SUMO AND UBIQUITIN LIGASES REGULATE
HEPATOCYTE NUCLEAR FACTOR – 1 BETA
TRANSCRIPTIONAL ACTIVITY

Approved by supervisory committee

NOTE: The top line is for the Supervising Professor's name. There should be as many lines as there are members of the committee. All signatures must be original and in ink. Adjust "Approved by Supervisory Committee" line upward if the committee list is very large.

Peter Igarashi, M.D.

Keith L. Parker, M.D., Ph.D.

Raymond J. MacDonald, Ph.D.

Andrew R. Zinn, M.D., Ph.D.

I DEDICATE THIS WORK TO FIREFIGHTERS AND THEIR SPOUSES.

ACKNOWLEDGMENTS

I would like to thank Drs. Kim Orth, James Chen, Thomas Carroll, Christopher Davis, Eric Olson, Kristen Lynch, Tyler Jacks, Ralph DiLeone, Zhiyuan Shen, Ke Shuai, Xin-Hua Feng, Hisato Saitoh, Howard Worman, Keiji Miyazawa, Kohei Miyazono, S. Stoney Simons, Jr., John R. Sedor, Serge Gisler, and Marco Pontaglio for their generous gifts of plasmids and antibodies over the course of my graduate career. I would like to thank Drs. Serge Gisler and Orson Moe for helping me perform the yeast two-hybrid screen. Especially, Dr. Gisler, whose patience and careful instruction helped me care for my "yeasty babies." I would like to thank all of the members of the Igarashi laboratory over the past five and a half years for their support, advice, and friendship. I would especially like to thank Sunshine Kuchkoltz, Yun Bai, Yun-Hee Choi, and Drs. Zhendong Ma, Scott Williams, Angus Sinclair, Thomas Hiesberger, and Fangming Lin. I would like to thank my dissertation committee Drs. Keith Parker, Raymond MacDonald, and Andrew Zinn. I appreciate the input they provided on my projects as I encountered various hurdles and their assistance in completing this body of work.

I am happy to thank my mentor Dr. Peter Igarashi for joining me on this long road. I am happy to say I am better scientist because of him, because he supported my independence and provided the tools for my education.

Lastly, I would like to thank my friends for their support over the years. I would like to thank the new members of my family, the Lavers, the Rothrocks, and especially Phil and Susan Horan, whom together have been wonderful additions to my life. I would like to thank my brother Justin, my grandmother Margaret, my parents Michael and Ranney, and my wife Caryn, who I am eternally grateful to for their unwavering love and support.

SUMO AND UBIQUITIN LIGASES REGULATE HEPATOCYTE NUCLEAR FACTOR – 1 BETA TRANSCRIPTIONAL ACTIVITY

by

BRIAN TIMOTHY MCNALLY

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
December 2006

SUMO AND UBIQUITIN LIGASES REGULATE
HEPATOCYTE NUCLEAR FACTOR - 1 BETA
TRANSCRIPTIONAL ACTIVITY

Publication No. _____

Brian Timothy McNally, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2007

Supervising Professor: Peter Igarashi, M.D.

Hepatocyte nuclear factor-1β (HNF-1β) is a homeodomain-containing transcription factor that regulates tissue-specific gene expression in the kidney, liver, and other organs. Humans with heterozygous mutations of HNF-1β develop maturity-onset diabetes of the young type 5 (MODY5) associated with cystic abnormalities of the kidney. A yeast two-hybrid screen was performed to identify proteins that interact with HNF-1β. The SUMO E2 ligase, Ubc9, SUMO E3 ligases, PIAS1 and PIASγ, and the ubiquitin E3 ligase, Arkadia, were isolated as putative interacting proteins. SUMOylation of transcription factors affects their subcellular localization, intracellular transport, stability, and transcriptional activity. Ubiquitination of transcription factors has been shown to alter their stability and transcriptional activity. Immunostaining of the adult mouse kidney showed that HNF-1β co-localized with Ubc9, PIAS1, and PIASγ in the nuclei of renal tubular epithelial cells. Immunostaining also showed that Arkadia localized to the nuclei of renal tubules. Overexpression of Ubc9, PIAS1, or PIASγ inhibited the activity of the *Pkhd1* promoter, a known HNF-1β target. Expression of catalytically inactive Ubc9 or PIASγ mutants also inhibited *Pkhd1* promoter activity, suggesting that repression of HNF-1β transcriptional activity is SUMOylation-independent. In contrast to the repressive effect of SUMO ligases, overexpression of Arkadia stimulated *Pkhd1* promoter activity in an HNF-1β-dependent fashion. Immunoprecipitation of epitope-tagged SUMO or ubiquitin resulted in co-precipitation of HNF-1β, indicating that HNF-1β is both SUMOylated and ubiquitinated *in vivo*. In summary, the E2 and E3 SUMO ligases Ubc9, PIAS1, and PIASγ repressed HNF-1β transcriptional activity via SUMOylation-dependent and -independent mechanisms. The E3 ubiquitin ligase Arkadia stimulated HNF-1β transcriptional activity. Together, these results demonstrated that HNF-1β is covalently modified by SUMO and ubiquitin, and

these modifications had opposing effects on HNF-1β transcriptional activity. In addition, insights into the regulation of HNF-1β and subsequent effects on target gene expression may lead to a greater understanding of the mechanisms that lead to renal cystogenesis.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

1. Igarashi, P., Shao, X., **McNally, B.T.**, Hiesberger, T. (2005) Roles of HNF-1β in kidney development and congenital cystic diseases. *Kidney Int.* 68(5): 1944-1947.
2. Hiesberger, T., Bai, Y., Shao, X., **McNally, B.T.**, Sinclair, A.M., Tian, X., Somlo, S., Igarashi, P. (2004) Mutation of hepatocyte nuclear factor-1B inhibits Pkhd1 gene expression and produces renal cysts in mice. *J. Clin. Invest.* 113(6): 814-825.

ABSTRACTS

1. Choi, Y-H., **McNally, B.T.,** Igarashi, P. (2006) Zyxin Family Proteins Regulate the Transcriptional Activity of HNF-1β. *J. Am. Soc. Nephrol.* 17: 362A.
2. **McNally, B.T.,** Gisler, S.A., Igarashi, P. (2006) SUMO and Ubiquitin Ligases Regulate Hepatocyte Nuclear Factor-1 Beta Transcriptional Activity. Third International Conference on Ubiquitin, Ubiquitin-like Proteins and Cancer. Houston, Texas.
3. **McNally, B.T.,** Gisler, S.A., Igarashi, P. (2005) SUMO Ligases Regulate Hepatocyte Nuclear Factor-1 Beta Transcriptional Activity. *J. Am. Soc. Nephrol.* 16: 21A.
4. **McNally, B.T.,** Chiang, J.A., Yang, P., MacDonald, R.J., Graff, J.M., Igarashi, P. (2003) Expression-Based Bioinformatic Screen: Using UniGene to identify kidney-enriched transcription factors. *J. Am. Soc. Nephrol.* 14: 23A.
5. Labeikovsky, W., **McNally, B.,** Unlu, M., Minden, J. (2000) *Drosophila* embryonic pattern repair: Analysis of embryos response to hyperplastic growth by Differential Gel Electrophoresis (DIGE). 41st Annual

Drosophila Research Conference (Pittsburgh, PA), Program and Abstracts: 614.

6. Mergliano, J., **McNally, B.,** Minden, J. (1999) Fate mapping of mitotic domain 9 by photoactivated gene expression. 40th Annual *Drosophila* Research Conference (Bellevue, WA), Program and Abstracts: 165B.

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

ABBREVIATIONS

HNF - Hepatocyte Nuclear Factor

MODY - Maturity-Onset Diabetes of the Young

ARPKD - Autosomal Recessive Polycystic Kidney Disease

POU - Pit-1, Oct-1, Oct-2, Unc86 domain

SUMO - Small Ubiquitin-like Modifier

PIAS - Protein Inhibitor of Activated Stat

ESRD - End-Stage Renal Disease

DCoH - Dimerization Cofactor of HNF1

DNA - Deoxyribonucleic acid

RCAD - Renal Cysts and Diabetes

UBL - Ubiquitin-like Protein

bp - Base Pair

SAE - SUMO1 Activating Enzyme

AMP - Adenosine Monophosphate

ATP - Adenosine Triphosphate

PDSM - Phosphorylation-Dependent SUMO Motif

NDSM - Negatively charged amino acid-Dependent SUMO Motif

SBD - SUMO-Binding Domain

PML - Promyelocytic Leukemia

HSF2 - Heat Shock Factor 2

Ubc - Ubiquitin-Conjugating enzyme

SF-1 - Steriodogenic Factor-1

AR - Androgen Receptor

Elk-1 - Ets-like transcription factor

GR - Glucocorticoid Receptor

Sox - SRY box

STAT - Signal Transducer and Activator of Transcription

ChIP - Chromatin Immunoprecipitation

CBP - Cyclic AMP response element-binding protein

P/CAF - p300/CBP-associated factor

HDAC - Histone Deacetylase

HAT - Histone Acetylase

GST - Glutathione-S-Transferase

EMSA - Electrophoretic Mobility Shift Assay

E - Embryonic Day

PCR - Polymerase Chain Reaction

HA - Hemagglutinin

cDNA - complimentary DNA

PBS - Phosphate Buffered Saline

ROG - Repressor of GATA

LPP - Lipoma-Preferred Partner

RACK - Receptor for activated C kinase

PLZF - Promyelocytic leukemia zinc finger

GCKD - Glomerulocystic kidney disease

FJHN - Familial juvenile hyperuricaemic nephropathy

Q-RT-PCR - Quantitative reverse transcriptase polymerase chain reaction

ANOVA - Analysis of Variance

Hadh - hydroxyacyl-Coenzyme A dehydrogenase, type II

GMEB - glucocorticoid modulatory element-binding proteins

Yif - Yip1 interacting factor

PKHD - polycystic kidney and hepatic disease

Zimp - Zinc finger-containing, Miz-1, PIAS-like protein

AD - Activation Domain

SNP - Single Nucleotide Polymorphism

CHAPTER ONE

Introduction & Literature Review

RENAL CYSTIC DISEASE AND DIABETES

The kidneys are essential organs that are responsible for maintaining physiological homeostasis by removal of waste products and regulation of water and electrolyte balance. End-Stage Renal Disease (ESRD) is a failure of the kidneys to maintain the homeostatic state and is often the endpoint resulting from chronic kidney disease that progressively destroyed renal function. ESRD patients must have a form of renal replacement such as dialysis or kidney transplantation to survive. The United States Renal Data System reported that as of December 31, 2004 there were over 472,000 people with ESRD in the United States. The cost of ESRD treatment from both public and private efforts in 2003 totaled over $ 27.3 billion, which illustrates that ESRD is a significant health care issue in the United States (1)[1].

A number of different disease conditions can result in ESRD, with varied prevalence rates (Table 1.1). ESRD can result from genetic conditions that predispose individuals to renal disease or from non-inheritable causes that damage the kidneys. The categories listed in Table 1.1 are compilations of different pathologies observed in patients with ESRD. For example, inclusion of a patient in the cystic disease category could result from the patient being diagnosed with a single disease of the over 17 known diseases that develop renal cysts. However, some disorders could be classified into two or more of the categories listed. For

[1] The data reported here have been supplied by the United States Renal Data System (USRDS). The interpretation and reporting of these data are the responsibility of the author(s) and in no way should be seen as an official policy or interpretation of the U.S. government.

instance, a single genetically homogeneous clinical syndrome, Renal Cysts and Diabetes (RCAD), is characterized by having features present in two categories. This autosomal dominant syndrome is associated with mutations in the gene Hepatocyte Nuclear Factor-1 Beta (HNF-1β).

TABLE 1.1

Cause of ESRD	Number of Patients
Diabetes	172,938
Hypertension	114,481
Glomerulonephritis	77,121
Cystic Kidney Disease	21,397
Other Urologic	14,298
Other Cause	47,584
Unknown Cause	18,468
Missing Disease	5,812
Total	472,099

HEPATOCYTE NUCLEAR FACTOR – 1 BETA

Protein Structure

HNF-1β, also named TCF2, LFB3, HNF1B, vHNF1, is a transcription factor that was originally identified based on its ability to interact with a specific sequence in the promoters of liver-enriched genes such as albumin, alpha- and beta-fibrinogen, and alpha 1-antitrypsin (2, 3). The sequence was also known to

bind a previously described protein HNF-1α, also referred to as HNF1 (4, 5). The HNF1 family of transcription factors comprises HNF-1α and HNF-1β, based on homology and DNA-binding sequence specificity.

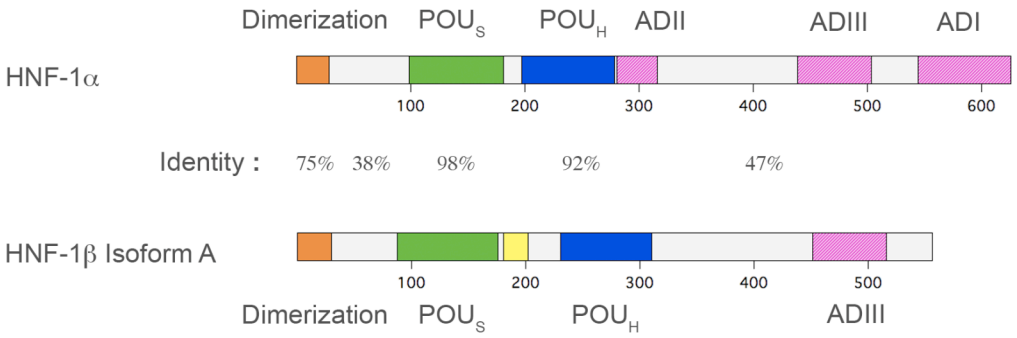


Figure 1-1. Domain Structures of HNF1 Family Members.
HNF1 family members have a conserved dimerization domain (orange) that facilitates the obligatory hetero- or homodimerization. The POU_S (green) and POU_H (blue) are responsible for DNA binding. There are three domains in the carboxyl terminus of HNF-1α that have been shown to confer transcriptional activity (pink). Only one of those domains is conserved in HNF-1β. HNF-1β has an alternatively spliced exon (yellow) that codes for an additional 26-amino acid that can recruit transcriptional cofactors. The identity between various domains of HNF-1α and HNF-1β is shown.

The members of the HNF1 family of transcription factors have a similar protein domain structure. The amino termini of HNF-1α and HNF-1β contain 32-amino acid dimerization domains, which facilitates obligatory hetero- or homo dimerization that is required for DNA binding. The dimerization domain is necessary and sufficient for dimerization. Although HNF1 proteins can dimerize in solution in the absence of any other molecules, *in vivo* the dimerization appeared to be regulated by the small protein named Dimerization Cofactor of HNF1 (DCoH). DCoH was originally identified by co-purification with HNF-1α and does not participate in DNA binding or have intrinsic transcriptional activity.

Two molecules of DCoH interact with the HNF1 dimers forming a tetrameric structure that is resistant to the exchange of individual subunits. DCoH is believed to stabilize the dimerized HNF1 complex for maximal transcriptional activity (6).

The HNF1 family members are classified as POU-homeodomain containing proteins. The POU domain is named for the first four transcription factors, Pit-1, Oct-1, Oct-2, and Unc86, in which it was identified. The POU domain is composed of two subdomains, an amino-terminal POU-Specific-domain (POU_S) followed by the POU-Homeodomain (POU_H). The POU_S and POU_H subdomains are separated by a variable linker. The POU_S and POU_H domains are responsible for sequence-specific DNA binding (7). At the amino terminus of the POU_H domain in HNF1 proteins is a cluster of basic amino acids that direct nuclear localization (8).

The carboxyl termini of HNF-1α and HNF-1β, while only 47% identical, contain transcriptional activation domains. Previous studies have demonstrated that HNF-1α and HNF-1β have different transactivational capacities, which could be a result of their different carboxyl termini (9, 10). In the case of HNF-1α, three regions were identified that contribute to transactivation. The three domains, named ADI, ADII, and ADIII, respectively, are enriched with the specific amino acids serine, proline, and glutamine. The ADI and ADIII domains combined together yielded transactivation *in vitro*, whereas ADI and ADII are involved in transactivation in an *in vitro* transcription assay (11). However, in HNF-1β only the glutamine-rich domain is present in the carboxyl terminus. The transactivation domain of HNF-1β was further defined using a series of GAL4 fusion proteins with carboxyl-terminal deletions, testing activity in GAL4-responsive promoter-reporter assays. The core transcriptional activation domain was shown between amino acids 352 and 483, which overlaps the glutamine-rich domain (12).

The HNF-1β isoforms generated by alternative splicing increase HNF-1β protein diversity. The first isoform, isoform A, contains a 26-amino acid-coding exon between the POU_S and POU_H, which is excluded in isoform B. Isoform A has a greater transactivational capacity in reporter assays (10). The amino-terminal half of isoform A was shown to interact with the co-activators CBP and P/CAF (13), whereas an amino-terminal half of isoform B was not able to interact with these proteins (12). The interactions between co-activators and isoform A and not isoform B may explain this different transcriptional activation. Isoform C is generated when a 5' splice site is repressed and an alternative poly-A site is used. The third mRNA isoform encodes a protein that contains the dimerization domain and both POU subdomains but has a unique 50-amino acid truncated carboxyl terminus. The A and B isoforms have relatively eqimolar ratios in the tissues tested, and the C isoform is expressed at lower levels. The A and B isoforms are the most well-studied, partly because the C isoform has been isolated only in rat (14).

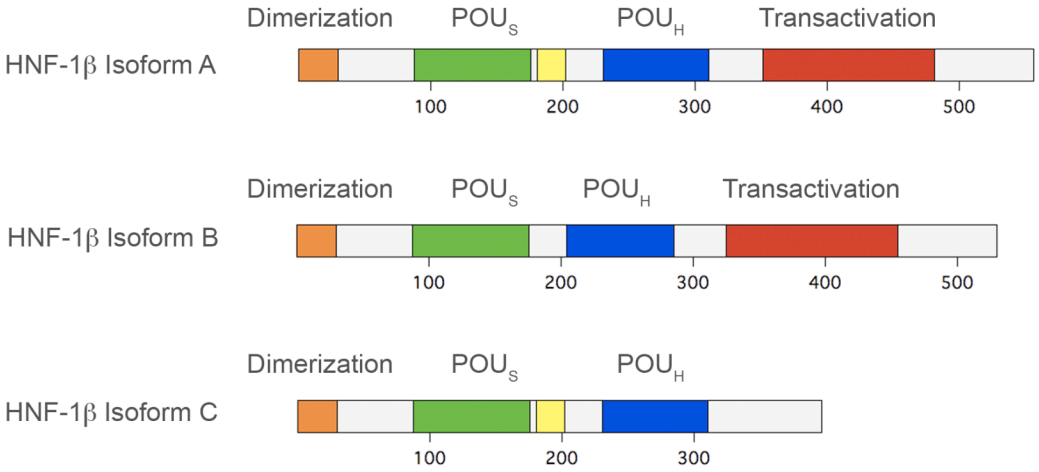


Figure 1-2. HNF-1β mRNA isoforms.
These three isoforms all contain an amino-terminal dimerization domain (orange) that mediates obligatory homo- or heterodimerization among HNF1 family members. The POU_S (green) and POU_H (blue) domains are responsible for DNA binding. An alternative exon (yellow) codes for 26-amino acids that may play a

role in transcriptional activation and protein-protein interactions. A carboxyl-terminal transactivation domain (red) was defined by deletion mapping. The isoform C does not use a splice site and creates an alternative carboxyl terminus that does not contain an activation domain.

Expression Pattern of HNF-1β

HNF-1β is expressed early in development through organogenesis and is expressed in the specialized epithelia of the adult kidney, liver, pancreas, and intestine. Initial characterization of HNF-1β expression in the developing mouse came from *in situ* hybridization experiments. This analysis detected HNF-1β transcripts as early as E6.5 in the visceral endoderm (15). HNF-1β transcripts were mainly observed in the extraembryonic endoderm, although not in the parietal endoderm. At E7.5, HNF-1β transcripts were detected in the columnar visceral endoderm and correlated with HNF-1 transcriptional target gene expression. The early HNF-1β expression pattern was further refined with the generation of a HNF-1β-targeted lacZ knockin mouse (16). In this mouse, β-galactosidase expression is under the control of the HNF-1β regulatory elements and was detected as early as E4.5 in the primitive endoderm. At E5.5, expression was detected in the parietal endoderm and the visceral endoderm but expression became progressively restricted to the columnar visceral endoderm. At E7.5, HNF-1β expression was detected in the emerging definitive endoderm. Subsequently, a high level of expression was detected in the neural tube and gut. The expression in the neural tube became restricted ventrally and to the roof plate and finally disappeared around E12. At E9.5, expression was detected in the hepatic and pancreatic precursors, as well as the epithelia in the gut.

In the kidney, lacZ expression was detected in the mesonephros at E10 (17). Expression was also detected in the ureteric bud, which emerged from the

Wolffian duct at E11.5. HNF-1β expression is detected throughout the subsequent stages of kidney development in the epithelia of the maturing nephrons. The Müllerian duct appears in conjunction with the development of the Wolffian duct. These ducts and their derivatives give rise to the genital tracts. In males, β-galactosidase is detected in the epithelia comprising the vas deferens, the prostrate, and the epididymis. In females, expression is observed in the oviduct and uterus. In the adult mouse, HNF-1β is expressed along the entire length of the nephron of the kidney, throughout the biliary system of the liver and in the endocrine cells of the pancreas.

HNF-1β in Humans

The initial discovery of a mutation in HNF-1β leading to disease in humans was based on the previous association of HNF-1α with Maturity-Onset Diabetes of the Young (MODY). MODY is a monogenic, autosomal dominant inherited form of diabetes mellitus that usually presents by the age of 25 with abnormal glucose-stimulated insulin secretion. Horikawa and colleagues screened 57 unrelated Japanese subjects with MODY for mutations in HNF-1β and DCoH (18). A R177X nonsense mutation was identified in one subject and was not observed in any unaffected control subjects. Pedigree analysis showed that the R177X mutation was present in family members that had MODY and nephropathy. This mutation is predicted to produce a truncated HNF-1β protein lacking the homeodomain and the carboxyl-terminal activation domain. The truncated protein was not able to stimulate a target promoter-reporter system and did not inhibit transcriptional activity of the wild-type protein. Therefore, the R177X mutation appeared to be a loss-of-function mutation, with a phenotype

that arises from haploinsufficiency of HNF-1β. Subsequently, mutations in HNF-1β associated with MODY were grouped together as MODY type 5.

Since the original discovery, a number of additional mutations in HNF-1β have been identified through screening patients with diabetes or severe renal disease. Mutations identified to date include splice site mutations, deletions, and missense and nonsense codon changes. The phenotype of patients with HNF-1β mutations has expanded since the originally identified mutation. The renal manifestations associated with MODY are varied and are often associated with cystic abnormalities such as simple cysts, multicystic renal dysplasia, and glomerulocystic kidney disease (GCKD). Other renal abnormalities include renal hypoplasia, agenesis, oligomeganephronia, and familial juvenile hyperuricemic nephropathy (FJHN). Genital malformations have been documented in a few cases of MODY, such as uterine hypoplasia or a bicornate uterus. However, recent studies have suggested that HNF-1β mutations in humans may not be confined to the MODY type 5 disorder. A recent survey showed that subjects with sporadic renal hypoplasia or dysplasia and chronic kidney disease may have mutations in HNF-1β (19). This study suggested that a much larger population of individuals could have kidney disease stemming from mutations in HNF-1β. A second study examined whether SNPs in MODY genes are associated with type II diabetes. This study demonstrated that a SNP within a noncoding region of the HNF-1β gene was associated with type II diabetes in a small population (20). Furthermore, HNF-1β may also play a role as a tumor suppressor gene in the context of either renal or ovarian cancer. Biallelic inactivation of HNF-1β has been identified in chromophobe renal cell carcinoma (21). Studies of ovarian cancer cell lines and primary ovarian cancer observed an epigenetic inactivation of HNF-1β (22, 23). Lastly, a study showed that a mutation in HNF-1β was associated with neonatal diabetes and intrauterine growth retardation (24). Taken

together these studies demonstrated that HNF-1β is a pleiotropic regulator of human health.

Model systems for HNF-1β Studies

To further understand HNF-1β function, a diverse set of strategies has been employed to understand the molecular mechanisms that lead to disease. The HNF1 family is an evolutionarily conserved gene family with representatives identified in most vertebrates. Therefore, researchers have investigated HNF-1β function in cell culture systems, frogs, zebrafish, mice, rats, and humans.

To assay the biochemical function of HNF-1β, cell culture systems have been employed in combination with promoter-reporter systems. Beginning with the initial identification of the R177X mutation, promoter-reporter assays have been used to examine the functional ramifications of mutations in HNF-1β. Promoter-reporter assays have described both loss-of-function mutations and gain-of-function mutations in HNF-1β. Loss-of-function mutations were identified by experiments that demonstrated that HNF-1β mutant protein is unable to activate target promoters and had no effect on wild-type HNF-1β. To better understand the molecular mechanism behind the loss-of-function phenotype additional experimental tests have been performed. Using immunofluorescencence microscopy, subcellular localization of wild-type HNF-1β and mutant proteins has been examined (8). The wild-type HNF-1β localized almost exclusively to the nucleus, and mutations that either delete or alter the sequence between amino acids 177 and 243 resulted in aberrant localization. Mutant proteins that localized to the cytoplasm do not affect the nuclear localization of wild-type protein. The ability of HNF-1β to hetero- or homodimerize is essential for function. Gel retardation experiments tested HNF-

1β mutant proteins' ability to dimerize. These experiments suggested that S151P, E101X, R177X, P159delT, Q243delC, R137-K161del mutant proteins do not heterodimerize with wild-type HNF-1β (8). DNA binding of HNF-1β mutant protein has been tested by electrophoretic mobility-shift assay (EMSA). A number of mutations have been identified in the POU_S and POU_H domains that abrogate DNA binding and resulted in a loss-of-function phenotype.

Some HNF-1β mutations appeared to act as gain-of-function mutations by producing a potent transactivator. The S36F mutation, identified in two families with diabetes, has a higher transactivational capacity in reporter assays (25, 26). Two mutations in HNF-1β that code for truncated proteins have been characterized as dominant-negative mutations (12, 27). These mutations lead to increased complexity when attempting to define the molecular mechanism leading to disease.

Promoter-reporter assays are useful for identifying the functional effects that mutations might have on HNF-1β but assume the mutant protein is produced. Haploinsufficiency may result from mutant transcripts being targeted to the nonsense-mediated decay pathway. Using a nested RT-PCR approach, investigators were able to assay for the presence or absence of mutant HNF-1β transcripts from isolated renal tubule cells. For some mutations, smaller amounts of mutant HNF-1β transcripts were detected compared to wild-type transcripts. This result suggested that those HNF-1β mutant transcripts are probably directed to the nonsense-mediated decay pathway (28).

The cell culture model system has provided insight into the molecular function of HNF-1β and the effect mutations might have on gene function. The cell culture experiments allowed for the general characterization of molecular function. Although cultured or primary cell lines are representative of tissues *in*

vivo, the experiments above do not model the physiological effects of HNF-1β mutations in the whole animal.

Knockout Mouse

A common genetic technique to study gene function is to generate a null mutant by removing, replacing or interrupting the coding sequence of a gene. The generation of an HNF-1β-deficient mouse was performed by two groups using similar strategies (16, 29). The targeting vectors designed by both groups replaced exon 1 of the mouse HNF-1β gene with the lacZ gene. Both groups' resulting heterozygous- and homozygous HNF-1β-deficient animals were virtually identical in phenotype. The heterozygote mice were normal in phenotype, in contrast to the human condition, and the homozygous null mice failed to survive past E7.5. Homozygous null animals have a loss of extraembryonic tissue or visceral endoderm and a disordered ectoderm at E6. To confirm that the visceral endoderm defect is responsible for the observed lethality and to examine subsequent developmental requirements for HNF-1β, tetraploid aggregation experiments were performed. This experiment generated chimeric embryos in which the extraembryonic tissue has a wild-type genotype, whereas the embryo has HNF-1β inactivated. The resulting chimeric embryos were rescued for the perimimplantation defect and survived to E9.5, and thus confirmed that HNF-1β is required for visceral endoderm formation. The chimeric embryos had neural tube defects, overgrowth of the neural folds, a large allantois, and distended pericardia. Lineage tracing of HNF-1β null cells by β-galactosidase expression suggested that HNF-1β inactivated cells were not excluded from any specific tissue. These studies described HNF-1β function in early development but did not examine the role of HNF-1β in subsequent organogenesis.

Conditional Knockouts

The traditional knockout strategy demonstrated an early developmental role for HNF-1β but yielded little insight into tissue-specific functions. The early lethality of null embryos precluded a more detailed analysis of HNF-1β function in organogenesis or adult organs.

To address the tissue-specific function of HNF-1β, conditional knockout strategies were employed to determine the function of HNF-1β in a given tissue. A conditional knockout strategy was designed using the Cre-lox recombination technology (30). Pontoglio and colleagues generated a mouse with HNF-1β exon 1 flanked by loxP sites and after crossing with a tissue-specific Cre recombinase mouse line a null allele of HNF-1β would be generated in that tissue (31). This "floxed" HNF-1β mouse were crossed to three Cre-expressing mouse lines. The *AlfpCre* transgene contains regulatory elements that drive expression in the hepatic bud beginning at E10. Therefore, when *AlfpCre* was crossed with the "floxed" HNF-1β, liver-specific inactivation of HNF-1β was generated. The resulting mice had severe growth retardation and jaundice. Inspection of the derivatives of the hepatic bud revealed gallbladder epithelial dysplasia and a scarcity of intrahepatic bile ducts. Lipid metabolism was altered in the HNF-1β-deficient liver perhaps because of reduced bile flow from the reduced number of intrahepatic bile ducts. This study provided the first analysis of HNF-1β function in the developing mouse liver (31).

The "floxed" HNF-1β mouse was also crossed to the RIP-Cre transgenic mouse line, thus inactivating HNF-1β in pancreatic β cells. The rat insulin promoter (RIP) drives expression in the pancreatic β cells but only weakly during pancreatic development. The mice with HNF-1β inactivated in the pancreatic β cells were phenotypically normal, except for impaired glucose tolerance (32). However, the RIP-Cre-HNF-1β mouse is a poor model of human subjects with

MODY type 5. The mice exhibited glucose intolerance only during a glucose tolerance test, whereas patients with MODY type 5 have fasting plasma hyperglycemia. Second, the onset of glucose intolerance in mice was consistent at two months of age, although the onset of diabetes in humans is highly variable. To further understand HNF-1β function in the pancreas, a study used tetraploid aggregation to rescue the early lethality associated with constitutive HNF-1β inactivation and allowed analysis of later stages in development (33). This chimeric mouse showed a complete absence of the ventral pancreatic bud and a paucity of the dorsal bud that led to complete pancreatic agenesis. Taken together, the RIP-Cre-HNF-1β mouse and the chimeric HNF-1β rescue mouse demonstrated a continued requirement for HNF-1β function in pancreatic development and function.

Kidney-specific inactivation of HNF-1β was tested by crossing the "floxed" HNF-1β mice with transgenic Ksp-cadherin Cre mice (34). The Ksp-cadherin promoter drives expression along the length of the nephron beginning from the earliest ureteric bud stages. The kidney-specific inactivation of HNF-1β led to a severe cystic phenotype. The cysts found along the length of the nephron were lined with HNF-1β inactive cells. The cystic phenotype observed in this model corresponded to the human renal cystic disease.

In addition to mouse studies with wild-type HNF-1β disrupted, MODY transgenic mouse lines were generated. Two transgenic animal lines were generated in which mutant HNF-1β was expressed specifically along the length of the nephron under the control of the Ksp-cadherin promoter. The mutant HNF-1β coding sequences chosen corresponded to two mutations described in MODY patients. Both transgenic mice were observed to have severe renal cysts (12, 27).

The MODY transgenic mouse and the kidney-specific HNF-1β knockout mouse both resemble the renal cysts found in patients. To examine the molecular

mechanism behind cystogenesis in these models, expression of known cystic disease-associated genes was assayed by Q-RT-PCR and *in situ* hybridization. In both studies, expression of *Pkhd1*, the gene associated with autosomal recessive polycystic kidney disease (ARPKD), had reduced expression in the mutant or transgenic mouse compared to wild-type (27, 34). Two HNF-1β binding sites 5' of the *Pkhd1* transcription start site were identified by chromatin immunoprecipitation (ChIP). Hiesberger and colleagues cloned the mouse *Pkhd1* proximal promoter and identified a HNF-1 site. The binding site, located at -49 to -62 bp from the transcription start site, was bound by HNF-1β *in vitro*. The proximal *Pkhd1* promoter was active in mIMCD-3 murine renal epithelial cells that endogenously expressed *Pkhd1* and *Hnf-1β*. Mutation of the HNF-1 site abolished expression of a promoter-reporter gene. Transient transfection of two truncated HNF-1β proteins acted in a dominant-negative fashion on *Pkhd1* expression by forming dimers with mutant and wild-type HNF-1β protein. The HNF-1β (A263insGG) mutant protein cannot bind DNA and prevented wild-type HNF-1β protein from binding in a dose-dependent fashion (27). The HNF-1β (P328L329fsdelCCTCT) mutant protein can bind DNA either as a homodimer or as a heterodimer with wild-type HNF-1β but not activate transcription because of the loss of the carboxyl terminus. These studies together identified the cystic disease gene, *Pkhd1*, as a transcriptional target of HNF-1β and linked together two genetic diseases in a common pathway.

UBIQUITINATION

Introduction

Ubiquitination is the biochemical ligation of the small protein, ubiquitin, to a target protein. Ubiquitination is a reversible post-translational modification that affects biochemical processes including endocytosis and transcriptional activity but most commonly protein stability. Ubiquitination can also modify the protein ubiquitin, thus forming polyubiquitin chains. Ubiquitin chain formation has increased the functional diversity of this post-translational modification. Perturbations of ubiquitination were associated with a number of disease processes including neurodegenation, muscle atrophy, and cancer.

Biochemical Pathway

Ubiquitin is a highly conserved 76-amino acid protein present in every eukaryotic cell. The ubiquitin protein is identical from insects to humans, and only three amino acids are different between human and yeast ubiquitin. Ubiquitin has a well-defined structure that is resistant to proteolysis. Ubiquitin is synthesized as a propeptide that is proteolytic processed to generate the mature 76-amino acid species. The cleavage is catalyzed by thiol proteases that reveal a glycine on the carboxyl terminus of ubiquitin.

Following cleavage, ubiquitin enters a multistep enzymatic pathway that delivers ubiquitin to the target protein. Initially, ubiquitin is activated by the E1 ligase in an ATP-dependent process. An ubiquitin-AMP intermediate is formed before the final E1-ubiquitin thiol ester is formed. Ubiquitin E2 ligases, of which 11 are known in humans, recognize and bind the E1-ubiquitin thiol ester. The

ubiquitin is transferred from the E1 ligase to the E2 ligase via another thiol ester linkage. The E2, often called the ubiquitin-carrier enzymes, then forms a complex with an E3 ubiquitin ligase. A conserved core in E2 ligases mediates the binding with the E3 ligase. In turn, the E3 ubiquitin ligases do not require a specific E2 ubiquitin ligase. There are an expanding number of ubiquitin E3 ligases, possibly totaling in the hundreds in the human genome. The E3 ligases have been grouped into three categories based on conserved domains. The first group consists of the HECT domain ligases that form a thiol ester bond with ubiquitin before transferring ubiquitin to the target protein. The second and third groups consist of RING finger domain-containing proteins or U-box-containing proteins. Both groups facilitate the transfer of ubiquitin to the substrate protein directly as opposed to forming an intermediate ubiquitin-E3 conjugate. The E3 ubiquitin ligases are responsible for target recognition, and they accomplish this by binding either directly to the substrate or via an adaptor protein.

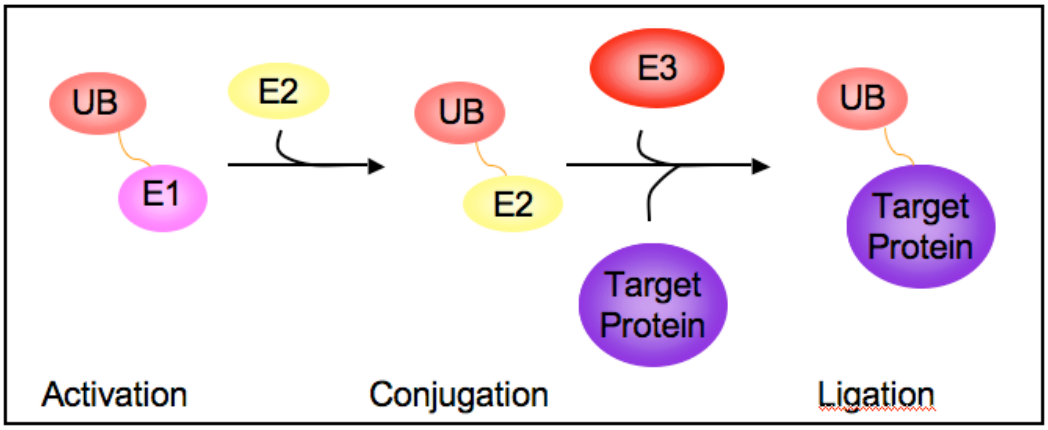


Figure 1-3. The Ubiquitination Pathway.

After proteolytic processing, ubiquitin enters a biochemical pathway by being conjugated to an E1 ligase in an ATP-dependent process. The ubiquitin is then transferred from the E1 ligase to the E2 ligase. The E2 ligase then forms a complex with an E3 ligase and the target protein. The ubiquitin is then transferred to the target protein directly or through an intermediate linkage with the E3 ligase.

After the ubiquitin is conjugated to the target protein, the ubiquitin molecule itself can be ubiquitinated, thus a chain of ubiquitin molecules is formed. The ubiquitin protein has seven lysines that can be ubiquitinated. Although all seven lysines may form chains, the Lysine-48 (K48) and Lysine-63 (K63) linkages are the best characterized. A polyubiquitin chain composed of four or more ubiquitin molecules linked via K48 will recruit the proteasome and facilitate the destruction of the target protein. The K63 chains have been linked to signal transduction and DNA repair pathways and not to target protein destruction. The ubiquitin chains can be removed from target proteins by ubiquitin-processing enzymes or ubiquitin carboxyl-terminal hydrolases. For K48 polyubiquitin chains, removal of the polyubiquitin chain from target protein occurs before destruction by the proteasome. The removal of K63 polyubiquitin chains is less well understood. Although ubiquitination of a target protein may lead to future ubiquitin chain formation, monoubiquitination is emerging as an important regulatory modification of membrane-bound receptors and transcription factors.

Role in Transcriptional Regulation

The connection between ubiquitination and transcription was originally made from the study of the first identified ubiquitin-conjugated proteins, histones. Since this initial observation, the field of ubiquitination has focused on the role in protein degradation, culminating in the awarding of the Nobel Prize in Chemistry to Hershko, Ciechanover, and Rose in 2004 for the discovery of ubiquitin-mediated protein degradation. However, in recent years, the connection between ubiquitination and transcriptional regulation has received new attention.

Transcription factors are regulated initially by activation of the protein, and subsequently the protein is deactivated. Destruction of the transcription factor is

an effective means of deactivation. This hypothesis was explored in two early studies. The first demonstrated that degradation of transcriptional activators by the proteasome correlated with activation potency (35). The second study demonstrated that the domains responsible for transcriptional activation overlapped with domains responsible for protein stability (36). These studies formed a foundation linking ubiquitin-mediated destruction with the intrinsic transactivational capacity of transcription factors.

The functional effects of transcription factor ubiquitination in higher eukaryotes were first described in 2003. The major histocompatibility complex (MHC) class II transactivator (CIITA) was shown bound to a promoter in an ubiquitinated state (37). In an impressive study, a component of the E3 ligase complex that ubiquitinates c-myc was demonstrated to also act as a transcriptional cofactor for c-myc (38). These studies and others enforced the concept that ubiquitination positively regulated transcription factor activity at the promoter.

In an effort to mechanistically understand the function of an ubiquitin linkage to a transcription factor, the LexA-VP16 artificial transcription factor was employed as a model system. The LexA DNA-binding domain is fused to the viral protein VP16 transactivation domain to create an artificial transcription factor. The LexA-VP16 transcription factor activates transcription of reporter genes containing LexA-binding sites in eukaryotic cells. By screening yeast deletion strains, Met30, an ubiquitin E3 ligase, was identified as a positive regulator of LexA-VP16 activity. Removal of Met30 function resulted in a loss of LexA reporter activity in the presence of LexA-VP16 protein. However, addition of LexA-VP16 fused to ubiquitin rescued reporter activity in a Met30 null background. This study demonstrated that monoubiquitination of a transcription factor was sufficient to promote transcription factor activity (39). In a subsequent study, p-TEFb, an important transcription elongation factor, showed increased recruitment to an ubiquitinated LexA-VP16 (40). These studies demonstrated that

monoubiquitination increased transcriptional activity perhaps by increased RNA polymerase elongation rates. However, another ubiquitin E3 ligase complex stimulated GAL4 turnover, GAL4-dependent transactivation, and GAL4 target gene cotranscriptional mRNA processing (41). This study suggested that monoubiquitination of transcriptional activators may act on multiple steps in transcription.

To place into context the effect of ubiquitination on transcriptional activation, the stability of the transcription factor must be examined. In a review by Kodadek and colleagues, three models were proposed for integrating the action of ubiquitination and transcription (42). The three models were the timer model, the black widow model, and the second-generation timer model. The timer model suggested that monoubiquitination of the transcription factor permits transcription initiation. The transcription factor remains active until a polyubiquitin chain containing four ubiquitins linked through K48 has formed. The polyubiquitinated transcription factor is then recognized by the proteasome and destroyed. Therefore, each transcription factor molecule has a finite lifetime in which to activate transcription (43, 44). The black widow model builds off the timer model by incorporating the results of a study performed on Gcn4. This study demonstrated that Gcn4, a yeast transcription factor, is phosphorylated by a Mediator complex component, Srb10, that is present on active promoters. Phosphorylation of Srb10 triggered the subsequent polyubiquitination of Gcn4 (45). The black widow model proposed that interaction of a transcription factor with the RNA polymerase complex triggers the ubiquitination and subsequent destruction of that transcription factor (46). Finally, the second-generation timer model added an additional layer of regulation. Ubiquitin chain elongation is a regulated balance between the elongation of the ubiquitin chains catalyzed by E4 ligases and shortening of the ubiquitin chain by deubiquitinating enzymes. Regulation of these rates could be initiated by polymerase complex or by

independent factors. This model allowed additional variables to be considered that may have been omitted previously.

The field of ubiquitinated transcription factors is still undeveloped. Many questions remain to be answered in this field with few defined molecular model systems. What steps of transcription require the ubiquitinated form of a transcription factor? When and where is a transcription factor ubiquitinated? Do alternative ubiquitin linkages play a role in this process? The understanding of these and other questions will allow for a better understanding of gene regulation *in vivo*.

SUMOYLATION

Introduction

SUMOylation is a reversible post-translational modification of proteins in which a Small Ubiquitin-like Modifier (SUMO) protein is covalently bonded to a target protein. The SUMO proteins belong to the Ubiquitin-like protein (UBL) family, and SUMOylation mirrors the ubiquitination enzymatic cascade (47). SUMO proteins and ubiquitin have a similar molecular mass, and their amino acid sequences are 20% identical. Both ubiquitin and SUMO are initially synthesized as propeptides that undergo a proteolytic cleavage yielding the mature protein containing a carboxyl-terminal glycine. The mature SUMO protein enters an enzymatic cascade that links SUMO to an E1 activating enzyme. The SUMO is then transferred to an E2 ligase before binding to the target protein and an E3 ligase. In this final complex, the SUMO is conjugated to the lysine in the target protein. Removal of the SUMO protein from the target protein is accomplished by a family of isopeptidases. SUMOylation has been shown to regulate protein complex formation, protein-DNA interactions, protein stability, subcellular localization, subnuclear localization, and transcription factor activity. Disruption of the murine SUMO E2 ligase, Ubc9, is lethal, emphasizing that SUMOylation is an essential biochemical modification (48).

Biochemical Pathway

The SUMOylation pathway is analogous to the ubiquitination pathway; however, differences between them merit further explanation. Ubiquitination involves a single protein, ubiquitin, whereas there are four SUMO proteins, SUMO-1, SUMO-2, SUMO-3, and SUMO-4. SUMO-1 shares 44% identity with

SUMO-2 and SUMO-3, although SUMO-2 and SUMO-3 are 86% identical (49). The SUMO-4 gene should be considered separately from the rest of the SUMO genes. The SUMO-4 gene is transcribed highest in the kidney; however to date no protein has been detected (50). Additionally, the putative SUMO-4 proprotein would be resistant to proteolytic maturation because of a unique proline-90 and thus would not be a substrate for SUMO conjugation (51).

The SUMO family proproteins enter the SUMO conjugation pathway after a proteolytic cleavage. The proteolytic cleavage is catalyzed by a family of isopeptidases called the SENP family (SUMO-specific proteases). The SENP family proteins are cysteine proteases with two functions, the first is a C-terminal hydrolase activity that processes the SUMO proprotein and the second is the cleavage of the isopeptide bond that links SUMO to the target protein. The catalytic core is conserved in the C-terminal domain of the SENP family, whereas the amino terminus is variable and may regulate subcellular localization and target specificity. The SENP enzymes discriminate between individual SUMO proteins. As an example, SENP-5 did not process SUMO-1 but cleaved SUMO-2 or SUMO-3 (52).

After proteolytic cleavage, the mature SUMO protein is conjugated to an E1 activating enzyme complex. In contrast to the ubiquitin pathway in which the E1 ligase is a monomer, the E1 is a heterodimeric complex that separates the adenylation and thioesterification activity into SAE1 and SAE2 subunits, respectively. The SAE1/SAE2 complex facilitates the formation of an adenylated SUMO protein by linking the C-terminus to AMP. Subsequently, the bond is broken and the same C-terminus forms a thiol ester bond with a cysteine in SAE2. The SUMO/SUMO E1 complex proceeds to interact with Ubc9, the SUMO E2 enzyme. Ubc9 is the only known SUMO E2 ligase identified to date.

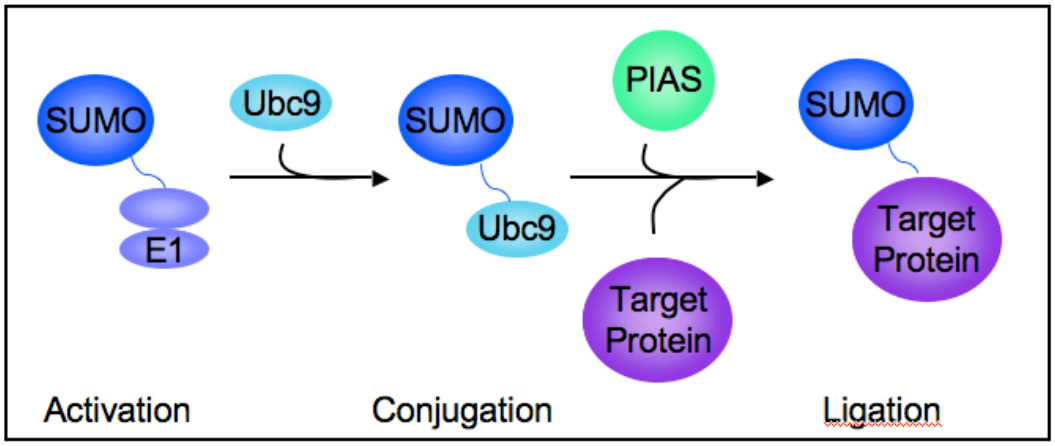


Figure 1-4. The SUMOylation Pathway.

The mature SUMO protein enters the SUMO modification pathway by conjugation to a heterodimeric E1 ligase in an ATP-dependent process. The SUMO is transesterified to Ubc9, the SUMO E2 ligase, yielding a thiol ester. The SUMO-Ubc9 conjugate then forms a complex with the target protein and a SUMO E3 ligase (such as the PIAS family proteins as shown above). The SUMO is then conjugated to a lysine in the target protein.

The SUMO is transesterified from the cysteine in SAE2 to cysteine-93 in Ubc9, yielding an Ubc9-SUMO thiol ester. Ubc9-SUMO complex catalyzes the formation of an isopeptide bond between the carboxyl terminus of SUMO and the amino group on a target lysine. Target proteins are recognized by Ubc9 via a consensus motif without any additional factors. This mechanism sharply contrasts with the ubiquitination pathway where target specificity is defined by the E3 ligase. The consensus target motif was first described as psi-K-X-E, where psi is a large hydrophobic amino acid and X represents any amino acid (53). Exceptions to the SUMO target site have been characterized, and not all consensus SUMO target sites are modified. Recently, two studies have further refined definition of SUMO consensus site (54, 55). Phosphorylation preceded SUMOylation for some transcription factors such as MEF2 and HSF family proteins. Further investigation identified a phosphorylation-dependent SUMO motif (PDSM) as an additional

SUMOylation consensus site. The PDSM is the conventional SUMOylation site followed by a SP phosphorylation motif, or psi-K-X-E-X-X-S-P (56). Sharrocks and colleagues identified acidic residues carboxyl-terminal to the target site as important thus yielding the negatively charged amino acid-dependent SUMOylation motif (NDSM) (57). The NDSM motif has been a successful predictive tool for identifying sites of SUMOylation.

Ubiquitin modification of proteins exists in three states, monoubiquitination, multi-ubiquitination, and poly-ubiquitination. Similarly, SUMO-modified proteins can be monoSUMOylated, multi-SUMOylated, and poly-SUMOylated. MonoSUMOylation consists of a single SUMO, SUMO-1, SUMO-2 or SUMO-3, attached to a target protein. Multi-SUMOylation is the addition of two or more SUMO proteins at different lysines on a single protein. PolySUMOylation is a chain of SUMO proteins that are linked to a single lysine. SUMO-2/3 contain consensus SUMO sites that are modified *in vivo*, whereas SUMO-1 cannot be SUMOylated (58). SUMO-1 can be incorporated into a polySUMOylation chain but SUMO-1 incorporation terminates chain elongation. The function of SUMO-2/3 chains is not well understood but may result in more robust transcriptional repression (59).

Four types of SUMO E3 ligases have been described. The first type is represented by a single large nuclear pore component enzyme called RanBP2/Nu358. RanBP2 is not homologous to any known ubiquitin E3 ligase. The RanBP2 SUMO E3 ligase activity resides in a 30 kDa domain. The domain consists of two 50-amino acid domains (IR1 and IR2) separated by a spacer (M). The IR1 domain bound Ubc9 with high affinity, and the IR2 domain bound SUMO-1 preferentially. RanBP2 does not interact with target proteins directly. RanBP2 is thought to be an allosteric co-factor that alters the structure of the Ubc9-SUMO-1 thiol ester, resulting in the transfer of SUMO-1 to the target lysine. The second type of SUMO E3 ligase is also represented by a single protein

Pc2. Pc2 is a component of the polycomb chromatin-modifying complex that mediates transcriptional repression. The Pc2 protein is akin to RanBP2; the SUMO E3 activity resides in a bipartite domain that is not homologous to ubiquitin E3 ligases. Additionally, the Pc2 catalytic domain does not bind the target protein but directly interacts with Ubc9. The Pc2 protein recruited substrate proteins via another domain, thus forming a platform for SUMOylation (60). The third type of SUMO E3 ligase is represented by the Protein Inhibitor of Activated STAT (PIAS) family of proteins. The PIAS family has at least four members in mammals, PIAS1, PIAS3, PIASx, and PIASγ. The PIAS proteins contain a SP-RING domain that is similar to the RING finger domain of ubiquitin E3 ligases. The PIAS family members contain several conserved protein motifs (Fig. 1-5). The first domain is an amino-terminal SAP domain, a domain named for the proteins: SAFA/B, ACINUS, and PIAS. The SAP domain facilitates interactions between the nuclear matrix and specific regions of DNA, perhaps leading to changes in chromatin structure. These interactions with the nuclear matrix could promote either activation or repression of associated downstream targets. Adjacent to the SAP domain is a PINIT domain. The PINIT domain may function together with the SP-RING domain to mediate the SUMO E3 ligase activity (61). The SP-RING domain is similar to the RING domain of the ubiquitin E3 ligases, except the SP-RING domain lacks the third and sixth cysteine residues (62). PIAS proteins contain a SUMO binding domain (SBD) that facilitates the interaction between PIAS proteins and SUMOylated proteins. The PIAS proteins are thought to promote interactions between the target proteins and Ubc9, thus promoting SUMO conjugation. However, additional functions for PIAS proteins are suggested by experiments that demonstrated PIAS proteins interact directly with SUMOylated proteins. Additionally, previous studies have showed that multiple PIAS proteins may interact with the same target protein allowing for functional

redundancy. This explanation is the most likely for the relatively mild phenotypes observed in the PIAS1 (63) and PIASγ (64, 65) knockout mice.

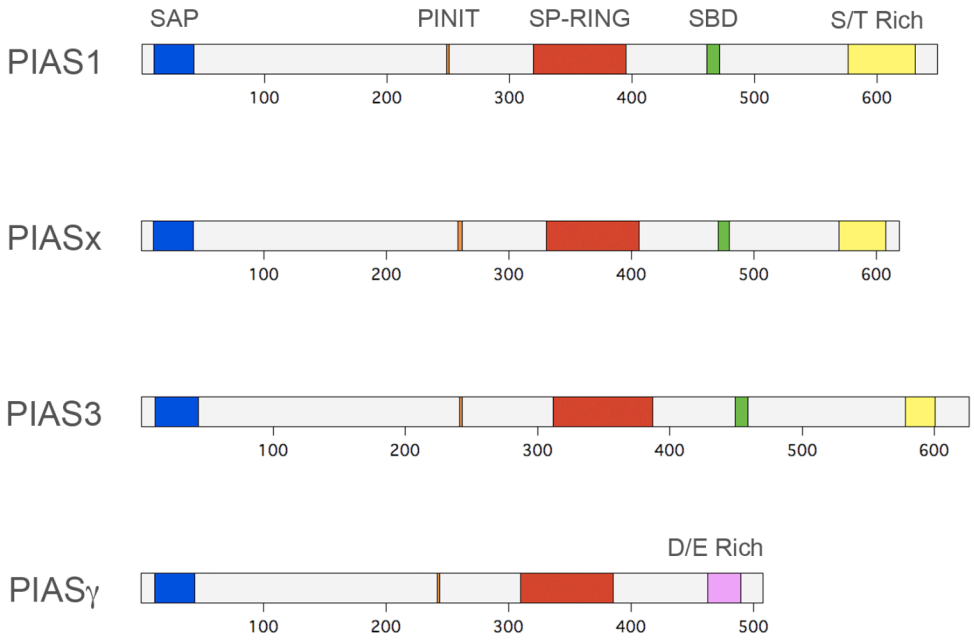


Figure 1-5. The PIAS family of proteins.
An evolutionarily conserved SAP domain (blue) facilitates interactions with the nuclear matrix. A PINIT (orange) and SP-RING (red) mediate the transfer of SUMO from Ubc9 to the target protein. PIAS family proteins may bind SUMO directly via a SUMO-binding domain (SBD) (green). Near the carboxyl terminus, the PIAS proteins have domains rich in Serine/Threonine (S/T) or Aspartate/Glutatmate (D/E).

The fourth type of SUMO E3 ligase is the Zimp family of proteins. The Zimp family has two members in mammals and has a single orthologue in flies. The Zimp proteins are named Zimp7 and Zimp10 for zinc finger-containing, Miz-1, PIAS-like protein on chromosome 7 or 10 (66). The Zimp proteins contain an SP-RING domain, the domain that harbors the SUMO E3 ligase activity in PIAS proteins. Zimp10 can enhance Androgen Receptor (AR) SUMOylation *in vivo* but not as efficiently as PIAS1 (67). The weaker SUMO E3 ligase activity may be

due to other structural elements that are absent in Zimp10, such as the PINIT domain. The Zimp proteins contain strong acidic transactivation domains and function as co-activators of AR-regulated transcription (67, 68). The Zimp transactivation activity may be a result of the interaction between Zimps and the SWI/SNF2 chromatin remodeling complexes. These PIAS-like proteins extend the functional diversity of SUMO E3 ligases.

SUMOylation is a reversible modification, and removal of SUMO protein is catalyzed by SENP family proteins. The regulation of the removal of SUMO from target proteins is not well understood, but SENP enzymes are tightly regulated. SENP enzymes localize to distinct subcellular locales and therefore interact with different sets of SUMOylated proteins. The SENP enzymes display substrate specificity for the conjugated SUMO, for instance SENP-5 preferred SUMO-2/3 conjugates versus SUMO-1 conjugates (52). SENP enzyme expression is regulated transcriptionally, which influences the quantity of SUMOylated protein. To highlight the importance of SENP function, overexpression of SENP1 has been associated with prostate cancer development (69).

SUMOylation alters behavior of target proteins by altering physical interactions. Studies of SUMOylation have demonstrated changes in protein complex assembly, DNA interactions, subcellular and subnuclear localization and effects on activity. However, to date the most common group of proteins that are SUMOylated are transcription factors. Generally, SUMOylation is thought to have a repressive effect on transcriptional activation; however there are examples of stimulatory effects.

Role in Transcriptional Regulation

Nuclear proteins are preferentially SUMOylated. Although SUMOylation has been shown to occur in the cytoplasm, substrate modeling *in vivo* demonstrated that nuclear targeting is required for SUMO modification (53). Studies have linked SUMOylation to various nuclear processes including the cell cycle, DNA topology, DNA repair, antagonism to ubiquitination, nuclear and subnuclear localization, and transcriptional regulation (70). The SUMO E2 and E3 ligases both positively and negatively regulate transcriptional activity, via both SUMOylation-dependent and -independent mechanisms.

SUMOylation of transcription factors affects protein stability, localization, and activity. SUMO modification of a transcription factor can alter both the subcellular and subnuclear distribution of a transcription factor. As an example, SUMO modification is required for Elk-1 to enter the nucleus (71). SUMO modification of Daxx and PML localized these proteins to PML bodies (72-74). Immunolocalization of PIAS proteins also showed a subnuclear focal pattern, and this localization is dependent on their E3 activity. However, co-localization studies of PIAS proteins and PML showed that there is an incomplete overlap of these proteins. This study suggested that there are nuclear bodies containing SUMOylated proteins which are not the transcriptionally silent PML bodies (75).

SUMOylation can target the same lysine as ubiquitination, thus providing a competitive model for regulation. In the case of the inhibitor of nuclear factor κB alpha, IκBα, SUMOylation blocked ubiquitination, which stabilized the protein and inhibited the transcriptional activity of NFκB (76). SUMOylation and ubiquitination competed for the same lysine on the pathogenic protein Htt that is associated with Huntington's disease (77). SUMOylation exacerbated neurodegeneration in a Drosophila model by stabilizing the Htt protein fragment that formed protein aggregates and repressed transcription. Ubiquitination destabilized the protein fragment and led to a reduction in protein aggregates. Mutation of this target lysine reduced disease pathology.

SUMO modification of proteins can alter DNA-protein and protein-protein interactions by inhibiting some associations and promoting novel interactions. The SUMOylation of HSF2 decreased the affinity for DNA but oligomerization was not affected (78). An example of disrupting protein-protein interactions was illustrated by the SUMOylation of the synergy control (SC) motif in SF-1. Some target promoters of SF-1, such as Müllerian inhibitory substance, are regulated by a number of transcription factor complexes. Mutation of a SUMOylated lysine in the SF-1 SC motif allowed for greater synergistic activation with Sox9, suggesting that SUMOylation inhibited this interaction-based activation (79). Chromatin immunoprecipitation (ChIP) experiments demonstrated that SUMO-modified Elk-1 had an increased association with HDAC-2. This association correlated with decreased histone acetylation of target gene promoters (80).

SUMO ligases are able to act as transcriptional cofactors, independent of their ability to SUMOylate target proteins. This bifunctionality appears to rely on non-covalent interactions with target proteins. A SUMO ligase may recruit other cofactors or delocalize the target protein. For example, Ubc9 modulated the transcriptional activation of the glucocorticoid receptor (GR). Simons and colleagues characterized Ubc9 as a transcriptional adaptor as defined by the total increase in GR transactivational capacity. Independence from SUMOylation was demonstrated by a similar effect using a catalytically inactive Ubc9 that bound GR and GMEB (81). Catalytically inactive Ubc9 was generated by mutation of cysteine-93 to a serine, thus preventing the formation of the SUMO-Ubc9 thiol ester but not interfering with the binding of either SUMO or target proteins. A similar phenomenon has been demonstrated for at least three other transcription factors, in which overexpression of a catalytically inactive Ubc9 has the same effect on promoter activity as the wild-type Ubc9 (82-84).

The PIAS proteins were originally identified as modulators of STAT transcriptional activity. Four mechanisms have been described whereby PIAS

proteins can affect transcription factor activity independent of SUMOylation. The first mechanism is relocalization of a transcription factor to a distinct subnuclear localization by a PIAS protein. PIAS1 relocalized Msx1 bound target promoters to the nuclear periphery where they are inactive (85). In addition, PIASγ mediated relocalization of LEF1 to PML bodies (75). The second mechanism is disruption of the transcription factor-promoter interaction. PIAS1 interfered with STAT1 DNA-binding (86). The third mechanism is cofactor recruitment. PIASγ negatively regulated AR transcriptional activity independent of SUMOylation (87). PIASγ interacted with HDACs, and this interaction was not dependent on SUMOylation activity. This study suggested that a PIAS protein could recruit a co-repressor without SUMOylation of the target protein. The final mechanism of a SUMOylation-independent effect by a PIAS protein is perhaps the least intuitive. In this case, PIASx acted as a transcriptional activator for Elk-1 by promoting its deSUMOylation and loss of HDAC-2 binding (88). Although the mechanism by which this transcriptional activation is accomplished is unknown, the SUMOylation activity of PIASx was dispensable.

CHAPTER TWO
SUMO Ligases Regulate HNF-1β

Introduction

Hepatocyte Nuclear Factor-1 beta (HNF-1β) is a member of the HNF1 family of transcription factors. Heterozygous mutations of HNF-1 transcription factors in humans are associated with Maturity-Onset Diabetes of the Young (HNF-1α type 3 and HNF-1β type 5)(89). Patients with MODY type 5 present with an array of renal malformations, most common are cysts along the length of the nephron leading to renal failure. In addition, biallelic inactivation of HNF-1β has been associated with development of renal cell carcinomas underscoring the importance of HNF-1β function(90).

HNF1 proteins contain three conserved protein domains: an amino-terminal dimerization domain that facilitates obligatory hetero- or homo-dimerization, and a POU_S and POU_H that are responsible for sequence-specific DNA-binding. HNF1 family members recruit co-activators to target promoters via their carboxyl terminal domains (12, 13). The transactivation domain is required for proper HNF-1β function *in vivo*. However, the transcription cofactor interactions previously described to require the carboxyl terminus are also mediated by a domain near the POU_S domain in the A isoform. Other factors may interact with HNF-1β through the carboxyl terminus either specifically or in conjunction with other domains within HNF-1β. Additionally, HNF1 family members are similar in the dimerization domain and DNA-binding domains but are divergent elsewhere, potentially explaining the variable activation strength on the same promoters. These divergent portions of HNF-1α and HNF-1β may foster

unique interactions with other proteins. The interaction of other proteins with HNF-1β may play a fundamental role in regulating activity with ramifications throughout development and adult metabolic homeostasis and disease.

Results

SUMO Ligases Interacted with HNF-1β

A yeast two-hybrid screen was performed to identify proteins that interact with HNF-1β. Two bait plasmids were constructed by fusing either the full-length mouse HNF-1β (isoform B) or a C-terminal fragment of HNF-1β (amino acids 264-532) to the LexA DNA-binding domain. Each bait construct was tested for expression and the inability to auto-activate the reporter system (data not shown). An adult mouse kidney cDNA library was screened with both bait proteins. The full-length bait and C-terminal bait screened approximately 11×10^6 and 11.6×10^6 clones and yielded 86 and 80 positive clones, respectively. Sixty-five clones were isolated to homogeneity, and 52 were sequenced. The cDNA sequences were then used to search the Genbank database to identify the putative interacting proteins. Multiple independent clones were identified as opening reading frames encoding the SUMO ligases, Ubc9, PIAS1, and PIASγ. Two clones encoded the SUMO E2 ligase, Ubc9, from amino acids 1-158 and 2-158. The full-length Ubc9 clone was isolated with the full-length bait, while the Ubc9 clone missing the first amino acid was isolated with the C-terminal bait. The full-length bait isolated the single PIAS1 cDNA clone encoding amino acids 2-652. The full-length bait also isolated the two clones of PIASγ that encode amino acids 1-507 and 6-507. All the Ubc9 (Fig. 1A) and PIAS protein clones (Fig. 1B) were re-transformed to verify they did not confer survival under histidine selection and lacZ expression by themselves.

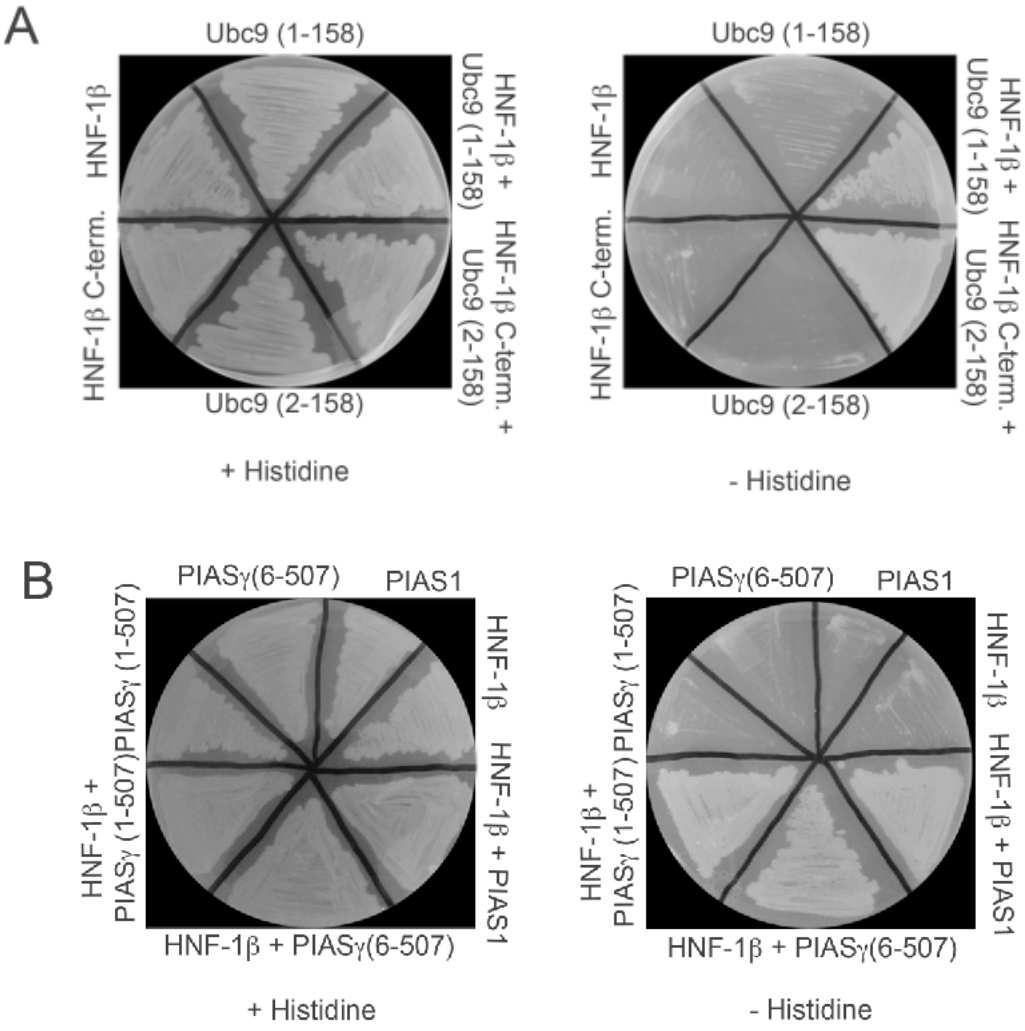


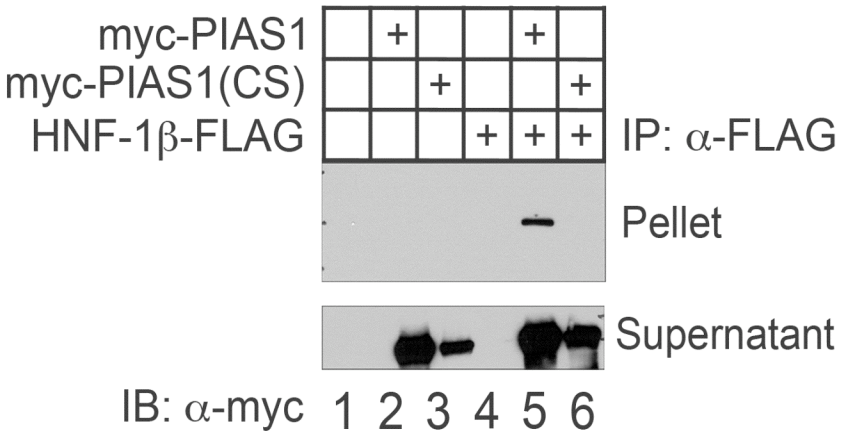
Figure 2-1. SUMO Ligases interacted with HNF-1β in a yeast two-hybrid.
A. Yeast were transformed with either full-length HNF-1β bait, the HNF-1β C-terminal bait, the isolated Ubc9 prey plasmids or both the bait and prey plasmids and were tested for growth under auxotrophic conditions. Yeast only survived in the absence of histidine when both bait and prey plasmids were present.
B. Yeast were transformed with either the full-length HNF-1β bait, the isolated PIAS1, the isolated PIASγ prey plasmids or both the bait and prey plasmids and were tested for growth under auxotrophic conditions. Yeast only survived in the absence of histidine when both bait and prey plasmids were present.

SUMO Ligases Interacted with HNF-1β in vivo

Co-immunoprecipitation experiments were performed to verify that HNF-1β interacted with the SUMO ligases *in vivo*. The co-immunoprecipatations were conducted with 293T cells that were cotransfected with epitope-tagged HNF-1β and epitope-tagged SUMO E3 ligases or mutant versions of the ligases. Immunoprecipitation of HNF-1β-FLAG resulted in co-precipitation of myc-PIAS1 (Fig. 2-2A) indicating that PIAS1 and HNF-1β form a complex *in vivo*. A PIAS1 (CS) mutant protein(91) contains three cysteine to serine mutations in the SP-RING domain which abolish the ability to enhance SUMOylation. This mutant protein was also tested for the ability to interact with HNF-1β. The myc-PIAS1 (CS) mutant was not detected in the anti-FLAG immunoprecipitates but was detected in the supernatant (Fig. 2-2A) indicating that the SP-RING is necessary for the interaction with HNF-1β.

293T cells were cotransfected with epitope-tagged HNF-1β and epitope-tagged PIASγ. Immunoprecipitation of FLAG-PIASγ resulted in co-precipitation of HA-HNF-1β (Fig. 2-2B). The PIASγ (AA) mutant contains the LXXLL coregulator signature motif mutated to LXXAA(92). The PIASγ (W363A) mutant contains a mutation in the SP-RING of PIASγ that is unable to enhance SUMOylation(87). The HA-HNF-1β was also co-precipitated in the presence of two PIASγ mutants, PIASγ (AA) and PIASγ (W363A) (Fig. 2-2B). These results indicated that PIASγ can form a complex with HNF-1β *in vivo* and mutation of the SP-RING domain and the LXXLL domain does not prevent this interaction.

A.



B.

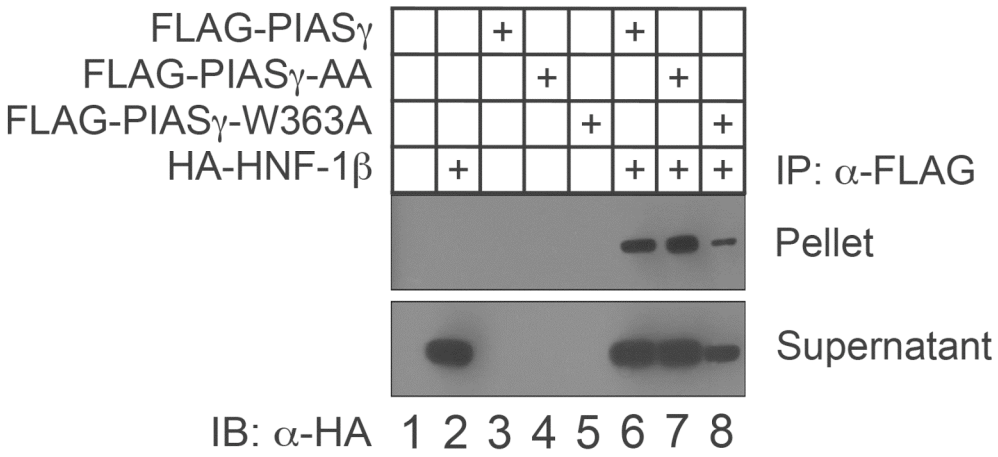


Figure 2-2. SUMO ligases interacted with HNF-1β *in vivo*.
A. 293T cells were transiently transfected with 4.0 μg of myc-tagged PIAS1 expression plasmid together with pcDNA3 or FLAG-tagged HNF-1β expression plasmid. Cells were lysed and subjected to immunoprecipitation with mouse monoclonal anti-FLAG antibodies. PIAS1 was detected by immunoblotting using an anti-myc antibody. B. 293T cells were transiently transfected with 4.0 μg of FLAG-tagged PIASγ expression plasmid together with pcDNA3 or HA-tagged HNF-1β expression plasmid. Cells were lysed and subjected to immunoprecipitation with mouse monoclonal anti-FLAG antibodies. HNF-1β was detected by immunoblotting using an anti-HA antibody.

SUMO Ligases and HNF-1β are co-expressed in the kidney

Co-localization studies were performed to validate potential interactions between the SUMO ligases and HNF-1β. Adult mouse kidney sections were immunostained with antibodies against HNF-1β and the SUMO ligases, Ubc9, PIAS1, and PIASγ. HNF-1β was observed in the nuclei of the tubular epithelium along the length of the nephron consistent with previous studies (34). Ubc9 immunostaining was observed in the nuclei of all cells when co-stained with DAPI (data not shown). Ubc9 and HNF-1β co-localization was detected in the tubular epithelium (Fig. 2-3 A-C). PIAS1 was detected in the nuclei of both tubular epithelial cells and interstitial cells. PIAS1 co-localized with HNF-1β in tubular epithelial cells (Fig. 2-3 D-F). PIASγ expression was detected in the nuclei of some epithelial cells and interstitial cells. PIASγ co-localized with HNF-1β in tubular epithelial cells (Fig. 2-3 G-I). Therefore, HNF-1β is expressed in renal tubular epithelial cells, and co-localization studies verified that all the SUMO ligases that interact with HNF-1β *in vitro* are also expressed in the same cells in the adult kidney.

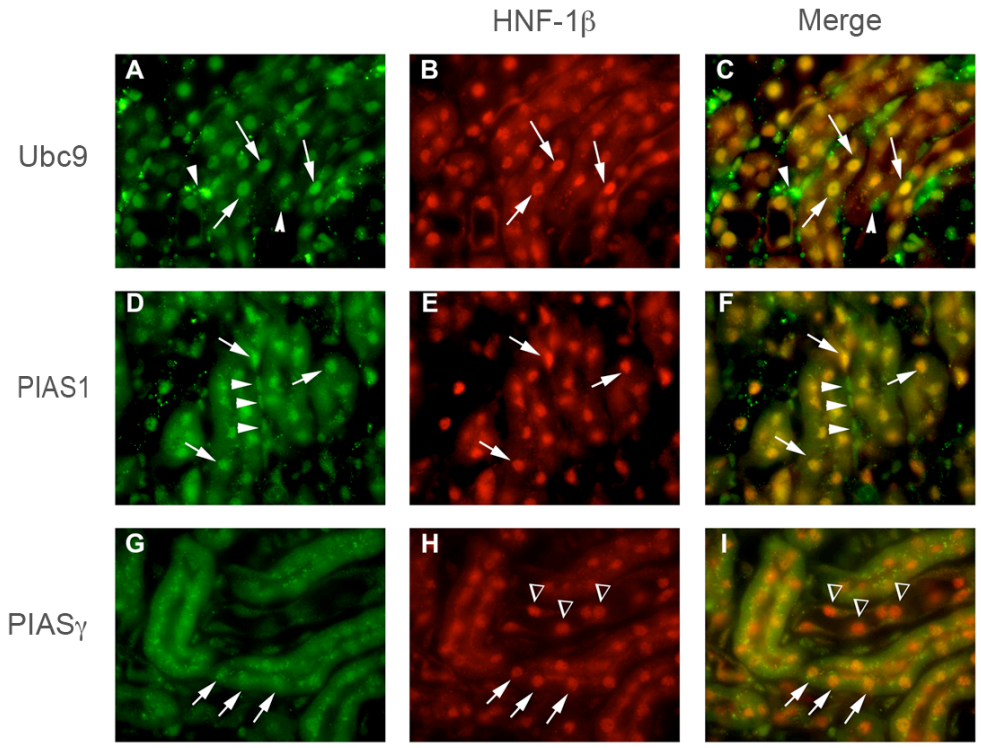


Figure 2-3. Co-localization of SUMO ligases with HNF-1β in the adult mouse kidney.
A-C. Adult mouse kidney medulla showed expression of Ubc9 (A), HNF-1β (B) and merged image (C). D-F. Adult mouse kidney medulla showed expression of PIAS1 (D), HNF-1β (E) and merged image (F). G-I. Adult mouse kidney medulla showed expression of PIASγ (G), HNF-1β (H) and merged image (I). Arrows identifies co-expressing cells. Arrowheads identify cells that expressed the SUMO ligase and not HNF-1β. Open arrowheads identify cells that expressed HNF-1β and not the SUMO ligase.

HNF-1β is SUMOylated

The SUMO E2 ligase, Ubc9, recognizes the consensus SUMO site ΨKXE in target proteins, where Ψ represents a large hydrophobic amino acid. Initially, the HNF-1β primary sequence was scanned using the online program SUMOplot

(http://www.abgent.com/doc/sumoplot) to identify potential SUMO sites in HNF-1β. Two potential SUMO sites were identified in HNF-1β located at K42 and K237. Subsequently, a bioinformatic search using the NDSM extended motif only predicted that K42 would be a suitable candidate for modification (57). A co-immunoprecipitation experiment was performed to test if HNF-1β is SUMOylated *in vivo*. mIMCD-3 murine renal epithelial cells were transfected with FLAG-tagged HNF-1β or empty pcDNA3 together with HA-tagged SUMO-1. The cells were then lysed and subjected to immunoprecipitation with an anti-HA antibody to isolate SUMOylated proteins. The precipitate was separated by SDS-PAGE and immunoblotted with an anti-FLAG antibody. An immunoreactive protein corresponding to HNF-1β was detected in the supernatant in cells that were transfected HNF-1β (Fig. 2-4A, lanes 6 and 8). An immunoreactive band was observed in the pellet only when HA-SUMO-1 and HNF-1β-FLAG were transfected together (Fig. 2-4A, lane 4). The immunoprecipitated protein migrated at a higher molecular weight corresponding to the estimated molecular weight of HNF-1β-FLAG and a single HA-SUMO-1. This result suggested that SUMO is covalently bound to HNF-1β and HNF-1β is SUMOylated *in vivo*.

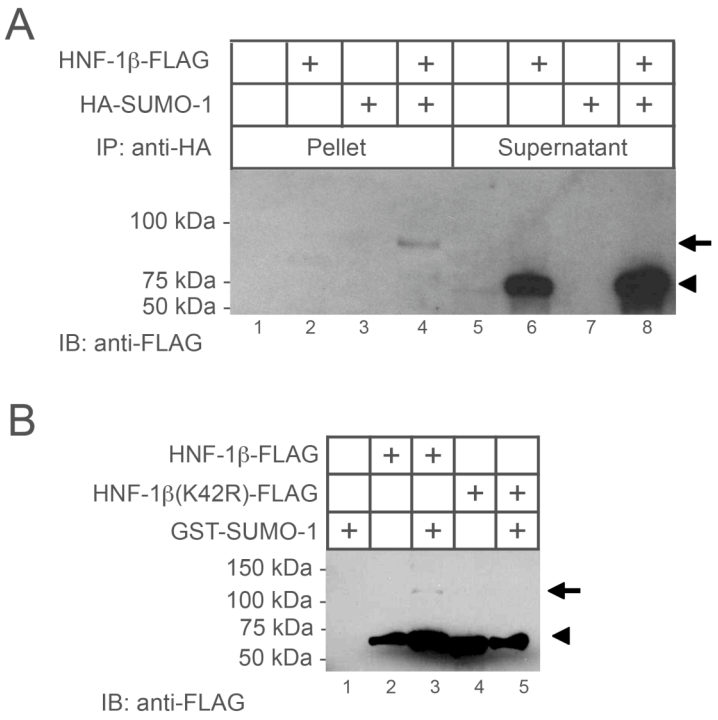


Figure 2-4. HNF-1β is SUMOylated on lysine-42.
A. mIMCD-3 cells were transiently transfected with plasmids encoding the indicated proteins. The cells were lysed, and HA epitope-tagged proteins were immunoprecipitated. The immunoprecipitates (lanes 1-4) and supernatants (lanes 5-8) were then analyzed by immunoblotting with an anti-FLAG antibody.
B. *In vitro* expressed HNF-1β-FLAG and HNF-1β (K42R)-FLAG were incubated with SUMO E1 ligase, Ubc9, and ATP either in the presence or absence of GST-SUMO-1. After incubation, the reactions were analyzed by immunoblotting using an anti-FLAG antibody.

An *in vitro* SUMOylation assay was used to confirm that HNF-1β is SUMOylated and to identify the site of SUMOylation. FLAG-tagged HNF-1β was *in vitro* translated using rabbit reticulocyte lysates and incubated in the presence of GST-SUMO-1, SUMO E1 ligase, and Ubc9. Following incubation, the reaction products were separated by SDS-PAGE and immunoblotted with an anti-FLAG antibody. An immunoreactive protein corresponding to HNF-1β was

detected in programmed lysate (Fig. 2-4A, lanes 6 and 8). An immunoreactive protein that migrated at a higher molecular mass was observed in lysates with GST-SUMO-1. This higher molecular weight species corresponded to the molecular weight of HNF-1β-FLAG and a single GST-SUMO-1. This result showed that HNF-1β could be modified by SUMOylation *in vitro*. Site-directed mutagenesis was performed to change the lysine-42 to arginine. The mutant HNF-1β plasmid was then transcribed, translated and tested in the *in vitro* SUMOylation assay. The HNF-1β(K42R) mutant sample was observed not to have a higher molecular weight after the reaction with GST-SUMO-1. This result indicated that the primary site of SUMOylation in HNF-1β is lysine-42.

SUMO Ligases repressed HNF-1β transcriptional activity

SUMO ligases have been shown to affect the transcriptional activity of many transcription factors (93). To determine the effect of Ubc9, PIAS1 and PIASγ on HNF-1β transcriptional activity, promoter-reporter assays were performed. The plasmid containing the *Pkhd1* promoter driving firefly luciferase was transiently transfected into mIMCD3 cells together with increasing amounts of SUMO ligase expression plasmid. Renilla luciferase expression plasmid was used to control for transfection efficiency. Increasing amounts of Ubc9 produced a dose-dependent reduction in *Pkhd1* promoter activity (Fig. 2-5A). To test whether the reduction in promoter activity required the SUMOylation activity of Ubc9, increasing amounts of a catalytically inactive Ubc9 mutant (C93S) were transfected into cells. The inactive Ubc9 showed a dose-dependent reduction similar to wild-type Ubc9, suggesting that Ubc9 repressed *Pkhd1* promoter independent of SUMOylation activity (Fig. 2-5A). Increasing amounts of PIAS1 produced a dose-dependent repression of *Pkhd1* promoter activity (Fig. 2-5B). PIAS1 (CS), a PIAS1 mutant that does not interact with HNF-1β, was unable to

repress *Pkhd1* promoter activity (Fig. 2-5B). Increased amounts of PIASγ produced a marked dose-dependent reduction in *Pkhd1* promoter activity. Two mutants of PIASγ were tested for their effects on *Pkhd1* promoter activity. PIASγ(AA) was unable to repress *Pkhd1* promoter activity, suggesting that the LXXLL domain is required for PIASγ-dependent repression. The PIASγ (W363A) mutant was able to repress *Pkhd1* promoter activity but with a diminished capacity compared to the wild-type enzyme (Fig. 2-5C). This result suggested that PIASγ can repress *Pkhd1* promoter activity through both SUMOylation-dependent and -independent mechanisms.

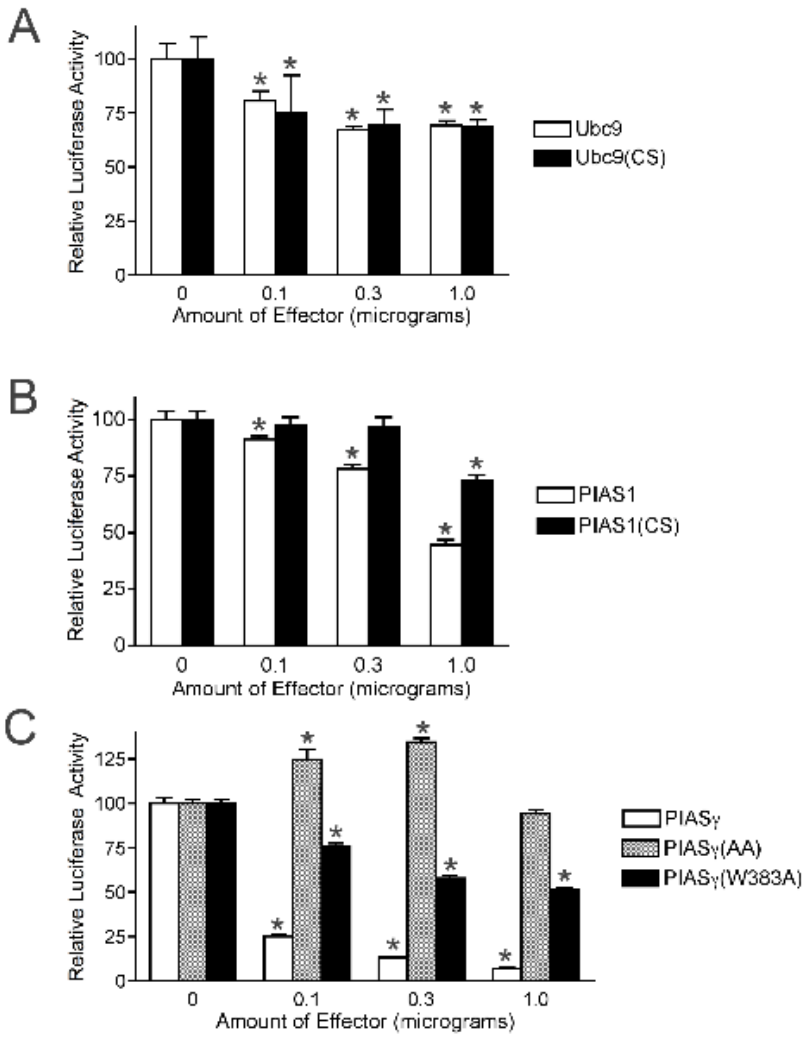


Figure 2-5. SUMO ligases repressed PKHD1 promoter activity.
A. mIMCD-3 cells were transiently transfected with 0.6 μg of *Pkhd1* promoter-reporter plasmid and increasing amounts of plasmids encoding wild-type Ubc9 (open) or mutant Ubc9 (closed). B. mIMCD-3 cells were transiently transfected

with 0.6 μg of *Pkhd1* promoter-reporter and increasing amounts of PIAS1 (open) or mutant PIAS1 (closed) expression plasmid. C. mIMCD-3 cells were transiently transfected with 0.6 μg of *Pkhd1* promoter-reporter and increasing amounts of PIASγ (open), PIASγ-AA (hatched) or PIASγ-W363A (closed) expression plasmid. 20 ng of a renilla expression plasmid were used as a transfection control. Cells were lysed after 48 hours and assayed for luciferase activity. Relative light output is shown as the mean (± S.D.) of six independent experiments. * $P < 0.01$ compared with no SUMO ligase plasmid added.

SUMOylation is not required for nuclear localization of HNF-1β

SUMOylation has previously been shown to regulate the nuclear localization of transcription factors(71, 94). To test whether HNF-1β must be SUMOylated to enter the nucleus, fusion proteins were constructed containing EGFP and wild-type HNF-1β or the SUMOylation-defective HNF-1β mutant (K42R). HNF-1β-EGFP fusion protein and the mutant HNF-1β(K42R)-EGFP protein localized to the nucleus of mIMCD-3 cells, as verified by DAPI co-staining (Fig. 5). These results demonstrated that the SUMOylation site of HNF-1β is not required for its nuclear localization.

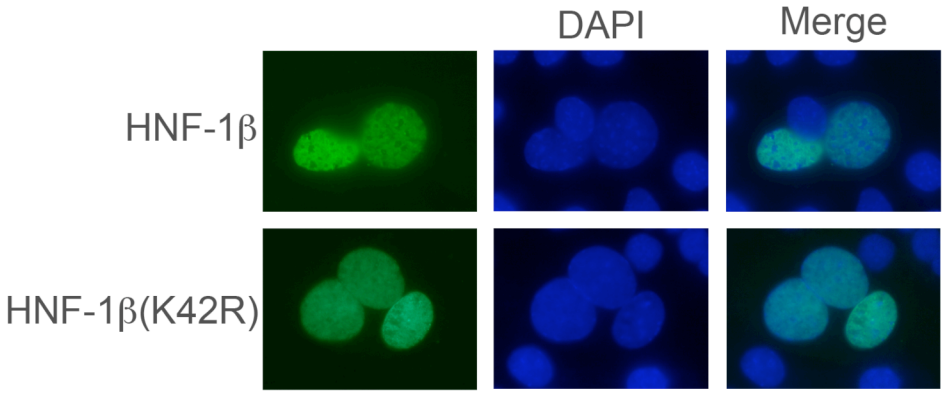


Figure 2-6. Lysine-42 is not required for nuclear localization of HNF-1β. mIMCD-3 cells were transiently transfected with either HNF-1β-EGFP or HNF-1β(K42R)-EGFP. Cells were then grown for 48 hours, fixed and then stained with

DAPI. Fluorescence microscopy shows HNF-1β and HNF-1β(K42R) in green and both localized to the nucleus shown in blue.

Mutation of the SUMO modification site stimulated HNF-1β transcriptional activity

Overexpression of SUMO ligases repressed HNF-1β transcriptional activity. However, the catalytically inactive mutants of Ubc9 and PIASγ also repressed HNF-1β activity. To understand the role of SUMOylation on the transcriptional activity of HNF-1β, GAL4 fusion proteins were constructed and used in promoter-reporter assays. GAL4 was fused to the wild-type HNF-1β or to HNF-1β (K42R). These plasmids were transfected into mIMCD3 cells with a GAL4-responsive luciferase reporter plasmid. The HNF-1β (K42R) fusion protein stimulated GAL4-responsive promoter activity approximately 3.5-fold greater compared to wild-type HNF-1β (Fig. 2-7).

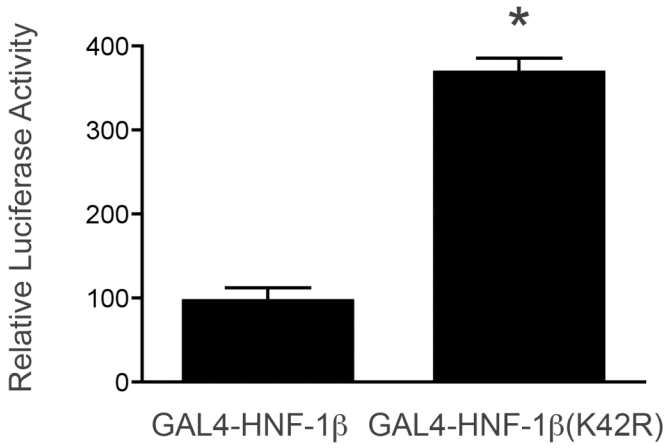


Figure 2-7. Mutation of Lysine-42 stimulated HNF-1β transcriptional activity.
mIMCD-3 cells were transiently transfected with 0.3 μg of a GAL4-responsive promoter luciferase reporter and either 0.3 μg of GAL4-HNF-1β expression vector or 0.3 μg of GAL4-HNF-1β(K42R) expression vector. 20 ng of a renilla

expression plasmid was used as a transfection control. Cells were lysed after 48 hours and assayed for luciferase activity. Relative light output is shown as the mean (± S.D.) of six independent experiments. * $P < 0.01$.

Discussion

HNF-1β is an essential transcription factor and is required for normal development (16, 29). The importance of HNF-1β is underscored by its involvement in renal dysfunction and disease. Previous studies have identified targets of HNF-1β, such as *Pkhd1*, that are also important in human disease (89). Few studies have addressed the mechanisms that regulate HNF-1β transcriptional activity. Therefore, identifying interacting partners might reveal insight into the regulation of HNF-1β. To identify proteins that physically interact with HNF-1β and potentially regulate target promoter activity, a yeast two-hybrid screen was performed with a kidney cDNA library using HNF-1β as bait. Multiple independent clones representing members of the SUMOylation pathway were identified as interacting partners of HNF-1β, including Ubc9, the SUMO E2 ligase, PIAS1 and PIASγ, SUMO E3 ligases.

The recent finding that SUMO-1 haploinsufficiency is associated with cleft lip and palate highlighted the tissue-specific effects potentially mediated by SUMO ligases (95). Previous analysis of SUMO and SUMO ligases did not specifically describe their pattern of expression in the kidney. Immunofluorescent localization demonstrated that HNF-1β and SUMO ligases co-localized in the epithelia of the nephron. Co-expression of HNF-1β and SUMO ligases confirmed that these proteins might have a relevant interaction *in vivo*.

SUMOylation of transcription factors has emerged as a common post-translational modification with diverse effects on protein localization. The data in this study demonstrated that HNF-1β can be SUMOylated, and the primary site of

SUMOylation is lysine-42. SUMOylation does not appear to be required for nuclear localization, as the HNF-1β(K42R) mutant still localized to the nucleus. Previous studies have shown that SUMOylated c-Myb localized to PML bodies(96). Subnuclear distribution of the HNF-1β(K42R) mutant appeared unaffected when compared to wild-type HNF-1β suggesting that SUMOylation does not regulate subnuclear localization of HNF-1β.

SUMO ligases and SUMOylation have been associated with repression of transcriptional activity. However, effects of SUMO ligases on transcriptional activity are not exclusively linked to SUMOylation, and a number of studies have demonstrated that SUMOylation-independent mechanisms have either stimulatory or repressive effects on the transcriptional activity of target proteins. Ubc9 repressed HNF-1β transcriptional activity and that repression is SUMOylation-independent. Ubc9 has been shown to act as a SUMOylation-independent co-repressor of GR, AR, TEL, and IE2(81, 97-99). PIAS1 also repressed HNF-1β transcriptional activity, and the mutant PIAS1(CS) had no effect on HNF-1β presumably because it did not interact with HNF-1β in co-immunoprecipitation experiments. PIASγ repression of HNF-1β transcriptional activity appeared to have two modes. Mutation of the SP-RING domain, which disrupts SUMOylation activity, partially inhibited PIASγ repression of HNF-1β transcriptional activity. This result suggested that PIASγ repressed HNF-1β via both SUMOylation-dependent and -independent means. Mutation of the LXXLL motif ablated the repressive activity of PIASγ, and suggested that this domain is responsible for the recruitment of negative regulatory factors to a target promoter. Future studies to identify the factor(s) that are recruited by this domain will better define the mechanism of repression. Mutation of lysine-42 relieved a repressive contribution to HNF-1β transcriptional activity. This result emphasized that SUMOylation repressed HNF-1β transcriptional activity.

In summary, this study described a new post-translational modification of HNF-1β. Three members of the SUMOylation pathway negatively regulated HNF-1β transcriptional activity. The mechanisms that regulate the interactions between HNF-1β and the SUMO ligases will contribute to the understanding of HNF-1β function in development and disease.

CHAPTER THREE
Arkadia Regulates HNF-1β

Introduction

Mutations in HNF-1β are associated with both RCAD and renal cell carcinomas. HNF-1β regulates gene expression via the transcriptional activation domain in the carboxyl terminus (12, 13). Previously, a candidate approach had examined potential mediators of HNF-1β transcriptional activity. Initial observations implicated histone acetyltransferases (HATs) and histone deacetylases (HDACs) (12, 13). Concurrently, an unbiased proteomic approach to identify specific cofactors that regulate HNF-1β transcriptional activity was performed. The identification of SUMO ligases as regulators of HNF-1β (described in Chapter 2) suggested that other post-translational modifications might be important regulators of HNF-1β function. The yeast two-hybrid screen also identified an ubiquitin E3 ligase as a putative HNF-1β-interacting protein. The experiments in this chapter described the identification of Arkadia, the ubiquitin E3 ligase, as a HNF-1β-interacting protein and described a role for Arkadia in HNF-1β activity.

Results

Arkadia Interacted with HNF-1β

As described in Chapter 2, a yeast two-hybrid screen was performed with HNF-1β as bait to screen a mouse kidney cDNA library. A single clone encoding

Arkadia was isolated with the full-length HNF-1β bait. Arkadia is a RING finger protein with ubiquitin E3 ligase activity. This clone was transformed into the yeast parental strain and did not auto-activate the reporter system, verifying the interaction was specific.

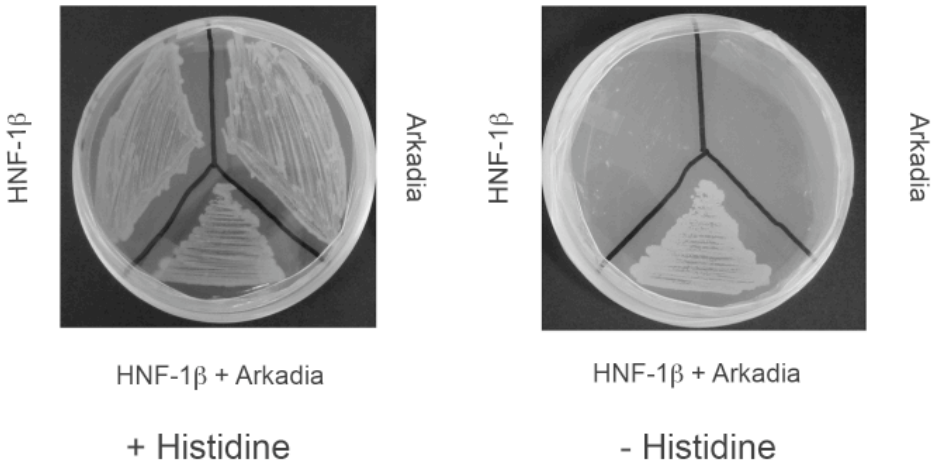


Figure 3-1. Arkadia interacted with HNF-1β in a yeast two-hybrid screen. Yeast were transformed with full-length HNF-1β bait, the isolated Arkadia prey plasmid, or both the bait and prey plasmids and were tested for growth under auxotrophic conditions. Yeast only survived in the absence of histidine when both bait and prey plasmids were present.

Arkadia Interacted with HNF-1β in vivo

Co-immunoprecipitation experiments were performed to verify that HNF-1β interacts with Arkadia *in vivo*. The co-immunoprecipatations were conducted with 293T cells cotransfected with epitope-tagged HA-HNF-1β and with epitope-tagged FLAG-Arkadia. Immunoprecipitation of Arkadia resulted in co-precipitation of HNF-1β and indicated that Arkadia and HNF-1β form a complex *in vivo* (Fig. 3-2). Mutant versions of Arkadia were tested for their ability to bind

HNF-1β. An Arkadia-ΔC mutant protein, which lacks the carboxyl-terminal RING domain, co-precipitated with HA-HNF-1β (Fig. 3-2). The Arkadia-CA mutant, which contains a cysteine to alanine mutation at residue 937 that abrogates the ubiquitin E3 activity, co-precipitated with HNF-1β (Fig.3-2). These results indicated that the RING finger of Arkadia is not required for the interaction with HNF-1β.

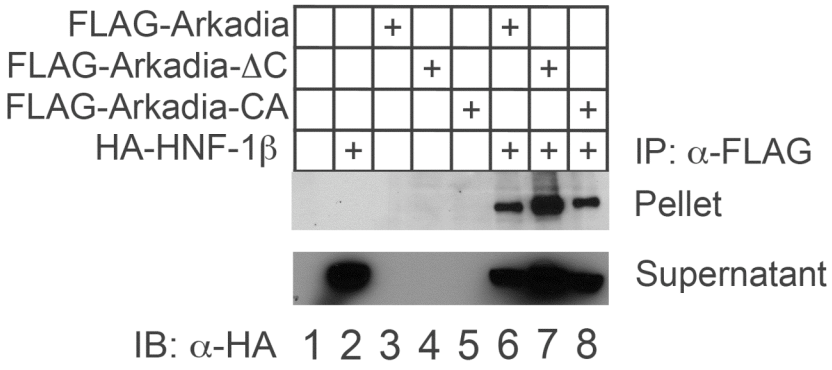


Figure 3-2. Arkadia interacted with HNF-1β *in vivo*.
293T cells were transiently transfected with 4.0 μg of plasmids encoding FLAG-tagged Arkadia, FLAG-tagged Arkadia-ΔC, or FLAG-tagged Arkadia-CA together with pcDNA3 or plasmid encoding HA-tagged HNF-1β. Cells were lysed after treatment with proteasome inhibitors for six hours and subjected to immunoprecipitation with mouse monoclonal anti-FLAG antibodies. HNF-1β was detected by immunoblotting using an anti-HA antibody.

Arkadia is expressed in renal tubules

Two previous studies examined Arkadia expression in the mouse suggested that Arkadia is ubiquitously expressed (100, 101). Although previous studies demonstrated that Arkadia is expressed in the kidney, a more detailed analysis was necessary to confirm a potential interaction with HNF-1β. Adult mouse kidney sections were immunostained with antibodies against Arkadia,

expression was observed in the nuclei of kidney cells (Fig. 3-3). Arkadia positive nuclei lined the nephron, and suggested that Arkadia was expressed in the tubular epithelium. As discussed in Chapter 1, HNF-1β is expressed in renal tubular epithelial cells, therefore localization studies confirmed expression of both Arkadia and HNF-1β in the epithelia of the adult kidney.

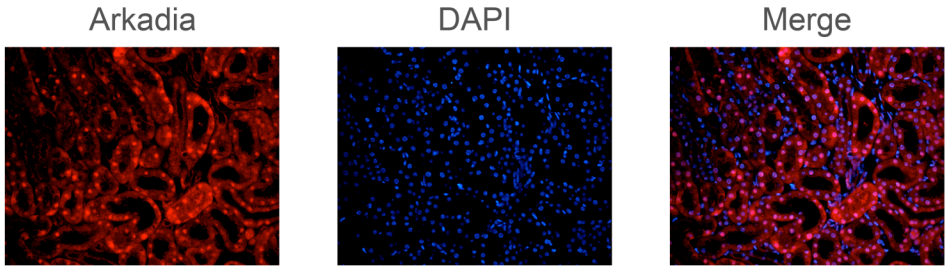


Figure 3-3 Arkadia localized to the nuclei of renal tubules.
Adult mouse kidney showed expression of Arkadia that was co-stained with DAPI.

HNF-1β is Ubiquitinated

To determine whether HNF-1β is a substrate for ubiquitination, an *in vivo* ubiquitination assay was performed. mIMCD-3 cells were cotransfected with FLAG-tagged HNF-1β or empty pcDNA3, together with HA-tagged ubiquitin. The cells were treated with proteasome inhibitors to block degradation of poly-ubiquitinated proteins, lysed, and immunoprecipitated with an anti-HA antibody to isolate ubiquitinated proteins. The precipitate was separated by SDS-PAGE and immunoblotted with an anti-FLAG antibody. An immunoreactive protein corresponding to HNF-1β was detected in the supernatant of cells transfected with an HNF–1β expression vector (Fig. 3-4 lanes 6 & 8). A series of immunoreactive proteins of increasing molecular weight were observed in the pellet only when HA-ubiquitin and HNF-1β-FLAG were transfected together (Fig.3-4, lane 4). These immunoprecipitated proteins had uniformly increased molecular weight,

indicative of poly-ubiquitination. An immunoreactive band corresponded to an unmodified form of HNF-1β was also observed in the anti-HA immunoprecipitate (Fig.3-4 lane 4). This unmodified HNF-1β co-precipitated with ubiquitinated HNF-1β, and suggested that modified and unmodified HNF-1β can dimerize *in vivo*.

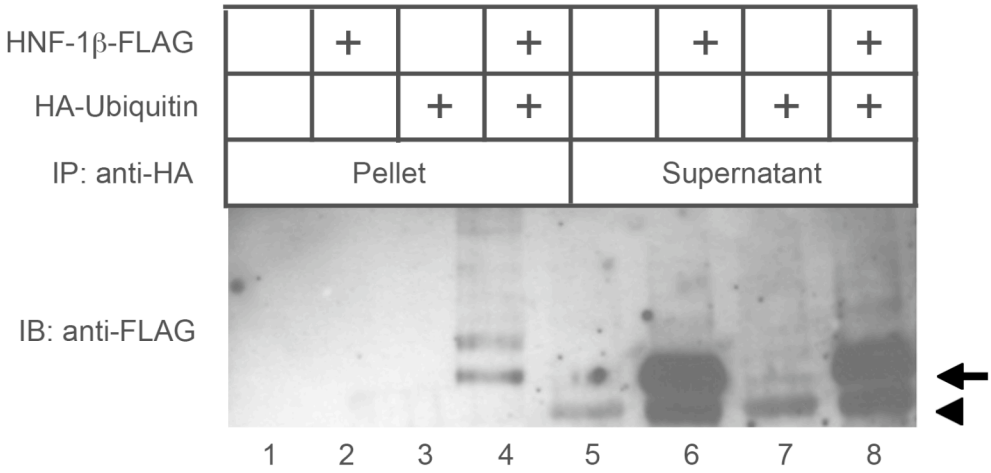


Figure 3-4. HNF-1β is ubiquitinated *in vivo*.
mIMCD-3 cells were transiently transfected with the indicated plasmids. The cells were lysed after six hours of proteasome inhibitor treatment, and HA epitope tagged proteins were immunoprecipitated. The precipitates (lanes 1-4) and supernatants (lanes 5-8) were then analyzed by immunoblotting with an anti-FLAG antibody. The arrowhead denoted a non-specific band and the arrow denoted the unmodified HNF-1β.

Arkadia stimulated HNF-1β transcriptional activity

Previous studies have shown that ubiquitination affects the activity of transcription factors, such as myc, Gal4, Gcn4 (reviewed in (42, 102)). To determine the effect of Arkadia on HNF-1β transcriptional activity, promoter-reporter assays were performed. The plasmid containing the *Pkhd1* promoter driving firefly luciferase was transiently transfected into mIMCD3 cells with increasing amounts of Arkadia expression plasmid. Renilla luciferase expression

plasmid was used as a transfection efficiency control. Increasing amounts of Arkadia induced a dose-dependent stimulation of *Pkhd1* promoter activity (Fig. 3-5). To test whether the stimulation in promoter activity required the RING finger of Arkadia, increasing concentrations of the mutant Arkadia-ΔC expression plasmid were transfected into cells. The Arkadia-ΔC did not stimulate the *Pkhd1* promoter, suggesting that the RING finger is required for stimulation of *Pkhd1* promoter activity. In a similar manner, the Arkadia-CA mutant was unable to stimulate promoter activity. Rather, increasing amounts of Arkadia-CA expression plasmid repressed *Pkhd1* promoter activity (Fig. 3-5). Taken together, these results indicated that Arkadia can stimulate the *Pkhd1* promoter in a dose-dependent fashion, and this stimulation required the RING domain and E3 ligase activity.

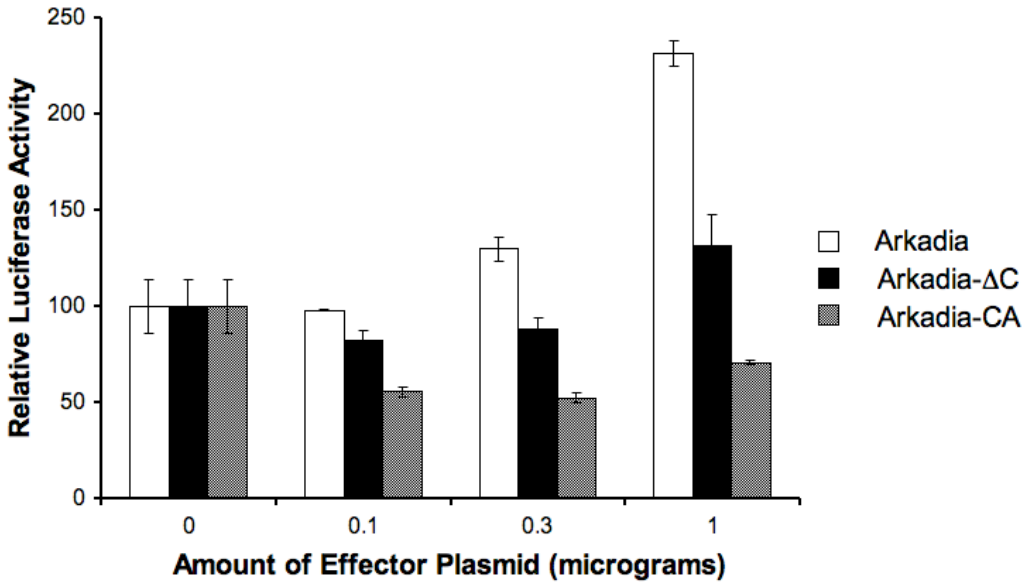


Figure 3-5. Arkadia stimulated *Pkhd1* promoter activity.
mIMCD-3 cells were transiently transfected with 0.6 μg of *Pkhd1* promoter-reporter plasmid and increasing amounts of Arkadia (open), Arkadia-ΔC (closed)

or Arkadia-CA (gray) expression plasmid. 20 ng of a renilla expression plasmid were used as a transfection control. Cells were lysed after 48 hours and assayed for luciferase activity. Relative light output is shown as the mean (± S.D.) of three independent experiments.

To examine whether Arkadia had a direct role on HNF-1β-dependent *Pkhd1* promoter activity, additional reporter assays were performed. mIMCD-3 cells were transiently transfected with wild-type *Pkhd1* promoter-reporter plasmid or a *Pkhd1* promoter-reporter containing a mutated HNF1 site.

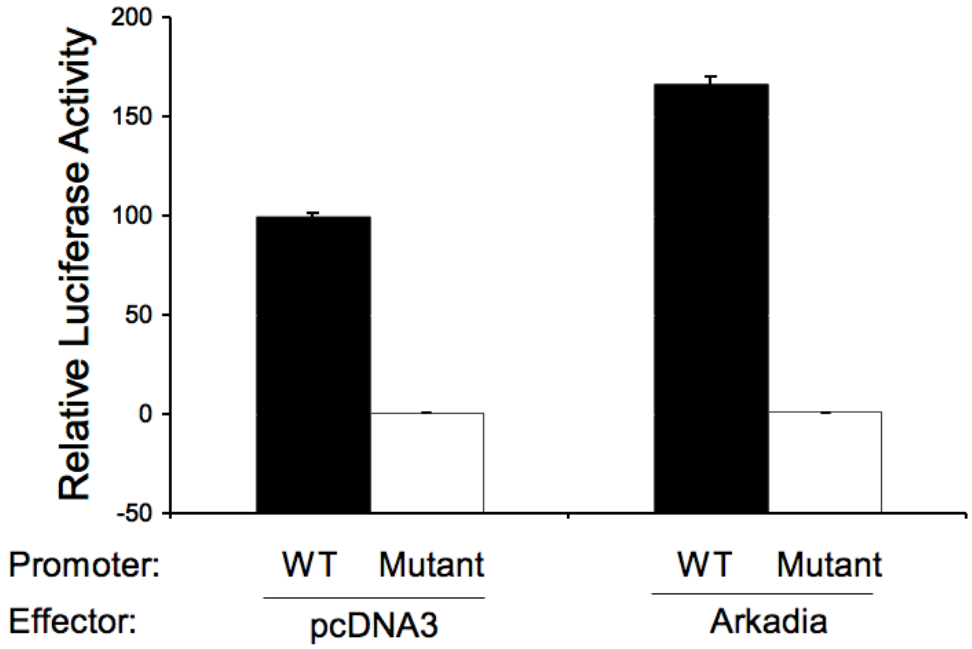


Figure 3-6. The HNF1 site in the *Pkhd1* promoter is required for stimulation by Arkadia.
mIMCD-3 cells were transiently transfected with 0.6 μg of either *Pkhd1* promoter-reporter or the Pkhd1 promoter containing a mutated HNF1 site in the presence of 1.0 μg of Arkadia expression plasmid (closed) or pcDNA3 (open). Cells were lysed after 48 hours and assayed for luciferase activity. Relative light output is shown as the mean (± S.D.) of three independent experiments.

The cells were cotransfected with either pcDNA3 or the Arkadia expression plasmid. Arkadia only stimulated the wild-type promoter and not the mutant promoter (Fig. 3-6). This result demonstrated that the HNF1 site is necessary for Arkadia-dependent stimulation of the *Pkhd1* promoter.

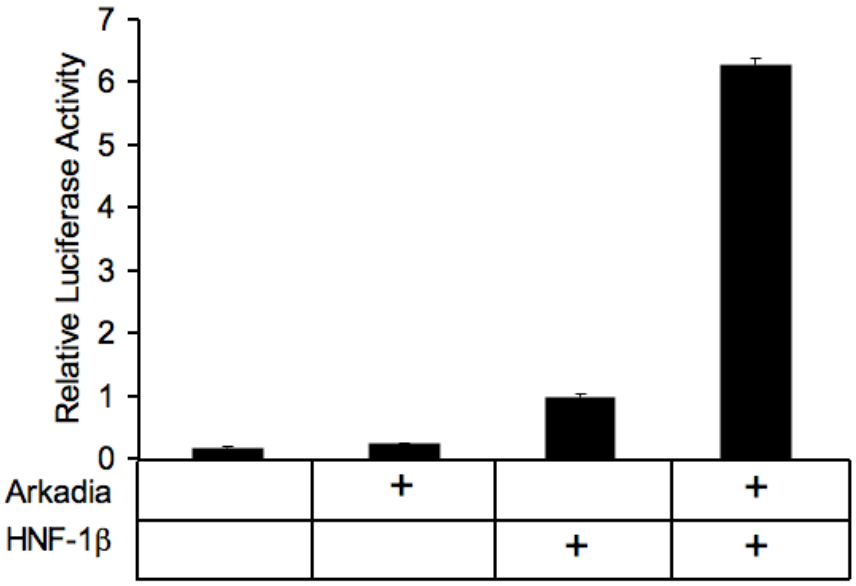


Figure 3-7. HNF-1β Protein is required for Arkadia-dependent stimulation of *Pkhd1* promoter activity.
HeLa cells were transiently transfected with 0.3 μg of *Pkhd1* promoter-reporter and 0.3 μg of Arkadia expression plasmid or HNF-1β expression plasmid or both plasmids using pcDNA3 to equalize for the total amount of transfected DNA. Cells were lysed after 48 hours and assayed for luciferase activity. Relative light output is shown as the mean (± S.D.) of three independent experiments.

HeLa cells, which do not endogenously express HNF-1β, were transiently transfected with the *Pkhd1* promoter-reporter plasmid and the Arkadia expression plasmid together with pcDNA3 or a HNF-1β expression plasmid. *Pkhd1* promoter

activity was stimulated in the presence of the HNF-1β expression plasmid alone. The addition of Arkadia only stimulated *Pkhd1* promoter activity in the presence of HNF-1β (Fig. 3-7). Therefore, HNF-1β is required for Arkadia to stimulate the *Pkhd1* promoter. Together, these results demonstrated that the HNF1 site and HNF-1β protein are required for Arkadia to stimulate *Pkhd1* promoter activity.

Discussion

HNF-1β is an essential transcription factor required for normal development(16, 29). Disruption of HNF-1β resulted in renal dysfunction and disease. *Pkhd1* was previously identified as a downstream target of HNF-1β, and proper *PKHD1* expression is also implicated in human disease(89). Few studies have addressed the mechanisms that regulate HNF-1β transcriptional activity. A yeast two-hybrid screen was performed using HNF-1β as bait to identify interacting proteins that potentially regulate HNF-1β transcriptional activity. A single clone representing Arkadia, an ubiquitin E3 ligase, was identified as a HNF-1β-interacting protein. Co-immunoprecipitation experiments showed that Arkadia and HNF-1β interact *in vivo*. Studies using mutant versions of Arkadia demonstrated that the RING domain and E3 ligase activity of Arkadia are not required for the interaction with HNF-1β. Arkadia expression was previously described in the kidney, but immunofluorescence studies showed that Arkadia is expressed in the tubular epithelia of the mature nephron.

Ubiquitination is most commonly associated with marking proteins for destruction by the proteasome. However, ubiquitination has been demonstrated to also have direct effects on transcription factor activity. Ubiquitination of transcription factors has emerged as a post-translational modification that increases transcriptional activity. The data in this study showed that HNF-1β could be ubiquitinated. Arkadia enhanced *Pkhd1* promoter activity in an HNF-1β-

dependent fashion. Stimulation of *Pkhd1* promoter activity required both the RING domain and ubiquitin E3 ligase activity of Arkadia. Arkadia required the presence of HNF-1β protein and a functional HNF1 site in the *Pkhd1* promoter. These data together suggested that Arkadia stimulated *Pkhd1* promoter activity via the ubiquitination of HNF-1β.

This study describes one of the first examples of an ubiquitin ligase enhancing activation of a mammalian transcription factor. The Gcn4 and Gal4 transcription factors have been reported to undergo ubiquitin-mediated activation (41, 103). In mammalian systems, CIITA has been shown to be a more potent transcription factor upon ubiquitination but an E3 ligase was not described (37). In perhaps the most well-characterized system, an F-box protein interacted with myc and acted as a transcriptional cofactor by targeting myc for ubiquitination by a SCF complex (36, 39, 104, 105). Ubiquitination of a transcription factor is thought to recruit transcriptional machinery.

Arkadia and HNF-1β represent a novel system to study ubiquitination of a transcription factor, using the endogenous catalytic E3 ubiquitin ligase and target protein. Additionally, HNF-1β and Arkadia may provide a useful model to study early developmental processes. HNF-1β is an essential regulator of development and loss-of-function resulted in early lethality (16, 29). Arkadia was first identified in a recessive lethal gene-trap screen that is phenotypically similar to homozygous HNF-1β knockout mice. Both Arkadia homozygous null and HNF-1β homozygous null embryos have visceral endoderm defects. This study and the studies that described the Arkadia and HNF-1β null mice suggested that a biochemical interaction between these two proteins might have ramifications in early development.

Arkadia is regulated transcriptionally and post-translationally by TGF-β signaling (101, 106). TGF-β treatment of HaCaT cells, a human keratinocyte cell

line, resulted in a transient reduction in Arkadia mRNA levels, whereas TGF-β treatment of Cos7 cells resulted in Arkadia no longer being restricted to the nucleus. Taken together, Arkadia regulation by TGF-β may have effects on HNF-1β transcriptional activity.

In summary, this study described a new post-translational modification for HNF-1β. The ubiquitin ligase Arkadia positively regulated HNF-1β transcriptional activity. Ascertainment of the mechanism of action of Arkadia and the regulation of its interaction with HNF-1β may be important in understanding HNF-1β function in development and disease.

CHAPTER FOUR
Conclusions and Recommendations

Summary

The molecular function of HNF-1β is still being characterized in the following ways: the identification of downstream targets, regulation of HNF-1β, and characterization of mutant forms of HNF-1β. The studies performed herein began with the hypothesis that the identification of HNF-1β-interacting proteins might give insight into the mechanisms governing HNF-1β function. The unbiased proteomic approach that was used to identify HNF-1β-interacting proteins has two fundamental strengths. The first advantage is an unbiased approach can yield unexpected or ignored candidates as potential interaction partners. The second is the initial candidates have a biochemical property that can be validated, namely forming a complex with HNF-1β. The interacting proteins chosen for further examination in this body of work reveal two novel post-translational modifications of HNF-1β with direct effects on transcriptional activity. These studies provide model systems for three areas of research as discussed below, and suggestions for future investigations are included.

Contributions to SUMOylation and Future Directions

SUMOylation has emerged as a common post-translational modification of nuclear proteins, especially transcription factors. As more examples of SUMOylated transcription factors have been described, exceptions to the early SUMOylation models seem to have increased. Initially, SUMOylation was

associated with repression of transcriptional activity. However, the ratio of SUMOylated to non-SUMOylated protein did not correlate well with observed effects. The SUMOylated fraction of a given transcription factor pool is often less than one percent. The predicted fraction of HNF-1β that is SUMOylated based on the *in vivo* SUMOylation assay would seem to agree with these previous reports. To address this Ronald Hay proposed a model where a SUMOylated protein has a "history of modification." (47) SUMOylation targets a given transcription factor to an inactive complex or state. After incorporation into the repressed state, the SUMO modification can be removed by SENPs without immediate re-activation of the transcription factor. The transcription factor must be activated by a second signal. A precedent for the "history of modification" model is ubiquitination of receptor proteins. Ubiquitination targets transmembrane receptors to membrane domains that will be endocytosed. In the process, the ubiquitin is removed and the receptor is endocytosed later. SUMOylated transcription factors bound to a target promoter could subsequently recruit chromatin modifiers to inactivate the promoter. SUMO could be removed from the transcription factor, and the chromatin modifications could maintain the repressed state. Experiments in this study do not specifically address the mechanism of SUMOylation-dependent repression of HNF-1β transcriptional activity. However, the HNF-1β(K42R) protein stimulated a promoter to a greater extent than the wild-type HNF-1β. This result agrees with the "history of modification" model because HNF-1β(K42R) increased transcriptional activity is greater than the expected fraction of SUMOylated HNF-1β.

The "history of modification" model is attractive for explaining SUMO-dependent effects of SUMO ligases on target proteins. However, as shown in the case of HNF-1β, SUMO ligases can have SUMOylation-independent effects as well. Ubc9 repression of HNF-1β transcriptional activity independent of

SUMOylation suggests that Ubc9 nucleates a co-repressor complex. A caveat to this model is that catalytically inactive Ubc9 retains the ability to bind SUMO, thus allowing a previously SUMOylated HNF-1β to recruit the mutant Ubc9 and form a similar complex as the wild-type Ubc9. This study did not address whether catalytically inactive Ubc9 required HNF-1β to be SUMOylated before forming a repression complex. To test this possibility, a promoter-reporter assay would test the effect of Ubc9(C93S) on the HNF-1β(K42R) mutant protein. In any case, the characterization of proteins that bind the Ubc9-HNF-1β complex could suggest the mechanism of repression.

PIASγ repressed HNF-1β transcriptional activity in both SUMOylation-dependent and -independent mechanisms. The SUMOylation-dependent mechanism for PIASγ repression would be part of the "history of modification" model. The independent mechanism required the LXXLL motif of PIASγ to repress HNF-1β transcriptional activity. The LXXLL motif was identified in proteins that bound the ligand binding domain of nuclear receptors and was characterized as a protein-protein interaction motif(107, 108). LXXLL motifs have been shown to recruit both co-activators such as CBP/p300 and co-repressors such as RIP140 and ETO (109). The LXXLL domain of PIASγ is required for repression of STAT1 (92). In order to understand the mechanism of repression, the proteins that interact with the LXXLL domain of PIASγ must be identified. A potential model for PIASγ-mediated repression would be PIASγ bound to HNF-1β and then recruits a co-repressor complex to the promoter.

SUMOylation is associated with transcriptional repression but in a few cases, it is associated with transcriptional activation. As an example, p53 is activated by SUMO-1 conjugation (110). In experiments designed to test the effects of SUMO ligases on *Pkhd1* promoter activity in the absence of HNF-1β, the promoter-reporter system described in Chapter 2 was tested in cell lines that

do not endogenously express HNF-1β. In contrast to the SUMO ligase-mediated repression of the *Pkhd1* promoter in mIMCD-3 cells, SUMO ligases stimulated *Pkhd1* promoter activity in a dose-dependent fashion in HeLa and 293T cells (data not shown). Previous experiments verified that HNF-1β was SUMOylated in both of these cell lines (data not shown). This reciprocal effect on promoter activity with only a change in the cells suggested that perhaps SUMOylation is a "contextual-switch." "Contextual switch" is defined here as a modification that altered the behavior of a target protein in a cell type-dependent fashion. The stimulation of the *Pkhd1* promoter by Ubc9, PIAS1, and PIASγ observed in these experiments suggested that a common pathway exists. There are at least two possible hypotheses that arise from this observation. The first is factors in HeLa and 293T cells disrupt the repression. The second hypothesis is the presence of factors in mIMCD-3 cells that mediate this repression. A "contextual-switch" may diversify the effects SUMOylation might have on a given transcription factor. For example, HNF-1β is expressed in numerous cell types in the kidney and other organs; however, SUMO ligase-mediated repression has only been tested in one cell type that endogenously expresses HNF-1β. Experiments to examine this "contextual-switch" will begin by determining if this is a biologically relevant observation or simply an effect of exogenous expression. However, if positive regulation of HNF-1β transcriptional activity by SUMO ligases were confirmed in a tissue that endogenously expresses HNF-1β, this would be a unique contribution to the field of SUMOylation.

The description of SUMOylation of HNF-1β is not surprising, considering a putative SUMOylation site in HNF-1β was identified *in silico* (57). However, the SUMOylation of HNF-1β might provide a good model system to understand the redundant functions of SUMO ligases on a given target protein *in vivo*. Experimental mouse models suggested that SUMO E3 ligases might be

redundant. The interactions of HNF-1β with the SUMO E3 ligases could be used as a model to better understand the nature of this redundancy. As examples, is the SUMO E3 ligase PIAS1 transcriptionally upregulated to compensate in the kidneys of PIASγ null animals? Does the amount of SUMOylated HNF-1β change in either of these knockout models? HNF-1β and SUMO ligases may also be a model system for the molecular function of SUMOylation. SUMOylated HNF-1β could provide a unique opportunity to understand a "contextual-switch" between stimulation and repression of transcriptional activity. The observations leading to this hypothesis may be unique and have not been actively addressed in the literature.

Contributions to Ubiquitination and Future Directions

The ubiquitination of transcription factors as related to transcriptional activation is an emerging field of research with few examples. Further narrowing the models available, there are perhaps three or four transcription factor-ubiquitin ligase pairs identified to date. The Arkadia-HNF-1β interaction provides a model system with potential consequences related to human disease. Questions in the field of ubiquitinated transcriptional activators need to describe the most fundamental characteristics. As an example, what types of polyubiquitin chains are formed on transcriptional activators? The current model in the literature suggests that the initial ubiquitination activates the transcription factor, triggering transcription. The ubiquitin chain is elongated over time with K48 linkages. The proteasome recognizes the transcription factor for destruction after three more ubiquitins have been added (42). However, an abstract at the 3rd International Conference on Ubiquitin, Ubiquitin-like proteins and Cancer by Dr. Cam Patterson presented results that suggested another model. Dr. Patterson described

ubiquitin ligases that activated a transcription factor with the addition of K63 linked polyubiquitin chains. Whereas, the rate-limiting step in generating an activated transcription factor is the initial ubiquitination, different ubiquitin chains may have consequences on subsequent modes of action. The ubiquitin chains formed on HNF-1β have not been analyzed, but ubiquitinated HNF-1β was not isolated in the absence of proteasome inhibitors, suggesting that K48 linkages would be the predominant form in the cell. Further questions in this field that the HNF-1β model could address are: Is the transcription factor ubiquitinated on the promoter or in the nucleoplasm? What is the mechanism of action? What are the dynamics of ubiquitination? Are there signals that regulate ubiquitination-mediated activation of transcription factors, chain type and chain elongation rates? Ubiquitination of HNF-1β by Arkadia has provided a model for studying all of these questions as they relate to ubiquitination of transcription factors.

Contributions to HNF-1β and Future Directions

The studies described here began with the goal of understanding HNF-1β function as related to human biology. Although the experiments described above allow HNF-1β to become a model substrate for two post-translational modifications, these studies describe two new regulatory mechanisms governing HNF-1β function. SUMOylation has not been previously described for either HNF1 family member. Ubiquitination of HNF-1α was described previously as it related to protein stability (111). The role these pathways play must be further understood in both normal and disease states.

Post-translational modifications alter the biochemical properties of target proteins. SUMOylation and ubiquitination are not the first post-translational modifications described for HNF-1β. HNF-1β was shown to be phosphorylated in

an intestinal derived cell line after treatment with okadaic acid, a phosphatase inhibitor (112). Phosphorylation of HNF-1β resulted in decreased DNA binding and subsequent reduction in target gene expression. These experiments demonstrated that HNF-1β activity could be regulated post-translationally, and suggested that either extra- or intracellular signaling pathways may regulate HNF-1β. The SUMOylation and phosphorylation of HNF-1β may act in series to affect transcriptional activity. Phosphorylation-dependent SUMOylation has been illustrated for a few transcription factors. Serine-49 of HNF-1β is in sufficient proximity to direct phosphorylation-dependent SUMOylation. Serine-49 resides in an Erk1 kinase phosphorylation motif (predicted by Scansite: http://scansite.mit.edu/) supporting the possibility of serine-49 phosphorylation regulating the SUMOylation of lysine-42. Together these two modifications might together regulate HNF-1β function.

There are at least two examples in which these post-translational modifications may play a role in human disease. First, mutations in HNF-1β, may affect the rate of SUMOylation or ubiquitination. The second mechanism might be extracellular signals that regulate the rate of HNF-1β SUMOylation or ubiquitination. Mutations of HNF-1β have been associated with RCAD, renal dysplasia, and pancreatic atrophy. Mutations in the coding sequence could alter the ability for SUMO or ubiquitin ligases to efficiently interact with HNF-1β. As an example, serine-36 of HNF-1β is mutated to a phenylalanine in two families with diabetes (25, 26). These studies demonstrated that the resulting HNF-1β protein has higher transcriptional activity than the wild-type HNF-1β. One might hypothesize that S36F may disrupt Ubc9 binding and SUMOylation of lysine-42, yielding a mutant protein with a greater intrinsic transactivation potential.

An example of extracellular signaling perhaps altering HNF-1β function in a disease process may involve reducing the level of ubiquitinated HNF-1β. The

amount of Arkadia in the cell may influence HNF-1β transcriptional activity as evidenced by promoter-reporter assays described in Chapter 3. Previous studies suggested that Arkadia protein localization and transcription are regulated by TGF-β and possibly Wnt signaling (101, 106). Therefore, TGF-β regulation of Arkadia, either transcriptionally or by relocalization of Arkadia protein, may affect HNF-1β target gene expression. In unilateral ureteral obstruction models (UUO), TGF-β signaling is increased. Preliminary experiments showed an increase in Arkadia expression in the tubule of a UUO model (113). The increase in Arkadia, as detected by immunofluorescent microscopy, may reflect a shift in Arkadia from the nucleus to the cytoplasm. Removal of Arkadia from the nucleus could result in diminished HNF-1β transcriptional activity.

The studies described in Chapters 2 and 3 define novel post-translational modifications for HNF-1β. Omitted from this discussion are the other identified proteins in the yeast two-hybrid using HNF-1β as a bait protein. Already several of these other proteins have been confirmed to interact with HNF-1β *in vivo* and have effects on HNF-1β transcriptional activity. Together, understanding the interactions and modifications of HNF-1β will assist in our understanding of human biology and disease.

CHAPTER FIVE

Materials and Methods

Plasmids - The FLAG epitope tagged HNF-1β expression plasmid and the 1.9 kb *Pkhd1* promoter firefly luciferase reporter plasmid were described previously(27). The yeast two-hybrid bait fusion proteins were produced by inserting the PCR products encoding the full-length HNF-1β and C-terminus of HNF-1β (amino acids 263-532) into the vector pBTM116(114). The HA epitope-tagged HNF-1β expression plasmid was generated by inserting a PCR product encoding the coding sequence of HNF-1β into pCMV-HA (BD Biosciences Clontech, Palo Alto, CA). The expression plasmid HA-SUMO-1 was a generous gift from Dr. Kim Orth (UT Southwestern, Dallas, TX). The expression plasmids pmycUbc9 and pmycUbc9C93S were generous gifts from Dr. Zhiyuan Shen (University of New Mexico Health Science Center, Albuquerque, NM). The expression plasmids FLAG-PIASγ, FLAG-PIASγ-AA(92), and FLAG-PIASγ-W363A were generous gifts from Dr. Ke Shuai (UCLA, Los Angeles, CA). The plasmids pGEX2TK-PIAS1 and pGEX2TK-PIAS1(CS) were generous gifts from Dr. Xin-Hua Feng (Baylor College of Medicine, Houston, TX). The myc epitope tagged PIAS1 and myc epitope tagged PIAS1(CS) expression plasmids were generated by inserting the coding sequence derived from pGEX2TK-PIAS1 and pGEX2TK-PIAS1(CS) into pCMV-myc (BD Biosciences Clontech). The expression plasmids FLAG-Arkadia, FLAG-ArkadiaΔC, and FLAG-Arkadia-CA were generous gifts from Dr. Miyazono and colleagues (University of Tokyo, Japan) (101).

Site-Directed Mutagenesis - Mutagenesis of lysine 42 in HNF-1β was performed with QuikChange (Stratagene) and primers 5'-

AATTTCGGGGTGAGGCTGGAGACACTG-3'and 5'-CAGTGTCTCCAGCCTCACCCCGAAATT-3'. The mutation was verified by DNA sequencing.

Yeast Transformation and Growth Selection - LexA-HNF-1β fusion vectors were transformed into *S. cerevisiae* (strain L40, genotype *MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::LexA-LacZ*). Yeast were grown in YPDA (2% Difco peptone, 1% yeast extract, 2% glucose, 0.1% adenine hemisulfate) or in synthetic minimal Trp dropout medium (SD-Trp) complemented with 0.1% adenine hemisulfate. For transformation, a culture was grown overnight to an OD600 of ~1 was centrifuged for 3 min. at 1200g, washed with 40 ml of distilled H2O, and resuspended in 1 ml of H2O. Aliquots of 50 ml were treated with 240 μL of PEG 3350 (50% w/v), 50 μL of ddH2O, 36 μL of 1 M LiOAc, 25 μL of single-stranded DNA (2.0mg/mL), and 1 mg of plasmid. Proper expression of the baits was confirmed by Western blotting of total cell lysates with antibody against LexA. As a control experiment, an autonomous activation of the L40 reporter genes by the baits was verified.

Library Screening - An adult mouse kidney cDNA library (Matchmaker, BD Biosciences Clontech) was used. A 100 mL culture in SD-Trp medium was inoculated, grown to OD546 greater than 2 at 30°C and used to inoculate 1 liter of YPDA to an OD600 of 0.2. The culture was propagated to an OD600 of 0.7, split up in quarters and pelleted at 4200g for 15 min at 4°C. The pellets were washed with 100 mL sterile ddH20 and centrifuged again. Each pellet was resuspended in 40 mL of ddH2O, transferred to 50 mL tubes, and repelleted at 1300g for 10 min at 4 °C. Each pellet was transfected separately using a scaled-up reaction mixture as above with 50 μl of cDNA (1mg/ml). After an incubation at 30°C followed by

a 30 minute heat shock at 42 °C, cells were harvested at 1900g for 3 min, washed with 80 mL of ddH2O, recombined in 20 mL of ddH2O and plated onto 50 14-cm Petri dishes. After 3 days, Trp/Leu/His prototrophies were tested for *LacZ* expression by a colony-lift assay, where the permeabilized cells transferred onto Whatman filters were overlaid with 0.2 mg/ml X-gal, in TBS (50 mM Tris-HCl pH 7.4,150 mM NaCl) and 0.8% agarose. Putative positive colonies were restreaked on selective medium and rescreened by colony-lift assay. Yeast DNA of true positives was isolated, and prey plasmids were rescued by transformation into KC8 cells. Insert sizes were checked by *Bgl*II digestion and subjected to sequencing.

Immunolocalization - Paraformaldehyde-fixed adult mouse kidney sections were stained with antibodies against the SUMO ligases Ubc9 (Santa Cruz Biotechnology, Santa Cruz, CA), PIAS1 (Santa Cruz Biotechnology), PIASγ (Santa Cruz Biotechnology), HNF-1β (gift from Dr. Marco Pontoglio, Pasteur Institute, Paris, France), and Arkadia (gift from Dr. Kohei Miyazono, University of Tokyo). Secondary antibodies were conjugated to Alexa Fluor 488 (Invitrogen, Carlbad, CA) or Alexa Fluor 594 (Invitrogen). Immunofluoresecence and microscopy was performed as described previously.

Cell Culture & Transfections - Mouse inner medullary collecting duct cells (mIMCD-3) and 293T cells were grown in DMEM containing 10% FBS and 1% penicillin and streptomycin. HeLa cells were grown in MEM containing 10% FBS and 1% penicillin and streptomycin. Reporter gene assays were seeded with 1.2 X 10^5 cells/well in six-well dishes. The cells were grown for 24 hours prior to transfection. Cells were transfected, using Effectene (Qiagen), with 0.6 μg of *Pkhd1* luciferase reporter plasmid and 0.02 μg of phRL-SV40 (Promega,

Madison, WI) as a transfection efficiency control. After 48 hours, cells were lysed in 500 μL of Passive Lysis buffer (Promega) for 15 minutes and then centrifuged. Twenty microliters of supernatant was added to a 96-well microplate, and firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Light output was measured by a Wallac VICTOR V multilabel counter (Perkin Elmer, Wellesley, MA).

Co-immunoprecipitations – 293T cells were transfected with 4.0 μg of expression plasmids using Effectene (Qiagen). Cells were harvested 48 hours later and lysed in 150 μL of BIP-Lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA, 10% glycerol, 1% NP-40) for 30 minutes on ice. Extracts were centrifuged for to remove cellular debris. Then the extract was diluted 10 fold in BIP Wash Buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA). The lysate was rotated at 4°C overnight with the antibody-coupled sepharose. The sepharose was washed three times by centrifugation (10 sec, 13,000 x g) using BIP Wash Buffer. The pellet and supernatant were analyzed by immunoblotting using an anti-myc (Sigma) or anti-HA antibody (Sigma).

In vivo SUMOylation Assay – mIMCD3 cells were transfected with 4.0 μg of expression plasmids using Effectene (Qiagen). Cell were harvested 48 hours later in SUMO lysis buffer and then sonicated. The lysate was then centrifuged before being diluted 10 fold with PBS + 0.5% NP-40. The diluted lysate was then rotated overnight with 40 μL of anti-HA-Agarose antibody at 4°C. The agarose was washed three times with PBS + 0.5% NP-40 and centrifugation. The pellet and supernatant were analyzed by immunoblotting using an anti-FLAG epitope antibody (Sigma).

In vivo Ubiquitination Assay – mIMCD3 cells were transfected with 4.0 μg of expression plasmids using Effectene (Qiagen). Cells were treated 36 hours later with proteasome inhibitors for 6 hours before the cells were harvested in lysis buffer and then sonicated. The lysate was then centrifuged before being diluted 10 fold with PBS + 0.5% NP-40. The diluted lysate was then rotated overnight with 40 μL of anti-HA-Agarose antibody at 4°C. The agarose was washed three times with PBS + 0.5% NP-40 and centrifugation. The pellet and supernatant were analyzed by immunoblotting using an anti-FLAG epitope antibody (Sigma).

In vitro SUMOylation Assay - The HNF-1β protein and mutant proteins were *in vitro* transcribed and translated using the TNT® Coupled Reticulocyte Lysate Systems (Promega) with rabbit reticulocyte lysate in 25 μL reactions according to the manufacturer's protocol. For the *in vitro* SUMOylation reaction, 2 μL of programmed lysate was added to 2 μl of Ubc9 (500ng/μL, LAE Biotech), 2 μL of GST-SUMO-1 (1 mg/mL), a generous gift from Dr. Kim Orth (UT Southwestern, Dallas, TX), 2 μL of 10x reaction buffer (LAE Biotech), 2 μL of human SAEI/SAEII (75 ng/μL, LAE Biotech), 2 μL of ATP (20 mM, Sigma) and 8 μL of ddH20. The samples were incubated at 37°C for 60 minutes.

Statistical Analysis – The results of the promoter-reporter assays are expressed as means with the error bar representing the standard deviations of six independent experiments in duplicate. The statistical significance between the means was done using the one-way ANOVA test with Dunnet's post-hoc test. Differences were considered significant at P values less than 0.01.

APPENDIX A

Clones Isolated in Yeast Two Hybrid Screen

Encoded Gene	Number of Independent Clones	Bait Used	Auto-Activation
Ubc9	2	Both	No
PIAS1	1	Full-length	No
PIASγ	2	Full-length	No
Arkadia	1	Full-length	No
Zyxin	1	Full-length	No
LPP	1	C-terminal	No
RACK1	1	C-terminal	No
PLZF	3	Both	Yes
ROG	1	C-terminal	Yes
MAN1	1	Full-length	No
β-galactosidase	1	C-terminal	No
Tcte1l	Not Determined	C-terminal	No
Yif1	2	Full length	No
DNase I	2	C-terminal	No
GMEB-1	1	Full-length	Not Determined
Fibulin 5	1	C-terminal	Not Determined
Hadh2	1	C-terminal	Not Determined

BIBLIOGRAPHY

1. U.S. Renal Data System National Institutes of Health, N.I.D.D.K.D., Bethesda, MD. 2006. USRDS 2006 Annual Data Report: Atlas of End-Stage Renal Disease in the United States.
2. Cereghini, S., Blumenfeld, M., and Yaniv, M. 1988. A liver-specific factor essential for albumin transcription differs between differentiated and dedifferentiated rat hepatoma cells. *Genes Dev* 2:957-974.
3. Baumhueter, S., Courtois, G., and Crabtree, G.R. 1988. A variant nuclear protein in dedifferentiated hepatoma cells binds to the same functional sequences in the beta fibrinogen gene promoter as HNF-1. *Embo J* 7:2485-2493.
4. Courtois, G., Morgan, J.G., Campbell, L.A., Fourel, G., and Crabtree, G.R. 1987. Interaction of a liver-specific nuclear factor with the fibrinogen and alpha 1-antitrypsin promoters. *Science* 238:688-692.
5. Courtois, G., Baumhueter, S., and Crabtree, G.R. 1988. Purified hepatocyte nuclear factor 1 interacts with a family of hepatocyte-specific promoters. *Proc Natl Acad Sci U S A* 85:7937-7941.
6. Mendel, D.B., Khavari, P.A., Conley, P.B., Graves, M.K., Hansen, L.P., Admon, A., and Crabtree, G.R. 1991. Characterization of a cofactor that regulates dimerization of a mammalian homeodomain protein. *Science* 254:1762-1767.
7. Chi, Y.-I., Frantz, J.D., Oh, B.-C., Hansen, L., Dhe-Paganon, S., and Shoelson, S.E. 2002. Diabetes Mutations Delineate an Atypical POU Domain in HNF-1[alpha]. *Molecular Cell* 10:1129-1137.
8. Bohn, S., Thomas, H., Turan, G., Ellard, S., Bingham, C., Hattersley, A.T., and Ryffel, G.U. 2003. Distinct molecular and morphogenetic properties of mutations in the human HNF1beta gene that lead to defective kidney development. *J Am Soc Nephrol* 14:2033-2041.
9. Rey-Campos, J., Chouard, T., Yaniv, M., and Cereghini, S. 1991. vHNF1 is a homeoprotein that activates transcription and forms heterodimers with HNF1. *Embo J* 10:1445-1457.
10. Ringeisen, F., Rey-Campos, J., and Yaniv, M. 1993. The transactivation potential of variant hepatocyte nuclear factor 1 is modified by alternative splicing. *J Biol Chem* 268:25706-25711.
11. Cereghini, S. 1996. Liver-enriched transcription factors and hepatocyte differentiation. *Faseb J* 10:267-282.
12. Hiesberger, T., Shao, X., Gourley, E., Reimann, A., Pontoglio, M., and Igarashi, P. 2005. Role of the hepatocyte nuclear factor-1beta (HNF-1beta)

C-terminal domain in Pkhd1 (ARPKD) gene transcription and renal cystogenesis. *J Biol Chem* 280:10578-10586.

13. Barbacci, E., Chalkiadaki, A., Masdeu, C., Haumaitre, C., Lokmane, L., Loirat, C., Cloarec, S., Talianidis, I., Bellanne-Chantelot, C., and Cereghini, S. 2004. HNF1beta/TCF2 mutations impair transactivation potential through altered co-regulator recruitment. *Hum Mol Genet* 13:3139-3149.
14. Bach, I., and Yaniv, M. 1993. More potent transcriptional activators or a transdominant inhibitor of the HNF1 homeoprotein family are generated by alternative RNA processing. *Embo J* 12:4229-4242.
15. Cereghini, S., Ott, M.O., Power, S., and Maury, M. 1992. Expression patterns of vHNF1 and HNF1 homeoproteins in early postimplantation embryos suggest distinct and sequential developmental roles. *Development* 116:783-797.
16. Barbacci, E., Reber, M., Ott, M.O., Breillat, C., Huetz, F., and Cereghini, S. 1999. Variant hepatocyte nuclear factor 1 is required for visceral endoderm specification. *Development* 126:4795-4805.
17. Coffinier, C., Barra, J., Babinet, C., and Yaniv, M. 1999. Expression of the vHNF1/HNF1beta homeoprotein gene during mouse organogenesis. *Mech Dev* 89:211-213.
18. Horikawa, Y., Iwasaki, N., Hara, M., Furuta, H., Hinokio, Y., Cockburn, B.N., Lindner, T., Yamagata, K., Ogata, M., Tomonaga, O., et al. 1997. Mutation in hepatocyte nuclear factor-1 beta gene (TCF2) associated with MODY. *Nat Genet* 17:384-385.
19. Weber, S., Moriniere, V., Knuppel, T., Charbit, M., Dusek, J., Ghiggeri, G.M., Jankauskiene, A., Mir, S., Montini, G., Peco-Antic, A., et al. 2006. Prevalence of mutations in renal developmental genes in children with renal hypodysplasia: results of the ESCAPE study. *J Am Soc Nephrol* 17:2864-2870.
20. Bonnycastle, L.L., Willer, C.J., Conneely, K.N., Jackson, A.U., Burrill, C.P., Watanabe, R.M., Chines, P.S., Narisu, N., Scott, L.J., Enloe, S.T., et al. 2006. Common Variants in Maturity-Onset Diabetes of the Young Genes Contribute to Risk of Type 2 Diabetes in Finns. *Diabetes %R 10.2337/db06-0178* 55:2534-2540.
21. Lebrun, G., Vasiliu, V., Bellanne-Chantelot, C., Bensman, A., Ulinski, T., Chretien, Y., and Grunfeld, J.P. 2005. Cystic kidney disease, chromophobe renal cell carcinoma and TCF2 (HNF1 beta) mutations. *Nat Clin Pract Nephrol* 1:115-119.
22. Tsuchiya, A., Sakamoto, M., Yasuda, J., Chuma, M., Ohta, T., Ohki, M., Yasugi, T., Taketani, Y., and Hirohashi, S. 2003. Expression profiling in ovarian clear cell carcinoma: identification of hepatocyte nuclear factor-1

beta as a molecular marker and a possible molecular target for therapy of ovarian clear cell carcinoma. *Am J Pathol* 163:2503-2512.
23. Terasawa, K., Toyota, M., Sagae, S., Ogi, K., Suzuki, H., Sonoda, T., Akino, K., Maruyama, R., Nishikawa, N., Imai, K., et al. 2006. Epigenetic inactivation of TCF2 in ovarian cancer and various cancer cell lines. *Br J Cancer* 94:914-921.
24. Edghill, E.L., Bingham, C., Slingerland, A.S., Minton, J.A., Noordam, C., Ellard, S., and Hattersley, A.T. 2006. Hepatocyte nuclear factor-1 beta mutations cause neonatal diabetes and intrauterine growth retardation: support for a critical role of HNF-1beta in human pancreatic development. *Diabet Med* 23:1301-1306.
25. Wang, C., Fang, Q., Zhang, R., Lin, X., and Xiang, K. 2004. Scanning for MODY5 gene mutations in Chinese early onset or multiple affected diabetes pedigrees. *Acta Diabetol* 41:137-145.
26. Yoshiuchi, I., Yamagata, K., Zhu, Q., Tamada, I., Takahashi, Y., Onigata, K., Takeda, J., Miyagawa, J., and Matsuzawa, Y. 2002. Identification of a gain-of-function mutation in the HNF-1beta gene in a Japanese family with MODY. *Diabetologia* 45:154-155.
27. Hiesberger, T., Bai, Y., Shao, X., McNally, B.T., Sinclair, A.M., Tian, X., Somlo, S., and Igarashi, P. 2004. Mutation of hepatocyte nuclear factor-1beta inhibits Pkhd1 gene expression and produces renal cysts in mice. *J Clin Invest* 113:814-825.
28. Harries, L.W., Hattersley, A.T., and Ellard, S. 2004. Messenger RNA transcripts of the hepatocyte nuclear factor-1alpha gene containing premature termination codons are subject to nonsense-mediated decay. *Diabetes* 53:500-504.
29. Coffinier, C., Thepot, D., Babinet, C., Yaniv, M., and Barra, J. 1999. Essential role for the homeoprotein vHNF1/HNF1beta in visceral endoderm differentiation. *Development* 126:4785-4794.
30. Igarashi, P. 2004. Kidney-Specific Gene Targeting. *J Am Soc Nephrol %R 10.1097/01.ASN.0000136298.09488.D7* 15:2237-2239.
31. Coffinier, C., Gresh, L., Fiette, L., Tronche, F., Schutz, G., Babinet, C., Pontoglio, M., Yaniv, M., and Barra, J. 2002. Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. *Development* 129:1829-1838.
32. Wang, L., Coffinier, C., Thomas, M.K., Gresh, L., Eddu, G., Manor, T., Levitsky, L.L., Yaniv, M., and Rhoads, D.B. 2004. Selective deletion of the HNF1{beta} (MODY5) gene in {beta} cells leads to altered gene expression and defective insulin release. *Endocrinology*.

33. Haumaitre, C., Barbacci, E., Jenny, M., Ott, M.O., Gradwohl, G., and Cereghini, S. 2005. Lack of TCF2/vHNF1 in mice leads to pancreas agenesis. *Proc Natl Acad Sci U S A* 102:1490-1495.
34. Gresh, L., Fischer, E., Reimann, A., Tanguy, M., Garbay, S., Shao, X., Hiesberger, T., Fiette, L., Igarashi, P., Yaniv, M., et al. 2004. A transcriptional network in polycystic kidney disease. *Embo J* 23:1657-1668.
35. Molinari, E., Gilman, M., and Natesan, S. 1999. Proteasome-mediated degradation of transcriptional activators correlates with activation domain potency in vivo. *Embo J* 18:6439-6447.
36. Salghetti, S.E., Muratani, M., Wijnen, H., Futcher, B., and Tansey, W.P. 2000. Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. *Proc Natl Acad Sci U S A* 97:3118-3123.
37. Greer, S.F., Zika, E., Conti, B., Zhu, X.S., and Ting, J.P. 2003. Enhancement of CIITA transcriptional function by ubiquitin. *Nat Immunol* 4:1074-1082.
38. von der Lehr, N., Johansson, S., Wu, S., Bahram, F., Castell, A., Cetinkaya, C., Hydbring, P., Weidung, I., Nakayama, K., Nakayama, K.I., et al. 2003. The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. *Mol Cell* 11:1189-1200.
39. Salghetti, S.E., Caudy, A.A., Chenoweth, J.G., and Tansey, W.P. 2001. Regulation of transcriptional activation domain function by ubiquitin. *Science* 293:1651-1653.
40. Kurosu, T., and Peterlin, B.M. 2004. VP16 and ubiquitin; binding of P-TEFb via its activation domain and ubiquitin facilitates elongation of transcription of target genes. *Curr Biol* 14:1112-1116.
41. Muratani, M., Kung, C., Shokat, K.M., and Tansey, W.P. 2005. The F box protein Dsg1/Mdm30 is a transcriptional coactivator that stimulates Gal4 turnover and cotranscriptional mRNA processing. *Cell* 120:887-899.
42. Kodadek, T., Sikder, D., and Nalley, K. 2006. Keeping transcriptional activators under control. *Cell* 127:261-264.
43. Gonzalez, F., Delahodde, A., Kodadek, T., and Johnston, S.A. 2002. Recruitment of a 19S proteasome subcomplex to an activated promoter. *Science* 296:548-550.
44. Conaway, R.C., Brower, C.S., and Conaway, J.W. 2002. Emerging roles of ubiquitin in transcription regulation. *Science* 296:1254-1258.
45. Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A., and Deshaies, R.J. 2001. Negative regulation of Gcn4 and Msn2

transcription factors by Srb10 cyclin-dependent kinase. *Genes Dev* 15:1078-1092.
46. Tansey, W.P. 2001. Transcriptional activation: risky business. *Genes Dev* 15:1045-1050.
47. Hay, R.T. 2005. SUMO: a history of modification. *Mol Cell* 18:1-12.
48. Nacerddine, K., Lehembre, F., Bhaumik, M., Artus, J., Cohen-Tannoudji, M., Babinet, C., Pandolfi, P.P., and Dejean, A. 2005. The SUMO pathway is essential for nuclear integrity and chromosome segregation in mice. *Dev Cell* 9:769-779.
49. Su, H.L., and Li, S.S. 2002. Molecular features of human ubiquitin-like SUMO genes and their encoded proteins. *Gene* 296:65-73.
50. Bohren, K.M., Nadkarni, V., Song, J.H., Gabbay, K.H., and Owerbach, D. 2004. A M55V polymorphism in a novel SUMO gene (SUMO-4) differentially activates heat shock transcription factors and is associated with susceptibility to type I diabetes mellitus. *J Biol Chem* 279:27233-27238.
51. Owerbach, D., McKay, E.M., Yeh, E.T., Gabbay, K.H., and Bohren, K.M. 2005. A proline-90 residue unique to SUMO-4 prevents maturation and sumoylation. *Biochem Biophys Res Commun* 337:517-520.
52. Gong, L., and Yeh, E.T. 2006. Characterization of a family of nucleolar SUMO-specific proteases with preference for SUMO-2 or SUMO-3. *J Biol Chem* 281:15869-15877.
53. Rodriguez, M.S., Dargemont, C., and Hay, R.T. 2001. SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J Biol Chem* 276:12654-12659.
54. Gregoire, S., and Yang, X.J. 2005. Association with class IIa histone deacetylases upregulates the sumoylation of MEF2 transcription factors. *Mol Cell Biol* 25:2273-2287.
55. Hietakangas, V., Ahlskog, J.K., Jakobsson, A.M., Hellesuo, M., Sahlberg, N.M., Holmberg, C.I., Mikhailov, A., Palvimo, J.J., Pirkkala, L., and Sistonen, L. 2003. Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. *Mol Cell Biol* 23:2953-2968.
56. Hietakangas, V., Anckar, J., Blomster, H.A., Fujimoto, M., Palvimo, J.J., Nakai, A., and Sistonen, L. 2006. PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc Natl Acad Sci U S A* 103:45-50.
57. Yang, S.H., Galanis, A., Witty, J., and Sharrocks, A.D. 2006. An extended consensus motif enhances the specificity of substrate modification by SUMO. *Embo J* 25:5083-5093.
58. Tatham, M.H., Jaffray, E., Vaughan, O.A., Desterro, J.M., Botting, C.H., Naismith, J.H., and Hay, R.T. 2001. Polymeric chains of SUMO-2 and

SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem* 276:35368-35374.

59. Holmstrom, S., Van Antwerp, M.E., and Iniguez-Lluhi, J.A. 2003. Direct and distinguishable inhibitory roles for SUMO isoforms in the control of transcriptional synergy. *Proc Natl Acad Sci U S A* 100:15758-15763.
60. Kagey, M.H., Melhuish, T.A., Powers, S.E., and Wotton, D. 2005. Multiple activities contribute to Pc2 E3 function. *Embo J* 24:108-119.
61. Takahashi, Y., and Kikuchi, Y. 2005. Yeast PIAS-type Ull1/Siz1 is composed of SUMO ligase and regulatory domains. *J Biol Chem* 280:35822-35828.
62. Schmidt, D., and Muller, S. 2003. PIAS/SUMO: new partners in transcriptional regulation. *Cell Mol Life Sci* 60:2561-2574.
63. Liu, B., Mink, S., Wong, K.A., Stein, N., Getman, C., Dempsey, P.W., Wu, H., and Shuai, K. 2004. PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. *Nat Immunol* 5:891-898.
64. Roth, W., Sustmann, C., Kieslinger, M., Gilmozzi, A., Irmer, D., Kremmer, E., Turck, C., and Grosschedl, R. 2004. PIASy-deficient mice display modest defects in IFN and Wnt signaling. *J Immunol* 173:6189-6199.
65. Wong, K.A., Kim, R., Christofk, H., Gao, J., Lawson, G., and Wu, H. 2004. Protein inhibitor of activated STAT Y (PIASy) and a splice variant lacking exon 6 enhance sumoylation but are not essential for embryogenesis and adult life. *Mol Cell Biol* 24:5577-5586.
66. Beliakoff, J., and Sun, Z. 2006. Zimp7 and Zimp10, two novel PIAS-like proteins, function as androgen receptor coregulators. *Nucl Recept Signal* 4:e017.
67. Sharma, M., Li, X., Wang, Y., Zarnegar, M., Huang, C.Y., Palvimo, J.J., Lim, B., and Sun, Z. 2003. hZimp10 is an androgen receptor co-activator and forms a complex with SUMO-1 at replication foci. *Embo J* 22:6101-6114.
68. Huang, C.Y., Beliakoff, J., Li, X., Lee, J., Li, X., Sharma, M., Lim, B., and Sun, Z. 2005. hZimp7, a novel PIAS-like protein, enhances androgen receptor-mediated transcription and interacts with SWI/SNF-like BAF complexes. *Mol Endocrinol* 19:2915-2929.
69. Cheng, J., Bawa, T., Lee, P., Gong, L., and Yeh, E.T. 2006. Role of desumoylation in the development of prostate cancer. *Neoplasia* 8:667-676.
70. Johnson, E.S. 2004. Protein modification by SUMO. *Annu Rev Biochem* 73:355-382.

71. Salinas, S., Briancon-Marjollet, A., Bossis, G., Lopez, M.A., Piechaczyk, M., Jariel-Encontre, I., Debant, A., and Hipskind, R.A. 2004. SUMOylation regulates nucleo-cytoplasmic shuttling of Elk-1. *J Cell Biol* 165:767-773.
72. Ishov, A.M., Sotnikov, A.G., Negorev, D., Vladimirova, O.V., Neff, N., Kamitani, T., Yeh, E.T., Strauss, J.F., 3rd, and Maul, G.G. 1999. PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. *J Cell Biol* 147:221-234.
73. Jang, M.S., Ryu, S.W., and Kim, E. 2002. Modification of Daxx by small ubiquitin-related modifier-1. *Biochem Biophys Res Commun* 295:495-500.
74. Zhong, S., Muller, S., Ronchetti, S., Freemont, P.S., Dejean, A., and Pandolfi, P.P. 2000. Role of SUMO-1-modified PML in nuclear body formation. *Blood* 95:2748-2752.
75. Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., and Grosschedl, R. 2001. PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. *Genes Dev* 15:3088-3103.
76. Desterro, J.M., Rodriguez, M.S., and Hay, R.T. 1998. SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. *Mol Cell* 2:233-239.
77. Steffan, J.S., Agrawal, N., Pallos, J., Rockabrand, E., Trotman, L.C., Slepko, N., Illes, K., Lukacsovich, T., Zhu, Y.Z., Cattaneo, E., et al. 2004. SUMO modification of Huntingtin and Huntington's disease pathology. *Science* 304:100-104.
78. Anckar, J., Hietakangas, V., Denessiouk, K., Thiele, D.J., Johnson, M.S., and Sistonen, L. 2006. Inhibition of DNA binding by differential sumoylation of heat shock factors. *Mol Cell Biol* 26:955-964.
79. Komatsu, T., Mizusaki, H., Mukai, T., Ogawa, H., Baba, D., Shirakawa, M., Hatakeyama, S., Nakayama, K.I., Yamamoto, H., Kikuchi, A., et al. 2004. Small ubiquitin-like modifier 1 (SUMO-1) modification of the synergy control motif of Ad4 binding protein/steroidogenic factor 1 (Ad4BP/SF-1) regulates synergistic transcription between Ad4BP/SF-1 and Sox9. *Mol Endocrinol* 18:2451-2462.
80. Yang, S.H., and Sharrocks, A.D. 2004. SUMO promotes HDAC-mediated transcriptional repression. *Mol Cell* 13:611-617.
81. Kaul, S., Blackford, J.A., Jr., Cho, S., and Simons, S.S., Jr. 2002. Ubc9 is a novel modulator of the induction properties of glucocorticoid receptors. *J Biol Chem* 277:12541-12549.

82. Kobayashi, S., Shibata, H., Yokota, K., Suda, N., Murai, A., Kurihara, I., Saito, I., and Saruta, T. 2004. FHL2, UBC9, and PIAS1 are novel estrogen receptor alpha-interacting proteins. *Endocr Res* 30:617-621.
83. Pan, X., Li, H., Zhang, P., Jin, B., Man, J., Tian, L., Su, G., Zhao, J., Li, W., Liu, H., et al. 2006. Ubc9 interacts with SOX4 and represses its transcriptional activity. *Biochem Biophys Res Commun* 344:727-734.
84. Poukka, H., Aarnisalo, P., Karvonen, U., Palvimo, J.J., and Janne, O.A. 1999. Ubc9 interacts with the androgen receptor and activates receptor-dependent transcription. *J Biol Chem* 274:19441-19446.
85. Lee, H., Quinn, J.C., Prasanth, K.V., Swiss, V.A., Economides, K.D., Camacho, M.M., Spector, D.L., and Abate-Shen, C. 2006. PIAS1 confers DNA-binding specificity on the Msx1 homeoprotein. *Genes Dev* 20:784-794.
86. Liu, B., Liao, J., Rao, X., Kushner, S.A., Chung, C.D., Chang, D.D., and Shuai, K. 1998. Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci U S A* 95:10626-10631.
87. Gross, M., Yang, R., Top, I., Gasper, C., and Shuai, K. 2004. PIASy-mediated repression of the androgen receptor is independent of sumoylation. *Oncogene* 23:3059-3066.
88. Yang, S.H., and Sharrocks, A.D. 2005. PIASx acts as an Elk-1 coactivator by facilitating derepression. *Embo J* 24:2161-2171.
89. Igarashi, P., Shao, X., McNally, B.T., and Hiesberger, T. 2005. Roles of HNF-1[beta] in kidney development and congenital cystic diseases. *Kidney Int* 68:1944-1947.
90. Rebouissou, S., Vasiliu, V., Thomas, C., Bellanne-Chantelot, C., Bui, H., Chretien, Y., Timsit, J., Rosty, C., Laurent-Puig, P., Chauveau, D., et al. 2005. Germline hepatocyte nuclear factor 1alpha and 1beta mutations in renal cell carcinomas. *Hum Mol Genet* 14:603-614.
91. Liang, M., Melchior, F., Feng, X.H., and Lin, X. 2004. Regulation of Smad4 sumoylation and transforming growth factor-beta signaling by protein inhibitor of activated STAT1. *J Biol Chem* 279:22857-22865.
92. Liu, B., Gross, M., ten Hoeve, J., and Shuai, K. 2001. A transcriptional corepressor of Stat1 with an essential LXXLL signature motif. *Proc Natl Acad Sci U S A* 98:3203-3207.
93. Gill, G. 2005. Something about SUMO inhibits transcription. *Curr Opin Genet Dev* 15:536-541.
94. Wang, J., Feng, X.H., and Schwartz, R.J. 2004. SUMO-1 modification activated GATA4 dependent cardiogenic gene activity. *J Biol Chem*.
95. Alkuraya, F.S., Saadi, I., Lund, J.J., Turbe-Doan, A., Morton, C.C., and Maas, R.L. 2006. SUMO1 haploinsufficiency leads to cleft lip and palate. *Science* 313:1751.

96. Dahle, O., Bakke, O., and Gabrielsen, O.S. 2004. c-Myb associates with PML in nuclear bodies in hematopoietic cells. *Exp Cell Res* 297:118-126.
97. Poukka, H., Aarnisalo, P., Karvonen, U., Palvimo, J.J., and Janne, O.A. 1999. Ubc9 Interacts with the Androgen Receptor and Activates Receptor-dependent Transcription. *J. Biol. Chem. %R 10.1074/jbc.274.27.19441* 274:19441-19446.
98. Chakrabarti, S.R., Sood, R., Ganguly, S., Bohlander, S., Shen, Z., and Nucifora, G. 1999. Modulation of TEL transcription activity by interaction with the ubiquitin-conjugating enzyme UBC9. *PNAS %R 10.1073/pnas.96.13.7467* 96:7467-7472.
99. Tomoiu, A., Gravel, A., Tanguay, R.M., and Flamand, L. 2006. Functional Interaction between Human Herpesvirus 6 Immediate-Early 2 Protein and Ubiquitin-Conjugating Enzyme 9 in the Absence of Sumoylation. *J. Virol.* 80:10218-10228.
100. Episkopou, V., Arkell, R., Timmons, P.M., Walsh, J.J., Andrew, R.L., and Swan, D. 2001. Induction of the mammalian node requires Arkadia function in the extraembryonic lineages. *Nature* 410:825-830.
101. Koinuma, D., Shinozaki, M., Komuro, A., Goto, K., Saitoh, M., Hanyu, A., Ebina, M., Nukiwa, T., Miyazawa, K., Imamura, T., et al. 2003. Arkadia amplifies TGF-beta superfamily signalling through degradation of Smad7. *Embo J* 22:6458-6470.
102. Muratani, M., and Tansey, W.P. 2003. How the ubiquitin-proteasome system controls transcription. *Nat Rev Mol Cell Biol* 4:192-201.
103. Lipford, J.R., Smith, G.T., Chi, Y., and Deshaies, R.J. 2005. A putative stimulatory role for activator turnover in gene expression. *Nature* 438:113-116.
104. Adhikary, S., Marinoni, F., Hock, A., Hulleman, E., Popov, N., Beier, R., Bernard, S., Quarto, M., Capra, M., Goettig, S., et al. 2005. The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. *Cell* 123:409-421.
105. Salghetti, S.E., Kim, S.Y., and Tansey, W.P. 1999. Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. *Embo J* 18:717-726.
106. Liu, W., Rui, H., Wang, J., Lin, S., He, Y., Chen, M., Li, Q., Ye, Z., Zhang, S., Chan, S.C., et al. 2006. Axin is a scaffold protein in TGF-beta signaling that promotes degradation of Smad7 by Arkadia. *Embo J* 25:1646-1658.
107. Heery, D.M., Kalkhoven, E., Hoare, S., and Parker, M.G. 1997. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* 387:733-736.

108. Torchia, J., Rose, D.W., Inostroza, J., Kamei, Y., Westin, S., Glass, C.K., and Rosenfeld, M.G. 1997. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* 387:677-684.
109. Plevin, M.J., Mills, M.M., and Ikura, M. 2005. The LxxLL motif: a multifunctional binding sequence in transcriptional regulation. *Trends Biochem Sci* 30:66-69.
110. Rodriguez, M.S., Desterro, J.M., Lain, S., Midgley, C.A., Lane, D.P., and Hay, R.T. 1999. SUMO-1 modification activates the transcriptional response of p53. *Embo J* 18:6455-6461.
111. Park, I.N., Cho, I.J., and Kim, S.G. 2004. Ceramide negatively regulates glutathione S-transferase gene transactivation via repression of hepatic nuclear factor-1 that is degraded by the ubiquitin proteasome system. *Mol Pharmacol* 65:1475-1484.
112. Carriere, V., Lacasa, M., and Rousset, M. 2001. Activity of hepatocyte nuclear factor 1alpha and hepatocyte nuclear factor 1beta isoforms is differently affected by the inhibition of protein phosphatases 1/2A. *Biochem J* 354:301-308.
113. Liu, F.Y., and Li, X.Z. 2006. The roles of Arkadia in renal tubular epithelial to mesenchymal transition. *Med Hypotheses* 67:1205-1207.
114. Gisler, S.M., Stagljar, I., Traebert, M., Bacic, D., Biber, J., and Murer, H. 2001. Interaction of the type IIa Na/Pi cotransporter with PDZ proteins. *J Biol Chem* 276:9206-9213.

VITAE

Brian Timothy McNally was born in Washington, D.C., on November 3, 1974, the son of Josephine Ranney Diamond McNally and Michael John McNally. After completing his work at Paint Branch High School, Burtonsville, Maryland in 1992, he entered Carnegie Mellon University at Pittsburgh, Pennsylvania. During the summer of 1994, he was employed in the laboratory of David C. Beebe M.D. at the Uniformed Services University of the Health Sciences (USUHS) Bethesda, Maryland. In the spring of 1995, he performed a research project in the laboratory of Charles Ettensohn Ph.D. at Carnegie Mellon. In 1996, he was employed as a research technician in the laboratory of Dr. Ishaiahu Shechter, Ph.D. at USUHS. He received the degree of Bachelor of Science with a major in biological sciences from Carnegie Mellon University in December of 1997. During the following two years, he was a biological research technician in the laboratory of Jonathan S. Minden Ph.D. at Carnegie Mellon University, Pittsburgh, Pennsylvania. On August 23, 2000, he entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. He was awarded the degree of Doctor of Philosophy on December 7, 2006. In 2005, he married Caryn Rothrock McNally of Greensboro, Georgia.

Permanent Address: 442 Mills Lane
Irving, Texas 75062