Our ***long-term goal*** is to understand the basic underlying molecular and biochemical mechanism of preservation injury in the kidney and to use this information to preserve kidneys better for clinical transplantation to expand the donor pool. The ***main objective*** of this proposal is to determine if ezrin, a sub-lamellar cytoskeletal protein, is involved in hypothermic preservation injury of kidneys and to explore the possible and likely mechanisms. Our ***Central Hypothesis*** is that ***Ezrin failure during cold ischemia causally contributes to renal preservation injury by membrane and mitochondrial mechanisms.*** This hypothesis will be tested further by addressing these specific aims:

1. To determine the preservation associated factors that cause ezrin failure (cytoskeletal dis-association) HYPOTHESIS: Protease activity and reduced site specific kinase activity significantly contribute to ezrin disassociation (ezrin failure) after cold storage and reperfusion. A proven cell model of preservation injury in the porcine renal epithelial cell line LLC-PK1 will be used. We will use various pharmacologic and molecular inhibitor strategies to test a causal role for proteases and site-specific ezrin phosphorylation pathways in ezrin failure. The ezrin specific activating pathways will include Rho Kinase and PKCí, which both promote the binding configuration of ezrin by site specific phosphorylation (T567). Treated groups of cells will be compared to appropriate control groups to determine the involvement of these pathways in ezrin failure by monitoring structural and biochemical outcomes that reflect both preservation injury and ezrin failure. We anticipate that inhibiting these pathways will reduce ezrin failure and improve preservation outcomes.

2. To determine the possible mechanisms of ezrin in cold ischemia preservation injury HYPOTHESIS: Ezrin failure contributes to the phenotype of preservation injury by membrane and mitochondrial mechanisms. We will first assess ezrin's membrane linker role in preservation injury by using our LLC-EZ mutant cell lines expressing site specific mutations of ezrin that activate (T567D) or suppress (T567A) linker function. Wild type cells serve as controls in our cell preservation model that uses metabolic, physiological, structural, and biochemical read outs. We predict that preservation injury will be attenuated with the "active" mutant and potentiated with the "inactive" mutant. This would support a membrane mechanism for ezrin. Next, we will determine ezrin specific binding to various mitochondrial fractions using antibody, histological, and molecular techniques to determine if ezrin binds to components of the mPTP. Finally, we will determine if ezrin modulates the function of the mPTP using recombinant ezrin proteins and ezrin mutant cell lines. We expect to detect binding of ezrin to one or more components of the mPTP including VDAC and ANT and this interaction may inhibit pore opening to reduce preservation injury. Ezrin may be in an "inactive" molecular configuration during mitochondrial binding since it probably migrates from the sub-lamellar sites during ischemia in that configuration.

3. To determine the role of ezrin failure in clinically relevant models of renal preservation injury. HYPOTHESIS: Expression of active or inactive ezrin mutants in genetically engineered knock-in mice modulates renal preservation injury by facilitating or blocking the membrane binding configuration of ezrin. We have created knock-in mice that express either T567A (inactive) or T567D (active) mutations of the ezrin protein to test this hypothesis in renal tubule or whole kidney preservation studies. One objective is to extend the cell observations to more complex integrated and translational systems like intact nephron segments and whole kidney transplants. Another objective is to further test the membrane linker hypothesis in the whole kidney during preservation. To that end, we will prepare proximal convoluted renal tubules from ezrin wild type and ezrin mutant mouse kidneys and study preservation injury in our traditional tubule models to determine molecular configurations of ezrin that protect the tubules from preservation injury. We will then extend these studies to mouse kidney IPK and transplant models to more clearly assess translation and clinical relevancy of our earlier findings in cell cultures. At the end of these studies, we will demonstrate that ezrin is causally involved in renal preservation injury in cell, renal tubule, and kidney transplant models. We also will have narrowed the possible mechanisms of how this occurs and extended the effect to translational models with clinical relevancy. Cytoskeletal proteins, like ezrin, are not just passive structural proteins that degrade as terminal epiphenomenon of reperfusion injury but are multifunctional molecules whose early derangement in ischemia causally contributes to the injury, probably through multiple and diverse mechanisms. Some of these mechanisms will be explored in this proposal and some novel innovative therapeutic options will be developed.