The role of estrogen sulfotransferase in ischemic acute kidney injury

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Acute kidney injury (AKI) is a sudden impairment of kidney function. It has been suggested that estrogens may protect mice from AKI. Estrogen sulfotransferase (*SULT1E1*/EST) plays an important role in estrogen homeostasis by sulfonating and deactivating estrogens, but the role of *SULT1E1* in AKI has not been previously reported.

In this dissertation, Wild-type (WT) mice, *Sult1e1* knockout (*Sult1e1* KO) mice, and *Sult1e1* KO mice with hepatic reconstitution of *Sult1e1* were subjected to a bilateral kidney ischemia-reperfusion model of AKI, in the absence or presence of gonadectomy. Kidney injury was assessed at biochemical, histological and gene expression levels. WT mice treated with the *Sult1e1* inhibitor triclosan were also used to determine the effect of pharmacological inhibition of *Sult1e1* on AKI. Results showed that AKI induces the expression of *Sult1e1* in a tissue- and sex-specific manner, inducing the hepatic expression of *Sult1e1* in both male and female mice, but the kidney induction of *Sult1e1* was only observed in male mice. A deeper investigation further demonstrated that (1) genetic knockout or pharmacological inhibition of *Sult1e1* protects mice of both sexes from AKI in a sex hormone-independent manner. (2) Moreover, the effect of *Sult1e1* on AKI is also tissue- and sex-specific, because transgenic reconstitution of *Sult1e1* to the liver of *Sult1e1* KO mice abolishes the protection in male mice, but not in female mice.

Finally, it was observed that the protective effect of *Sult1e1* ablation is possibly associated with increased vitamin D receptor signaling. Overall, this dissertation elucidates a novel function

of *Sult1e1* in the pathogenesis of AKI. *Sult1e1* inhibitors may have their therapeutic utility in the clinical management of AKI.

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List of Abbreviations

4-OHT	4-hydroxytamoxifen
ß-gal	ß-galactosidase
AKI	Acute kidney injury
ALT	Alanine aminotransferase
AR	Androgen receptor
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BUN	Blood Urea Nitrogen
Calcidiol	25-hydroxycholecalciferol
Calcitriol	1,25-dihydroxycholecalciferol
CAR	Constitutive Androstane Receptor
Ccnd1	Cyclin D1
CEEs	Conjugated equine estrogens
CDCA	Chenodeoxycholic acid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CKD	Chronic kidney disease
cSNPs	Coding single nucleotide polymorphisms
CLP	Cecum ligation and puncture
CYP	Cytochrome P450
Cyp24a1	Cytochrome P450 family 24 subfamily A member 1, enzyme that deactivates calcidiol and calcitriol
Cyp2r1	Cytochrome P450 Family 2 Subfamily R Member 1 or Vitamin D 25-hydroxylase

CXCL1	Chemokine ligand-1
db/db	Diabetic mouse model
DCNP	2,6-dichloro p-nitrophenol
DEX	Dexamethasone
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DMEs	Drug metabolizing enzymes
DOPA	Dihydroxyphenylalanine
E_1	Estrone
E_2	Estradiol
eGFR	Glomerular filtration rate
EE	Ethynylestradiol
ER	Estrogen receptor
Sult1e1/EST	Estrogen Sulfotransferase
Fgg	Fibrinogen gamma chain
GEO	Gene Expression Omnibus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
GST	Glutathione S-transferases
GW3965	Liver X Receptor agonist
GW4064	Oxymethyl-5-isopropylisoxazole
HFD	High fat diet
HIF-1	Hypoxia-inducible factor 1
HRT	Hormone replacement therapy
HKC-8	Human kidney proximal tubular
HNF4α	Hepatocyte nuclear factor 4α
IFNγ	Interferon gamma

IGF-1	Insulin-like growth factor-1
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-10	Iinterleukin 10
IL-18	Interleukin 18
IRF-1	Interferon regulatory factor-1
KDIGO	Kidney disease: improving global outcomes
Ki	Uncompetitive inhibitory constant
Kic	Competitive inhibitory constant
KIM-1	Kidney injury molecule-1
KO	Knockout
KOLE	Transgenic mouse strain obtained from mating KO and LE mice
Lap	Liver-enriched activator protein
LE	Transgenic strain that overexpresses *Sult1e1* artificially in liver only
LIRI	Liver ischemia-reperfusion injury
LPS	Lipopolysaccharide
L-FABP	Liver fatty acid binding protein
LXR	Liver X receptor
M1	*Ttrans*-resveratrol-3-O-sulfate
M2	*Trans*-resveratrol-4-O-sulfate
MCP-1	Monocyte chemoattractant protein-1
MIP-2	Macrophage inflammatory protein 2
MPA	Medroxyprogesterone acetate
NAT	N-acetyltransferase
NGAL/Lcn2	Neutrophil associated lipocalin 2
NET	Norethindrone (NET)
NRF2	Nuclear factor erythroid 2-related factor 2
Oae	Male obe mice with adipose reconstitution of *Sult1e1*

Ob/ob	Genetic mouse model of obesity and type 2 diabetes
Obe	Female ob/ob mice with a *Sult1e1* ablation
PAPSS1	3'-Phosphoadenosine 5'-Phosphosulfate Synthase 1
PAPSS2	3'-Phosphoadenosine 5'-Phosphosulfate Synthase 2
PAS	Periodic acid Schiff
PBS	Phosphate buffer saline
PC	Partition coefficient
PCB	Polychlorinated biphenyls
PCB-OHs	Hydroxylated metabolites of PCBs
PG	Progesterone receptor
PGC1α	Peroxisome proliferator-activated receptor-γ coactivator 1α
PPARγ	Peroxisome proliferator-activated receptor gamma
PXR	Pregnane X Receptor
SULT	Sulfotransferase
KRT	Kidney replacement therapy
RCTs	Randomized controlled trials
RIF	Rifampicin
RORα	Retinoid-related orphan receptor alpha
ROS	Reactive oxygen species
SERMs	Selective estrogen receptor modulators
STAT3	Signal transducer and activator of transcription 3
STAT5b	Signal transducers and activators transcription 5b
STS	Steroid sulfatase
SULT	Sulfotransferase
T2DM	Type 2 diabetes mellitus
TCBOPOP	CAR agonist
TGF-ß	Transforming growth factor beta
TUNEL	Terminal transferase dUTP nick-end labeling

UGT	UDP-glucuronosyltransferase

Preface

When I was first interviewed by my advisor, Dr. Wen Xie, I knew my chances were very small, but he surprisingly accepted me as his student and I am so grateful to him. His continued support, guidance, and mentorship throughout my Ph.D. training made me stronger and more confident. He always gave me independency and trusted my instincts. This dissertation would not have been possible without his encouragement, advice, motivation, and inspiration.

Whenever needed, I knew I could always count on the support of Dr. Youhua Liu and Dr. Dong Zhou. They patiently helped me when I was struggling with experiments in the kidney field and their lab was always open to me. Countless times they trusted me with their equipment and I am profoundly thankful to them. I would also like to thank the other members of my dissertation committee, Dr. Christian Fernandez, Dr. Robert Gibbs, and Dr. Thomas Nolin for their time and valuable feedback. I would like to thank all current and former members in the Xie Lab for their help, support, and friendship. I also would like to acknowledge all the collaborators who contributed to the research illustrated in this dissertation.

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My sincere gratitude also goes to my beloved parents. When I was eight years old my father gave me a toy microscope. I was very curious about all the insect slides it contained. After a few days, my fascination was growing more and I was punching my own finger to observe my blood under the microscope. Little did he know that gift would change my life forever. From early on, my mother further identified that I was very eager to learn other languages. Although my parents struggled a lot financially and private English courses were very expensive, they always put their children first and sacrificed for our education. I owe my family everything, and it's gracious to observe how every single step of their generosity along this journey has contributed to this moment right now.

Above all, I'm grateful to God. In research, sometimes our data cannot be easily understood, and neither can faith.

This dissertation is dedicated to God, my family, relatives, and my friends.

1.0 Introduction

1.1 AKI

Acute kidney injury (AKI) is defined by the Kidney Disease: Improving Global Outcomes (KDIGO) criteria as an abrupt impairment of kidney function manifested by high levels of serum creatinine (1.5–1.9 times baseline, or ≥ 0.3 mg/dl) and/or reduced urine output (0.5 ml/kg/h for 6–12 hours) (**Table 1**) [1]. The AKI syndrome is common in critically ill patients and is associated with increased length of hospitalization, morbidity, chronic kidney disease, and mortality [2]. The etiology of AKI includes pre-kidney (loss of blood flow to the kidney), intrinsic (direct damage to the organ), and post-kidney (obstruction of the lower urinary system) causes (**Table 2**) [1-3].

Table 1: Staging of AKI: KDIGO.

STAGE	SERUM CREATININE	URINE OUTPUT
1	1.5-1.9 times baseline OR ≥ 0.3 mg/dl (≥ 26.5 nmol/l) increase	< 0.5 ml/kg/h for 6-12 hours
2	2.0-2.9 times baseline	< 0.5 ml/kg/h for ≥ 12 hours
3	3.0 times baseline OR increase in serum creatinine ≥ 4.0 mg/dl (≥ 353.6 μmol/l) OR initiation of kidney replacement therapy (KRT) OR, in patients < 18 years, decrease in eGFR to < 35 ml/min per 1.73 m_2	< 0.3 ml/kg/h for ≥ 24 hours OR anuria for ≥ 12 hours

Adapted from [1, 4].

Table 2: Causes of AKI.

CAUSES OF AKI		
PRE-KIDNEY	**INTRINSIC**	**POST KIDNEY**
Ischemia Hemorrhage Heart attack Heart disease Liver failure Severe burns/dehydration Use of NSAIDs Severe allergic reaction	Blood clots in the kidney's veins and arteries Cholesterol deposits Glomerulonephritis Antibiotics Certain chemotherapies Toxins (e.g. alcohol) Heavy metals Cocaine	Urinary tract obstruction at any site downstream the kidney. (e.g. stones, tumor, and prostatic enlargement)

Adapted from [1, 4].

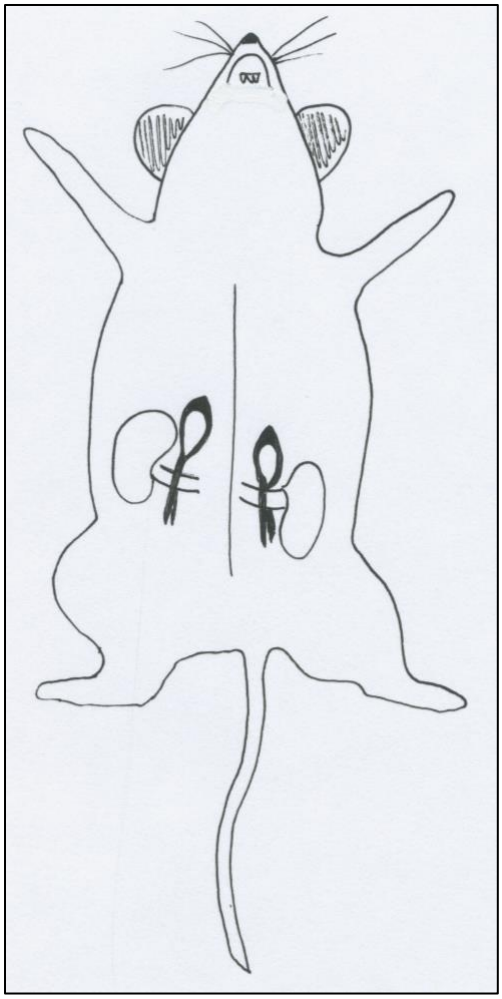

Figure 1: Illustration of bilateral kidney ischemia-reperfusion.

Understanding the pathophysiology of AKI will facilitate the development of novel strategies to manage this disease. The bilateral kidney warm ischemia-reperfusion is a widely used mouse model of AKI [5]. For this approach, a midline incision is performed dorsally in a mouse anesthetized with 90mg/kg ketamine and 10mg/kg xylazine. Using heatpad or heatblock, the animal's body temperature is controlled at 37ºC. Left and right kidney pedicles are then obstructed for 30 minutes using metallic clamps, as in **Figure 1**. During ischemia, the kidneys turn into a darker color. Mice are closely monitored throughout that period and dryness is avoided by covering the abdominal cavity with phosphate buffer saline (PBS) embedded gauze. Once the 30 minutes

have been reached the clamps are removed, the kidneys are reperfused with blood flow and gradually turn into a red color, darker than natural [6] due to inflammatory activation.

Inflammation plays an important role in AKI pathophysiology. In the initial phase of AKI, renal vascular endothelial cells affected by IRI interact with injurious agents that disrupt the endothelial wall. This loss of cellular adhesion results in increased vascular permeability and enables leukocyte (neutrophil, macrophage, lymphocyte, natural killer cells, and dendritic cells) infiltration [7]. Next, reactive oxygen species (ROS) stimulate the production of interferon regulatory factor-1 (IRF-1) –a transcription factor that stimulates expression of proinflammatory genes– in the S3 portion of renal tubular cells [8]. Kidney injury marker-1 (KIM-1), an immunoglobulin superfamily cell surface molecule produced in epithelial tubule cells after IRI is then rapidly increased. Kim-1 converts epithelial cells into phagocytes that engulf apoptotic and necrotic cells [9]. Tubule cells and leukocytes then release proinflammatory cytokines, such as interferon-gamma (IFNγ), Interleukins (IL) 2,10, and 6, and transforming growth factor-beta (TGF-ß) [10, 11]. Cytokines, complement, ROS, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) system, and toll-like receptor (TLR)-related pathways stimulate chemokines, such as chemokine ligand-1 (CXCL1), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 2 (MIP-2), and monocyte chemoattractant protein-1 (MCP-1) [10-12]. All these contribute to the resulting AKI injury.

The clinical management of AKI is comprised of fluid support and agents to prevent life-threatening nutritional and hemodynamic changes, but no specific pharmacological treatment for AKI has been approved [1]. To avoid acidosis, electrolyte alterations, uremia, and fluid accumulation, the KDIGO criteria suggest discontinuing agents toxic to kidneys, controlling fluid overload, monitoring disease markers, and consuming a total of 20 to 30kcal/kg/day in patients of any AKI stage. At later stages, it is suggested to evaluate the medication regimen, consider admission at the intensive care unit (ICU), and artificial kidney filtration. Finally, at the last stage, subclavian catheters should be avoided because of the high incidence of subclavian stenosis in this group [1] (**Table 3**).

Table 3: AKI Management.

AKI STAGE			
HIGH RISK	**STAGE 1**	**STAGE 2**	**STAGE 3**
Discontinue all agents toxic to kidneys when possible			
Ensure volume status and pressure			
Consider functional hemodynamic monitoring			
Monitor serum creatinine and urine output			
Avoid hyperglycemia			
Consider alternatives to radiocontrast procedures			
	Non-invasive diagnostic workup		
	Consider invasive diagnostic workup		
		Check for changes in drug dosing	
		Consider kidney replacement therapy	
		Consider ICU admission	
			Avoid subclavian catheters if possible

Adapted from [1, 4].

Besides its kidney effect, AKI has been reported to affect many distal organs, including the liver. The accumulation of uremic toxins, an imbalance between acid and alkaline molecules, electrolyte imbalance, inflammation, oxidative stress, and neurohormonal dysfunction are responsible for impairing the function of organs such as the brain, immune system, intestine, lung, liver, and heart (**Figure 2**) [13, 14].

AKI may, sometimes, resolve without complications. In a cohort study, 26.6% of AKI patients recovered within a 7-days hospitalization. However, relapses were common and their 1-year mortality after hospital discharge reached 10% [15]. Therefore, AKI commonly progresses to chronic kidney disease (CKD). CKD development was observed in 24.6% of a cohort followed-up for three years after hospital admission [16]. Moreover, controlled CKD may once again turn acute. Consequently, both AKI and CKD may progress to kidney failure. The complications of kidney failure comprise, especially, the cardiac and pulmonary systems. It is estimated that kidney failure has a prevalence of approximately 2100 per million people in the US population and it is expected that by 2030 this prevalence may increase up to 70% [17]. Therefore, it is crucial to find a specific drug target to treat AKI.

Each year in the United States, the need for dialysis is increased by 10% in comparison to the previous year. This high incidence is especially associated with male sex, black race, and older age. Sepsis, cardiac complications, and mechanical respiration were responsible for a third of cases that required dialysis [18]. Continuous kidney replacement therapy (CRRT), the leading form of RRT, comprises hemofiltration, which stands for treatment with a replacement fluid that is combined with the blood and enables clearance, this solution is then ultrafiltered by a semipermeable membrane at an effluent flow rate of 20–25 ml/kg per h and returned to the patient [19]. Nonetheless, the overall patient survival rate is below 60%, especially among pediatric patients [20].

Kidney transplantation is a common outcome in kidney failure patients. Although the number of successful transplants are increasing each year, approximately four thousand people die annually on the wait-list [21]. However, up to three years after transplanting the new organ, approximately 11% of patients develop new episodes of AKI, principally those at the early stages

of CKD. As far as 90 days after an AKI hospitalization of kidney transplanted patients, transplant loss for any reason, death with a working transplant, and death-censored transplant failure happened to 26.3%, 11.4%, and 14.9% of AKI patients, respectively [22]. These issues can be partially accounted for by subclinical kidney injury in deceased donors. Reese *et al.* (2016) demonstrated that five urinary biomarkers of AKI, such as NGAL, interleukin 18 (IL-18), KIM-1, microalbumin, and liver-type fatty acid-binding protein (L-FABP) were elevated in 9% of these donors while creatinine was still low. The transplant loss can also be accounted for by stressful events preceding organ donation that lead to kidney damage, such as brain trauma, hypotension, and administration of compounds toxic to kidneys during hospitalization. Moreover, organ recipients of a kidney with AKI acquire delayed graft function and have a 40% increased risk of graft loss within 1 year of transplantation, as the allograft damage culminates with immune cell infiltration, inflammation, interstitial fibrosis, tubular atrophy, and glomerulosclerosis [21]. Therefore, there are many risks associated with kidney replacement therapy and kidney transplantation. Identifying and treating AKI at the early stages is paramount to prevent serious complications that may become irreversible.

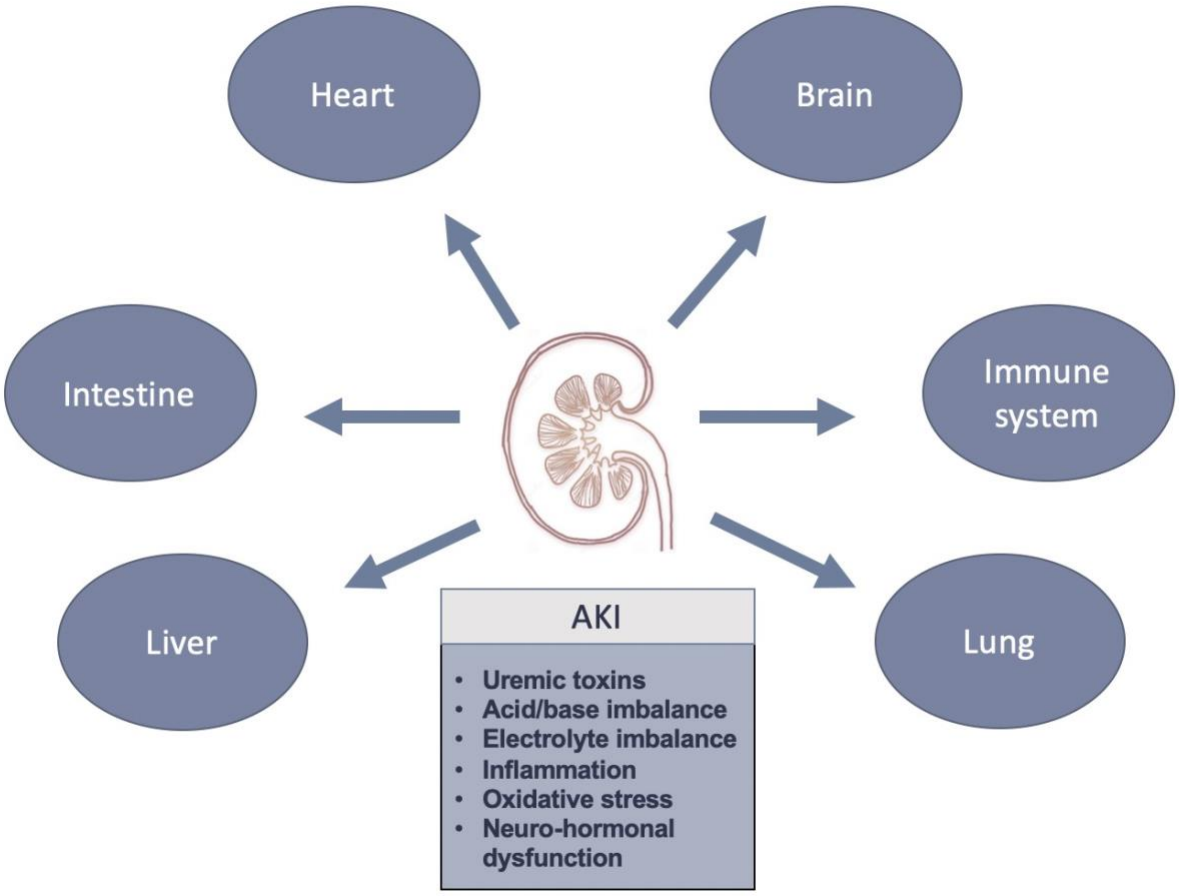

Figure 2: The impact of AKI on distant organs.

Adapted from [14].

Understanding how AKI impairs distant organs at early stages is paramount to prevent the occurrence of complications. In the liver, AKI may lead to increased levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and impaired activity of multiple drug metabolizing enzymes (DMEs) via increased inflammation, apoptosis, and oxidative stress [23-25]. Most of the reported effects of AKI on DMEs are focused on the phase I cytochrome P450 enzymes [25], whereas the AKI effect on the expression and activity of the phase II conjugating enzymes is largely unknown.

1.2 Phase II enzymes

Metabolism is a crucial mechanism to inactivate and excrete both endobiotics and xenobiotics. This process occurs in the gut, liver, and kidneys, and is divided into hydrolytic and oxide redox (Phase I) reactions, as well as conjugation reactions (Phase II). The phase I enzymes are responsible for N- and O-dealkylation, aliphatic and aromatic hydroxylation, N- and S-oxidation, and deamination of any lipophilic compounds. Ester hydrolases and the cytochrome P450 enzymes are involved primarily in the hydroxylation, the latter being the most important and extensively studied phase I enzymes [26].

The phase II enzymes also play a crucial role in drug metabolism. These enzymes are responsible for the conjugation of oxidized or hydrolyzed compounds, therefore making them more hydrosoluble and suitable for excretion. Phase II enzymes are mostly transferases that transfer small molecular weight, organic donor molecules such as 3-phosphoadenosine 5'-phosphosulfate (PAP)-sulfate, glutathione, UDP-glucuronic acid, or acetyl-coenzyme A [26-29]. These conjugation reactions are catalyzed by PAPS-sulfotransferase (*SULT*), glutathione *S*-Transferase (GST), UDP-glucuronosyltransferase (UGT), and N-Acetyltransferase (NAT).

1.2.1 Sulfotransferases, their functions and tissue distributions

Sulfotransferases (*SULT*) comprise a gene family of enzymes responsible for catalyzing reversible sulfation of low molecular weight compounds via the transfer of a negatively charged sulfonate group (SO3-) from the universal donor 3-phosphoadenosine 5'-phosphosulfate (PAPS) to a nucleophilic group of their substrates [30-36]. PAPS is produced by the reaction between inorganic sulfate, uptake from the extracellular medium to the cytosol [35], and two molecules of

ATP, which can be mediated by both ATP sulfurylase and two forms of adenosine 5'-phosphosulfate kinase (APS kinases), PAPSS1 and PAPSS2 [30-32, 36]. Both PAPS and APS kinases are conserved among species and their absence culminates with lethality because sulfotransferases are vital for homeostasis [36, 37]. *SULT* enzymes are divided into membrane-bound, Golgi-residing [34, 38], and soluble cytosolic enzymes [34]. The Golgi-located *SULTs* conjugate proteins, carbohydrates, and proteoglycans, whereas the cytosolic enzymes sulfate essentially small hydrophobic molecules, such as phenols, xenobiotics (including drugs, dietary chemicals, and environmental contaminants [36, 39]) and steroids [34, 36, 39]. Although some sulfated chemicals remain metabolically active, sulfation is majorly a vital step for detoxification and reduction of biological activity, as it increases hydrosolubility, enabling the molecule to be excreted from the body via urine and/or bile [39]. At least thirteen isoforms of human cytosolic *SULTs* have been identified [30, 33, 40], but out of those, *SULT*1 and *SULT*2 families are responsible for sulfonating the largest number of xeno and endobiotics, making them the most important isoforms for drug metabolism [30, 33, 34, 36]. Their isoforms comprise phenol sulfotransferases (*SULT1A1* and *SULT*1A2), catecholamine phenol sulfotransferase (*SULT*1A3/4), thyroid hormone sulfotransferase (*SULT1B*1), estrogen sulfotransferase (*SULT1E1*), and hydroxysteroid sulfotransferase (*SULT*2A1) [39]. For the substrates, *SULT* 1A1 and 1A2 are responsible for the metabolism of phenolic compounds, whereas *SULT*1A3/4 is responsible for the conjugation of catecholamines. *SULT1B*1 conjugates the thyroid hormone substrates, tyrosine, and DOPA, whereas *SULT*2A1 and *SULT1E1* have steroid substrates, with estrogens as the preferred substrates of *SULT1E1* [41]. The expression of *SULT*1 and *SULT*2 isoforms vary among tissue types, and the expression is subject to the regulation by tissue development and hormonal influence [30, 33, 34].

Among *SULT*S, *SULT1A1* is one of the most studied *SULT* isoforms. It has been suggested that polymorphisms in its gene may have accounted for variations in inter-individual susceptibility to cancers [30, 32, 33] due to the fact that *SULT1A1* activates environmental mutagens and carcinogens found in well-done meat [30, 33, 40]. A study conducted by Riches *et al.* analyzed the expression of all major human *SULT*S within different organs. Their results demonstrated that out of all human hepatic *SULT* enzymes, 53% of them are *SULT1A1*, followed by *SULT*2A1 (27%), *SULT1B*1 (14%), and *SULT1E1* (6%). In the gastrointestinal tract, *SULT1B*1 accounted for 36% of all *SULTs*, followed by *SULT1A3* (31%), *SULT1A1* (19%), *SULT1E1* (8%), and *SULT2A1* (6%). In the same report, the authors also observed that *SULT1E1* was the main isoform expressed in the lung (40%), with a lower expression of *SULT1A1* (20%), *SULT1A3* (19%), *SULT1B1* (12%), and *SULT*2A1 (9%). Meanwhile, *SULT1A1*, *SULT1B1*, and *SULT1A3* were abundantly expressed in the kidney, constituting 40, 31, and 28% of all *SULTs*, respectively [39]. Although the kidney expression of *SULT1E1* was not detectable in the study, Miki *et al.* have previously shown that this enzyme is also expressed in the tubular cells of the human kidney, and in several other tissues, including trachea, lung, esophagus, spleen, pancreas, adkidney gland, thyroid, urinary bladder [42], as well as placenta, testis, and ovaries [43, 44]. On the other hand, the evaluation of *SULT* tissue distributions in mice showed some discrepancies compared to the human isoforms. As reported by Alnouti and colleagues, the *Sult1e1* mRNA was only expressed in the gonadal organs [45]. In contrast to their findings, several studies have since been published showing that *Sult1e1* is expressed in mouse extragonadal tissues, such as the liver and adipose tissue [46-49], whereas recent findings suggest it may also be expressed in the mouse kidney [50].

1.3 *SULT1E1*

The circulating estrogens are predominantly synthesized in premenopausal women's ovaries. Upon menopause, the ovary discontinues the estrogen production, but extragonadal tissues such as the breast [51], adipose tissue, and brain maintain the production of estrogens. In males as well as females, testosterone and androstenedione can serve as substrates of the brain and testis *CYP19A1* enzyme (aromatase) to synthesize estrogens [52]. Most, if not all, the cellular effects of estrogens are mediated by the nuclear receptors estrogen receptor alpha (ERα) and -beta (ERß) with a high ligand-receptor binding affinity (Kd ˜1nM) [53-55]. Although estrogens, namely estradiol (E2) and estrone (E1), have been reported to be the substrates of multiple *SULTs* including *SULT1A1* and SULT2A1 [56], *SULT1E1* exhibits the highest affinity for these hormones, especially the 3-hydroxyl position of E_2 [30, 55], to which it binds with a Michaelis-Menten constant (*Km)* of 0.27 nM and with a turnover number (*kcat)* of $10s^{-1}x10^3$/nM respectively [57, 58]. This sulfoconjugation of estrogens can be reversed by the deconjugation reaction catalyzed by the steroid sulfatase (STS) [59].

In COS-1 cells, *SULT1E1* was able to sulfoconjugate dehydroepiandrosterone (DHEA), but with a low affinity of 850 nM [60]. Interestingly, at the concentration of 1.8 nM E_1 is sulfonated by *SULT1E1*; however, at 40 nM this compound inhibits the *Sult1e1* activity [58, 61]. The structure of *SULT1E1* is majorly formed by an α/ß motif that comprises a ß sheet of five parallel ß-strands involved by two lateral α-helices and a preserved helix, which accommodate the PAPS binding site [62]. Petrotchenko and colleagues demonstrated that through van der Waals interactions, E_2 firmly adheres to the Tyr-81/Phe-142 residues of *SULT1E1* and is placed horizontally in the cylindrical hydrophobic binding pocket. The enzyme then transfers the 5'-sulfate of PAPS to the

3’-phenolic hydroxyl group of E_2 [57]. Mutations in these residues lead to reduced E_2 sulfonation [57].

A ligand-binding study performed by Zhang *et al*. showed that two different molecules of E_2 may independently attach to the *SULT1E1* binding pocket, either via an allosteric or a catalytic site, suggesting a random Bi Bi mechanism with two dead-end complexes [55]. The resulting sulfonated estrogens are more hydrosoluble, the 1-octanol/water partition coefficients (PC) of E_2 and E_2-sulfate are 490 and <0.01, respectively [33, 63]. Therefore, sulfonated estrogens are unable to bind the receptors located inside the hydrophobic nuclear envelope and thus lose their hormonal activity. **Figure 3** depicts the major findings regarding the structure and physiological role of *SULT1E1*.

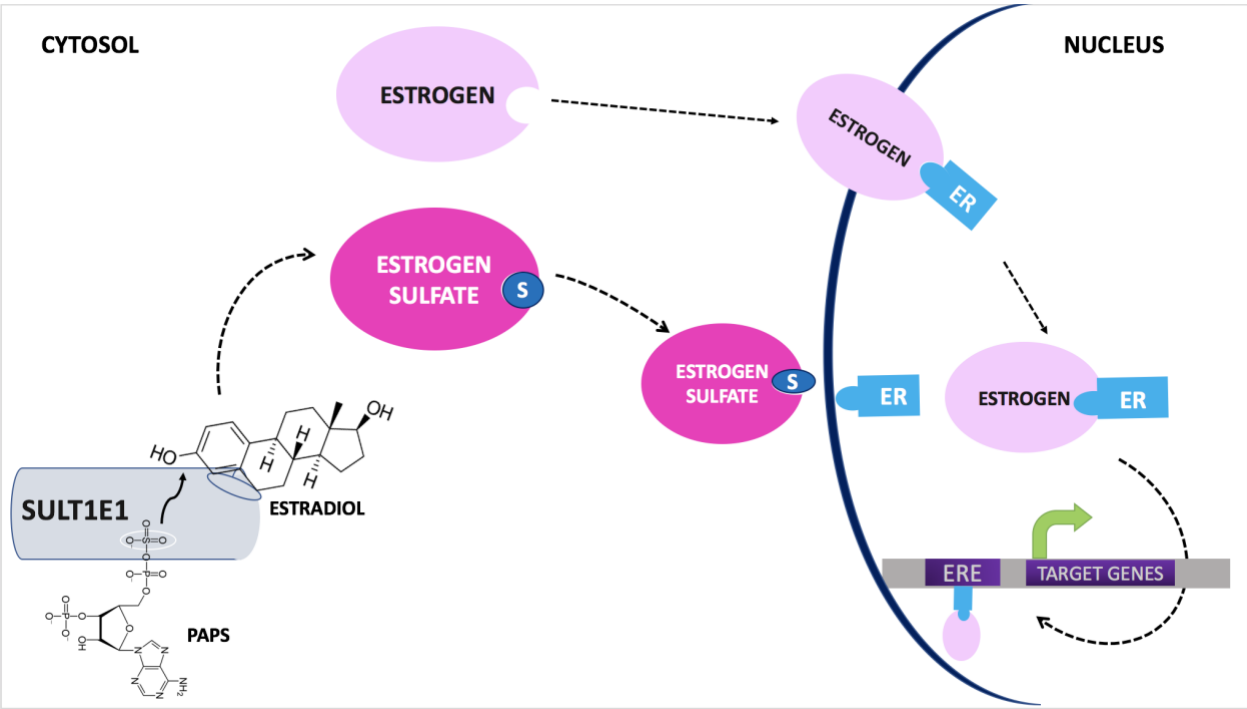

Figure 3: Physiological role of *SULT1E1* in the sulfoconjugation of estrogens.

Endogenous or synthetic estrogens bind to either the catalytic or allosteric domains of the *SULT1E1* dimer. Two molecules may bind independently. *SULT1E1* catalyzes the transfer of a sulfate group from the universal donor 3′- phosphoadenosine-5′-phosphosulfate (PAPS) to the 3′-hydroxy group of E_2. Upon sulfonation,

estrogens become hydrosoluble and unable to bind to the estrogen receptor (ER) α nor β. Consequently, unlike the parent estrogens, the sulfonated estrogens cannot translocate to the nucleus and cause ER and estrogen response element (ERE) mediated regulation of target genes.

SULT1E1-mediated sulfoconjugation and deactivation of estrogens is a reversible reaction because the hormonally inactive estrogen sulfates can be desulfonated and re-activated by STS. STS is responsible for catalyzing the hydrolysis of steroid sulfates and generating hydroxysteroids. This enzyme is present in several tissues, especially in the liver, where the metabolism of circulating steroid hormones mainly happens [64]. Therefore, *SULT1E1*-mediated sulfoconjugation and deactivation of estrogens is a reversible reaction, because the hormonally inactive estrogen sulfates can be desulfonated and re-activated by STS. In patients with chronic inflammatory liver diseases, inflammation-mediated activation of NF-kB in hepatocytes stimulates STS, and consequently the levels of circulating estrogens rise, which mitigates this inflammatory response [65]. Moreover, STS has been implicated in a sex-linked role in energy homeostasis, because transgenic overexpression of human STS in adipose tissue or liver of male and female mice resulted in different responses to high-fat diet (HFD)-induced obesity and type 2 diabetes mellitus (T2DM). In female mice, the inflammatory profile and metabolic functions were improved due to increased estrogenic activity whereas in male mice the metabolic response was worsened [66, 67].

1.3.1 Transcriptional regulation of *SULT1E1* by nuclear hormone receptors and its implications in drug-hormone interactions.

The expression of *SULT1E1* is subject to the transcriptional regulation by nuclear hormone receptors, a family of ligand-dependent transcriptional factors. The regulation of *SULT1E1* by nuclear receptors provides a mechanism for the drug/hormone-hormone interactions that lead to compromised estrogen activities. Several classes of drugs may indirectly modulate *SULT1E1* activity. For instance, glucocorticoids, such as dexamethasone (Dex), have been shown to reduce estrogenic activity *in vivo* and *in vitro* by increasing the expression of *SULT1E1*. The induction of *Sult1e1* and the resultant inhibition of estrogen activity by Dex were consistent with previous reports that glucocorticoids can inhibit estrogen responses [68-73]. Treatment with Dex attenuated the estrogen-induced uterine expression of insulin-like growth factor-I (*IGF-I*) [72]. DEX also blocked the stimulatory effect of estrogen on MCF-7 cell proliferation [71, 73]. Mechanistically, Dex interacts with the glucocorticoid receptor (*GR*), which acts as a transcriptional factor that promotes the upregulation of its transcriptional target, *Sult1e1*; consequently, *Sult1e1* induction is responsible for dramatically reducing the levels of active estrogens [71]. Similarly, cholesterol-derived oxysterols, or synthetic agonists such as GW3965, bind to the isoforms α and ß of the liver X receptor (LXR); this ligand-receptor complex, in turn, stimulates the transcription of hepatic *Sult1e1*, resulting in an increased estrogen sulfonation and decreased estrogen activity [74].

The regulation of *SULT1E1* by nuclear receptors can be both sex-specific and species-specific. As an example of sex-specificity, in female mice, the constitutive androstane receptor (*CAR*) agonist, TCBOPOP, was shown to induce liver *Sult1e1*, whereas in male mice the induction was not observed [75]. As an example of species specificity, although the regulation of *Sult1e1* by *Lxr* has been reported in mice, the same regulation is yet to be verified in humans. In addition to

the positive regulation, some nuclear receptors are associated with a decreased activity of *Sult1e1*. Retinoid-related orphan receptor alpha (*RORα*) is a negative regulator of *Sult1e1* in hepatocytes, therefore, the effects of agonists, such as cholesterol- and lipid-sulfates, and antagonists of *RORα* can modulate the activity of this steroid enzyme [76]. Another nuclear receptor agonist that downregulates *Sult1e1* is the antibiotic rifampicin, a known activator of the human pregnane X receptor (*PXR*). Rifampicin represses the transcription of *Sult1e1* in hepatocytes via interaction with hepatocyte nuclear factor 4α (*HNF4α*) [77]. Recently, Wang and colleagues also showed that, in HepG2 cells, the farnesoid X receptor (FXR) agonists, 3-(2,6-Dichlorophenyl)-4-(3'-carboxy-2-chlorostilben-4-yl), oxymethyl-5-isopropylisoxazole (GW4064) and chenodeoxycholic acid (CDCA), indirectly downregulated *SULT1E1* via the prevention of peroxisome proliferator-activated receptor-γ coactivator 1α (*PGC1α*) binding to *HNF4α* [78]. The role of nuclear receptors in *Sult1e1* regulation is summarized in **Figure 4**.

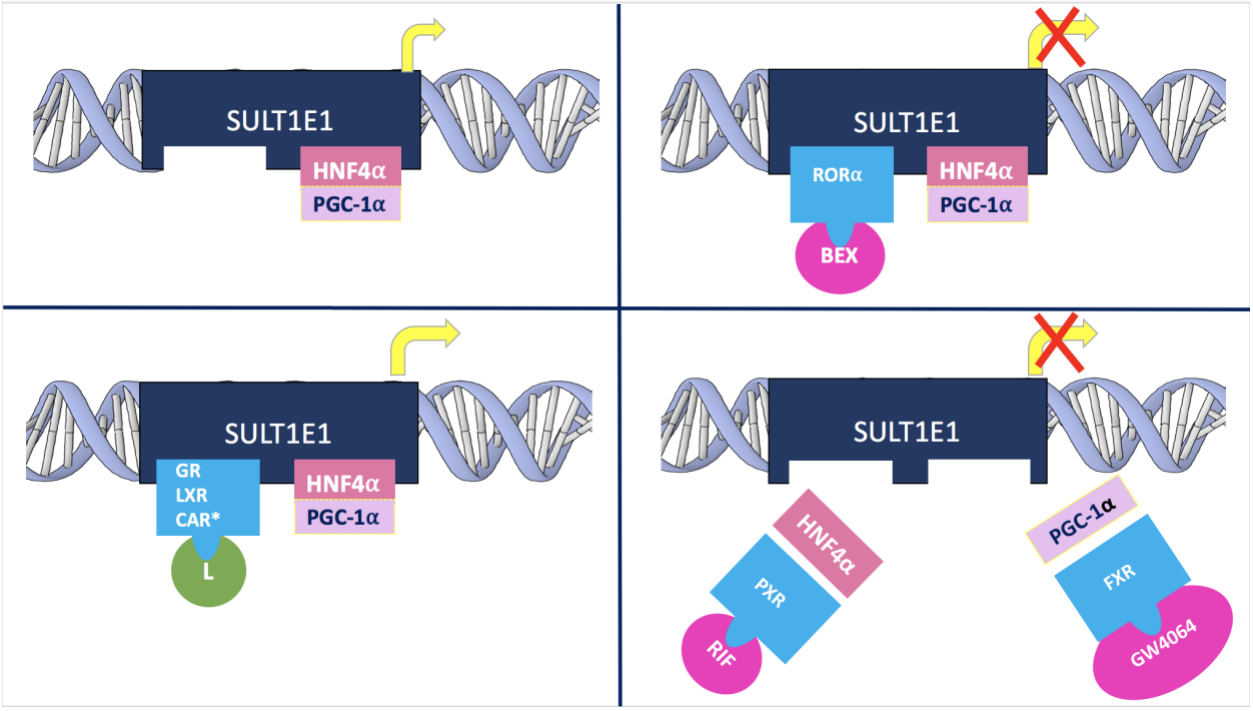

Figure 4: Regulation of *SULT1E1* by nuclear receptors.

Normally, *SULT1E1* has a low expression in some tissues, such as the liver. Upon the presence of a ligand (L), that can be either dexamethasone, GW3965, or TCPOBOP, the nuclear receptors *GR*, *LXR*, or *CAR*, respectively, can bind to the promoter region of the *Sult1e1* gene and increase its expression in the liver. * indicates that the induction only occurs in livers of female mice. In contrast, retinoid-acid related orphan receptor alpha (*RORα*) suppresses the expression of *Sult1e1* in the presence of its agonists Bexarotene (BEX). Similarly, upon the binding of its agonist rifampicin (RIF), pregnane X receptor (*PXR*) binds to the transcription factor hepatocyte nuclear factor 4α (*HNF4α*), which culminates with the downregulation of *Sult1e1*. Additionally, agonists for farnesoid X receptor (*FXR*), such as GW4064, prevent the binding of PGC1α to HNF4α, also leading to EST downregulation.

1.3.2 Metabolism of estrogenic drugs by *SULT1E1*

1.3.2.1 Estrogenic drugs that are *SULT1E1* substrates

Synthetic oral contraceptives are widely used among fertile women. Norethindrone (NET) and ethynylestradiol (EE) are the major active compounds in this medication group [79]. Cytochrome P450 (CYP) enzymes are responsible for the phase I metabolism of endo- and xenobiotics. *CYP3A4* and *CYP2C9*, followed by *CYP2C8*, *CYP2C19*, and *CYP3A5*, are the major enzymes responsible for oxidizing EE into 2-hydroxy-EE, which accounts for more than 90% of all metabolites [79]. Sulfation at the 3-O group of 2-hydroxy-EE accounts for up to 60% of EE's first-pass metabolism.

The proportion of intestinal over hepatic *SULTs* effective in this metabolism is approximately 2:1 [80]. Inhibition analysis with the *SULT1A1* inhibitor quercetin and the *SULT1E1* inhibitor 2,6-dichloro p-nitrophenol (DCNP) showed that *SULT1E1* is the *SULT* enzyme with the highest affinity for EE, with a Km value of 6.7 nM, being responsible for 75-80% of the

sulfoconjugation [61]. Moreover, the metabolism of EE may be altered when co-administered with agents that reduce its plasma levels, such as rifampicin [81], or agents that increase EE levels, such as acetaminophen, fluconazole, and ascorbic acid[82-84]. On the other hand, the acetylenic group of EE may act as an inhibitor of several CYP enzymes [85, 86], such as *CYP2B1*, *CYP2B6*, and *CYP3A4* [87, 88]. Additionally, in the human intestinal mucosa, EE was shown to indirectly inhibit sulfation considerably, as in the case of the progestogen (progesterone) oral contraceptive desogestrel, whose sulfation was inhibited by up to 48% in the presence of EE [89].

Selective estrogen receptor modulators (SERMs) inhibit the effects of estrogens in breast tissue. Tamoxifen and Raloxifene (Evista®) are SERMs widely used to decrease the risk of developing hormone-receptor-positive breast cancer in susceptible postmenopausal women. Sulfation assays demonstrate that, at therapeutic levels, *SULT1E1* has a high affinity for the tamoxifen metabolite, 4-hydroxytamoxifen (4-OHT), with a Km of 0.2 μM. Kinetic assays of raloxifene sulfation showed most *SULT* enzymes recognized this substrate; nonetheless, *SULT1E1* was the only *SULT* able to generate raloxifene monosulfates and disulfates. An affinity docking algorithm further demonstrated that in both rings of the molecule, the nucleophilic hydroxyls are placed in a crucial region for catalysis, predicting many possible interactions at two different positions. The analysis suggests this enzyme has a flexible active site that adjusts to accommodate reactive groups [90].

Hormonal replacement therapy (HRT) is broadly used in postmenopausal women to reduce uncomfortable symptoms of menopause, including hot flashes, disturbed sleep, and vaginal dryness. Tibolone is a synthetic steroid used as an HRT agent to modulate bone loss, menopause symptoms, and libido, possibly due to a selective sulfatase inhibition. Once absorbed, Tibolone binds to ER, progesterone receptor (PG), and androgen receptor (AR), and is rapidly metabolized

into 3α-OH and 3ß-OH-tibolone, which can be further metabolized into Δ_4-tibolone. Tibolone metabolism occurs mainly by steroid *SULTs*. *SULT1E1* sulfoconjugates the 3-OH position of both 3α-OH-tibolone and 3ß-OH-tibolone with a Km of 2.1 μM and 6.6 μM, respectively [90, 91].

Among estrogenic drugs, the clinical use of conjugated equine estrogens (CEEs) also involves *SULT1E1* and the estrogen re-activating enzyme STS. As a natural formulation of extraction from pregnant mares' urine, CEEs are one of the most prescribed estrogen production for postmenopausal HRT either alone or in combination with a progestin. CEEs are not a single estrogen but a complex containing 10 different estrogens in their sulfate esters, with estrone sulfate and equilin sulfate as the main constituents [92]. Since estrogen exerts its biological effect only in its unconjugated form, *SULT1E1* and STS are reasonably believed to be involved in CEEs metabolic process.

Similar to E2, which is also widely used in HRT, CEEs have been proven to benefit postmenopausal women, such as improvement of osteoporosis, with no increased risk of cardiovascular disease and invasive breast cancer [93, 94]. However, several randomized controlled trials (RCTs) have revealed significant declines in cognitive function as well as a higher incidence of probable dementia in patients receiving CEEs alone or in combination with medroxyprogesterone acetate (MPA) compared with placebo [95-97], whereas transdermal E2, in comparison to the placebo, was found played no effect on cognition [98]. Additionally, in postmenopausal women with an increased risk of Alzheimer's disease, continued or discontinued use of estradiol could improve attention/working memory/processing speed ($P = 0.04$) and verbal memory ($P = 0.01$) domains compared with continued or discontinued CEE use for 2years [99]. On the other hand, as the enzyme activating sulfated estrogen, STS also highly expresses in the brain. Evidence has shown that an STS inhibitor is related to the up-regulation of endogenous

dehydroepiandrosterone sulfate (DHEAS) which acts as γ-aminobutyric acid$_A$ receptor antagonists, resulting in a memory-enhancing effect [100]. Although these results might provide a possible link between estrogen sulfate and *SULT1E1*/STS in mental disorders, there is no solid evidence to support this view. The potential association and underlying mechanism need to be further evaluated.

1.3.2.2 Chemicals that inhibit the *SULT1E1* activity

Polychlorinated biphenyls (PCBs) are environmental pollutants with estrogenic or anti-estrogenic properties that have gained increasing attention due to their effects on animal reproduction and sexual development. Human exposure to PCBs has been associated with an increased incidence of testicular cancer and diminished semen quality and sperm counts [101]. It has been suggested that hydroxylated metabolites of PCBs (PCB-OHs) exert most of the hormonal properties of these compounds [102]. Kester *et al* investigated if PCB-OHs were inhibitors of E_2 metabolism and discovered that low concentrations of PCB-OHs (0.1 nM) were sufficient to bind to and inhibit human *SULT1E1* with an affinity higher than the endogenous estrogens. Their results suggest that PCB-OHs may increase local estrogenic activity in reproduction-related organs by suppressing *SULT1E1*-mediated estrogen sulfation and deactivation. Moreover, the authors observed that a hydroxyl group in the *para* position of PCB-OHs with two nearby chloride substituents was required for interacting with the *SULT1E11* enzyme and that the inhibitory effect was increased per the number of halogen groups in the molecule.

Phenolic OH groups in PCB-OHs were also shown to be non-competitive inhibitors of E_2 sulfation since they do not bind to the active site, but the allosteric site of *SULT1E1* [103]. Similarly, using recombinant human *SULT* enzymes, Miksits and colleagues demonstrated that although *SULT1A1* was the major enzyme, *SULT1E1* had a minor role in the sulfoconjugation of

3,4',5-trihydroxy-*trans*-stilbene (Resveratrol) [104, 105]. This is a polyphenol chemical present in the herb *Polygonum cuspidatum*, that among many functions also has estrogenic activity. The substrate inhibition profiles of the resveratrol metabolites, *trans*-resveratrol-3-O-sulfate (M1), and *trans*-resveratrol-4'-O-sulfate (M2) on *SULT1E1* had a Ki value of 3.37 μM and 13.1 μM, respectively [104].

Triclosan, also known as Irgasan, is another established *SULT1E1* inhibitor. Triclosan is a chlorinated phenolic compound that was used as an anti-microbial agent in hand soap and other personal care agents [106-109]. Triclosan has been detected in human blood, urine, and breast milk [110, 111]. People who accidentally ingested 4 mg of Triclosan presented 22 to 47% of the unconjugated molecule in plasma [112]. Stoker *et al*. evaluated the effects of Triclosan in female Wistar rats. They found that in pubertal mice this agent resulted in a premature vaginal opening, whereas in weaning mice Triclosan changed the degree of reproduction development and increased uterine response to EE [113]. In sheep, Triclosan was reported as a potent inhibitor of placental *Sult1e1* by competing with E_2 molecules for the enzyme's substrate-binding site with a competitive inhibitory constant (Kic) of 0.09nM[58]. Besides the competitive inhibition, Triclosan also displays an uncompetitive inhibition of the E_2-Sult1e1 interaction, with an uncompetitive inhibitory constant (Ki) of approximately 5.2 nM. In the same study, another PCB, 4'OH-CB79, also demonstrated competitive E_2- *Sult1e1* inhibition with a Ki of 0.89 nM[58]. **Table 4** summarizes all *Sult1e1* substrates and inhibitors and their enzyme binding affinity.

Table 4: Binding affinity of substrates and inhibitors of Estrogen Sulfotransferase (*Sult1e1*) within different species.

COMPOUND	APPROXIMATE AFFINITY (nM)	SPECIE	ROLE	REFERENCE
17ß-Estradiol (E_2)	0.27	Mouse	Substrate	[57]
Estrone (E_1)	1.8	Sheep	Substrate	[58]
Estrone (E_1)	40	Human	Inhibitor	[61]
Ethynylestradiol	6.7	Human	Substrate	[61]
4-hydroxytamoxifen	200	Human	Substrate	[90]
DHEA	850	Human	Substrate	[60]
3*α*-OH-Tibolone	2,100	Human	Substrate	[91]
3ß-OH-tibolone	6,600	Human	Substrate	[91]
Tibolone	19,500	Human	Substrate	[91]
4'OH-CB79	0.89	Sheep	Competitive Inhibitor	[58]
Triclosan	0.09	Sheep	Competitive Inhibitor	[58]
Triclosan	5.2	Sheep	Uncompetitive Inhibitor	[58]
***Trans*-resveratrol-3-O-sulfate (M1)**	3,370	Human	Inhibitor	[104]
***Trans*-resveratrol-4'-O-sulfate (M2)**	13,100	Human	Inhibitor	[104]

1.3.3 Disease effect on the expression and activity of *SULT1E1*

1.3.3.1 *SULT1E1* in human diseases

In humans, postmenopausal women receiving HRT have a higher risk of presenting serious side effects like pulmonary embolism, stroke, coronary heart disease, and cancer [114-117]. Cancer-focused studies have shown that the activity of *SULT1E1* has been correlated with a reduction in breast, endometrial, and ovarian cancer recurrence and improved survival [118-121], whereas *SULT1E1*-negative breast tumors may be associated with a poor prognosis due to a rise in *in situ* estrogens [42, 122]. These phenomena are in accordance with the finding that an increased sulfation of E_2 has been linked to decreased proliferation rates of hormone-sensitive malignant cells [123]. Endometriosis is manifested by abnormal growth of endometrial tissue ectopically of the uterus. Biopsy specimens of women with endometriosis presented a diminished expression of *SULT1E1* and an augmented expression of STS in accordance with the dependence of endometriosis on female sex hormone [124].

Variations in the activity of *SULT1E1* are responsible for differences in inter-individual response to hormonal-related diseases. Expression of *SULT1E11* in the human liver, although showing no sex-differences, presents significant variations between alcohol consumers, as well as among different individuals, where it can vary up to 25-fold. The causes behind such variations are not fully elucidated but it is believed they could happen as a result of *SULT1E1* polymorphisms and exogenous administration of estrogens [125]. Three nonsynonymous *SULT1E1* coding single nucleotide polymorphisms (cSNPs) have been characterized in COS-1 cells. Constructs containing the cSNPs evidenced a decline in *SULT1E1* activity, which suggest such polymorphisms may be partly responsible for the advancement of estrogenic diseases and metabolic alterations of estrogenic drugs [126].

Using genomic DNA extracted from buccal samples, Rebbeck and collaborators conducted a population based case-control study that evidenced an association between the chance of developing endometrial cancer and the G → A polymorphism at position -64 (−64G>A; rs3736599) of *SULT1E1*'s promoter region [119]. Although only a few studies have been conducted so far to understand the origin of such variations, lack of sex-specific expression changes suggest the role of *SULT1E1* in homeostasis may go beyond a simple estrogen inactivation.

1.3.3.2 *Sult1e1* in rodent disease models

The genes encoding *SULT1E1* are highly conserved in humans and mice, because the mouse *Sult1e1* shares 77% homology in amino acids with the human enzyme [43]. As a result, various mouse models have been used to further understand the impact of diseases on this enzyme, and *vice-versa*. Noticeably, the use of *Sult1e1* loss of function and gain of function models permitted the advancement of studies regarding the role of this enzyme in estrogen homeostasis and disease pathogenesis.

1.3.3.3 *Sult1e1* in estrogen homeostasis and reproduction

In animals, experiments using female *Sult1e1* null mice showed the importance of this enzyme as a regulator of estrogen levels, especially during pregnancy as the fetal loss was a common feature and the surviving offspring were smaller and had excessive levels of estrogens [127].

1.3.3.4 *Sult1e1* in adipocyte differentiation

The adipose tissue plays an important role in lipid storage, energy balance and insulin response; nonetheless, the mechanisms surrounding adipogenesis are not fully understood. We reported that *Sult1e1* was highly expressed in 3T3-L1 pre-adipocytes and at the time of cellular differentiation to mature adipocytes this expression was decreased considerably. Furthermore, upon *Sult1e1* overexpression in 3T3-L1 cells, adipocyte differentiation was diminished due to an *ERK1/2 MAPK*-dependent inhibition of insulin signaling, whereas *Sult1e1* ablation in adipocytes conferred differentiation. The enzymatic activity of *Sult1e1* was required for the inhibitory effect of *Sult1e1* on adipogenesis, because an enzyme-dead *Sult1e1* mutant failed to inhibit adipocyte differentiation. An *in vivo* investigation using transgenic female mice overexpressing *Sult1e1* specifically in adipose tissue further confirmed that the adipocytes' diameters were reduced. Interestingly, physiological concentrations of E_2 had little effect on 3T3-L1 differentiation. Their results suggest that *Sult1e1* is a negative regulator of adipogenesis in an estrogen-independent manner. The authors used transient transfection and luciferase reporter gene assay to examine other candidate substrates for *SULT1E1*, such as thyroid hormones, testosterone, glucocorticoids, and peroxisome proliferator-activated receptor gamma (*PPARγ*) ligands, but none of them were shown to be metabolized by this enzyme [128]. As such, the *SULT1E1* substrate(s) responsible for the effect of *Sult1e1* on mouse adipocyte differentiation remain to be defined.

Curiously, the effect of *Sult1e1* in adipocyte differentiation is species specific. We conducted a study using pre-adipocytes isolated from obese and non-obese subjects, combined with *Sult1e1* loss of function and gain of function manipulations. Our results showed that *Sult1e1* positively regulates adipogenesis via loss of estrogenic activity, and that the enzyme expression is

positively correlated with the body mass index. Moreover, human adipogenesis was affected by estrogen treatment [48].

1.3.3.5 *Sult1e1* in metabolic disease

Type 2 Diabetes Mellitus (T2DM) is a metabolic syndrome associated with insulin resistance. The diabetic mouse model (db/db) presents a liver induction of *Sult1e1* [129]. We showed that the hepatic expression of *Sult1e1* was also markedly induced in the ob/ob mice, another genetic model of obesity and type 2 diabetes. In determining the functional relevance of *Sult1e1* and its regulation by metabolic disease, we showed that ablation of *Sult1e1* in female ob/ob (termed obe) mice resulted in improved metabolic function due to a rise in hepatic estrogenic activity, as ovariectomy abolished the protection. Interestingly, the effect of *Sult1e1* ablation on obesity and type 2 diabetes was sex-specific, because *Sult1e1* ablation in male ob/ob mice worsened their phenotype, which was accounted for by the ß-cell loss due to the boosted macrophage infiltration and inflammation in the white adipose tissue (WAT) [130].

We initially thought the loss of expression and induction of hepatic *Sult1e1* in the male obe mice was responsible for the worsened metabolic function. In a follow-up study, we were surprised to find that transgenic reconstitution of *Sult1e1* in the adipose tissue, but not in the liver, attenuated diabetic phenotype in obe males. Mechanistically, adipose reconstitution of *Sult1e1* in obe mice resulted in reduced local and systemic inflammation, improved insulin sensitivity, and increased energy expenditure. At the molecular level, adipose induction of lipocalin-2 (Lcn2) in male obe mice with adipose reconstitution of *Sult1e1* (oae mice) may have contributed to the inhibition of inflammation, because the level of Lcn2 was negatively associated with TNF-α expression, and treatment of differentiated adipocytes with Lcn2 antagonized TNF-α-responsive inhibition of insulin signaling. The metabolic benefit of adipose reconstitution of *Sult1e1* was sex-specific,

because adipose reconstitution of *Sult1e1* in obe females had little effect. Interestingly, although reconstitution of *Sult1e1* in obe males improved metabolic phenotype, these mice were not protected from ß cell mass loss. Their results suggest *Sult1e1* is crucial for WAT homeostasis in an estrogen and ß cell-independent manner [131].

1.3.3.6 *Sult1e1* in liver injury induced by sepsis and ischemia-reperfusion

Sepsis is a major cause of mortality in the intensive care unit (ICU). Although sepsis and its associated inflammation are known to decrease the expression and activity of many drug-metabolizing enzymes, we found that upon bacterial lipopolysaccharide (LPS) treatment or subjecting mice to the cecum ligation and puncture (CLP), the hepatic expression of *Sult1e1* was highly upregulated via the activation of the NF-kB pathway. The mouse *Sult1e1* gene was established as a NF-kB target gene. The sepsis-responsive induction of *Sult1e1* was sufficient to compromise the estrogen activity. Interestingly, not only sepsis can induce *Sult1e1*- the expression and activity of *Sult1e1* can impact the clinical outcome of sepsis. Specifically, we showed that *Sult1e1* ablation or pharmacological inhibition of *Sult1e1* by Triclosan sensitizes mice to sepsis-induced death in an estrogen dependent manner. Mechanistically, *Sult1e1* ablation attenuates sepsis-induced inflammatory responses due to compromised estrogen deactivation, leading to increased sepsis lethality. The reciprocal regulation of inflammation and *Sult1e1* may represent a yet to be explored mechanism of endocrine regulation of inflammation, which has an impact on the clinical outcome of sepsis [49].

Liver ischemia-reperfusion injury (LIRI) is another liver injury condition that can regulate *Sult1e1*. *Sult1e1* has also been studied in inflammation-based conditions. LIRI is caused by hepatic blood flow blockage or reduction and is a common feature after organ transplantation, abdominal surgeries, massive trauma, hemorrhagic- and cardiogenic shock. LIRI is directly associated with

oxidative stress and inflammation. We reported that LIRI induced *Sult1e1* in the mouse liver, and that upon *Sult1e1* ablation the female mice were protected from the injury in an estrogen dependent manner, whereas the male mice were further sensitized in an androgen-dependent manner. The LIRI responsive induction of *Sult1e1* is dependent on the nuclear factor erythroid 2-related factor 2 (Nrf2), but independent of the hypoxia-inducible factor 1 (HIF-1). *Sult1e1* was established as a Nrf2 target gene [46].

1.3.3.7 *Sult1e1* in cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive condition characterized by mutations in both copies of the cystic fibrosis transmembrane receptor (CFTR) and manifested by pulmonary abnormalities. However, loss of CFTR is often linked to distant organ injury. The liver is one of the organs affected, which accounts for the high mortality observed in children with CF [132, 133]. Additionally, animal models of CF, characterized by both CFTR-deficient and CFTR KO mice, do not present pulmonary disease but manifest CF-associated gastrointestinal and reproduction comorbidities, such as severe growth retardation and mediocre weight gains [134]. Furthermore, CF patients present growth shortfalls that are associated with a decline in IGF-1 plasma levels [135, 136]. Li and colleagues have demonstrated that hepatic *Sult1e1* is induced in CFTR KO mice [137] and that in *SULT1E1*-transfected HepG2 cells, *SULT1E1* played a role in inhibiting both growth hormone-mediated signal transducers and activators transcription 5b (STAT5b) phosphorylation and insulin-like growth factor-1 (IGF-1) synthesis in an estrogen-dependent aspect [123]. As a result of estrogenic decline, the expression of the hepatic detoxifying enzymes, glutathione S-transferase P1 and carbonic anhydrase, are also downregulated in CFTR mice [137]. In addition, Falany and colleagues showed that co-transfection of HepG2 hepatocytes with human MMNK-1 cholangiocytes transfected with CFTR siRNA resulted in *SULT1E1* induction in an

LXR-dependent manner due to changes in cholesterol biosynthesis [138]. Therefore, *SULT1E1* induction in CF patients may be responsible for growth retardation and indicate a disrupted paracrine regulatory mechanism that may help elucidate the reasons behind CF-dependent liver damage. A general association between disease onset and *SULT1E1* regulation is summarized in **Table 5**.

Table 5: Disease onsets that were shown to regulate Estrogen Sulfotransferase (*SULT1E1)* expression within different species.

DISEASE STATE	EST REGULATION	SPECIE	REFERENCE
Endometrial Cancer	Downregulated	Human	[118]
Ovarian Cancer	Downregulated	Human	[120]
Breast Cancer	Downregulated	Human	[121]
Endometriosis	Downregulated	Human	[124]
Metabolic disease	Upregulated	Mouse	[130]
Sepsis	Upregulated	Mouse	[139]
Ischemia-reperfusion	Upregulated	Mouse	[140]
Cystic Fibrosis	Upregulated	Mouse	[137]

1.3.3.8 *Sult1e1* in AKI

Animal studies have suggested that estrogens may protect mice from AKI [141] [142, 143]. Epidemiology studies suggested that women below the age of menopause are believed to be protected due to the anti-inflammatory effects of estrogens [144-146] whereas the male sex can be an independent risk factor for AKI [18, 147-150]. It is unclear whether *Sult1e1* plays a role in the pathogenesis of AKI and if so, whether the effect of *Sult1e1* on AKI is sex hormone dependent.

1.4 Hypothesis and specific aims

Acute kidney injury (AKI) is defined as an abrupt impairment of kidney function. The acute ischemic AKI model was established by clamping the pedicle vessels of both kidneys to block the blood flow for 30 minutes. Besides its effect on kidneys, AKI has been reported to affect many distal organs, including the liver. Studies suggest estrogens may protect mice from AKI and that women below the age of menopause are believed to be protected due to the anti-inflammatory effects of estrogens. Moreover, male sex can be an independent risk factor for AKI. *SULT1E1* is the enzyme that exhibits the greatest affinity for estrogens, especially the 3-hydroxyl position of E_2. *Sult1e1* was the most highly induced gene in the livers of AKI mice, and this gene was also induced in the kidneys of male mice, but not female.

Hypothesis: Estrogen sulfotransferase (*Sult1e1*/EST) exacerbates ischemic acute kidney injury due to inactivation of estrogens.

Specific Aim 1: To abolish *Sult1e1* expression in AKI mice and investigate estrogenic response.

For the specific aim 1, eight-week old male and female *Sult1e1* knockout (*Sult1e1* KO) mice as well as WT mice of similar age treated with IP injections of corn oil or 10mg/kg or 50mg/kg of triclosan were analyzed. These mice were challenged with AKI or the sham surgery, and were sacrificed 24 h after. Kidney injury markers, such as serum creatinine, blood urea nitrogen, kidney NGAL expression, PAS and TUNEL staining, and the inflammatory marker IL-6 were significantly decreased in *Sult1e1* KO mice and triclosan-treated WT. Therefore, they exhibited a marked protection from AKI.

For a second part of this specific aim, male and female *Sult1e1* KO that underwent gonadectomy at 4-5 weeks were also used. Ovariectomized or castrated *Sult1e1*1 KO mice were

also protected from AKI, as determined by the aforementioned markers, demonstrating the effects mediated by *Sult1e1* ablation are androgen- and estrogen-independent.

Specific Aim 2: To compare the importance of liver and kidney *Sult1e1* for AKI development and investigate the injury mediator.

Knowing hepatic *Sult1e1* is induced by AKI, for the first part of specific aim 2, we wanted to determine whether the hepatic expression of *Sult1e1* distantly contributed to the pathogenesis of AKI. For this purpose, we reconstituted the expression of *Sult1e1* tissue-specifically to the liver by using the KOLE mice. In the male mice, restoration of liver *Sult1e1* was sufficient to re-sensitize KOLE mice to AKI, whereas female KOLE were still protected from the injury, indicating the importance of liver *Sult1e1* for males and a sex-difference that does not rely on sex hormones.

For the second part of specific aim 2, since the kidney protective effect of *Sult1e1* ablation was sex hormone-independent, we went on to determine whether the metabolism of other endogenous substrates may have been responsible for kidney protection. In this effort, we performed Affymetrix microarray analysis comparing the transcriptomic profile in the kidneys of WT AKI and *Sult1e1* KO AKI mice. Microarray showed altered expression of several genes involved in vitamin D metabolism and cell proliferation in the *Sult1e1* KO AKI group, such as *Cyp24a1* and *Cyclin D1*, which were confirmed at the mRNA and protein level. These results suggested that the increased VDR signaling may have contributed to the kidney protective effect of *Sult1e1* ablation, despise *SULT1E1* not recognizing active vitamin D, or calcitriol, as a substrate.

1.5 Dissertation outlines

The contents of the dissertation include:

Chapter I. Introduction is a concise description of AKI; and a literature review of how estrogen sulfotransferase mediates the metabolism of estrogenic drugs and how it interferes in the pathogenesis of diseases. It also includes overall research hypothesis, and specific aims.

Chapter II. Methods with general research approaches.

Chapter III. *Sult1e1* in ischemic AKI mice is a complete research report that describes the experimental results of Aims 1 and 2, discussion and conclusion.

Chapter V. Summary is a conclusive overview of the strategies currently used in the management of late AKI stages, as well as safety studies regarding triclosan, and future work.

2.0 Methods

Animals. WT C57BL/6 mice were purchased from the Jackson laboratories (Bar Harbor, ME). The creations of *Sult1e1* KO [151] and liver-specific Lap- *SULT1E1*/EST (LE) transgenic mice [139] were reported previously. The LE transgenic mice express the human *SULT1E1/EST* transgene in the liver under the control of the hepatocyte-specific liver-enriched activator protein (Lap) gene promoter. The KOLE mice, which are *Sult1e1* KO mice with the liver-specific reconstitution of *SULT1E1*, were created by cross-breeding the *Sult1e1* KO and LE transgenic mice as we have previously reported [152]. In the KOLE mice, the *Sult1e1* KO allele and LE transgenic allele were independently genotyped by PCR. Mice were maintained in a temperature-controlled animal facility at the University of Pittsburgh. The use of animals complied with the guidelines established by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Ischemia-Reperfusion Model of AKI. To induce AKI, both kidneys were clamped to block blood flow for 30 minutes. After ischemia, clamps were released to start reperfusion, and mice were sacrificed after 1, 2, 6, 24, or 72 hours. Blood samples were collected by cardiac puncture. Kidneys and livers were harvested for analysis. The sham surgery is a midline incision.

Histological Analysis. The kidney and liver tissues were fixed in 10% neutral buffered formalin for 24 hours and then dehydrated, embedded in paraffin, sectioned at 4 mm, and stained with periodic acid Schiff (PAS), and counterstained with hematoxylin. Immunostaining was performed on paraffin sections. Slides were incubated overnight with the primary anti- *SULT1E1* antibody (12522-1-AP) from Proteintech (Rosemont, IL), anti- CD3 antibody (MAB4841) from R&D Systems (Minneapolis, MN), anti-Cyp24a1 antibody (ab203308), anti-Ki67 antibody

(ab66155) from Abcam (Cambridge, MA), or anti-neutrophil gelatinase-associated lipocalin (NGAL) antibody (MAB1857) from R&D Systems. Antibodies were diluted to 1:100 (liver) or 1:50 (kidney) and incubated in humid chambers overnight at 4°C. Specimens were then treated with fluorescence- or biotin-conjugated secondary antibodies. Terminal transferase dUTP nick-end labeling (TUNEL) staining was performed by using the *In Situ* Cell Death Detection Kit from Roche (Mannheim, Germany).

Serum Biochemical Analysis of Creatinine, BUN, ALT, IL-6, and 17ß-E2. Creatinine levels were measured with the QuantiChrom Creatinine Assay Kit from BioAssay System (Hayward, CA). BUN was measured by using the QuantiChrom Urea assay kit (Cat #DIUR-100) from BioAssay Systems (Hayward, CA). The ALT levels were analyzed with the Stanbio ALT kit from Laboratory (Boerne, TX). The concentrations of IL-6 were measured by an enzyme-linked immunosorbent assay kit from R&D Systems. Serum levels of 17ß-E2 were measured by the Center for Research in reproduction at the University of Virginia using the ELISA (Calbiotech) kit.

Cell cultures. Primary hepatocytes were isolated from 12-week-old male WT mice by liver perfusion [153]. Hepatocytes were seeded onto type 1 collagen-coated dishes in William E medium containing 5% FBS until confluent. After 24 hours, the medium was replaced with William E medium supplemented with vehicle or IL-6 (70 ng/mL) for 24 hours before cell harvesting. Human kidney proximal tubular (HKC-8) cells were cultured in DMEM-F12 and 5% FBS until confluent. The cells were then treated with vehicle or IL-6 (35 ng/mL) for 24 hours before cell harvesting.

RNA Isolation, Quantitative Real-Time PCR, Northern Blot, and Affymetrix Microarray Analysis. Total RNA was isolated using the TRIZOL reagent from Invitrogen

(Carlsbad, CA). Total RNA was treated with RNase-free DNase I, and the resultant DNA-free RNA was used to synthesize single-strand cDNA. Real-time PCR was performed on an ABI 7300 Real-Time PCR System from Applied Biosystems (Foster City, CA). The primer sequences are listed in **Table 6**. Melting curve analysis was performed to determine the specificity of amplification. Gene expression was normalized to the expression of the control cyclophilin gene. Northern blot analysis using a ^{32}P-labeled cDNA probe was performed as previously described [154]. Affymetrix microarray analysis was performed at the High Throughput Genome Center at the Department of Pathology, University of Pittsburgh. Microarray data were first quantile normalized across samples. The probe-level intensities were then mapped to gene-level expression. If multiple probes are mapped to the same gene, only the probe with largest inter quartile range will be kept. Then the top 200 up-regulated and 200 down-regulated genes were selected based on log2 fold change. These differentially expressed genes were used as input for Ingenuity Pathway Analysis (IPA)®, with significant pathways defined by FDR=5%.

Table 6: qRT-PCR primer sequences.

Mouse genes	Forward	Reverse
Car	GGAGGACCAGATCTCCCTTC	GTGGAGGATCGACTCCAAAA
Cyp24a1	GAGGAAGAAGCCCTGACCTT	TGCAGGGCTTGACTGATTTG
Fgg	GTGCTGGCTGTAAAGAGCTG	TGGGCAGAAACTACCGAATCT
Il-6	TCCTCTCTGCAAGAGACTTCCATCC	GGGAAGGCCGTGGTTGTCACC
Lxr	GCCTCAATGCCTGATGTTTC	CTGCATCTTGAGGTTCTGTCTTC
Ngal	AATGTCACCTCCATCCTGGT	ATTTCCCAGAGTGAACTGGC
Stat5a	GCTCAGCGCCCACTTCA	GACTCTGCACCACGCCTGT
Sult1e1	GCCAAAGATGTCGCCGTTTC	AACCATACGGAACTTGCCCT

Transient Transfection and Luciferase Reporter Gene Assay. The pCMX-VDR, tk-VDRE [155] and pCMX- *SULT1E1* [140] constructs were described previously. HEK293T cells were transiently transfected with pCMX-VDR and tk-VDRE plasmids, with or without the co-transfection of pCMX- *SULT1E1* plasmid, using Lipofectamine 2000 from Invitrogen. pCMX-ß-gal plasmid was added as an internal control to monitor the transfection efficiency. After transfection, cells were treated with vehicle or calcitriol (10 nM) for 24 hours. The luciferase activity was normalized to the ß-gal activity.

Statistical Analysis. All data are expressed as the mean ± SD. Statistical analysis was performed using Student's *t*-test or one-way analysis of variance where appropriate. Differences between groups were considered statistically significant at $P < 0.05$. Multiple comparisons were evaluated by one-way analysis of variance followed by Tukey's multiple comparison tests.

3.0 Results

3.1 AKI induces the hepatic expression of *Sult1e1* in both male and female mice, but induces the kidney expression of *Sult1e1* only in male mice

The ischemic AKI model was established by clamping the kidney pedicle vessels of both kidneys to block the blood flow for 30 minutes. The AKI-induced kidney injury in male mice was confirmed at the biochemical, gene expression, and histological levels (**Fig. 5**). We used microarray to determine the effect of AKI on hepatic and kidney gene expression in mice. The microarray datasets have been submitted to the NIH Gene Expression Omnibus (GEO) under the GEO records GSE138995, GSE138996, and GSE138997. The microarray results showed that *Sult1e1* was the most highly induced gene in the livers of AKI mice, and the induction of hepatic *Sult1e1* by AKI in both male and female mice was verified by qRT-PCR (**Fig. 6A**) and Northern blotting (**Fig. 6B**). The AKI induction of hepatic *Sult1e1* was also confirmed by immunofluorescence (**Fig. 6C**). A basal expression of *Sult1e1* was also detected in the kidneys of both male and female mice, but interestingly, AKI induced kidney expression *Sult1e1* in male mice, but not in female mice as shown by qRT-PCR (**Fig. 6D**) and immunofluorescence (**Fig. 6E**).

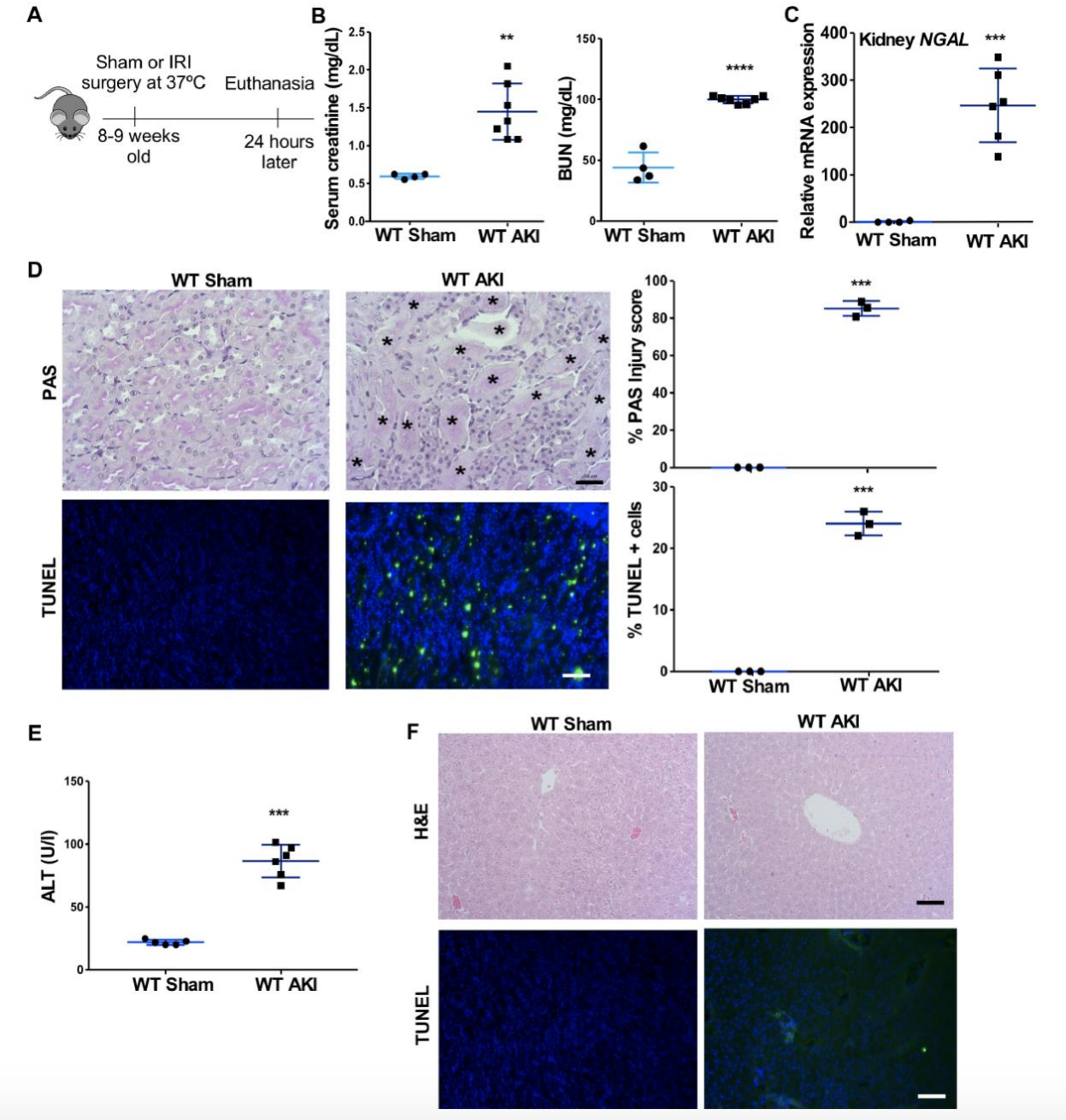

Figure 5: Establishment of the bilateral kidney ischemia reperfusion model of AKI.

(A) Schematic representation of the ischemic AKI model. (B-F) WT male mice were subject to the 30-min ischemic AKI, and the mice were sacrificed 24 h after the surgery. Shown are serum levels of creatinine and BUN (B), kidney mRNA expression of *NGAL* (C), kidney histology (D, with asterisks indicating tubular damage), serum ALT level (E), and liver histology (F). n=7 for each group. Scale bars are 50 µm. Results are presented as the mean ± SD. ***, P < 0.001; ****, P < 0.0001, compared with the sham group.

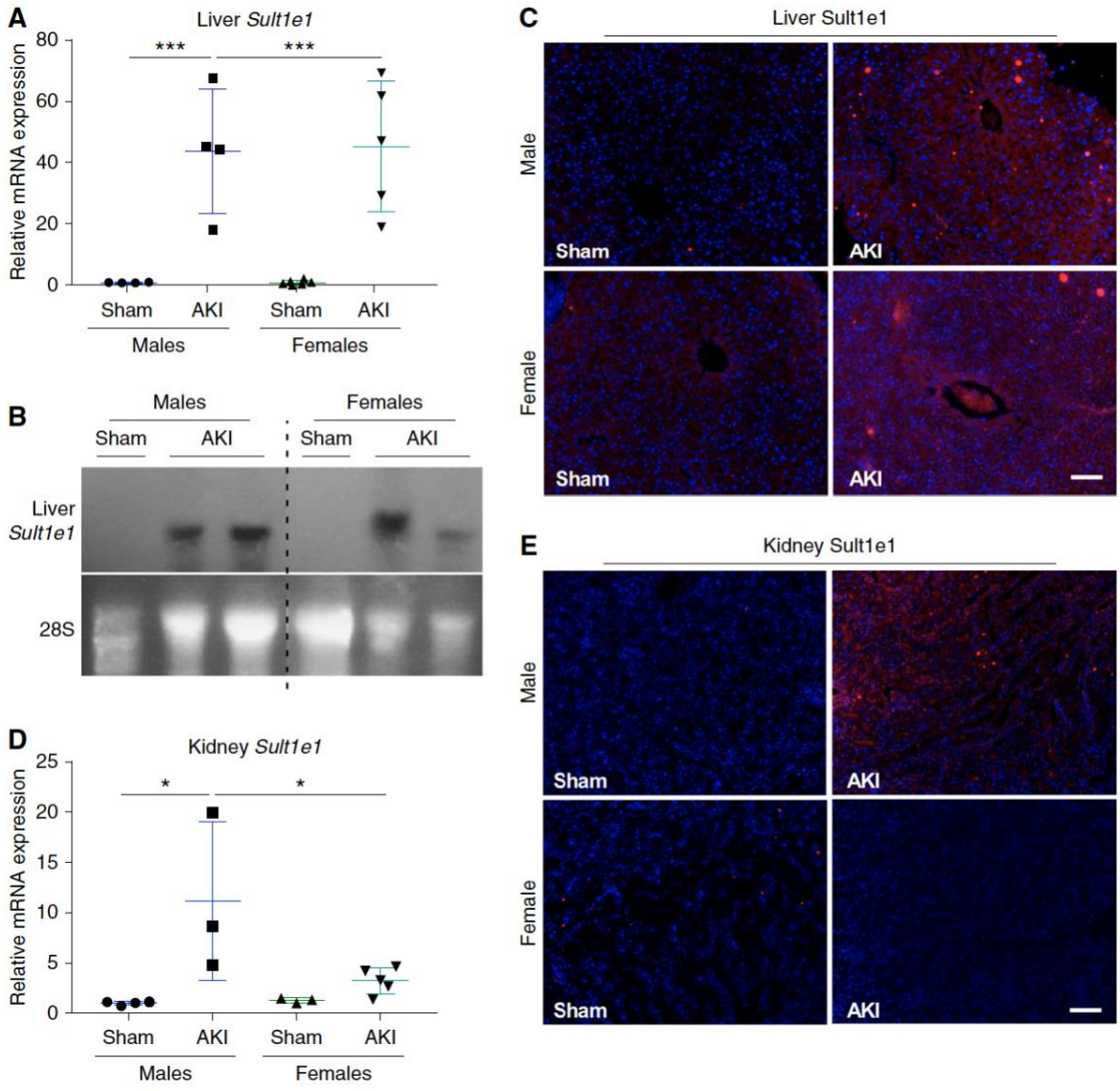

Figure 6: AKI induces the hepatic expression of *Sult1e1* in both male and female mice, but induces the kidney expression of *Sult1e1* only in male mice.

Mice were subject to the 30-min ischemic AKI or the sham surgery, and mice were sacrificed, and tissues were harvested 24 h after the surgery. (A-B) Hepatic mRNA expression of *Sult1e1* in male and female mice as shown by qRT-PCR (A) `(line is too long in Panel A)` **and Northern blot analysis (B). 28S RNA was included as the loading control in Northern blotting. (C) Immunofluorescence staining showed hepatic expression of *Sult1e1* in male and female mice. (D and E) Kidney expression of *Sult1e1* in male and female mice was evaluated by qRT-PCR (D) and immunofluorescence (E). n=7 for each group. Scale bars are 50 μm. Results are presented as the mean ± SD. *, P < 0.05; ***, P < 0.001, compared to the Sham groups.**

3.2 Inflammation is a potential mechanism for AKI responsive induction of *Sult1e1* in the liver

The *Sult1e1* gene has been reported to be positively regulated by nuclear receptors constitutive androstane receptor (*CAR*) [75] and liver X receptor α (*LXRα*) [74]. The expression of *Car* was suppressed as we have previously reported [23], whereas the expression of *Lxrα* was not affected by AKI (**Fig.7A**), suggesting that the AKI-responsive induction of hepatic *Sult1e1* was nuclear receptor independent. We have previously reported that the expression of *Sult1e1* can be induced by inflammation in sepsis and the *Sult1e1* gene is a transcriptional target of NF-kB [139]. Ischemic AKI is known to trigger local and systemic inflammatory responses [156], so we speculated that the AKI responsive inflammation and subsequent secretion of inflammatory cytokines into the circulation may have contributed to the hepatic induction of *Sult1e1*. Indeed, AKI induced kidney and hepatic expression of *Il-6* (**Fig. 7B**), and increased the circulating level of Il-6 (**Fig. 7C**). Time course analysis showed that the induction of hepatic *Il-6* and Il-1β preceded the induction of *Sult1e1*, in that the hepatic expression of *Il-6* and Il-1β started increasing 1 h after AKI (**Fig. 7D**), whereas the expression of *Sult1e1* did not increase until 6 h after AKI (**Fig. 7E**). The kidney infiltration of cluster of differentiation 3 (CD3)+ cells, a surface marker of T cells, was increased upon AKI, consistent with the inflammatory response of the kidney (**Fig. 7F**). *In vitro*, treatment with Il-6 induced the expression of *SULT1E1* in primary hepatocytes (**Fig. 7G**) and in the human kidney proximal tubular (HKC-8) cells (**Fig. 7H**). Together, these results suggested that AKI may distantly induce the expression of *Sult1e1* in the liver as a result of AKI-responsive inflammation as summarized in **Fig. 7I**.

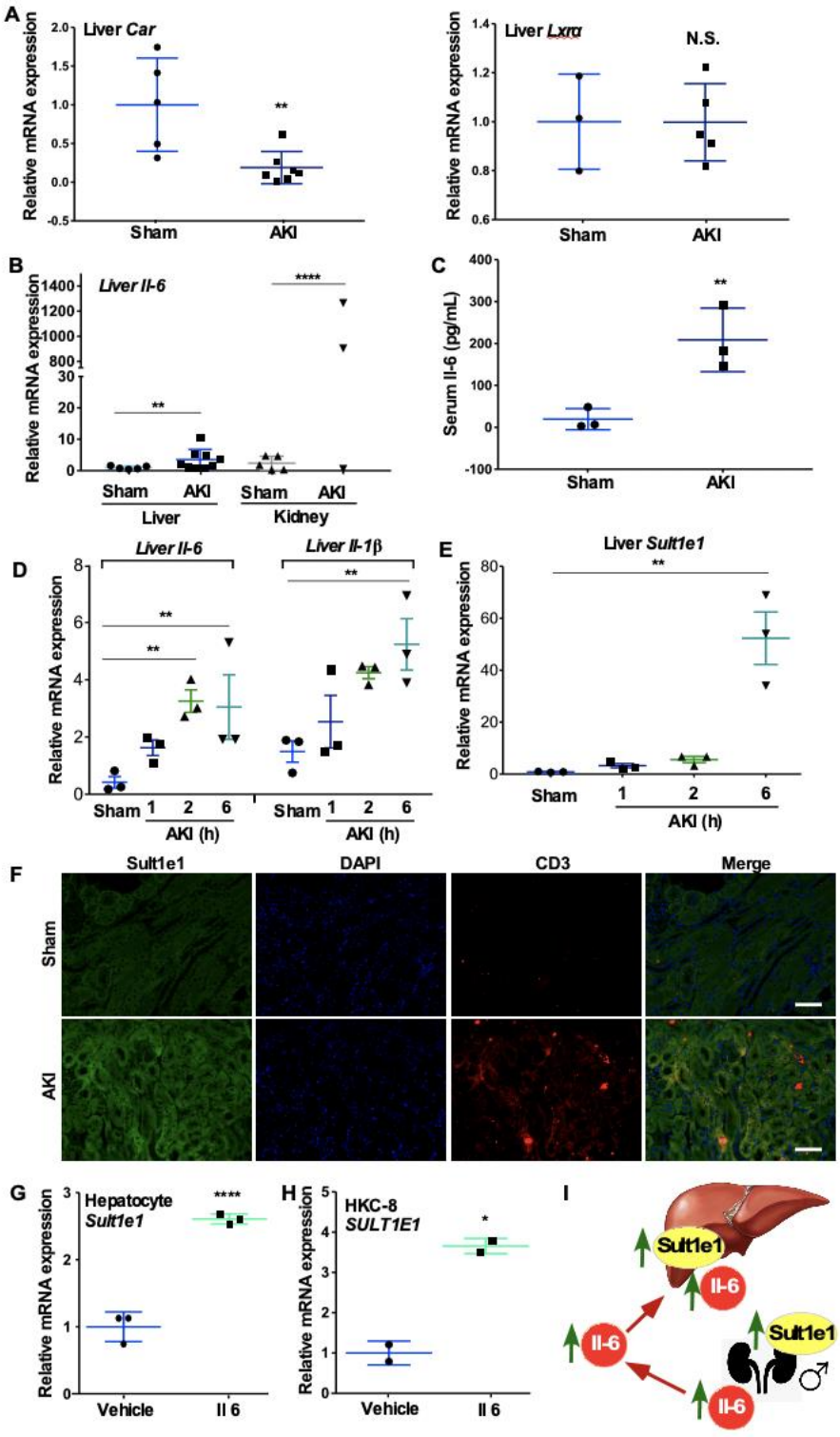

Figure 7: Inflammation is a potential mechanism for AKI responsive induction of *Sult1e1* in the liver.

(A-F) Mice are the same as described in Figure 1. Shown are hepatic mRNA expression of *Car* and *Lxrα* (A), hepatic and kidney mRNA expression of *Il-6* (B), serum level of Il-6 measured by ELISA (C), time course of

hepatic expression of *Il-6* and *Il-1β* (D) and *Sult1e1* (E), and immunofluorescence of *Sult1e1* and CD3 (F). (G and H) The expression of *SULT1E1* in primary hepatocytes (G) and HKC-8 cells (H) treated with vehicle or Il-6. (I) Proposed model of Il-6-mediated distal regulation of hepatic *Sult1e1* by AKI. Scale bars are 50 μm. Results are presented as the mean ± SD. *, P < 0.05; **, P < 0.01; ****, P < 0.0001, compared with the sham groups.

3.3 Genetic ablation or pharmacological inhibition of *Sult1e1* protects mice from AKI

Male sex is an independent risk factor for AKI and it's believed females are more resistant because of estrogens [144, 145, 157]. To determine the functional relevance of *Sult1e1* and its regulation by AKI, in accordance to aim 1, we challenged eight-week old female *Sult1e1* knockout (*Sult1e1* KO) mice with AKI or the sham surgery, and the mice were sacrificed for analysis 24 h after. The *Sult1e1* KO mice exhibited a marked protection from AKI, including the abolishment of AKI-responsive increase of serum level of creatinine and blood urea nitrogen (BUN) (**Fig. 8A**), attenuation of AKI-responsive induction of kidney injury marker gene neutrophil gelatinase associated lipocalin 2 (*NGAL*) (**Fig. 8B**), of kidney *Il-6* (**Fig. 8C**), and kidney histology (**Fig. 8D**). The protective effect of *Sult1e1* KO was not sex-specific, because the male *Sult1e1* KO mice were also protected from AKI, as evidenced by serum creatinine (**Fig. 9A**) and BUN levels (**Fig. 9B**). NGAL was also decreased at both the mRNA (**Fig. 9C**) and protein (**Fig. 9D**) levels. At the histological level, the *Sult1e1* KO mice subjected to AKI showed fewer tubular injury and less apoptosis, as shown by PAS staining and TUNEL assay, respectively (**Fig. 9E**). Moreover, the AKI-responsive kidney (**Fig. 9F**) and hepatic (**Fig. 9G**) induction of *Il-6* was largely normalized in *Sult1e1* KO mice, so was the circulating level of Il-6 (**Fig. 9H**). The kidney protective effect of *Sult1e1* ablation remained obvious 72 h after the ischemic AKI, which was evidenced by lower

levels of serum creatinine and BUN (**Fig. 10A**), decreased kidney expression of *NGAL* (**Fig. 10B**), and reduced kidney tubular injury and apoptosis (**Fig. 10C**).

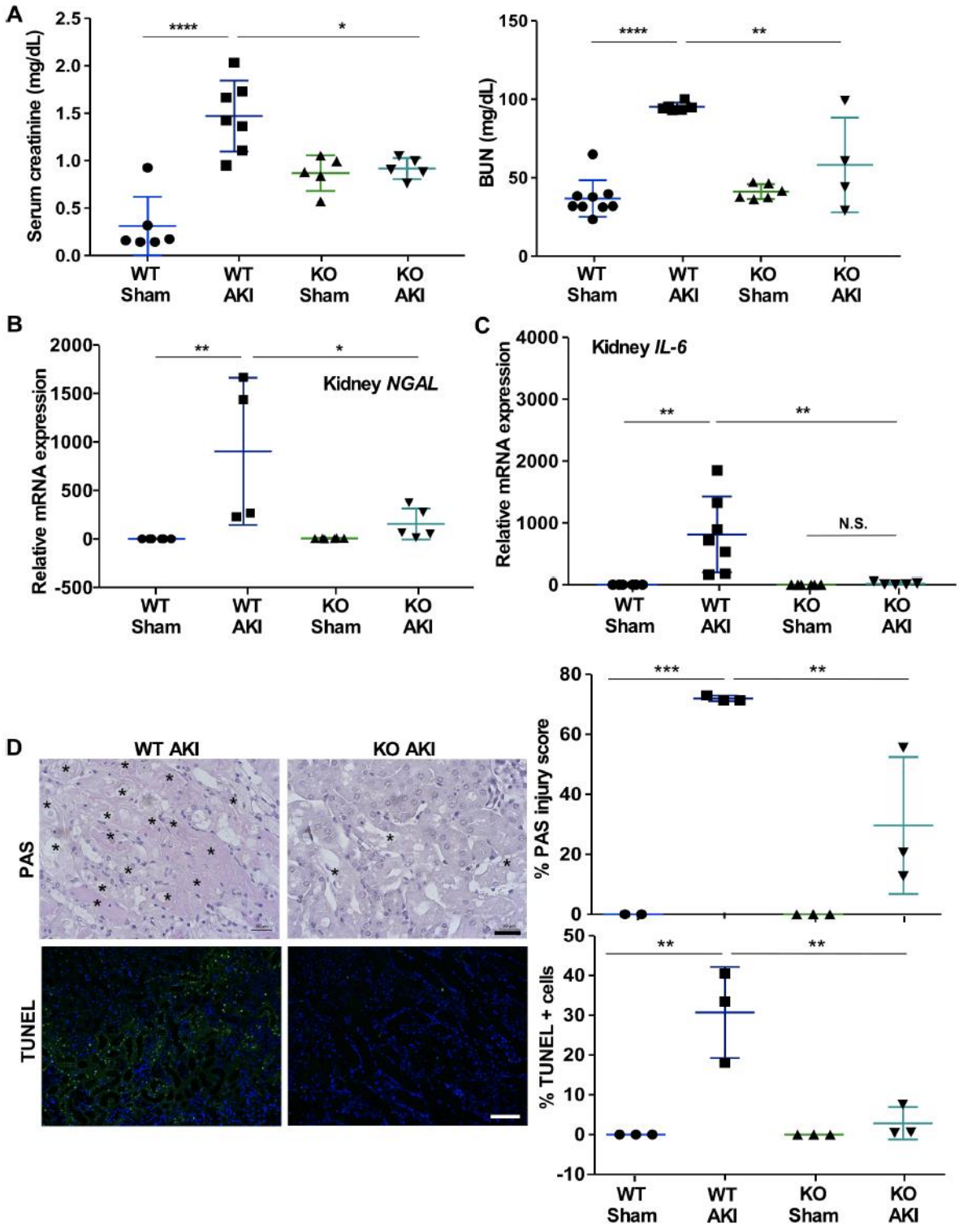

Figure 8: Knockout of *Sult1e1* protects female mice from AKI.

(A-D) WT and *Sult1e1* KO female mice were subjected to the 30-min ischemic AKI, and the mice were sacrificed 24 h after the surgery. Shown are serum levels of creatinine and BUN (A) (any difference WT vs KO in sham?), kidney mRNA expression of *NGAL* (B) and *Il-6* (C), and kidney histology (D, with asterisks indicating tubular damage). n=4 for each group. Scale bars are 50 μm. Results are presented as the mean ± SD. *P < 0.05; **, P < 0.01; ***, P < 0.001; the comparisons are labeled.

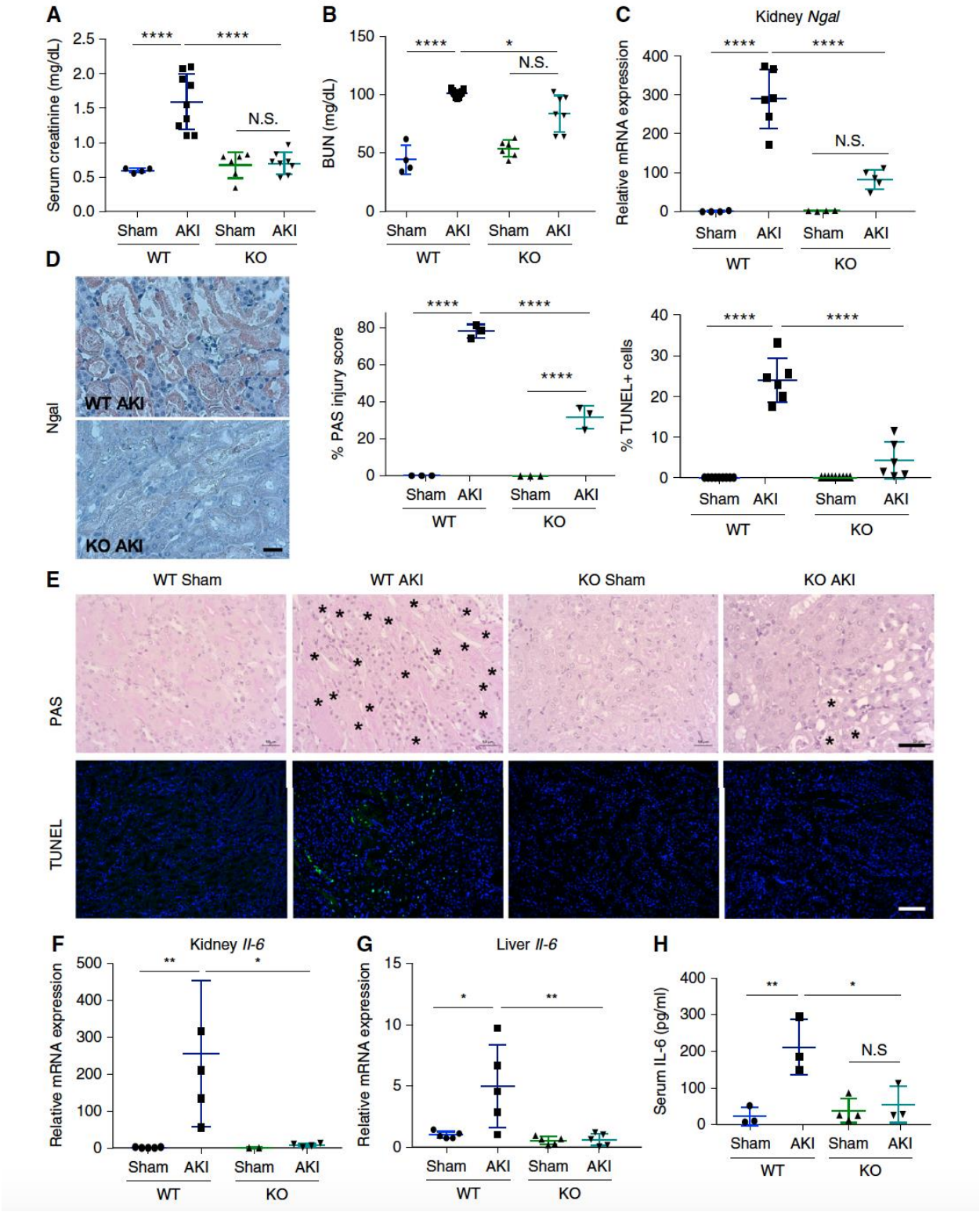

Figure 9: Knockout of *Sult1e1* protects male mice from AKI.

(A-H) WT and *Sult1e1* KO males were subject to 30-min ischemic AKI or the sham surgery, and the mice were sacrificed 24 h after the surgery. Shown are serum level of creatinine (A), BUN (B), the mRNA (C) and protein (D) expression of kidney NGAL, histology as shown by PAS staining and TUNEL with the quantifications shown on the top right (E, with asterisks indicating tubular damage), kidney and hepatic mRNA expression of *Il-6* (F-G), and serum level of Il-6 (H). n=3-6 for each group. Scale bars are 50 μm. Results are presented as the mean ± SD. *, P < 0.05; **, P < 0.01; ****, P < 0.0001; N.S., statistically not significant, with the comparisons labeled.

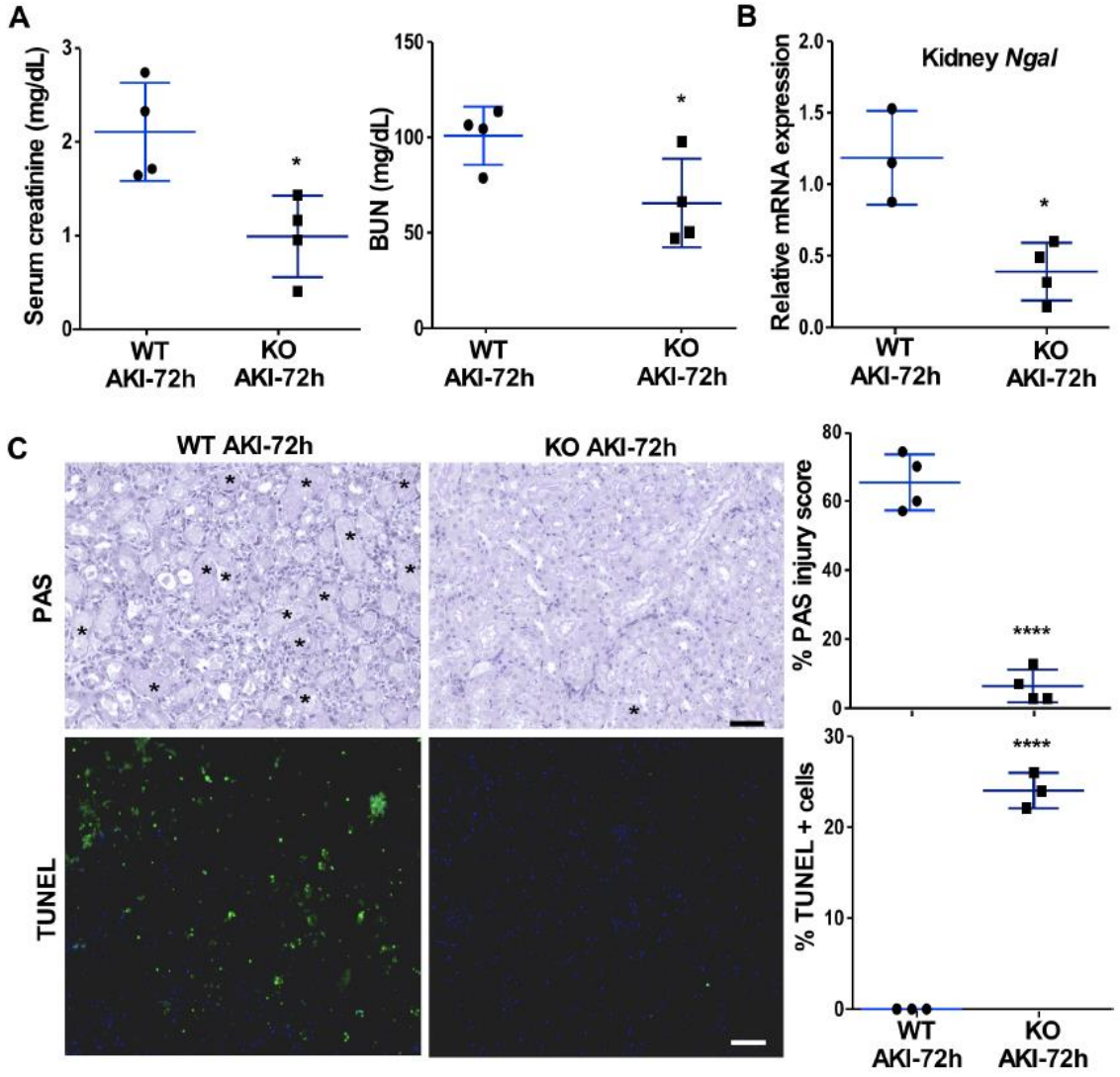

Figure 10: Kidney protective effect of *Sult1e1* ablation 72-hours post AKI.

(A-C) WT male mice were subjected to the 30-min ischemic AKI, and the mice were sacrificed 72 h after the surgery. Shown are serum levels of creatinine and BUN (A), kidney mRNA expression of *NGAL* (B), and kidney histology (C, with asterisks indicating tubular damage). n=4 for each group. Scale bars are 50 μm. Results are presented as the mean ± SD. *P < 0.05, compared with the WT AKI-72h groups.

As also proposed in Aim 1, in an independent pharmacological model of *Sult1e1* inhibition, WT mice were treated with triclosan, an efficient pharmacological inhibitor of *Sult1e1[58]*, as outlined in **Fig. 11A**. Consistent with results from the *Sult1e1* KO mice, treatment of female mice with 50 mg/kg dose of the *Sult1e1* inhibitor triclosan was effective in attenuating AKI-responsive kidney injury in female mice (**Fig. 11B-D**). Treatment of male mice with triclosan at 10 mg/kg or 50 mg/kg (**Fig. 12A**) also attenuated AKI, as shown by decreased serum creatinine and BUN levels (**Fig. 12B**), and kidney expression of *NGAL* (**Fig. 12C**). Triclosan-treated mice also presented improved histology (**Fig. 12D**), and decreased kidney and hepatic expression of *Il-6* (**Fig. 12E**).

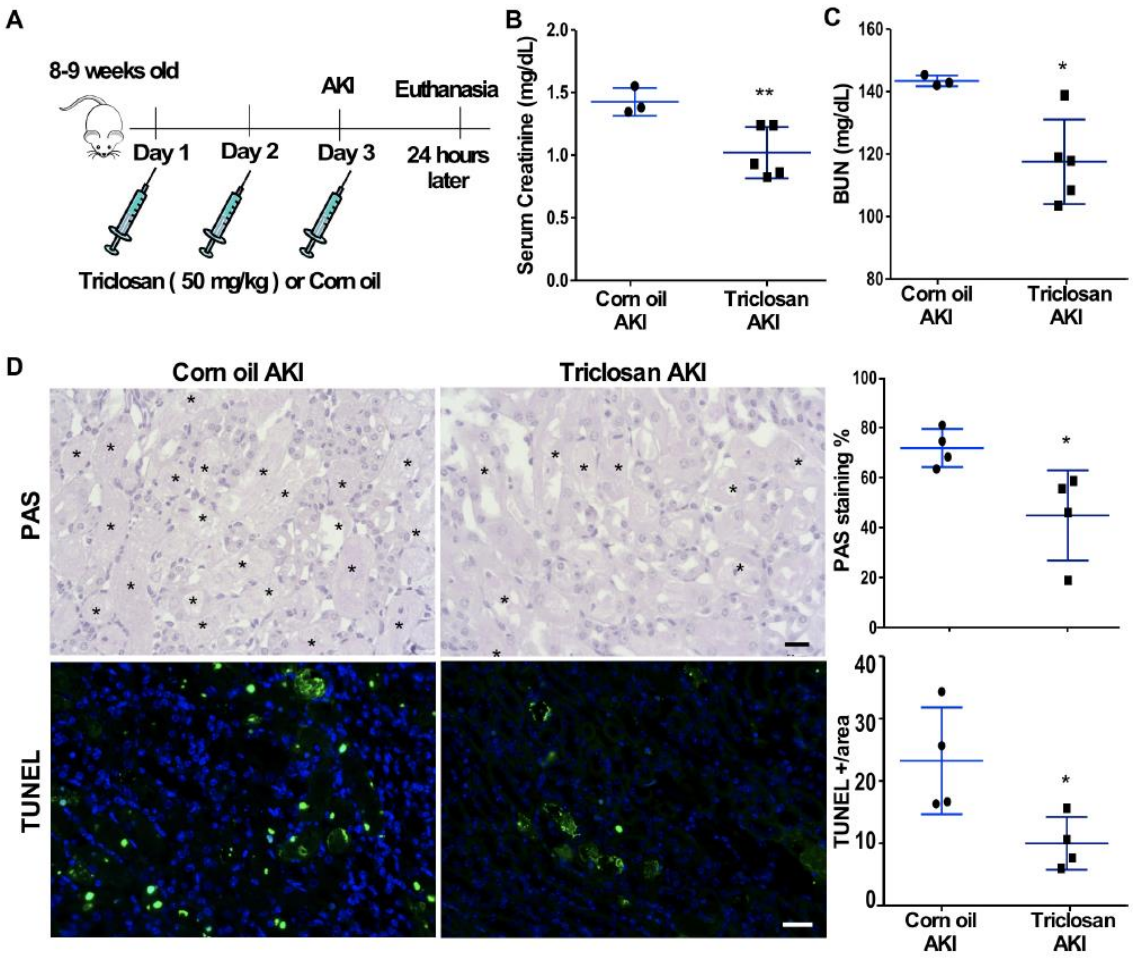

Figure 11: Treatment with triclosan protects WT female mice from AKI.

(A) Schematic representation of the triclosan (50 mg/kg) regimen. (B-D) Female mice were treated with three daily i.p. doses of triclosan or the vehicle corn oil before being subjected to the AKI surgery. Shown are serum levels of creatinine (B) and BUN (C), and the kidney histology (D, with asterisks indicating tubular damage). n=4 for each group. Scale bars are 50 µm. Results are presented as the mean ± SD. *P < 0.05; **, P < 0.01, compare to the corn oil AKI groups.

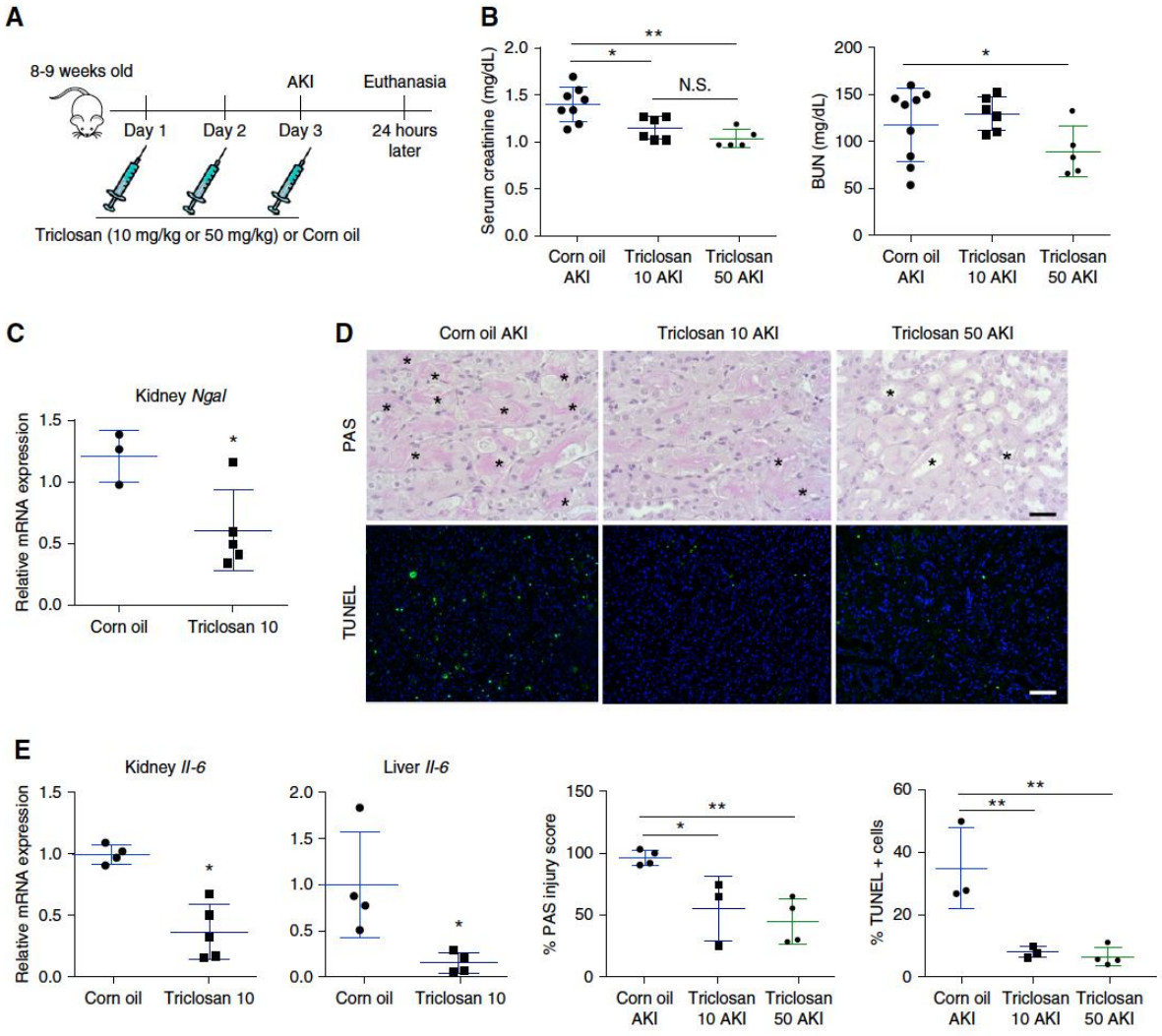

Figure 12: Pharmacological inhibition of *Sult1e1* protects male mice from AKI.

(A) Scheme of triclosan treatment. WT male mice received three doses of triclosan (10, or 50 mg/kg) or the vehicle corn oil before being subject to the 30-min ischemic AKI, and the mice were sacrificed 24 h after the surgery. (B-E) Shown are serum level of creatinine and BUN (B), kidney mRNA expression of *NGAL* (C), histology as shown by PAS staining and TUNEL with the quantifications shown on the bottom right (D, with asterisks indicating tubular damage), and kidney and hepatic mRNA expression of *Il-6* (E). n=4-8 for each group. Scale bars are 50 μm. Results are presented as the mean ± SD. *, P < 0.05; **, P < 0.01, compared to the corn oil groups, or the comparisons are labeled.

3.4 The kidney protective effect of *Sult1e1* ablation is estrogen- and androgen-independent

Since the primary function of *Sult1e1* is sulfonating and deactivating estrogens [62, 158], and administration of pharmacological doses of E_2 after cardiac arrest protected male mice from AKI [141], we wanted to know whether the kidney protective effect of *Sult1e1* ablation in male mice was estrogen dependent. To our surprise, the serum levels of E_2 (**Fig. 13A**) were not significantly altered in male *Sult1e1* KO AKI mice. The kidney expression of estrogen responsive gene *Stat5* [159] was not affected either (data not shown). Additionally, the expression of aromatase, the enzyme that converts testosterones into estrogens, was undetectable in the liver or kidney (data not shown). As outlined in Aim 1, castration or ovariectomy were then performed on male or female *Sult1e1* KO mice to determine whether the kidney protective effect of *Sult1e1* ablation was androgen and estrogen dependent, respectively. In this experiment, castration or ovariectomy was performed on *Sult1e1* KO mice four weeks prior to the AKI surgery as outlined in **Fig. 13B**. Both castrated *Sult1e1* KO males and ovariectomized *Sult1e1* KO females remained protected from AKI as their sham surgery counterparts, as shown by serum levels of creatinine, BUN, and histology (**Fig. 13C-F**). The effectiveness of the ovariectomy surgery was confirmed by decreased uterine weight as percentages of the body weight in ovariectomized mice (**Fig. 13G**). These results suggested that the kidney protective effect of *Sult1e1* ablation was sex hormone-independent.

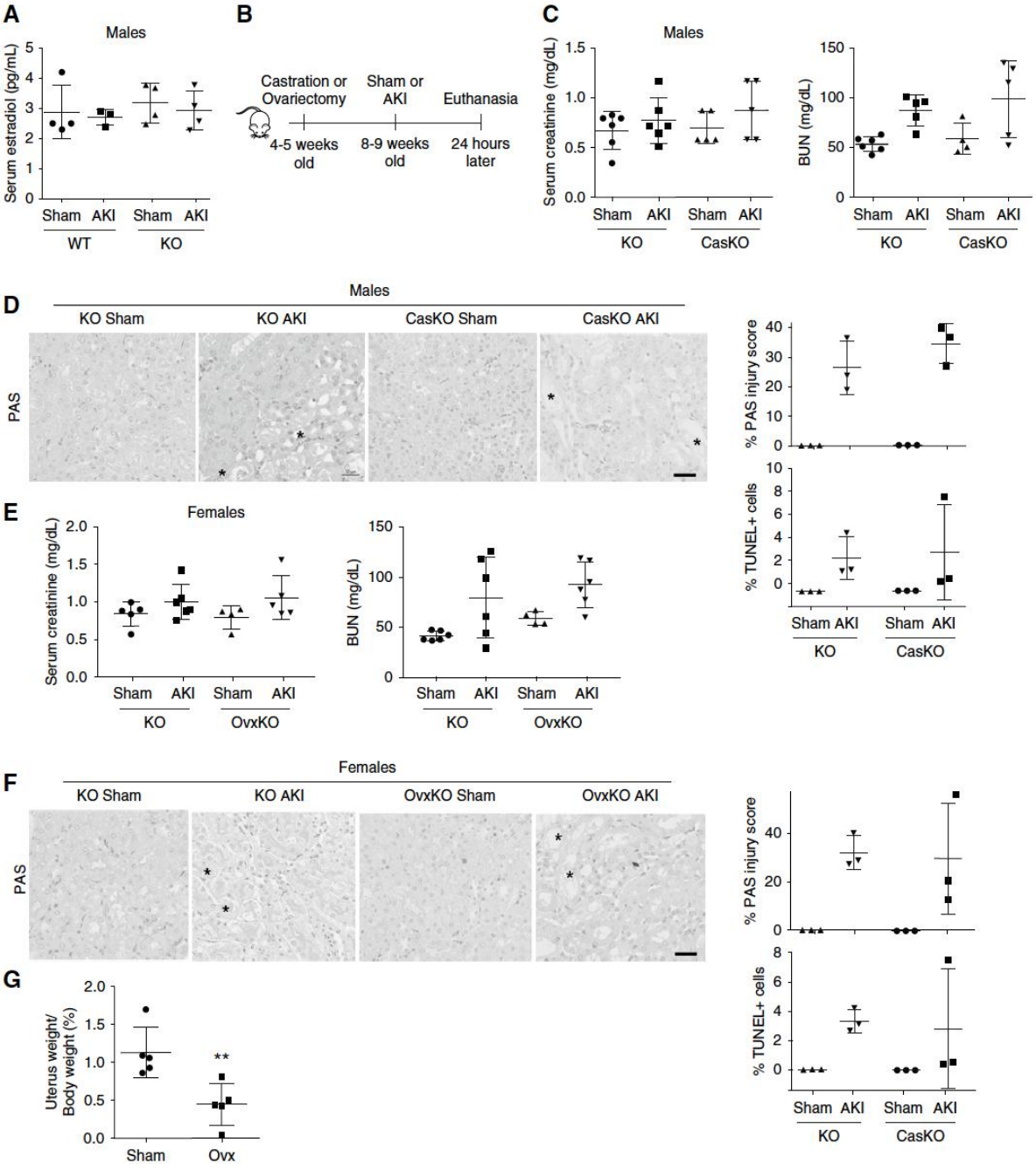

Figure 13: The kidney protective effect of *Sult1e1* ablation is estrogen- and androgen-independent.

(A) Mice are the same as described in Figure 3A. Shown are the serum level of E_2. (B) Scheme of castration or ovariectomy followed by kidney ischemic AKI. (C and D) Intact *Sult1e1* KO male mice or castrated *Sult1e1* KO males were subjected to Sham surgery or AKI surgery. Shown are serum levels of creatinine and BUN (C), and kidney histology as evaluated by PAS and TUNEL staining with their quantifications shown on the right (D, with asterisks indicating tubular damage). (E and F) Intact *Sult1e1* KO female mice or

ovariectomized *Sult1e1* KO females were subjected to Sham surgery or AKI surgery. Shown are serum levels of creatinine and BUN (E), and kidney histology as evaluated by PAS and TUNEL staining with their quantifications shown on the right (F, with asterisks indicating tubular damage). (G) Uterine weight as percentages of the body weight in female mice subjected Sham surgery or ovariectomy. n=4-6 for each group. Scale bars are 50 μm. Results are presented as the mean ± SD.

3.5 Hepatic *Sult1e1* is required for AKI injury in male, but not in female mice

Knowing hepatic *Sult1e1* is induced by AKI, we wanted to determine whether the hepatic expression of *Sult1e1* distantly contributed to the pathogenesis of AKI. For this purpose, we reconstituted the expression of *SULT1E1* tissue specifically to the liver by using the KOLE mice. KOLE mice were generated by cross-breeding the *Sult1e1* KO mice with the Lap- *SULT1E1/EST* (LE) transgenic mice that express the human *SULT1E1* transgene exclusively in the liver under the control of the liver-enriched activator protein (Lap) gene promoter [139] as outlined in **Fig. 14A**. The KOLE mice overexpress *Sult1e1* in the liver. Upon IRI, expression in the livers of male mice are further increase whereas in female the expression remains similar as Sham-operated female mice (**Fig. 14B)**. Despite the liver expression, KOLE mice resemble a *Sult1e1* KO background in other organs, including the kidney, which was verified by qRT-PCR (**Fig. 14C**). In the male mice, restoration of liver *Sult1e1* was sufficient to re-sensitize KOLE mice to AKI, as shown by serum levels of creatinine and BUN (**Fig. 14D**), kidney mRNA expression of *NGAL* (**Fig. 14E**) and *IL-6* (**Fig. 14F**), and histology (**Fig. 14G**). These results suggested that hepatic *Sult1e1* is required for male mouse's sensitivity to AKI. Interestingly, the effect of hepatic reconstitution of *Sult1e1* on AKI was sex specific, because the female KOLE mice remained

efficiently protected from AKI, as shown by serum levels of creatinine and BUN (**Fig. 14H**) and histology (**Fig. 14I**).

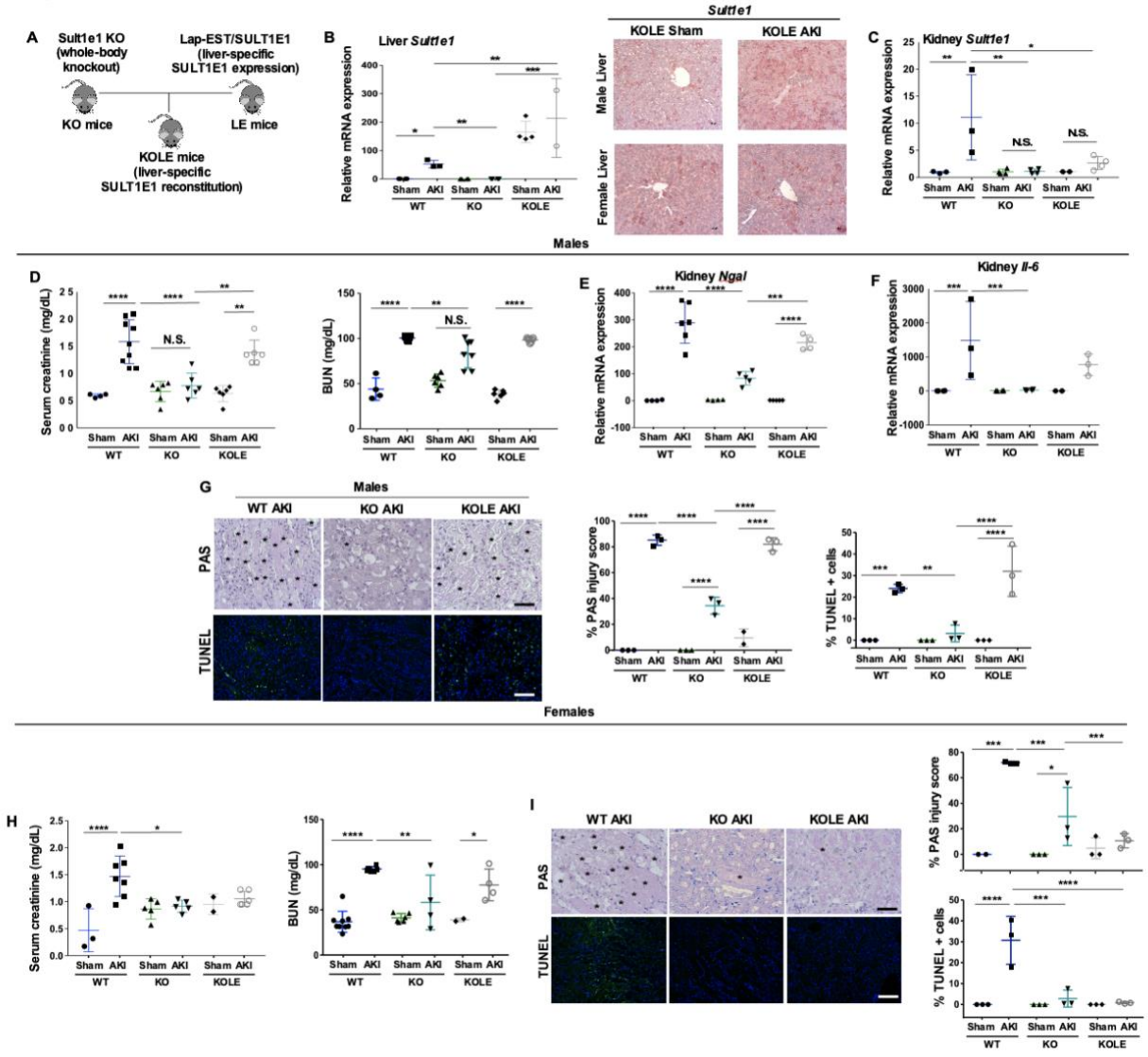

Figure 14: Hepatic *Sult1e1* is required for AKI injury in male, but not in female mice.

(A) Schematic representation of the KOLE mice that were created by breeding the liver specific Lap-*SULT1E1/EST* (LE) transgene into the *Sult1e1* KO background. (B) Reconstitution of *SULT1E1* in the liver and (C) lack of reconstitution in the kidney was confirmed. (D-G) Male mice of indicated genotypes were subjected to the 30-min ischemic AKI, and the mice were sacrificed 24 h after the surgery. Shown are serum levels of creatinine and BUN (D), kidney mRNA expression of *NGAL* (E) and *Il-6* (F), and kidney histology as shown by PAS staining and TUNEL with their quantifications shown on the right (G), with asterisks

indicating tubular damage). (H and I) Experiments were the same as described in (D-G) except that female mice were used. Shown are serum levels of creatinine and BUN (H) and kidney histology as shown by PAS staining and TUNEL with their quantifications shown on the right (I, with asterisks indicating tubular damage). n=6 for each group. Scale bars are 50 μm Results are presented as the mean ± SD. *P < 0.05, **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; N.S., statistically not significant, with the comparisons labeled.

3.6 The protective effect of *Sult1e1* ablation is associated with kidney regulation of vitamin D metabolizing and cell cycle genes

Since the kidney protective effect of *Sult1e1* ablation was sex hormone-independent, we went on to determine whether the metabolism of other endogenous substrates may have been responsible for kidney protection. In this effort, we performed Affymetrix microarray analysis comparing the transcriptomic profile in the kidneys of WT AKI and *Sult1e1* KO AKI mice. Microarray showed altered expression of several genes involved in vitamin D metabolism and cell proliferation in the *Sult1e1* KO AKI group (**Fig. 15**).

The inductions of *Cyp24a1* and *Ccnd1* and suppression of *Fgg* were verified by qRT-PCR (**Fig. 15A**). *Cyp24a1*, which encodes a vitamin D metabolizing enzyme, is a known vitamin D receptor (VDR) target gene [160]. The expression of *Ccnd1*, which encodes Cyclin D1, has been reported upregulated at early rises of vitamin D [161] and may promote cell proliferation [162]. *Fgg*, which encodes fibrinogen, has been shown to have an inverse correlation with vitamin D levels [163]. This gene expression profile suggested that the VDR signaling was enhanced in the kidneys of *Sult1e1* KO mice upon AKI. Administration of the active form of vitamin D calcitriol has been shown to improve several animal models of AKI [164, 165], and calcitriol is currently in clinical trials for the treatment of AKI [166]. These results suggested that the increased VDR

signaling may have contributed to the kidney protective effect of *Sult1e1* ablation. The induction of Cyp24a1 and Cyclin D1 in *Sult1e1* KO AKI kidneys and the loss of these inductions in KOLE AKI kidneys were confirmed by immunofluorescence (**Fig. 15B and 15C**), which correlated to the AKI protection and re-sensitization in these two genotypes, respectively. The increased kidney expression of Cyclin D1 was also verified by Western blotting (**Fig. 15D**). We speculate that the increased VDR signaling and the induction of Cyclin D1 may have contributed to the post-AKI kidney repair. Indeed, the kidney immunostaining of *Ki67*, a cell proliferation marker, was increased in *Sult1e1* KO AKI kidney and this effect was attenuated in KOLE AKI kidney (**Fig. 15E**). Our luciferase reporter gene assay results showed that overexpression of *SULT1E1* did not affect the activity of calcitriol in inducing the VDR responsive reporter activity (**Fig. 15F**), suggesting that calcitriol is not a direct substrate of *SULT1E1*.

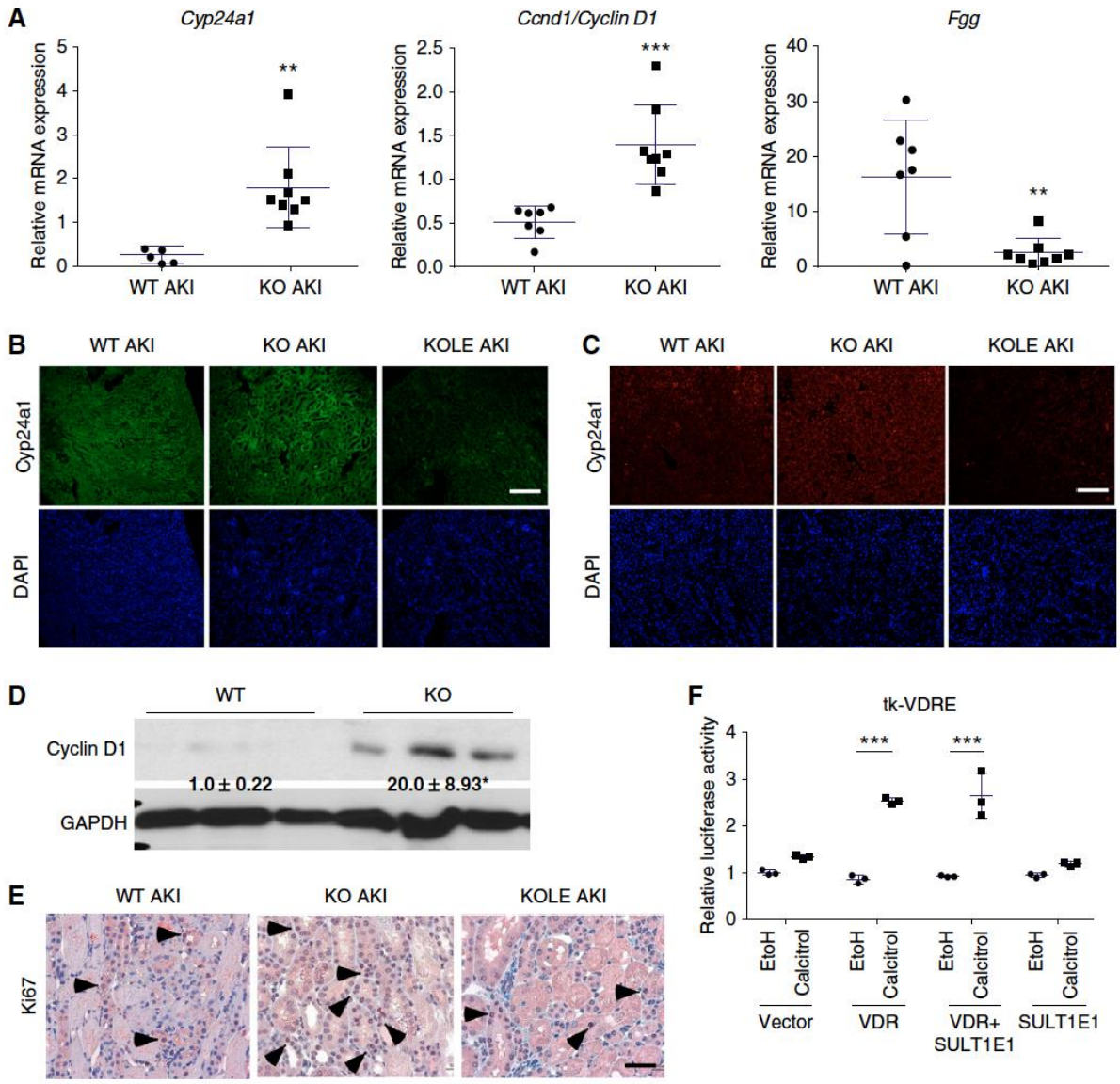

Figure 15: The protective effect of *Sult1e1* ablation is associated with kidney regulation of vitamin D metabolizing and cell cycle genes.

(A) Kidney mRNA expression of *Cyp24a1*, *Ccnd1*, and *Fgg* in WT and *Sult1e1* KO mice subjected to 30-min ischemic AKI. (B and C) The kidney expression of Cyp24a1 (B) and Cyclin D1 (C) in WT, *Sult1e1* KO, and KOLE mice subjected to 30-min ischemic AKI was shown by immunofluorescence. (D) The kidney expression of Cyclin D1 protein in WT and *Sult1e1* KO mice subjected to 30-min ischemic AKI was shown by Western blotting with the signal quantifications labeled. (E) Immunostaining of Ki67 in the same groups, with arrowheads indicating positive staining. (F) 293T cells were transfected with a VDR reporter gene tk-VDRE

in the presence of the co-transfection of VDR plasmid and *SULT1E1* plasmid alone or in combination. Transfected cells were treated with vehicle (ethanol) or Calcitriol (10 μM) for 24 h before cell lysis and luciferase assay. n=3-8 per group. Scale bars are 50 μm. Results are presented as the mean ± SD. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001, compared to WT AKI (A), or the comparisons are labeled (F).

3.7 Discussion

The sex-specific effect of *Sult1e1* on AKI is interesting. First, the regulation of *Sult1e1* by AKI is sex-specific. AKI induced the hepatic expression of *Sult1e1* in both male and female mice. However, the kidney induction of *Sult1e1* by AKI only occurred in males, but not in females. Interestingly, the effect of *Sult1e1* ablation and reconstitution was also sex-specific. Although both male and female *Sult1e1* KO mice were protected from AKI, the reconstitution of *Sult1e1* in the liver of male KOLE mice abolished the protective effect, whereas the female KOLE mice remained protected from AKI. Sex-specific effects of *Sult1e1* ablation and/or reconstitution were also observed in our previous studies in the context of metabolic disease. We reported that *Sult1e1* ablation protected female ob/ob mice from obesity and type II diabetes, but sensitized male ob/ob mice to metabolic syndrome [130]. When the expression of *Sult1e1* was reconstituted in the adipose tissue of *Sult1e1* deficient ob/ob (obe) mice, the reconstitution effect was sex-specific, because the adipose reconstitution of *Sult1e1* improved the metabolic function of male obe mice, but had little effect on the female obe mice [131, 167]. Interestingly, in both AKI and metabolic disease, the effect of *Sult1e1* reconstitution was uniformly obvious in males, but not in female mice. The male-specific effect of adipose reconstitution of *Sult1e1* was explained to be due to the high basal expression of *Sult1e1* in the adipose tissue of male mice [131]. The basal expression of *Sult1e1* in the liver is low in both sexes, so the mechanism underlying the male-specific effect of

the liver constitution of *Sult1e1* on AKI remains to be understood. The sex-specific effect of *Sult1e1* ablation was also observed in a mouse model of liver ischemia-reperfusion induced liver injury in that *Sult1e1* ablation conferred protection to female mice, whereas the male mice were further sensitized [140]. The effect of *Sult1e1* reconstitution on ischemia and reperfusion-induced liver injury remains to be tested.

The tissue-specific effect of *Sult1e1* on AKI is equally interesting. In male mice, although the AKI responsive induction of *Sult1e1* was observed in both the liver and kidney, our results suggested that loss of *Sult1e1* in the liver, but not in the kidney, was responsible for the renoprotection because reconstitution of *Sult1e1* in the liver was sufficient to abolish the protection. Immunostaining of KOLE samples suggested a sex-difference in *Sult1e1* expression upon IRI. This may have reflected on a lack of AKI effect in female KOLE mice. The role of basal and AKI inducible expression of kidney *Sult1e1* in AKI-induced kidney injury in male mice remains to be defined. In female mice, although the loss of *Sult1e1* in the liver was not responsible for the protection because the liver reconstitution of *Sult1e1* had little effect, we cannot conclude that the loss of *Sult1e1* in the kidney accounted for the renoprotection. It will be interesting to know whether the reconstitution of *Sult1e1* in the kidney of female *Sult1e1* KO mice will affect the protective effect of *Sult1e1* ablation.

The tissue-specific effect of *Sult1e1* was also observed in our previous study in the ob/ob mice. The ob/ob mice exhibited liver-specific up-regulation of *Sult1e1* [130]. *Sult1e1* ablation in ob/ob (obe) males worsened the metabolic phenotype [130]. Interestingly, the transgenic reconstitution of *Sult1e1* in the adipose tissue of male obe mice attenuated the metabolic phenotypes, including decreased local and systemic inflammation, improved insulin sensitivity, and increased energy expenditure [131, 167]. In contrast, the reconstitution of *Sult1e1* in the liver

failed to improve the metabolic function of obe males [131]. These results suggested that although the hepatic *Sult1e1* is markedly induced in ob/ob mice, it was not the loss of hepatic expression and induction of *Sult1e1* that was responsible for the worsened metabolic function in obe males.

Another interesting finding of this study is the estrogen and androgen independence of the AKI protective effect of *Sult1e1* ablation because the protective effect was intact in *Sult1e1* KO mice subject to ovariectomy or castration. The estrogen independence was a surprise, considering that a primary function of *Sult1e1* is to regulate estrogen homeostasis, and estrogens have been suggested to be AKI protective in animals [141-143] and humans [18, 149]. Indeed, our previous study showed that the metabolic benefit of *Sult1e1* ablation in female ob/ob mice was estrogen-dependent, because the metabolic benefit was abolished upon ovariectomy [130]. The protective effect of *Sult1e1* ablation on ischemia-reperfusion induced liver injury in female mice was also estrogen-dependent [140]. The androgen independence was also a surprise because prior studies suggested that castration ameliorates AKI, whereas testosterone administration worsens it [143]. In addition, the sensitizing effect of *Sult1e1* ablation on ischemia-reperfusion induced liver injury in male mice was also androgen-dependent, because the sensitization was abolished upon castration [140]. It remains to be understood why the sex hormone dependence of the *Sult1e1* effect varies among disease models. Nevertheless, the sex hormone independence of the AKI protective effect of *Sult1e1* ablation suggested that *Sult1e1* substrates other than estrogens might have been responsible for the *Sult1e1* effect on AKI.

Our gene profiling analysis suggested that increased vitamin D signaling may have contributed to the kidney protective effect of *Sult1e1* ablation, because kidney expression of several VDR responsive genes, such as *Cyp24a1*, *Ccnd1*, and *Fgg*, was affected in *Sult1e1* KO mice upon the AKI challenge. The enhanced VDR signaling may have explained the protective

phenotype because the administration of calcitriol has been shown to improve several animal models of AKI [164, 165], and calcitriol is currently in clinical trials for the treatment of AKI [166]. Furthermore, calcitriol and VDR signaling have been reported to promote cell proliferation, especially in cancerous and damaged cells [168]. As such, increased VDR signaling may have promoted cellular recovery and tissue repair after AKI, a notion supported by our observations of increased *Ki67* and Cyclin D1 expression in AKI *Sult1e1* KO mice. Calcitriol is also known for its anti-inflammatory activity [169], which could also have contributed to the renoprotection. Since we showed calcitriol is not a *Sult1e1* substrate, the mechanism by which *Sult1e1* ablation or inhibition increases VDR signaling remains to be understood. We recognize that both VDR activation and cyclin D1 induction in AKI *Sult1e1* KO mice were associations. Future studies are necessary to determine whether these two events are required for the AKI protective effect of *Sult1e1* ablation.

Among the limitations, although we have identified interesting crosstalk between the liver and kidney, and showed clearly the loss of hepatic *Sult1e1* was responsible for the AKI protective effect of *Sult1e1* ablation, future studies are necessary to identify the mediators released from the liver that affect0 the kidney injury. Our microarray IPA analysis showed the kidneys of *Sult1e1* KO AKI mice had the highest positive z-score for the ataxia telangiectasia mutated (ATM) pathway (**Fig. 16**). A positive z-score indicates this pathway was activated. The ATM pathway is activated in the presence of DNA damage and stimulates DNA repair, DNA recombination, and cell-cycle control [170, 171]. Checkpoint kinase 2 (Chk2), the main effector of ATM kinase, is in charge of cell cycle regulation and also controls calcitriol formation [172]. Future studies are necessary to determine whether the ATM signaling could be a possible mediator for the renoprotection. The sex-specific effect of the hepatic reconstitution of *Sult1e1* on *Sult1e1* KO AKI

mice also remains to be better understood. IPA analysis of our microarray results suggest that the livers of WT female, but not male mice had a positive z-score for the vitamin D receptor/retinoid X receptor (VDR/RXR) pathway (**Fig. 17 and 18**), but its significance in female sensitivity to AKI remains to be defined.

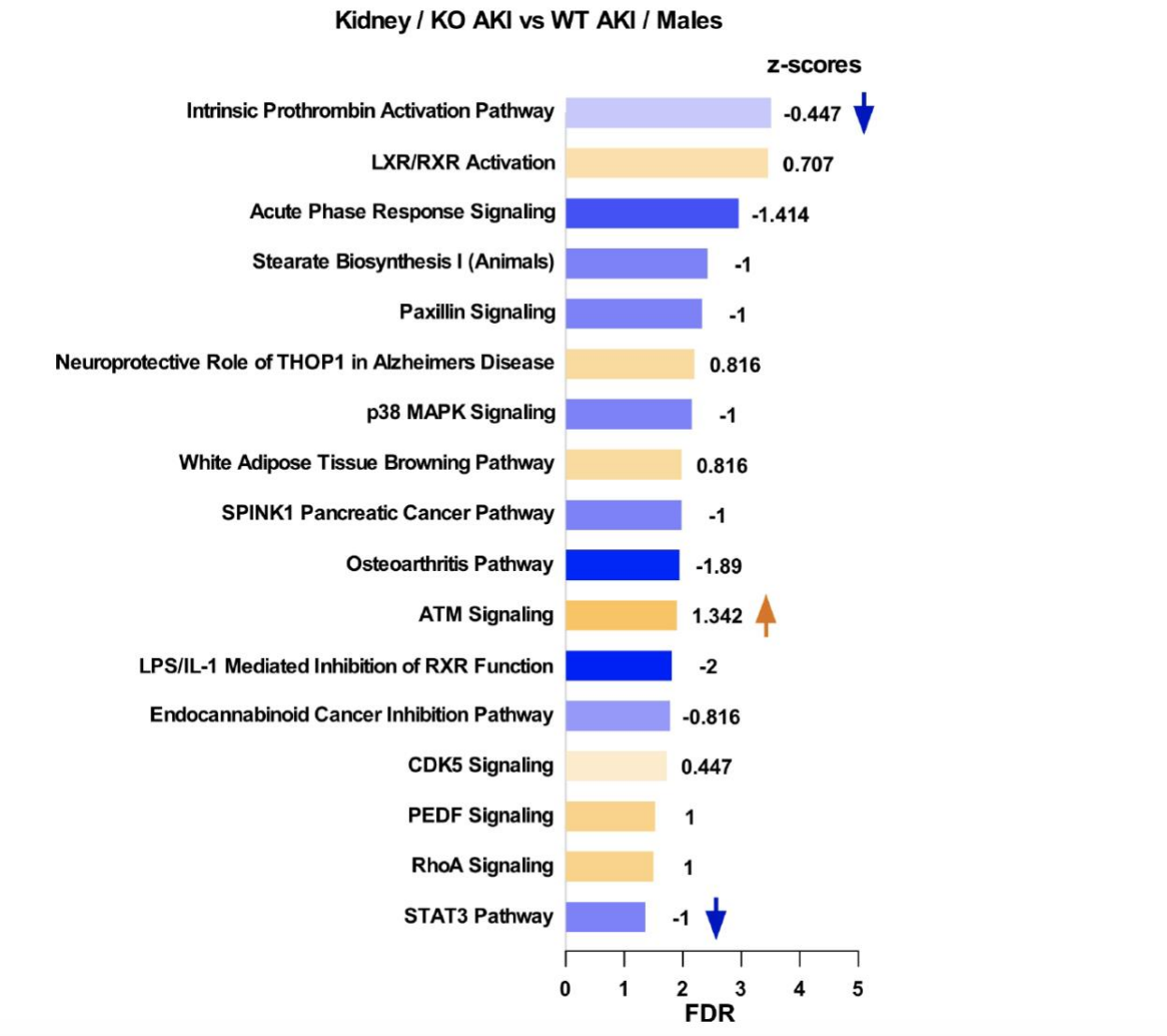

Figure 16: Ingenuity pathway analysis (IPA) of microarray results.

Shown are z-scores and false discovery rate (FDR) of male kidney (KO AKI vs WT AKI). Several up-regulated (orange arrows) and down-regulated (blue arrows) pathways are highlighted.

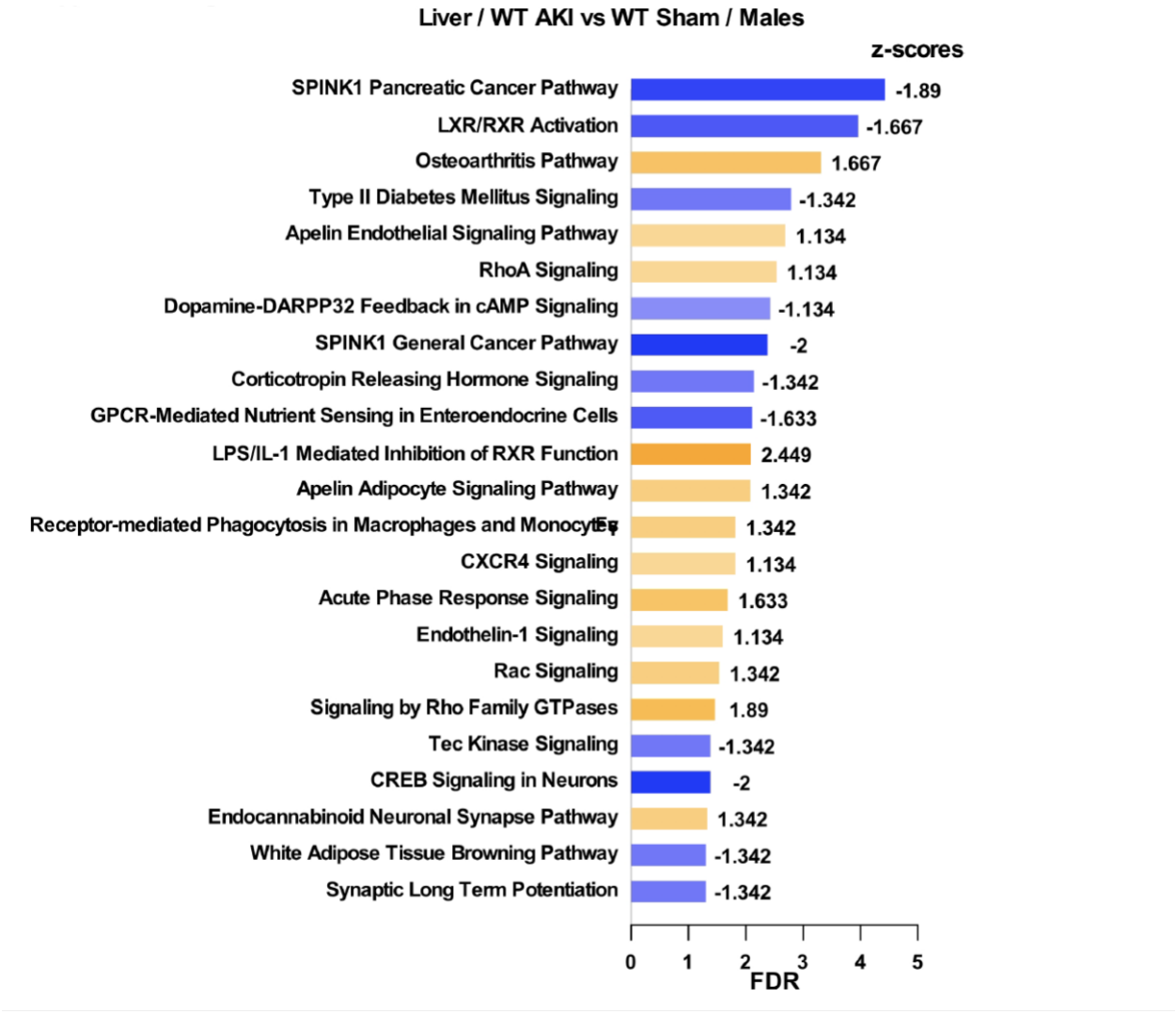

Figure 17: Ingenuity pathway analysis (IPA) of microarray results in male mice.

Shown are z-scores and false discovery rate (FDR) of male liver (WT AKI vs WT Sham). Several up-regulated (orange arrows) and down-regulated (blue arrows) pathways are highlighted.

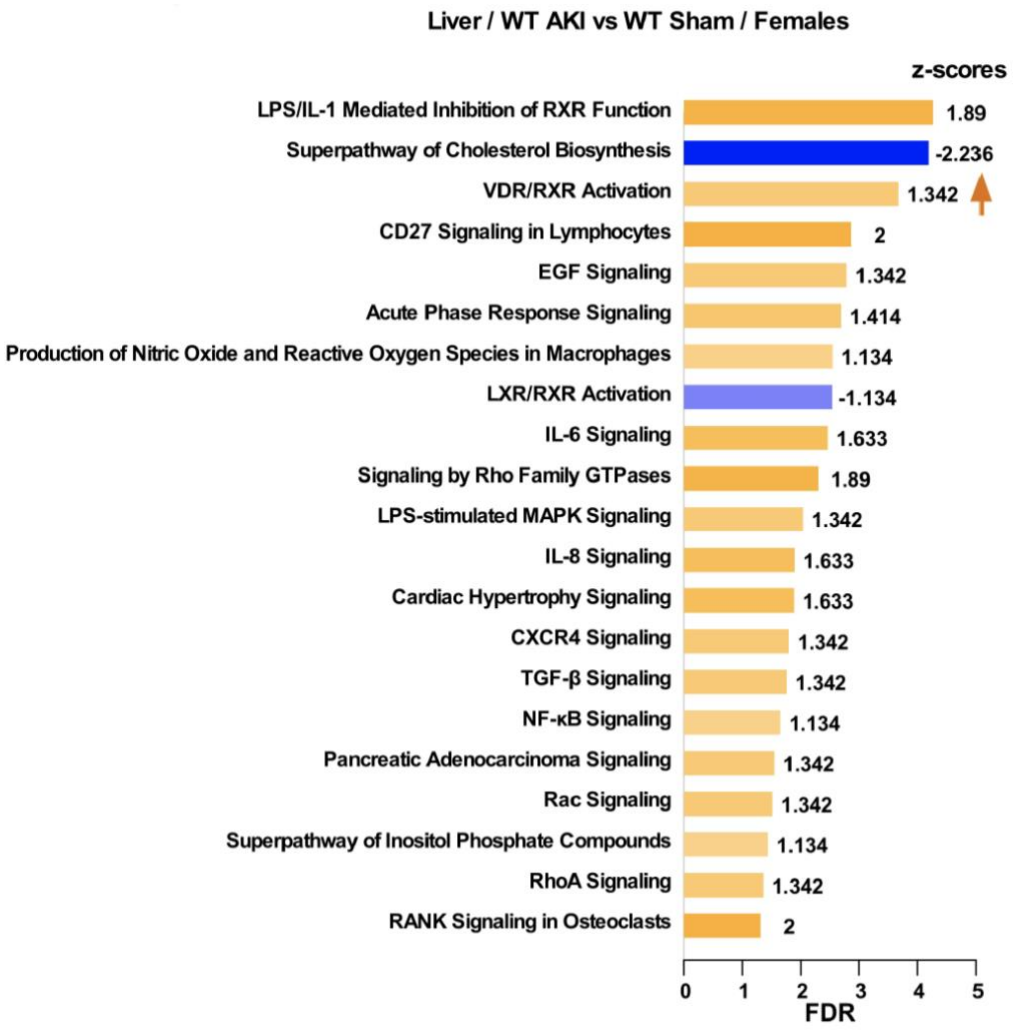

Figure 18: Ingenuity pathway analysis (IPA) of microarray results in female mice.

Shown are z-scores and false discovery rate (FDR) of female liver (WT AKI vs WT Sham). Several up-regulated (orange arrows) and down-regulated (blue arrows) pathways are highlighted.

In summary, we have uncovered a tissue- and sex-specific role of *Sult1e1* in kidney ischemic AKI. The hepatic expression of *Sult1e1* is required for an animal's sensitivity to ischemic AKI in males. Pharmacological inhibition of *Sult1e1* may represent a novel approach for the clinical management of AKI.

4.0 Summary

4.1 Final considerations

SULT1E1 has long been appreciated as a phase II metabolizing enzyme whose primary function is to sulfonate and deactivate estrogens, so *SULT1E1* is implicated in the metabolism of estrogenic drugs, including drugs used in oral contraceptives and hormone replacement therapy (HRT).

In several estrogen-dependent carcinomas, cancer cells activate mechanisms to decrease the expression of the deactivating enzyme *SULT1E1* and increase the expression of the re-activating enzyme, providing a mechanism for the initiation and progression of estrogen-dependent cancers. Furthermore, patients with polymorphisms that lead to enzyme inactivation have a correlation with a worse prognosis.

However, the physiological function of *SULT1E1* remains to be fully elucidated yet, as several studies have suggested both estrogen-dependent and -independent roles of this enzyme in physiology and pathophysiology. Early studies based on the use of *Sult1e1* null mice suggested a role of *Sult1e1* in reproduction. In the past 10 years, results from the Xie laboratory and other groups have pointed to the functions of this enzyme beyond reproduction. The expression of *Sult1e1* can be regulated by nuclear receptors and diseases. *Sult1e1* is implicated in adipogenesis and in several mouse models of inflammation-driven conditions such as sepsis, diabetes mellitus, cystic fibrosis, and ischemia-induced injuries, like ischemic AKI.

AKI therapy solely involves the management of its complications or the prevention of AKI causative factors. This dissertation shows the important role of *Sult1e1* in the development of AKI

in mice. The high incidence of this disorder worldwide calls our attention to the necessity of a strict therapy that may reverse the injured kidney state or prevent it from reaching more serious stages. The most successful strategies currently used in the clinic for patients that develop late stages are RRT, and the last resort is kidney transplantation. However, these strategies are associated with risks, high cost, organ availability, and lack of any long-term benefits.

4.2 Dissertation highlights

This dissertation features the potential use of Sult1e1 inhibitors, such as triclosan, as an AKI preventive agent in mice. Since the 1960s, triclosan has been widely used topically as a broad-spectrum antimicrobial agent found in a myriad of household products, such as toothpaste, soaps, deodorants, and lotions [106-109]. This molecule is also known for its anti-inflammatory potency, as it is believed it inhibits prostaglandin formation [173, 174]. The safety of oral administration of triclosan has also been evaluated previously [106]. The lethal dose (LD50) of oral triclosan in adult mice, dogs, and rats varies from 3,750mg/kg to more than 5,000mg/kg [175]. Although the intravenous toxicity can be seen in doses above 30mg/kg, regarding intraperitoneal injection this lethality is only reached in doses approximate to 1,090 mg/kg [176]. Moreover, male and female *Sprague Dawley* rats fed with 1000 parts per million (ppm) triclosan for a period of two years did not present liver toxicity nor any type of organ damage. This treatment did not affect reproduction and offspring either. Finally, human safety studies demonstrated that people exposed to toothpaste or mouth rinses containing 0.06% to 0.6% of triclosan within a period of 12 weeks had no adverse effects [175]. In our study, male and female C57bl/6 mice were treated with intraperitoneal injections of triclosan, with doses ranging from 10mg/kg to 50mg/kg daily for a total of three

consecutive days prior to bilateral kidney ischemia-reperfusion. This dose regimen was sufficient to protect mice from AKI and was greatly below the maximum tolerated dose in this species.

Ablation or Inhibition of Sult1e1 protected mice from ischemic AKI. During its initial phase, IRI stimulates endothelial cell disruption and the release of proinflammatory mediators from endothelium and tubule cells, such as IL-6. Our results demonstrate that protected mice presented low levels of IL-6. This could be due to increased resistance to IRI and consequently decreased endothelial damage. Another possible explanation is that Sult1e1 played a direct impact on controlling the release of IL-6. Xu et al (2013) demonstrated that HUVEC cells treated with SULT1E1 siRNA had downregulation of IL6 expression. Those authors concluded that SULT1E1 knock-down in these endothelial cells suppressed inflammation and lipid metabolism via PPARγ expression, in an estrogen-dependent and -independent manner [177]. Similarly, we demonstrated that the protection conferred to our Sult1e1 KO and Triclosan-treated WT was estrogen-independent. However, no meaningful changes were found in PPARγ regulation in our study, suggesting that loss of SULT1E1 may suppress inflammation through different mechanisms.

The species-specificity of the *SULT1E1* functions is very challenging. It remains to be determined whether many of the disease effects on *Sult1e1*, including AKI, can be recapitulated in humans, an area that warrants more studies.

4.3 Future directions

This dissertation demonstrates that augmented *Sult1e1* possibly decreases vitamin D signaling and consequently minimizes the repairing mechanism at the tubule cells. Our gene profiling analysis suggested that increased vitamin D signaling in *Sult1e1* KO may have

contributed to the kidney protective effect. Administration of calcitriol has previously shown to improve several animal models of AKI [164, 165], and calcitriol is currently in clinical trials for the treatment of AKI [166]. Furthermore, calcitriol and VDR signaling have been reported to promote cell proliferation, especially in cancerous and damaged cells [168]. As such, increased VDR signaling may have promoted cellular recovery and tissue repair after AKI, a notion supported by our observations of increased *Ki67* and Cyclin D1 expression in AKI *Sult1e1* KO mice. Calcitriol is also known for its anti-inflammatory activity [169], which could also have contributed to kidney protection.

There are still many gaps in this study. The real substrate that mediates AKI has not been found yet. Also, since we showed calcitriol is not a *SULT1E1* substrate, the mechanism by which *Sult1e1* ablation or inhibition increases VDR signaling remains to be understood. It's also possible that other vitamin D metabolites or moieties, such as the calcitriol precursor, namely 25(OH)-cholecalciferol (calcidiol) –which is activated in the liver by *Cyp2r1* and has a longer half-life–, may be mediating this protective effect. Moreover, we recognize that both VDR activation and cyclin D1 induction in AKI *Sult1e1* KO mice were associations. Future studies are necessary to determine whether these two events are indeed required for the AKI protective effect of *Sult1e1* ablation. VDR KO mice undergoing kidney ischemia-reperfusion could be used as a model to analyze the association of this receptor with cyclin D1 activity in the context of AKI.

It is paramount to investigate molecules in the ATM signaling pathway that are vitamin D responsive. RNA-sequencing, which is more robust and sensitive than microarray, could be used to analyze genes regulated in the livers of *Sult1e1* KO mice, in comparison to results obtained from their kidneys, as the mediator is probably being produced in the liver and migrating to the kidneys.

The bidirectional relationship between *Sult1e1* expression and AKI must also be addressed. Hepatic *Sult1e1* is induced in *db/db* mice in comparison to control, suggesting diabetes, hyperlipidemia, and obesity regulate *SULT1E1* expression [129]. In human vascular smooth muscle cells, incubation with IL-1ß leads to an upregulation of SULT1E1. Moreover, severe atherosclerotic aorta samples from women had enhanced SULT1E1 expression in comparison to mild atherosclerotic samples [178]. Although *IL-1ß* was significantly increased in our model at an early time-point, the acute phase mediator *IL-6* was the only pro-inflammatory molecule that increased in both liver and kidney at the 24h time-point. Hence, *IL6* is likely responsible for inducing *Sult1e1* in the kidneys and livers – likely in both Kupffer cells and hepatocytes – of AKI mice. However, it is still not clear by what mechanism *IL6*, or other inflammatory molecules, stimulate *Sult1e1* expression in the liver, kidneys, and possibly other organs. Activation of *IL6*-related pathways, such as the signal transducer and activator of transcription 3 (*STAT3)*, should be investigated. Moreover, ROS can also regulate *SULT1E1* expression, as they may either induce the expression of this enzyme via *Nrf2* [179] or inactivate it via oxidized glutathione [180]. Trans-signaling *IL6* has also shown to work synergistically with ROS in AKI [181]. Therefore, a ROS inhibitor and a *STAT3* inhibitor could be administered in different groups of mice prior to IRI to observe the real inflammatory pathway responsible for AKI induction.

Likewise, the mechanism by which liver *Sult1e1* is crucial for AKI development in males, and why this cannot be observed in females, should also be addressed. Loss of liver *Sult1e1* resulted in a low inflammatory response in males whereas, in females, other organs may have contributed to this effect.

Intraperitoneal administration of triclosan daily for three consecutive days was sufficient to protect mice from AKI, and although the compound is overall safe, limitations to its use can

also be encountered. Triclosan may deplete the placenta's ability to provide estrogen to the fetus, culminating with poor fetal growth and development [58] and also spontaneous abortion [182]. Furthermore, Triclosan has been detected in human blood, urine, and breast milk [110, 111]. People who accidentally ingested 4 mg of Triclosan presented 22 to 47% of the unconjugated molecule in plasma. In pubertal female Wistar rats, this agent resulted in a premature vaginal opening, whereas in weaning mice triclosan changed the degree of reproduction development and increased uterine response to EE [113]. Local triclosan administration has been previously suggested to treat infections and prevent systemic absorption [183]. Similarly, in AKI, local triclosan administration would be needed to prevent concerning effects in other organs, as *SULT1E1* presents tissue-specific effects.

This work also demonstrates the ischemic AKI protection effect conferred by *Sult1e1* ablation is male and female hormone-independent. However, since gonadectomy was not evaluated in WT and KOLE mice that underwent IRI, this may be an overstatement. Other experiments are needed to thoroughly investigate the hormone-independence and hormonal feedback response. It's important to consider that males and females present genetic differences that may have also contributed to our results. Additionally, membrane-bound estrogen receptors may act through genetic-independent intracellular pathways. Treatment of a human endothelial cell line with E2 culminated with nitric oxide stimulation via PI3-kinase activation of the Akt pathway in a gene transactivation-independent ER signaling [184]. Finally, aromatase is also known to be expressed in other organs, such as the brain [185], suggesting it is still possible that estrogens are mediating the protective effect in *Sult1e1* KO and triclosan-treated WT mice. It's paramount to include AKI mice groups treated with a systemic aromatase inhibitor and an estrogen receptor antagonist in our analysis.

Sex-differences were observed in the results obtained from KOLE mice. Immunostaining of these samples suggested a sex-difference in *Sult1e1* expression upon IRI. This may have contributed to a lack of AKI effect in female KOLE mice. But it is still not clear why a greatly induced *Sult1e1* expression in livers of female KOLE had no further phenotype effect, hence, no increased kidney injury, whereas a not so dramatic increase in livers of female WT was sufficient to establish AKI. Although these results in KOLE mice suggest that an enzyme saturation is likely occurring, further studies are paramount to understand this effect. Additionally, bioinformatics comparisons between male and female kidney-liver crosstalk will also be important to clarify what molecule is directly mediating the harmful effect of ischemia and culminating with AKI. Finally, because these studies are based in a mouse-model of AKI, evaluation of *SULT1E1* expression in livers and kidneys from deceased humans and correlation with any degree of kidney damage will provide crucial information regarding the importance of this enzyme in human beings and may uncover novel therapeutic targets that will benefit millions of those afflicted by kidney disorders.

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