injected animal caps expressed the pan-neural marker ncam1 and caps from noggin RNA injected embryos were used to show neural induction of ncam1 as a positive control. The asterisk (*) labels the 300nt band (1kb ladder): nt = neural tube, nc = notochord, ov = otic vesicle. Scale bar in BB equals 190μm (A-B, E-F, I-J, M-N, Q-R, U-V, Y-Z) and 95 μm (C-D, G-H, K-L, O-P, S-T, W-X, AA-BB).
REFERENCES


KRIEG, P. A., VARNUM, S. M., WORMINGTON, W. M. & MELTON, D. A. 1989. The mRNA encoding elongation factor 1-α (EF-1α) is a major transcript at the midblastula transition in Xenopus. Developmental Biology, 133, 93-100.


ZHANG, P., LI, J., TAN, Z., WANG, C., LIU, T., CHEN, L., YONG, J., JIANG, W., SUN, X., DU, L.,
ZHAO, B., WANG, Q., DU, J., LUO, S., XIA, J. & CHEN, Y. G. 2012. PICK1 promotes caveolin-
ZHAO, B., WANG, Q., DU, J., LUO, S., XIA, J. & CHEN, Y. G. 2012. PICK1 promotes caveolin-
ZI, J., SU, P., LI, D., TSANG, S., DUAN, E. & WANG, F. 2010. High-Efficiency Induction of
Neural Conversion in Human ESCs and Human Induced Pluripotent Stem Cells with a Single Chemical
Inhibitor of Transforming Growth Factor Beta Superfamily Receptors. Stem Cells, 28, 1741-1750.