Characterizing Murine Compensatory Renal Growth Following Unilateral Nephrectomy

by

Shulamit Ahuva Diena

A thesis submitted in conformity with the requirements
for the degree of Master of Science

Department of Molecular Genetics
University of Toronto

Characterizing Murine Compensatory Renal Growth Following Unilateral Nephrectomy

Shulamit Ahuva Diena

Master of Science

Department of Molecular Genetics
University of Toronto

2021

Abstract

Following the surgical removal of a single kidney, the remaining kidney shows a striking ability to compensate for this loss by rapidly initiating an acute growth program, coupled with increased function. The organ size change halts once normal function is restored, but the signals regulating this change in kidney size remain unknown. This work describes murine compensatory renal growth on the level of organ size, function, and cellular changes up to 28 days post-surgery. The data suggests that the extent of remnant kidney growth may be related to the ratio of kidney weight-to-body weight prior to surgery, potentially hinting at a predictive factor of postoperative renal mass recovery and implicating a size sensing mechanism in this process. We also developed an image processing pipeline to analyze kidney cross-sections, and identified trends in nuclear size and density changes, suggesting the contribution of both hypertrophy and hyperplasia to compensatory renal growth.

Acknowledgments

I would like to thank my supervisor Dr. Ran Kafri for his support, encouragement, and guidance throughout this project, and since my first day at the Kafri lab. Thank you for inspiring me to ask bigger questions and never lose sight of the curiosity and mystery that drives scientific inquiry. Many thanks to Dr. Mathieu Lemaire for your critical role in collaboration on this project, for driving me to think about scientific questions from all angles (particularly the ones I never considered), and for your valuable feedback on this work. Thank you to my committee members, Drs. Jim Dennis and Janet Rossant for your advice and the new viewpoints you have brought to this project.

Endless thank yous to the past and present members of both the Kafri and Lemaire labs for your help and support in every possible area. You have provided technical assistance, proofreading, coding, teaching, and stepping in whenever I needed an extra pair of hands, but thank you even more so for your patience, support, encouragement, and friendship. I am so grateful to have met you and to have worked with you all. I would specifically like to thank Dr. Nish Patel for his guidance from the day I started at the Kafri lab. I could not have done this work without his incredible training and patience. Additional thank you to Ceryl Tan for taking me in as a fledgling undergrad and for help in every single area of science, particularly my endless MATLAB questions. Thank you to the staff at LAS for all your help and patience with the animal work, and most importantly to the expert Dr. Jing Wu for showing me the ropes.

Finally, this would not have been possible without the tremendous support of my family and friends. Thank you for putting up with the late nights, early mornings, busy days, and all the talk about science. Thank you for listening to presentations, quelling anxieties, and celebrating statistical significance successes. Thank you for being the biggest cheerleaders out there and for encouraging me to continue pursuing science, even when it was challenging or tedious. It takes a village to raise a scientist, and thus this work is in part yours as well.

This work was supported by a SickKids Research Training Competition (RESTRACOMP) Graduate Scholarship.

Table of Contents

List of Figures

List of Appendices

Abbreviations and Acronyms

CRG	Compensatory renal growth
CRH	Compensatory renal hypertrophy
DT	Distal tubule
GFR	Glomerular filtration rate
mTORC1	Mammalian/mechanistic target of Rapamycin complex 1
Post-op	Post-operative
Pre-op	Pre-operative
PT	Proximal tubule
RRC	Renal reserve capacity
sCr	Serum creatinine
SFK	Solitary functioning kidney
UNx	Unilateral nephrectomy

Chapter 1
Introduction

1 Introduction

1.1. Background-Compensatory Renal Hypertrophy

1.1.1 Renal plasticity

The relationship between physiological input and organ output is critical to proper organismal function, as it ensures homeostasis and appropriate function in the face of changing conditions. To facilitate this feedback, complex molecular mechanisms and pathways act to relay cellular signals and regulate organ functions. In particular, the response of the kidney to environmental changes is extremely important to organismal health, as the kidney contributes to and maintains many homeostatic conditions in the body. It is critical for removal of toxins, waste clearance, and water balance, and is central in maintaining blood pressure, osmolarity, and pH. This ensures optimal hemodynamic conditions for nutrient and oxygen transport.[1] Strikingly, in response to increased demands, not only does the kidney upregulate its function, but it has been established for over a century that this upregulation is often coupled with increases in organ size, known as compensatory renal growth (CRG).

Acute changes in renal mass, typically due to the removal of a kidney (unilateral nephrectomy, UNx) trigger a 20-35% increase in remnant kidney volume that is responsible for rapid restoration to sufficient kidney function.[2–6] It is important to note that there is significant variability in the compensatory response noted in clinical studies. Conversely, when adult kidneys are transplanted to pediatric patients, they show a volume reduction of up to 26% over several months to accommodate the recipients' bodies.[7,8] In addition to growth of renal tissue, an important corollary of CRG is improved kidney function of the remnant kidney. with accompanying changes in kidney function (typically identified by measuring glomerular filtration rate, or GFR). It is unlikely that a single kidney would reach 200% of its filtration capacity, as this would be energetically costly as well as difficult to maintain without incurring damage.[9] However, the human remnant kidney has

been shown to increase its function to 60-90% of its pre-operative levels[4–6] which is enough to maintain healthy waste removal and hemodynamic balance.

Surgical reduction of mass is not the only process that can induce dramatic changes in kidney size or function. It is well documented that the kidney has remarkable plasticity, particularly during pregnancy and in response to high protein diet. Renal plasma flow can increase up to 80% and GFR up to 50% during pregnancy, which contribute to increases of 1-1.5 cm in length and up to 30% in renal volume during pregnancy. This enlargement generally restores back to normal size in the first week to several months postpartum.[10–12] Individuals with a single kidney still show approximately 25-30% increases in GFR during pregnancy--despite their already elevated single-kidney function brought on by prior nephrectomy or transplant.[13]Animal research suggests that the capacity for renal hypertrophy is very extensive: combining both UNx and repeated pregnancies does not exhaust the hypertrophic response.[14] Similarly, high protein diets alone can increase kidney size and renal function, and in conjunction with UNx or further reduction to renal mass, can induce even greater growth in the compensatory response.[15–17] These examples may be seen as more "natural" stimuli which demonstrate the tight link between renal function and size.

1.1.2 Renal function

While the coupling of size and function seems intuitive, the physiological architecture of the kidney renders major hypertrophic growth challenging. One must account for the diversity in cell types that create the complex structures in the kidney and enable their functions. To appreciate this complexity, one can consider the flow of blood through each of the several hundred thousand to one million nephrons in each kidney (**figure 1**).[18] These nephrons are the central filtration pipeline: a complex network of blood vessels surround tubule tissue, where solutes and water are moved back and forth between blood and the waste fluid known as filtrate. This process uses both active and passive transport depending on the region and the substance(s) moving between the fluids. It involves complex fluid dynamics, and both osmotic pressure and chemical gradients. As these liquids move through their respective pathways in the nephron, the filtrate becomes concentrated and the tubules converge into larger structures to allow the concentrated filtrate (urine) to be excreted via the bladder, while necessary liquid and solutes return to the blood via the vasculature.[1]

Briefly, blood from the body enters the afferent arteriole, which leads into a structure known as the Bowman's capsule. As the blood moves through the glomerulus, water, and solutes that are

smaller than 60-70kDa[19] are transferred from the blood into the Bowman's capsule largely indiscriminately, generating the first filtrate. The blood exits the glomerulus via the efferent arteriole, which branches out into a mesh-like network wrapping around the tubules, known as the peritubular capillaries. The Bowman's capsule opens into the segment known as the proximal tubule (PT), where reabsorption of these large molecules back into the blood can begin in a controlled manner. This reabsorption combats the blind filtration in the glomerulus. At the PT, nearly 2/3 of the previously filtered fluid is reabsorbed back into the blood.[20] In addition to water, larger molecules such as glucose and amino acids are returned to the blood, and ions including sodium, calcium, potassium, and bicarbonate are exchanged.[21,22] As the filtrate moves to the next segment, the descending loop of Henle, water is reabsorbed[23], while sodium, chloride, and potassium are resorbed in the ascending loop. This complex absorption profile requires the cooperation of molecular components such as transport proteins, in addition to biophysical alterations which produce suitable changes in pressure and osmolarity for these exchanges to take place. Additional ion exchange takes place in the next segment, the distal convoluted tubule, along with pH and calcium regulation.[1] At this point, the filtrate composition is close to ideal, and the few final ion and water adjustments take place as it moves through the collecting duct system, and eventually into the bladder and out of the body.

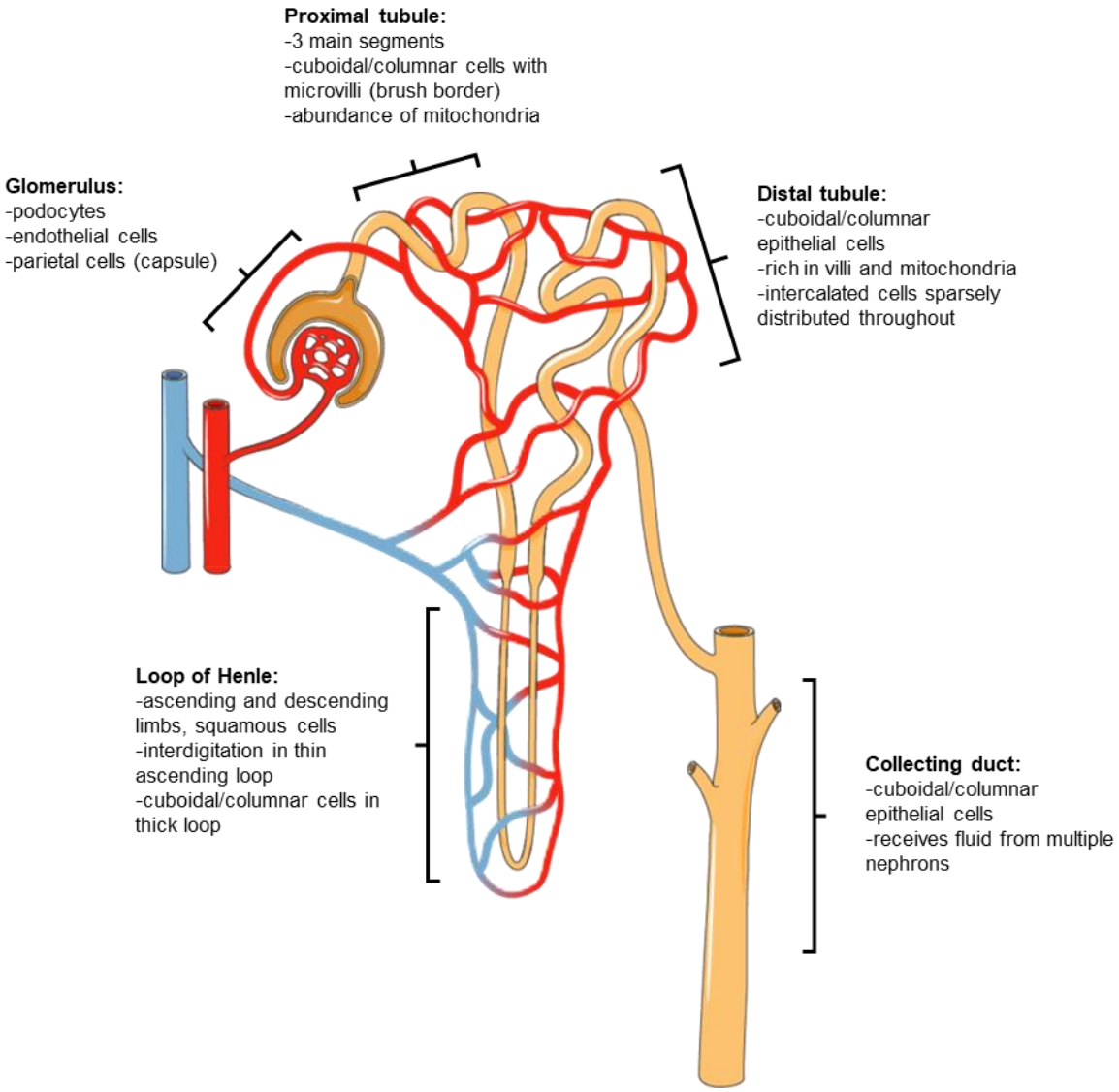

Figure 1. General overview of the nephron and its segments. The kidney is comprised of a complex system of tubules and vasculature divided into functional segments. Each segment is made up of different cell types, which contribute to its intricate architecture and highly specified function. Image modified from Servier Medical Art.[1,24]

1.1.3 Hyperplastic growth in CRH

Many animal studies have explored compensatory growth following UNx and determined that there are typically large increases in the protein-to-DNA ratio without concomitant increases in DNA or mitotic activity. [25–28] On that basis, cell hypertrophy-rather than hyperplasia-was suggested as the primary mechanism of growth, but few studies have quantified the contributions of these two patterns of growth directly. One approach to do so is to measure the incorporation radiolabelled thymidine in newly synthesized DNA. By applying this method to adult male mice subjected to UNx, Johnson and Roman showed that hyperplasia is responsible for ~25% of the size increase, and that this activity peaks at 48 hours post-UNx.[25] While 25% is a significant fraction of growth, the process of renal compensatory growth is typically referred to as compensatory renal hypertrophy, or CRH because of the larger contribution from cell hypertrophy. In contrast to the 75% hypertrophy-25% hyperplasia finding, more recent experiments using incorporation of thymidine analog BrdU suggest no contribution of hyperplasia in the adult male C57Bl/6 mouse at 4 and 7 days post-UNx, and a potentially small hyperplastic contribution in the adult male Sprague-Dawley rat. Their experiments showed that the proliferation in rats correlates with increases in CDK2-cyclin E, suggesting that these cells may proceed into the synthesis phase of the cell cycle.[26] However, these authors did not examine proliferation at 48 hours post-UNx as the previous study did, and thus may have missed this window. Further work by Azurmendi *et al*. in Wistar rats showed a 36% increase in cell number at two months post-UNx.[29] This underscores that there may be a species difference in the compensatory response with proliferation in rats but not in mice. It also begins to hint and the complex variability in the literature and findings on this subject.

1.1.4 Hypertrophic mechanisms of CRH

The notion that the contribution of proliferation to the compensatory response is lesser than hypertrophic growth has been reinforced through findings in later studies exploring mechanistic elements and regulators of CRH. A central hypothesis put forth by Liu and Preisig is that hypertrophy results from the activation of a mitotic signal which is terminated or overridden before the cell can enter the S phase of the cell cycle. As such, the cell grows in the G1 phase of the cell cycle but does not divide, remaining “stuck” in an oversized state. This cell cycle-dependent mechanism is demonstrated in adult male C57Bl/6 mice by increased levels of CDK4-cyclin D

following UNx, a complex active in early G1 and necessary for cell cycle progression. However, it is accompanied by decreases in CDK2-cyclin E, which is expressed later in G1 and required to proceed past the restriction point and into S phase, thus precluding entry into the rest of the cell cycle and the process of cell division.[26,30]

One of the key regulators of cell size and growth is the mammalian/mechanistic target of Rapamycin, or mTOR pathway, notably complex 1--the mTORC1 component. A highly conserved pathway, mTOR has roles in a host of cellular processes including growth, division, metabolism, and nutrient sensing, among others.[31] mTOR's control of growth is achieved through two arms: firstly, when phosphorylated on multiple residues by mTORC1, the eukaryotic translation initiation factor (eIF) 4E-binding protein 1 (4E-BP1) releases the eIF4E factor. This factor is then free to enable cap-dependent translation. In the second arm, mTORC1's activation of the S6 kinases1/2 (p70 S6K or S6K) allows S6K to phosphorylate ribosomal protein s6 (rpS6). In turn, rpS6 recruits the 40S ribosomal subunit for translation of the 5'-terminal oligopyrimidine tract (5'TOP) mRNA, a class of mRNA which encodes components of the translational machinery.[32–34] In line with this critical growth role, UNx activates upregulation of both the S6K and 4E-BP1 arms in the remaining kidney. UNx under treatment with mTORC1 inhibitor Rapamycin decreases the pathway's response and shifts the polysome profile towards monosomes in adult male DBA/2 mice.[32] Further experiments cemented the role of mTORC1 and the S6K arm in CRH by showing that deletion of S6K1 dampens the compensatory renal response in adult C57Bl/6 male mice by nearly 70%, and adding treatment with Rapamycin does not reduce this further.[35] Consistent with previous studies relating to cell cycle, these experiments also showed that UNx is followed by increases in cyclin D1 and decreases in cyclin E1. This cell cycle regulatory response is slightly dampened in FVB/NJ background-mice with non-phosphorylatable rpS6.[36] However, these studies do not explore the S6K-independent growth, which may correspond to an mTORC1-independent arm, or potentially to the ~25% contribution of cell division to CRH identified by Johnson and Roman.[25]

1.1.5 Age- and sex-based differences in CRH

It is well established that sex hormones play critical roles in modulating or modifying biological processes, and these sexual dimorphisms are often overlooked given studies of only one sex. Interestingly, sex differences in renal biology are quite evident, with lower rates of end-stage renal

disease in women, and greater susceptibility of males to kidney injury in animal models, with some research indicating that female sex hormones may have a renoprotective effect.[37] If sexual dimorphisms are mediated by gonadal steroids, they may also present as differences between immature and mature animals. This is indeed the case in CRH. In juvenile rats, there is a significant contribution of hyperplasia to CRH, beginning at 18 hours post-UNx and abating by 72 hours post-UNx, evident by increased ^{3}H-thymidine/BrdU staining in the cortical tubules and overall greater DNA content. These changes are associated with increased mRNA levels of immediate-early (pro-proliferative) genes c-fos and c-jun in the male weanling Wistar rat.[38] Juvenile female rats also show significant hyperplasia at 24 hours post-UNx, as do adult female Wistar rats to a lesser degree. In contrast, the hyperplastic response is absent in adult male rats at this time point. There is evidence of slight proliferation in adult males beginning later, at 72 hours post-surgery, but the details of this have not been thoroughly dissected.[38,39]

Further studies in adult Wistar rats determined that male animals show significantly more compensatory growth. For example, at 2 months post-UNx, remnant kidney weight in males increased by 114$\pm$7% whereas in females female kidney weight increased by only 57$\pm$4%. Interestingly, CRH in males was accompanied by morphological hallmarks of pathology.[40] In another study of UNx in the same strain, after 2 months, remnant kidney weight had increased in males by 50%, but only by 15% in females.[29] Similarly, at two months post-UNx, females and castrated male Wistar rats show glomerular volumes that were 29% and 26% respectively smaller than nephrectomized intact (non-castrated) males.[41] These studies suggest that testosterone may be the stimulus which shifts the compensatory response to predominantly hypertrophic response, but also may accelerate the pathologies associated with CRH such as tubular damage. Castration inhibits the increases in glomerular volume in nephrectomized male rats, but interestingly does not inhibit the increases in glomerular volume associated with normal aging in male rats (absent in female rats). This suggests that male sex hormones may play different roles in nephrectomy versus aging.[41,42] This is further underscored by the fact that ovariectomized adult female rats supplemented with testosterone showed an increased CRH response compared to controls, but those supplemented with estrogen or a cholesterol vehicle did not. [40] Despite the exploration of this gender disparate phenomenon in rats in earlier years, few such studies have been conducted in mice, and contemporary groups elucidating mechanistic details still focus largely on male mice due to their more robust response to UNx. As such, many of the sex differences in this phenomenon

remain undefined, and there is a lack of longitudinal studies which explore changes at further time points after surgery.

1.2 Cellular growth and cell size heterogeneity

The last century of studies on CRH has provided a plethora of biochemical details and proposed mechanisms for CRH. Many molecules have been suggested as modulators of the compensatory response, including tight junction protein zona-occludens 2, growth hormone, IGF-1, angiotensin, TGF-beta, and a yet-identified "renotrophic factor".[40,43–47] A recent series of studies from JK Chen and colleagues has found significant evidence for the role of the S6 kinase arm of the mTORC1 pathway, as well as role of the class III PI3 kinase in modulating the degree of hypertrophy. They suggest that increased amino acid influx into the remaining kidney due to increased blood flow triggers compensatory hypertrophy through PLD-1 and phosphatidic acid-mediated recruitment and activation of mTORC1.[27,28,48–50] However, whether amino acids are the specific initiator of the response, rather than growth being a by-product of this physiological change (increased blood flow and therefore an increased amino acid influx) is unclear. A definitive understanding of how this response is truly initiated and terminated has yet to emerge, as cellular growth requires tightly regulated feedback circuits to avoid inappropriate size changes.

These findings provide a critical backdrop for the question of cell size regulation, which affects systems far smaller and greater than the kidney. When measuring cell size in different cell types, it is evident that while sizes of different cell types vary greatly, the size variation within a particular cell type is remarkably narrow. It is well established that growth pathways such as mTOR are critical for cellular accumulation of biological mass, and numerous physiological signals have been characterized which promote or inhibit mTORC1 activity. However, it remains unknown how these pathways are set to specific size values or rates of growth. Quantitatively, *how is a given, common set of pathways programmed to specify a particular cell size for different organs, tissues, and cell types?* In the case of CRH, what triggers the activation of this response pathway or causes it to terminate? How are broad signals integrated in a cell-specific manner to maintain this size homogeneity?

Work from our lab suggests a model in which cultured mammalian cells can set and maintain a fixed and physiologically relevant size, or "target size", and can sense and correct their size when

it deviates from this goal. This maintains a population of similarly-sized cells with minimal size variance. Using time lapse microscopy, we have shown that cultured mammalian cells maintain a very narrow range of sizes when they transition from the G1 to S phase of the cell cycle, despite the heterogeneity of sizes at birth. This relationship between size and cell cycle progression also manifests as a negative correlation between cell size and G1 proportion, suggesting that cells which are "born" at smaller sizes spend a longer time in the G1 (growth) phase of the cell cycle, where they can grow until they have reached an appropriate size to proceed to division, and vice versa (**figure 2a**).[51,52] To investigate cell size homeostasis, we previously performed a large-scale drug screen to identify candidates which are involved in the coordination of G1 length and cell size.[51] Analysis of these results and the compounds' effects on this coordinated generated a categorial distinction of 'sensor' mechanisms and 'dial' mechanisms. 'Sensors' are defined as genes or molecules that are selectively activated in cells that are larger or smaller than their target size. By contrast, we define 'dials' as cellular mechanisms which function to determine a cell's appropriate target size value (**figure 2a, 2b**). Inhibition of sensor genes/proteins results in an increase in size variance in the population, as the cells have lost their ability to identify that they have diverged from the appropriate size, and a disruption of the coordination between G1 length and cell size (**figure 2b**). Conversely, inhibition of a dial maintains a population with low size heterogeneity, but overall, the cells are a different size than they would typically be. Their target size appears to have been changed, so while the relationship between G1 proportion and cell size is largely intact (suggesting that cells can still sense and control their size), their target size has been "dialled" up or down to a different value (**figure 2**).

As in the case of single cells, the process of kidney hypertrophy presents questions not only as to the mechanisms that drive cellular growth, but also to how the extent of cellular growth is regulated to meet functional demands. How is the growth of single cells coordinately regulated to maintain kidney architecture during the course of hypertrophy? Is kidney hypertrophy regulated by 'dial' mechanisms that determine the appropriate extent of hypertrophy and 'sensor'; mechanisms that ensure that cells have grown to the appropriate amount? Where do these changes take place, and how are they balanced with hyperplastic growth if it is indeed present? Given the structural and cellular complexity of the kidney, which is critical to for its function, it is tempting to speculate that a similar cell-autonomous signaling network regulates compensatory kidney growth in response to UNx. Such a system would ensure that kidney structure and cellular integrity are

maintained both during and after the growth process. This work aims to establish the UNx model system to begin to explore the phenomenon of cell size heterogeneity, sensing, and control on an organ-wide level. Cell and cell structure sizes have been shown to scale to each other, but how these increases in cell size correlate with wet weights following UNx has not been explored in detail. Current literature quantifying single structure or single cell data in this area is scarce. Most studies focus on smaller regions of the kidney or specific cell types, so global trends in size changes have yet to be established at a single tubule or cell resolution. In a model where even slight size changes can dramatically impact the 3-dimensional nature of renal structures, these morphological changes and variability in size will provide interesting insights into the nature of cell growth in the context of organ growth.

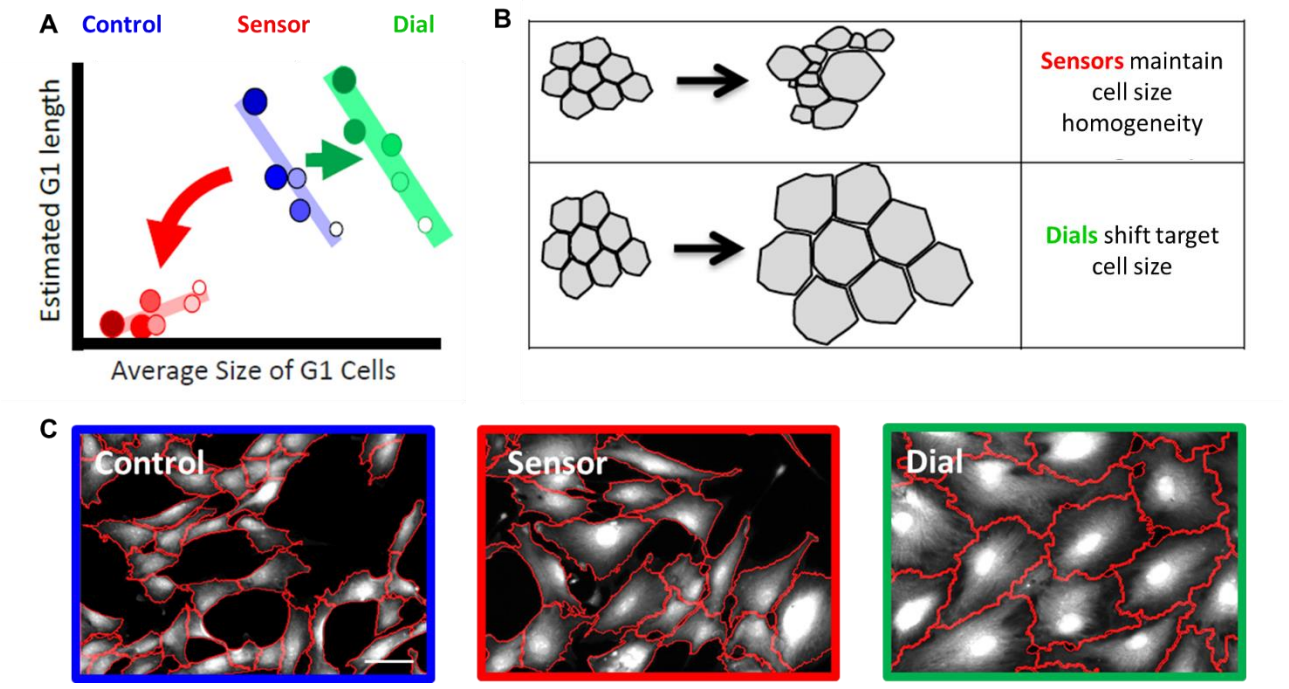

Figure 2. **Schematic of the negative correlation between G1 proportion as a proxy for G1 length, and average cell size.** A, B) Perturbations that disrupt the relationship between size and G1 length are characterized as "sensor" perturbations, and those that shifted the coordination to different values are characterized as "dials". Phenotypically, disrupting sensors result in a loss of cell size homogeneity, while perturbing dials result in homogeneously smaller or larger cell sizes across the population. C) Representative images of RPE1 (epithelial cells) correspond to different types of perturbations, and red lines delineate computationally segmented cell boundaries. Courtesy of and modified with permission from Ceryl Tan.

The primary regions which have received more coverage in the literature are the proximal tubules and the glomeruli: the proximal tubules make up over 40% of renal epithelial cells and greater than 65% of epithelial cell protein mass in the mouse, so they are often selected for as the focus of growth studies.[53] In adult male C57Bl/6 mice, Liu and Preisig showed increases of ~22% and ~30% in protein:DNA ratio of proximal tubule lysate at 4 and 7 days post-UNx, and Xu *et al*. showed a similar increase of just below 30% after 7 days in adult male FVB/NJ mice.[26,36] Glomerular area and volume are easily measured from tuft diameter, and similar to changes in kidney weight, studies in adult nephrectomized Wistar rats show larger increases in mean

glomerular volume (M_{GV}) in males than females. For example, at two months post-UNx, male M_{GV} increased by 126±13.4%, whereas in females it increased by only 20.2±16.1% (found to be not statistically significant).[40] Both glomerular and proximal tubular area of adult male FVB/NJ, C57Bl/6, and BALB/cByJ background mice have been shown to increase in UNx compared to sham operated animals, but the percentage increases have not been included.[28,36,48] A study in nephrectomized male Sprague-Dawley rats showed increases in the luminal and outer diameters of proximal (17% and 14% increase) and distal tubules (12% and 10% increase) compared to control animals. Combined with increases in tubule length measured by microdissection, these changes amount to significant size increases in renal structure and tubule surface area.[54] Overall, various approaches to quantifying changes in renal structures have been attempted over the years-particularly in rats-but a comprehensive picture is not yet complete.

1.3 Clinical and physiological relevance of cellular growth control

As demand for kidneys continues to be of the highest among organs, a large portion of donated organs comes from live donors, yet still the waiting lists are long.[55] There are several factors which rule out potential donors, primarily pre-existing medical conditions, high BMI, and age, because of their association with increased risk of poor outcome for the donors.[56] Clinically, a better understanding of CRH is critical to develop better outcome prediction for donor screening: to protect those who are at risk by donation, or enable others who may previously have been turned down from donating. Moving deeper, mechanistically CRH is a delicate balance—while a lack of renal growth puts an individual at risk for insufficient renal function, if this loss triggers selective hypertrophy and hyperfiltration it becomes pathological. Selective growth can cause the overuse of specific renal structures, leading to atrophy, and a chain reaction of further hypertrophy and atrophy of nephrons-ultimately to highly damaged kidneys.[9,57] If disproportionate growth due to hypertrophy of specific renal structures or cell populations is indeed the case, it is important to explore the growth pattern of these populations. This can allow for anticipation and prevention of pathological hypertrophy which may drive kidney failure, as well as studies of what other functional or morphological changes the kidney undergoes to avoid non-sustainable overwork.[58]

Identifying the structures or cell populations which are prone to growth enables a better understanding of the mechanism of CRH, and the ability to develop therapeutic interventions to modulate its extent. Such intervention could be used to upregulate CRH where it is insufficient, or

conversely, to prevent the pathologies which may accompany it. These interventions may also be suitable to those with pre-existing conditions which render their baseline kidney function lower. The generation of new nephrons in the human kidney ceases around the 36th week of pregnancy and in mice at postnatal day 3, after which point the kidneys grow by increasing their cell size.[59–61] In some animal species, individuals with conditions causing a congenital solitary functioning kidney (SFK) early on in development can compensate for this losses by developing more nephrons in this single kidney.[62] However, it is unclear whether renal mass is increased by nephrogenesis or hypertrophy in humans with a SFK[63], and if these two methods would contribute to different functional outcomes later in life.[64]

A final issue to consider is the class of mTOR inhibitors which are used as immunosuppressants such as sirolimus and everolimus.[65] Can kidney transplant recipients use such a class of drugs, or will this jeopardize the growth response that is seen in recipients of a single or smaller kidneys?[66] Can mTOR inhibitors as chemotherapeutic agents be used in conjunction with nephrectomy as treatment for renal cancers? More broadly, how do these classes of drugs affect the developing or standard kidney function? An understanding of the role of growth pathways and hyperplasia will continue to shed light on these basic biological questions and their clinical applications, and ultimately to better outcomes for the broad group of renal diseases.

1.4 Outstanding questions addressed by the MSc research

1.4.1 Trajectory of gross organ growth (renal mass) and function (creatinine)

While studies on CRH have been conducted for decades, they are often cross-sectional, focusing on a single timepoint after nephrectomy, typically not extending past 10 days post-surgery. Animals have a remarkable ability to heal quickly, but this short timeframe leaves unanswered the question of whether compensatory growth is completed within just a few days, or whether it continues for longer. This also limits our understanding of how kidney function changes throughout the growth process. When do these changes in size and function plateau, or return to normal? This work explores the process of CRH at multiple time points, ranging from 24 hours to 28 days to delineate the trajectory of organ growth and restoration of kidney function following UNx.

As in many scientific studies, previous work on CRH was largely conducted on male animals, with limited work on females or the potential sex discrepancies in this process. This work includes both male and female mice in order to explore how sex modulates this phenomenon, thereby providing a more complete picture of CRH.

1.4.2 Trajectory of size changes on a single cell level

Perhaps one of the most critical aspects of CRH is that it has been determined to be a primarily hypertrophic, as opposed to hyperplastic phenomenon. Multiple lines of evidence including protein:DNA ratio, and labelled nucleotide incorporation suggest that the bulk of growth following UNx happens due to increases in individual cell size, with evidence for a smaller hyperplastic component.[25,26,36] One of the potential biases of using this ratio is that when considering hyperplastic growth, the addition of new cells will add (relatively) more protein than DNA, contributing to an overall increase in protein:DNA levels. To determine whether there are changes in cell size by this method, quantification of the number of units of DNA would be required to rule out the contribution of hyperplasia or increased cell number. Much of the work on CRH uses global changes in protein levels from kidney homogenate as a proxy metric for changes in cell size. However, the limited direct quantification of cell or structure size changes is a notable gap in the field, especially in mouse models. The effects of cell size changes on a tubular system should influence the characteristics of the major kidney structures, including tubular length, diameter, and lumen size. For example, tubule diameter could change as a function of altered lumen size (expansion or dilation), or because of altered tubule cell sizes.

It remains surprising that this phenomenon concerning cell size changes has yet to be measured via imaging at a single-cell level. Several studies have used *in vitro* experiments to demonstrate size changes within specific cell populations or experiment with molecular drivers of this phenomenon, but these types of experiments isolate the cell from its home environment in the kidney, and thus may be missing out on signals or changes which are context-specific (to the cell as part of an organ).

In addition to providing quantitative information about cell growth, analysis of CRH on a single cell level can provide insight to a fascinating and critical structural question; that of whether the growth seen following UNx is isometric (equal in all cell types), or allometric (with different cell

types showing different extents of growth). The type of growth the kidney undergoes will have a direct impact on the structure and morphology of this complex organ, potentially affecting its functional capabilities as the process of CRH continues and after its completion. Changes in one area can result in further structural or functional adaptation in a different segment, which may have implications for hyperfiltration. Given that existing literature suggests that the majority of changes take place in the proximal tubule (allometric growth), one must consider how this affects the kidney microanatomy. How do these downstream segments, which presumably remain the same size as they were, accommodate the increased blood flow from the proximal tubules which are no longer proportionally sized? If these later segments are required to upregulate their function significantly, is this functional increase sustainable or does it overwork these cells? Are these structures subject to injury due to increased flow or pressure which they are not suitably sized for? What genetic or molecular changes would accompany such a process? Additionally, quantitation of these size changes is particularly lacking in female animals. It remains unknown, and is of great interest, to know how these gross organ metrics correlate with cell size changes.

1.4.3 Possibility of prediction of extent of growth, based on pre-operative factors

Live kidney transplantation is possible because most donors exhibit sustained near-normal kidney function after kidney donation: the phenomenon is due at least in part to compensatory renal growth of the remnant kidney. Of critical clinical importance is the ability of a kidney donor to recover and resume a normal and healthy life with a solitary kidney. Therefore, potential donors are put through rigorous testing before being approved to donate, to ensure their own good health following surgery. Roughly ~20% of live donors do not show CRH following donation, thus increasing their own risk of developing kidney disease later in life.[3] Typically, donors are ruled out due to pre-existing medical or renal conditions, obesity, and age, but few other factors can predict how a donor will respond. Understanding this phenomenon at a cellular and mechanistic level can provide further insights into interventions that could help improve CRH if documented to be suboptimal.

Overall, I aim to implement UNx-induced hypertrophy as a model to begin to explore cellular growth and size in an *in vivo*, organ-scale model. I aim to develop a picture of organ growth and function throughout CRH to shed light on the process of CRH, and correlate these changes with changes in cell or nuclear sizes. Further, I plan to use this comprehensive picture of the process of

CRH retrospectively: to explore whether there are other characteristics which correlate with or can predict optimal or suboptimal hypertrophy and functional recovery in mice, which may reflect similar traits in human donors.

Chapter 2
Materials and Methods

2 Materials and Methods

2.1 Animal surgery and associated procedures

All animal experiments were performed under Animal Care Committee (ACC) guidelines in accordance with the Sick Kids Lab Animal Services (LAS) Facility protocol #47113 and LAS standard operating procedures.

2.1.1 Animal procurement and husbandry

Adult male and female C57Bl/6J (strain #000664) mice were ordered at pre-specified ages (8-12 weeks, operated on from 10-16 weeks of age) from Jackson Laboratories (Maine, USA), and were housed at the SickKids Research Institute LAS facility. Animals were acclimatized to LAS for a minimum of 5 days prior to any procedures, given free access to a standard LabDiet 5058 (irradiated chow) and acidified water throughout the duration of the experiments, and were on a 14:10 light dark cycle.

2.1.2 Blood collection

The first of blood samples to measure creatinine levels were collected at least 3 days prior to surgery. Approximately 75-100 uL of blood was collected by accessing the submandibular vein via sterile needle puncture (maximum of 10% of animal's blood volume, based on approximation of 0.08 mL blood/10 g animal weight, per LAS guidelines). Animals were given saline fluid to replace 1:1 the approximate volume of blood removed via subcutaneous injection. Upon recovery, the animals were provided with soft food and water (HydroGel cat #70-01-5022, DietGel 31M cat #72-08-5022, both ClearH_2O); sunflower seeds were also given as "treats". Creatinine levels were also measured from samples collected at pre-specified post-operative endpoints via cardiac puncture under isoflurane anesthesia.

2.1.3 Nephrectomy and sham surgery

Animals were anesthetized with 3% isoflurane for induction and 1-2% for maintenance during the surgical procedure. Animals were given subcutaneous injection of both slow-release buprenorphine (0.5-1.0 mg/kg, Chiron, 0.6 mg/mL solution) for post-operative pain management and 1 mL of sterile 0.9% NaCl to maintain stable hydration. After ensuring a surgical plane of anesthesia, the left flank was cleanly shaved and disinfected with 70% ethanol and iodine solution to ensure an aseptic incision site. The incision was made with scissors on the left dorsum of the mouse, slightly lateral to the spine and about midway along the animal. The right kidney is not used because it is partially hidden under the right lobe of the liver and in closer proximity to other organs. The left kidney was exteriorized by applying gentle pressure at the retroperitoneal space flanking the incision. Then, the kidney and associated structures (renal artery, renal vein and ureter) were severed from the animal. To do so, a silk suture of size 5-0 was passed under the three structures, as close as possible to the kidney, and tied with a loose surgical knot. Another loose knot was tied ~5-10 millimetres away from the first one using the same procedure. Then, both knots were tightened before completing the nephrectomy completed by cutting in between the two knots. The vascular/ureteral stomp remnant was then inserted in the renal cavity, and the muscles and cutaneous layers were sutured back together with Vicryl sutures of size 5-0. SHAM surgery consisted of anesthesia, flank incision, externalization of the left kidney through the incision, return of the kidney to the renal cavity, and closing of the wound. All surgical procedures were performed under sterile conditions. Animals were recovered to ambulation on heating pads and returned to their home cages with soft food and water, and monitored twice daily for 3 days post-surgery.

2.1.4 Serum creatinine measurement

Following collection, blood was allowed to clot at room temperature for ~1 hour and centrifuged at 5000 RCF for 10 minutes. Serum was aliquoted and frozen at -80ºC for later measurement. Serum creatinine levels were quantified by the Toronto Centre for Phenogenomics (TCP) Pathology Core staff using the Bio-Rad Liquid Assayed Multiqual products, Cat. 694 and 696.

2.2 Processing of murine renal tissue

2.2.1 Organ weights

Immediately following surgery or removal at endpoint, the whole kidneys were gently decapsulated[29,35,36,40,67–69] with forceps to remove excess blood, perinephric fat and weighed.

2.2.2 Histological preparation and staining

Immediately following removal and weighing, whole kidneys were fixed by immersion in 20 mL of 4% paraformaldehyde (PFA) in PBS for 48 hours. Then the kidneys were transferred to 20 mL of 70% ethanol for dehydration and storage. 4 um paraffin-embedded sections were dewaxed by xylene clearance and ethanol dehydration, followed by antigen retrieval in 1M sodium citrate buffer (~pH 6). Sections were blocked in CAS-block (Invitrogen/Life Technologies, cat #00-8120) for 10 minutes, followed by 30+ minutes of incubation with primary antibody diluted in CAS-block at room temperature, and overnight incubation at 4ºC. The following antibody dilutions were used: anti-human E-cadherin (mouse monoclonal, BD Biosciences cat #610182)-1:100, anti-rat Aquaporin-1 (rabbit polyclonal, Abcam cat #15080)-1:500, anti-human Calbindin-(rabbit monoclonal, Cell Signalling D1I4Q) 1:500/1:800, anti-human phospho-S6 ribosomal protein (rabbi monoclonal, Cell Signalling cat #CS4858)1:250/1:400. After 2xPBS-T and 1xPBS washes (5 minutes each), sections were incubated for 2-3 hours at room temperature in secondary antibodies in 1% bovine serum albumin in PBS (goat α-rabbit Alexa Fluor 555 or goat α-mouse Alexa Fluor 647) at a dilution of 1:500. Following a 5-minute PBS wash, sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI, suspended in DMSO) diluted in PBS for 10 minutes at room temperature. All incubations were performed in a dark, humidified chamber. Finally, samples were washed in PBS and mounted using an aqueous mounting medium (Immco/Trinity Biotech, cat #2505).

2.2.3 Imaging of murine renal tissue sections

Due to their large size, whole kidney sections were imaged in tiles on the Zeiss Axio Observer.Z1 at 20x magnification and stitched using the Zeiss Zen 2 (Blue edition) Software. CZI images were exported to TIFF format for MATLAB image analysis as described below.

2.3 Computational analysis to systematically measure key renal structures

CZI images from Zeiss Zen Pro 2.0 software were exported to TIFF images used for analysis and quantification. Analysis was done to quantify structures from images of DAPI staining and GFP autofluorescence, which is innately present due to lipofuscin in the kidney as well as PFA autofluorescence.[70–72] The following steps of processing and analyses were applied to all images. Because of the variability in image brightness and section size, relative values and percentiles, as opposed to absolute intensity values, were used to ensure consistent and accurate selection of tissue and nuclear areas.

Assessing cell size within a tissue section is both technically and computationally challenging. Much work has gone into determining methods by which to measure cell size including assessing cell borders, and nuclear distances. While we continue to optimize our analysis methods to better incorporate more information from the kidney images, for this work we have chosen to use nuclear size as a proxy for cell size[73,74]; or rather to explore the changes in nuclear size as they relate to changes in kidney size and function.

2.3.1 Obtaining tissue section area and nuclear area

To discriminate kidney section from background, the median intensity value of all pixels in the GFP channel was used as the threshold which selected for the majority of the tissue. After removing small background or debris structures from the image, the tissue areas which met the intensity threshold were connected and holes were filled to create a mask that defined the region within the image that is to be included in the image analysis (**figure 3a**, first and second panels).

To delineate the borders of nuclei and cells, we implemented the ‘seed-based watershedding’ algorithm[75], whereby islands of nuclear intensity (DAPI) were used to identify nuclei as ‘seeds’. Because of the presence of intensely bright chromocenters of condensed DNA[76] (**figure 3b**) in the nuclei, they required multiple smoothing steps to improve identification of the entire nucleus. DAPI images were smoothed based on the average intensities of 10 surrounding pixels, and the resulting image was subtracted from the original DAPI image. This result was further blurred by Gaussian smoothing and filtered again to include pixels above an appropriate intensity and size

value. The pixels selected in these transformations were binarized (**figure 3c**). To determine whether nuclei are included in the tissue regions, we used the following three criteria: nuclei must fall within the defined tissue area, must be of suitable size and intensity based on smoothing), and must be greater than the 20^{th} percentile of intensity of all the nuclei in the original image.

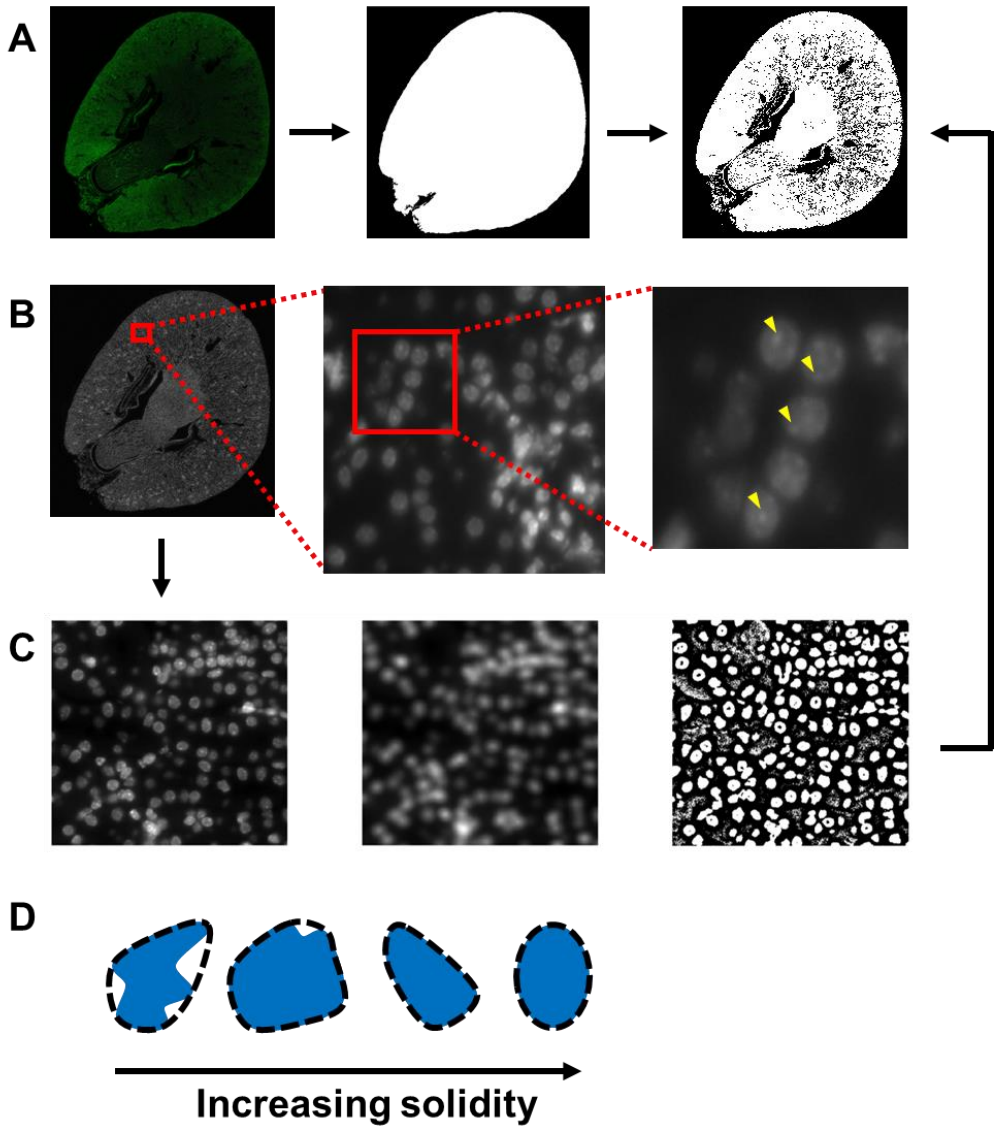

Figure 3. Using segmentation of images for automatic identification of nuclei in murine kidney tissue: A) Tissue area is selected by pixel intensity threshold of the GFP (tubule) image, and binarized to determine the general tissue area. DAPI-stained nuclei within this tissue area can be seen in the third panel. B) Bright chromocentres are visible in nuclei (indicated by yellow arrowheads). C) Nuclei are smoothed by blurring and then size and intensity thresholding to create a binarized image. D) Schematic of nuclear solidities, whereby the ratio of convex hull (black dashed line) and area (blue) is increasing.

2.3.2 Computation of nuclear size and density

Once suitable parameters/thresholds have been defined for nuclei and section area, the image can be processed to determine various size-related parameters for each image. Firstly, nuclei which meet the intensity threshold assigned above are selected. Their size in pixels, cartesian and polar coordinates (relative to the center of the kidney section), and intensity are computed and compiled. We also compute solidity, or smoothness of shape, to help determine whether an object is a true nucleus or debris. Solidity is the relationship or ratio between the convex hull of an image to its filled region. Hence, the more irregularly or roughly shaped an object in the image is, the lower its solidity is (**figure 3d**). In the kidney images, there is generally a negative correlation between nuclear size and solidity. These very large "nuclei" which are highly irregularly shaped (low solidity), are likely groups of nuclei which the image processing could not detect as separate structures and instead grouped together; they may also be artefacts of the image processing. Using a high solidity threshold provides more certainty that a structure is a true nucleus. These nuclear characteristics are important in determining the spread of nuclear sizes and the regions of kidney tissue they correspond to. Nuclei were filtered by a solidity score of 0.95 to 1.0. Within this population, we selected nuclei with average pixel intensities and nuclear areas between the 10^{th} and 90^{th} percentile (of all those found in that kidney section) to eliminate outliers. (Similar values held true even when the solidity score threshold was reduced as low as 0.5).

The size of the kidney section can vary between right and left kidneys and between animals, due to natural variation and the exact location in the kidney where the organ section is cut. As such, overall nuclear number based on the entire kidney section will fluctuate. To develop a normalized value that to use to compare between kidneys, we calculated nuclear density per unit of 10,000 pixels. This was determined by dividing the total kidney area in pixels, by 10,000, to obtain the number of 10,000-pixel units, and the total number of nuclei were divided by this number of units.

2.3.3 Identifying tubular lumina and non-cell area

To ensure consistency between the nuclear and GFP images, the same mask that was generated for the nuclear processing was used to select for the tubule area defined above was used to assign tissue area (**figure 3a**, middle panel). The uneven fluorescence due to autofluorescence from both the kidney tissue (lipofuscin) and PFA fixation (**figure 4a**), makes it challenging to use intensity thresholds to separate true tissue from background signal or tubular lumen. As such, the tubular images require normalization of pixel intensities. Due to the large size of the images and the technical limitations in processing the full image (processing such large images as whole units requires tremendous computing power), block processing was used to break the image into sections of defined sizes and analyze it in these smaller parts. To reduce computation time, the smoothing filter was iteratively (loop) implemented row-wise and column-wise, across intensity profiles.

In the first stage, GFP images were smoothed using a moving average filter which smooths according to the 200 nearby pixels. The second stage calculated the ratio of the smoothed to non-smoothed image by dividing pixel intensities of the original (unsmoothed) images and the smoothed outputs (**figure 4b**). This procedure resulted in a smoothed and uniform representation of the tubular tissue. In addition to increasing uniformity of the tubular tissue intensity, this normalization also increases the contrast between tubular tissue and the lumens or peritubular capillaries (which present as the space between tubules or non-cell area), which can then be used to define thresholds for tubular tissue versus lumen or non-cell area (**figure 4c**). These non-cell areas were also subtracted from the total image area to obtain a general size of the renal tissue.

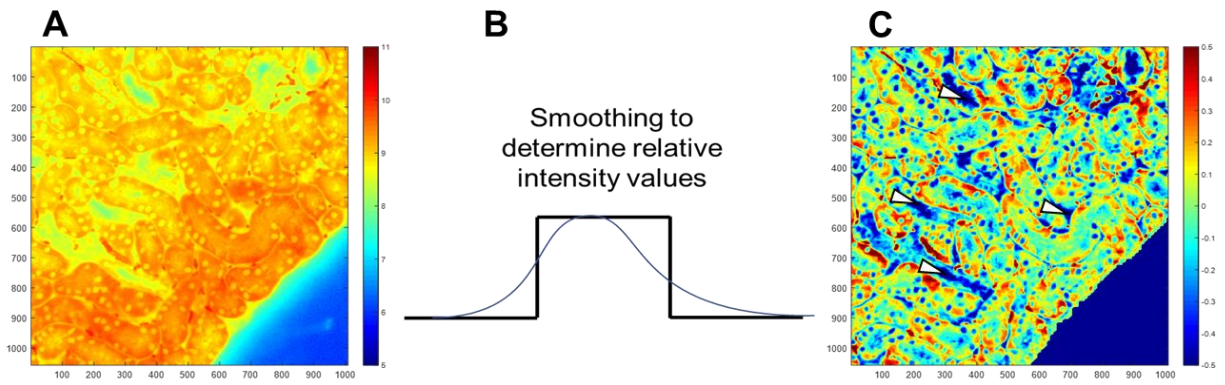

Figure 4. Normalization of GFP signal for tubule identification: A) Renal tissue autofluorescence and fixation produce uneven intensity artefacts, visualized by the colour gradient on the heatmap. The range of pixel intensities from low to high is represented by the colour changes from blue to red. B) Pixel values are corrected using smoothing to determine relative intensity values. C) Corrected pixel intensities display a normalized distribution of pixel intensities which allows for distinction between tubule and non-tubule tissue using intensity thresholding. Example of tubule edges and lumens can be visualized near the white arrowheads.

Chapter 3
Results

3 Results and Discussion

3.1 Results: Characterizing gross changes in kidney weight and their correlation with functional changes (creatinine)

3.1.1 Overview

In the existing CRH literature, study designs often use weight-matched animals as controls to nephrectomized ones—whereby the hypertrophied kidney from a nephrectomized animal is compared to the ipsilateral (corresponding) kidney of age- and weight-matched controls (sham or no surgery). While this matching is an important element, our analyses show that there can be significant differences in the kidney sizes within and between animals. As an added level of control, this study compared the weights of kidneys from the same animal, i.e. how the nephrectomized (removed) kidney compares to the remnant kidney (allowed to hypertrophy). (**figure 5**) The assumption is that kidneys from a given animal are likely more similar in size at baseline than those ipsilateral kidneys from different animals, particularly if those animals differ in weight. This should reduce some confounding factors that are due to the comparison of different animals who may have kidneys of different size to begin with, as well as the influences of environment (such as diet) and genetics which will vary between animals. However, even within the same animal, the right and left kidney sizes/weights are usually not exactly equal. This initial inequality can either artificially inflate or mask some of the effects seen when comparing the weights of two kidneys, and likely contributed to what appears to be significant growth in some of the sham control animals. As such, the ideal way to determine the magnitude of growth is measuring both kidneys before surgery in a non-invasive manner such as using MRI or ultrasound. Unfortunately, due to an equipment issue with our imaging collaborators, I was unable to complete this part of the study, and as such cannot rule out the possibility that the differences in kidney sizes pre- and post-operatively were influenced by the initial kidney sizes--which may not have been exactly equal.

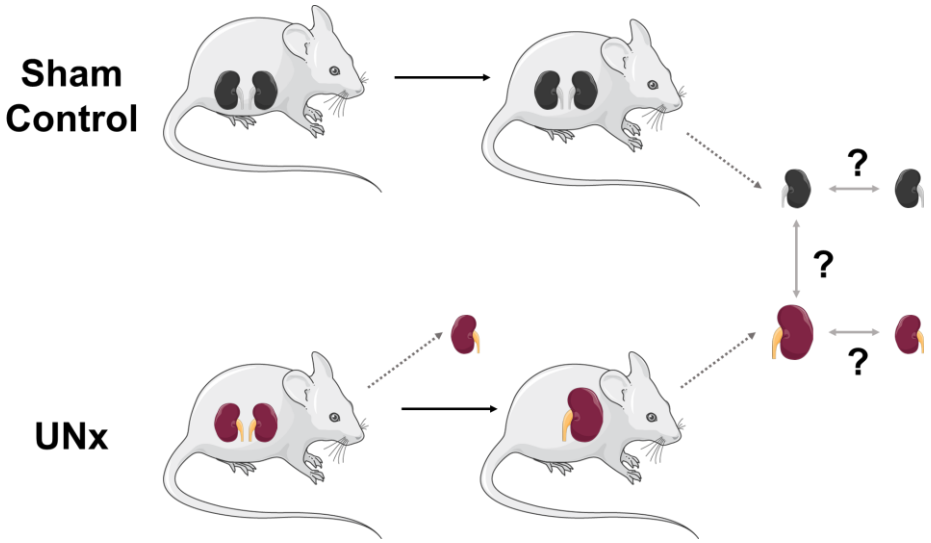

Figure 5. **Schematic of experimental protocol:** Left kidneys from adult (10-16 week) C57Bl/6 mice are removed (experimental animals) or externalized (sham animals), and right kidneys are allowed to grow for a defined time period. CRH is assessed by comparing the remnant kidneys to nephrectomized kidneys, as well as to kidneys from sham-operated animals. Images modified from Servier Medical Art.[77,78]

$\Delta Weight = Weight\ at\ Endpoint - Pre\text{-}op\ Weight$

$1.1.\ Weight\ Correction\ Factor\ (WCF) = \Delta Weight / Pre\text{-}op\ Weight$

$1.1.1.\ Kidney\ weight\ correction = Nephrectomised\ Kidney\ Weight + (Nephrectomised\ Kidney\ Weight \times WCF)$

$1.1.2.\ Theoretical\ Post\text{-}op\ Creatinine = Pre\text{-}op\ Creatinine + (Pre\text{-}op\ Creatinine \times WCF)$

Equation Set 1: Calculation of weight correction factor; this helps account for the growth of an animal over time and its impact on kidney weight and serum creatinine values.

3.1.2 CRH correlation with time post-surgery

Our analysis shows that the extent of growth observed for kidneys of nephrectomized male mice was proportional to the time elapsed since surgery (**figure 6a**). This relationship persisted even when applying a novel correction factor that accounts for the expected amount of normal kidney growth that should have been observed during that timeframe in the absence of nephrectomy (**equation 1.1.1**). Additionally, this study extended the time in which kidneys were allowed to grow beyond the typical maximum times in the existing literature exploring CRH in mice (7-10 days before sacrificing the animals). This suggests that the compensatory growth period may extend longer than previous studies have explored. Interestingly, both males and females showed a decrease in growth (negative slope) between days 2-4 and days 7-14 post-UNx (**figure 6a and 6b**). The steepest increase in growth was between 4 to 7 days and the slightest from days 21-28 post-UNx, suggesting that growth may be petering out at this stage.

In addition to the complexity and variability that the female hormonal cycle can add to outcomes in female animal experiments, it is documented in the literature that females tend to undergo less hypertrophy than males, and as such, few studies include female animals in their experiments.[27,40] To address this discrepancy, I included female animals in all experimental time points. Indeed, the female mice showed considerably less hypertrophy than their male counterparts at all time points. Additionally, whereby the male animals showed a significant correlation between growth time and extent of weight increase in the remnant kidney, the female animals showed this trend only weakly, with small fluctuations in weight between time points (**figure 6b**). These results underscore the need for further studies exploring the compensatory response in female animals to determine whether CRH in females follows a different trend or is influenced more strongly by different factors.

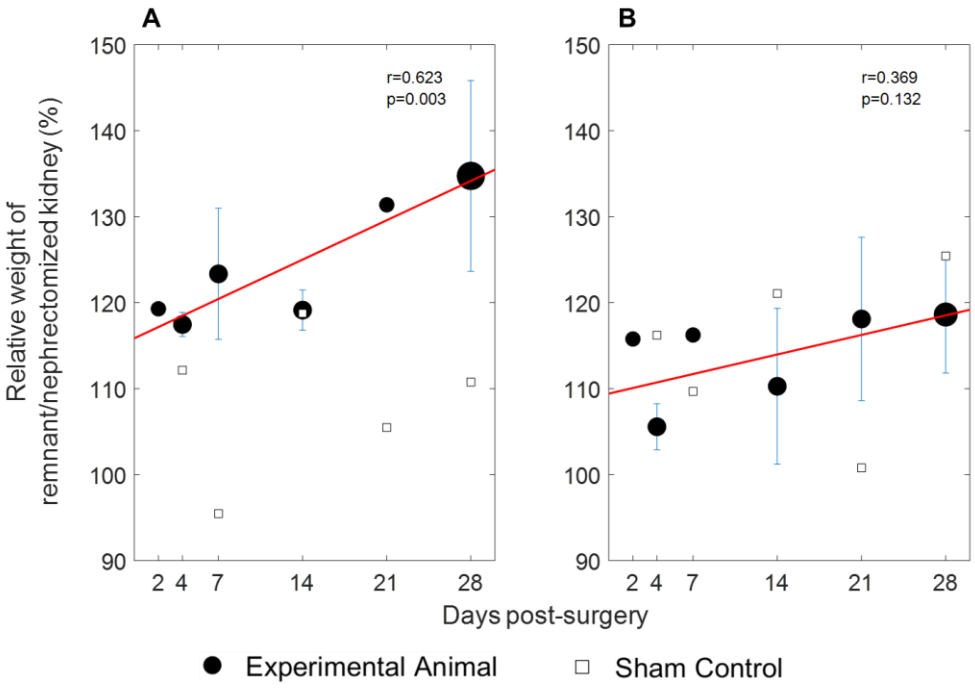

Figure 6. Increases in remnant kidney wet weight over time in males and females: Relationship between increases in remnant (right) kidney weight and time post-surgery, presented as mean values in males (A) and females (B). Relative kidney weights were measured as: remnant kidney weight/weight-corrected nephrectomized kidney weight. *n*=3 or more animals for all data points with error bars, error bars represent standard deviation, *n*=2 for those without. Pearson's correlation (r) and p-values are calculated from raw data. Marker size reflects sample size for time point.

3.1.3 Resected kidney mass negatively correlates with remnant kidney growth in females and some cohorts of males

An alternative lens to consider the phenomenon of CRH through is that of a potential requirement of a certain ratio of renal mass to body mass. Much like other biological and organ systems, it is known that kidney function scales to demand. This is seen in the renal response to non-surgical stimuli such as pregnancy[10–12,14] or high protein diet[15–17], and in the natural scaling of organ size to body size: larger animals need larger kidneys to filter a larger blood volume. Put simply, following UNx, an animal with small kidneys in relation to its body size would require more growth of the remaining kidney than a similarly sized animal with larger kidneys. As such, I wanted to explore whether an animal's initial kidney mass had any impact on the extent of the growth of the remnant kidney.

To understand whether this was a factor at play in the process of CRH, I analyzed the data in accordance with the ratio of initial kidney weight to body weight; exploring whether there was a relationship between the weight of the removed (left) kidney as a percentage of the animal's body weight, and the extent of CRH (**figure 7a and 7b**). Percent body weight was calculated by dividing the weight of the left kidney by the animal's pre-operative weight. There was a striking negative correlation between remnant kidney growth, and nephrectomized kidney-to-body weight in the female cohort, suggesting that remnant kidney growth may be related to the ratio of nephrectomized (removed) kidney to body weight. While this relationship was not statistically significant in the cohort of male animals *overall*, it was evident and significant within some groups of animals at several time points (**appendix figure A1**). For example, at days 4 and 7 post-UNx, there was a negative correlation between the ratio of removed kidney weight-to-body weight, and increase in remnant kidney weight, but this relationship was not statistically significant ($p>0.05$). However, at 28 days post-UNx there was a statistically significant negative correlation between the two ($p=0.037$). This may reflect a trend that is visible in larger sample sizes, as the 28-day post-UNx group had a larger sample size than the others. It may also suggest that it takes more time for this trend to become evident in males, and the factor or gene which is responsible for this relationship needs more time to mediate it in males than females. Overall, this result is consistent with the idea that an animal with smaller kidneys relative to its body size may require more growth than an animal of the same body size with larger kidneys.

Without a non-invasive imaging modality such as MRI and ultrasound, it is impossible to rule out that the remnant kidney may have been larger than the nephrectomized kidney to begin with. In such a case, the apparent size of the remnant kidney may be inflated or masked. These discrepancies can be seen in the sham operated animals who appear to show some "growth" of the remnant kidney (white boxes on **figure 6a and 6b**), but the low number of sham controls in this study make it difficult to conclude whether the sham surgeries induce some compensatory response or are simply subject to this right-left discrepancy. These differences carry over to inequalities in the percentage of body weight which the left and right kidney occupy (**figure 7c and 7d).** However, the ratio of remnant (right) kidney weight to body weight was consistently larger in nephrectomized animals than sham-operated animals, implying that UNx likely induced increases in size of the remnant kidney beyond the expected right-left discrepancies. While these values are subject to variability, UNx animals typically show a remnant kidney-to-body weight ratio of greater than 0.6%, whereas sham or unoperated animals generally don't reach this threshold. The difference in kidney-to-body weight ratio between the left and right kidneys in sham or unoperated animals does not often exceed 0.065%, whereas in nephrectomized animals it can differ by greater than 0.1%.

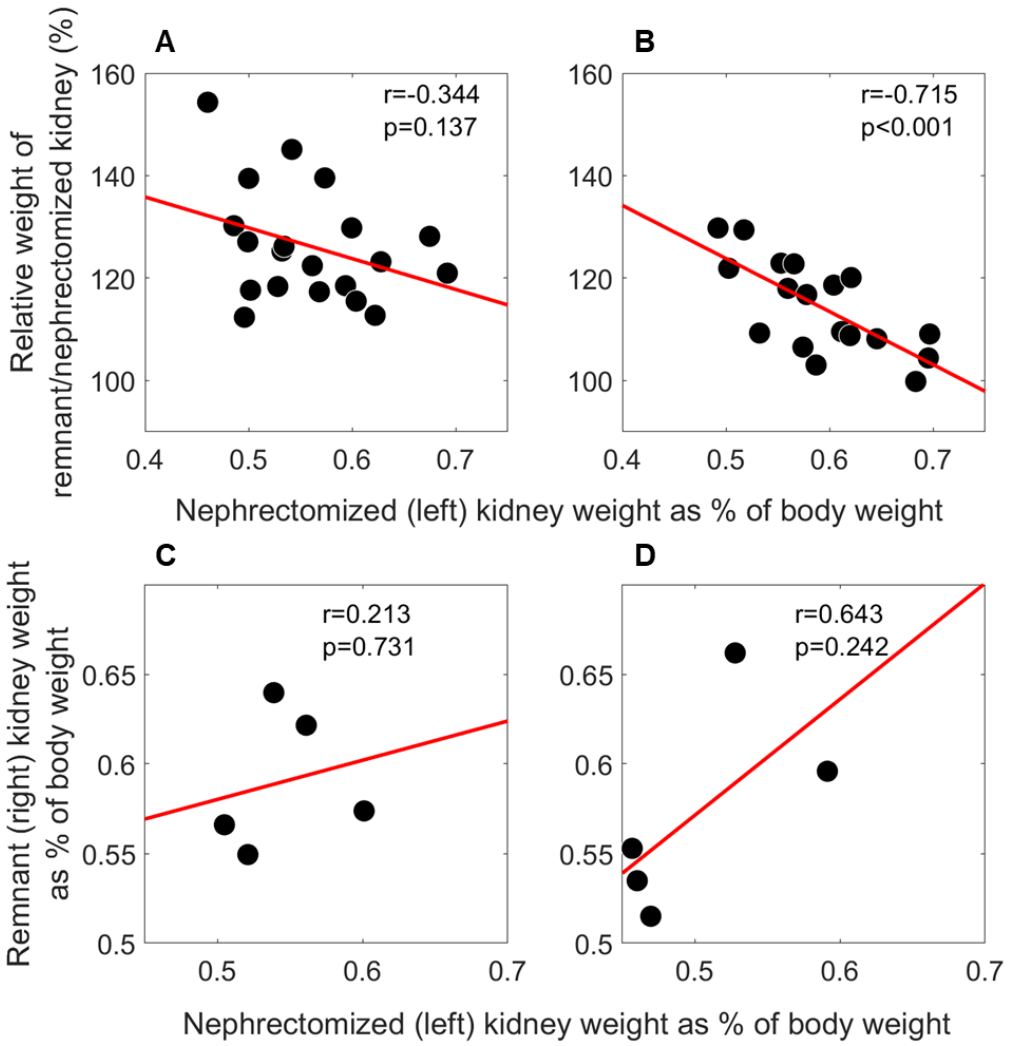

Figure 7. Correlation between initial kidney-to-body weight of nephrectomized (left) kidney, and extent of remnant (right) kidney growth at all time points in nephrectomized males (A) and females (B). Animals with smaller left kidney wet weight:body weight ratio showed greater relative growth of the remnant kidney. *n*=20 for males and *n*=18 for females. Pearson's correlation (r) and p-values are calculated from raw data. C), D) Relationship between wet kidney:body weight of (left) kidney and kidney:body weight of remnant (right) kidney in sham-operated males (C) and females (D), at all time points. *n*=5 for males and females. Pearson's correlation (r) and p-values are calculated from raw data.

3.1.4 Serum creatinine levels decrease with time post-surgery

The ultimate "goal" of CRH is to restore kidney function to a level which is comparable to pre-operative levels, or sufficient to maintain homeostasis. As such, a key metric of "successful" CRH is restoration of kidney function, which can be measured by comparing serum creatinine (sCr) levels measured both pre- and post-operatively. Creatinine is a breakdown product of muscle creatine that is excreted by the kidneys. As such, significant changes in longitudinal serum creatinine levels can be used as a proxy for changes in kidney filtration because muscle mass is unlikely to drive this phenomenon significantly during the time frame of the experiment.[79] I nevertheless applied a correction factor based on weight gain to account for the predicted change in serum creatinine over time under normal circumstances i.e. in an animal with two kidneys (**equations 1.1 and 1.1.2**; this correction is more important for later time points, particularly at day 28 post-UNx where animals will have likely gained more weight). This value was compared to the true post-operative serum creatinine level to determine the change in kidney function after surgery (post-op sCr/weight-corrected pre-op sCr). As expected, these corrections had greater effects on the animals who showed more dramatic weight differences between surgery and endpoint.

While the creatinine values and ratios ranged, both the male and female cohorts showed an overall downward trend in the ratio of post-op to pre-op serum creatinine levels (**figure 8**). This indicates that after the expected initial rise in sCr, restoration of function is well established by day 14 to 21 post-UNx. sCr ratio was inversely related to the extent of remnant kidney growth, suggesting that kidney growth does contribute to restoration of kidney function. However, given that renal mass did not increase in proportion to the functional restoration levels, it is likely that increases in renal mass are not the sole driver of restoration of renal function. Because function was measured through static measurements of sCr, collected at a single moment, it is possible that the particular sample collected at that time simply had a higher or lower level of sCr than average. Several animals showed extremely low post-to-preop sCr ratios ($\leq$0.5), which were due to uncharacteristically low or high sCr values at either or both time points (pre- or post-op collection), which strongly influences this ratio (raw data values **appendix figure A2**). These extreme values may explain the low ratios, which suggest not only fully restored, but improved kidney function post-operatively.

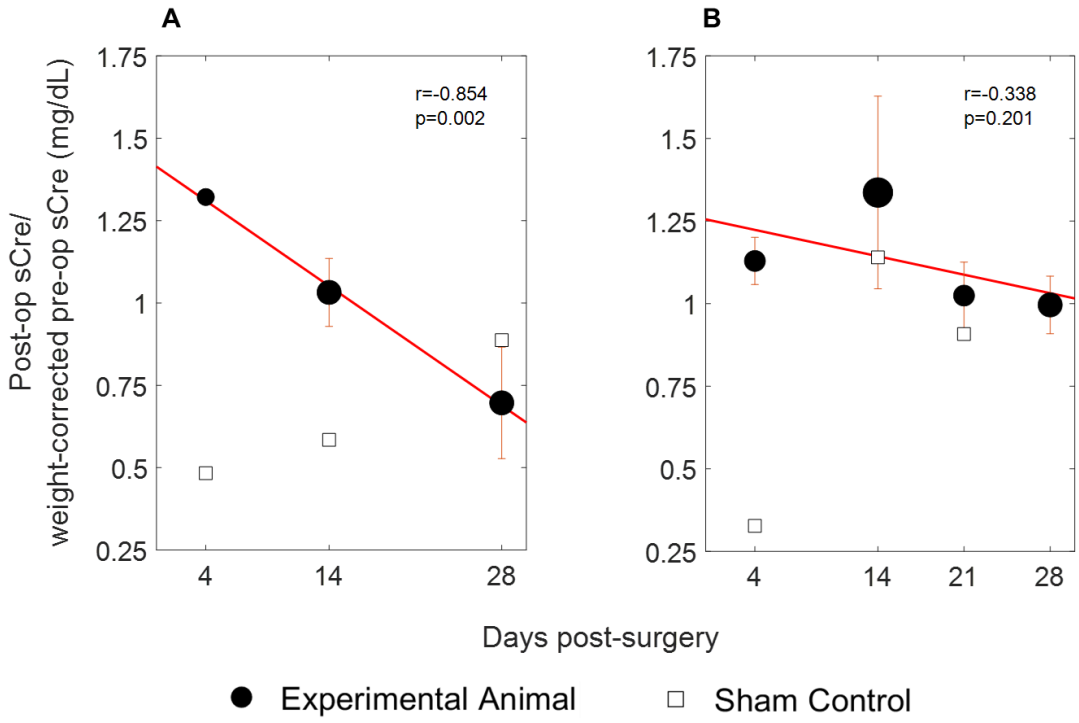

Figure 8. Ratio of post-operative to weight corrected pre-operative serum creatinine levels (mg/dL), in A) males and B) females. Ratios close to 1 suggest restored kidney function following surgery. *n*=3 or more animals for all data points with error bars and *n=2* for the others, error bars represent standard deviation. Pearson's correlation (R) and p-values are calculated from raw data.

3.2 Characterizing cellular changes and their correlation to gross organ changes

3.2.1 Exploring nuclear size and density changes in the kidney

Changes in wet kidney weight correlated strongly with time post-UNx in the male animals and weakly in females. Surprisingly, changes in nuclear size used as a proxy for cell size, showed differing trends. In males, the relative difference in mean nuclear size (mean nuclear size of the remnant kidney divided by mean nuclear size of the nephrectomized kidney *100) decreased with time post UNX (**figure 9a**). In males, the smallest relative nuclear sizes were evident at 14 days post-UNx and the left and right nuclear sizes restored near equal to each other by 28 days. These results were highly unexpected given the existing literature suggesting that compensatory renal growth is strongly hypertrophic, and as such I expected to see a sustained increase in the ratio of remnant-to-nephrectomized nuclear sizes. The increase in nuclear size at earlier time points may suggest initial cell hypertrophy followed by later cell division, or it may be indicative of the nuclear growth preceding division. The female cohort showed a spread of nuclear size changes, without an evident trend, and many females showed larger increases in nuclear size than the male animals (**figure 9b**). The majority of the females showed a larger relative nuclear size difference at 14 days, but also restored near to equal by 28 days, and there was not a strongly identifiable trend in the changes in nuclear area ratios over time (**figure 9b**).

To identify whether there is a hyperplastic component that may explain the smaller than anticipated nuclear size, I compared the change in mean nuclear size to average nuclear density (per units of 10000 pixels). The male cohort showed a negative relationship between nuclear size ratios and nuclear density ratios (**figure 9c)**. The combination of decreasing relative nuclear sizes, together with increased nuclear density may suggest that nuclear sizes are decreasing because of a proliferative response. However, the opposite trend is evident in the female cohort. The majority of the female mice showed either increased relative nuclear density with increasing nuclear area ratios (upper right quadrant of **figure 9d**), or roughly equivalent nuclear density ratio with increased nuclear size (lower right quadrant of **figure 9d**). These results again show sex-based differences in the compensatory response, and suggest that changes in both nuclear (cell) size and number contribute to the changes seen in CRG.

Interestingly, there is also a positive correlation in both sexes between the sizes of lumen or non-cellular space and nuclear density, which may indicate that widening lumina in tubules or peritubular capillaries are pushing cells more closely together, thus increasing their density. Larger lumina may also be able to hold more liquid (filtrate or blood), and thus contribute to increased kidney wet weights.

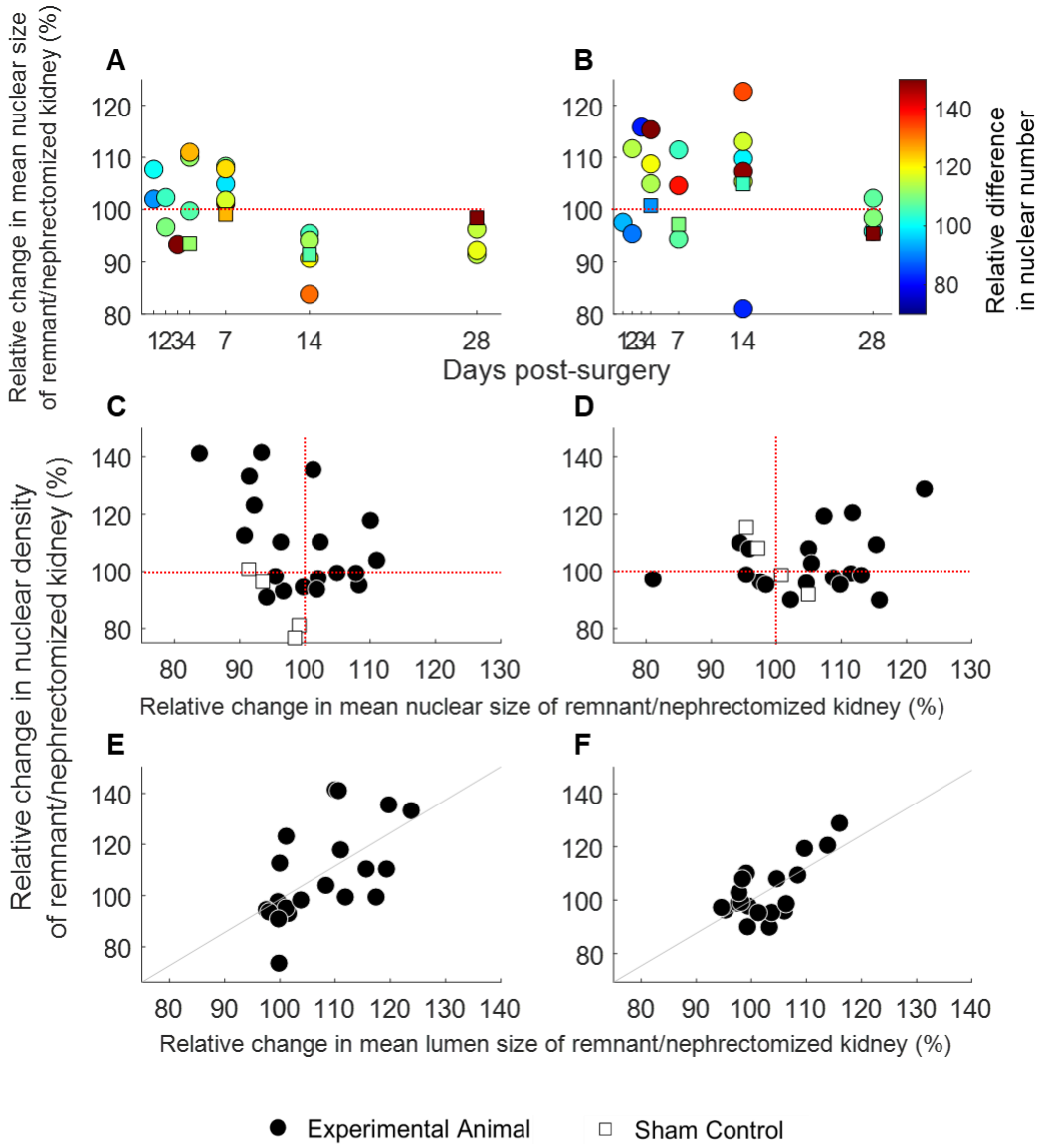

Figure 9. Relationship between relative mean nuclear size, and time post-UNx in male (A) and female (B) mice. Nuclear size changes were calculated by dividing the mean nuclear area in the right (remnant) kidney to the mean nuclear area in the left (nephrectomized) kidney. The color bar in panels A and B represents the relative number of nuclei in the kidneys (right nuclear number divided by left nuclear number). Similarly, nuclear density (number of nuclei per 10000 pixels) in the right kidney was divided by nuclear density in the left kidney to obtain a relative value for changes in nuclear density in males (C) and females (D) over time post-UNx. The dashed lines are simply to help visualize the magnitude of these different changes relative to 100%, where these metrics would be if right and left kidney values were equal. E) Changes in nuclear density as compared to changes in mean lumen/non-cellular compartment size in males and F) females. n=19 for females and n=20 for males.

3.2.2 Mapping additional changes in renal cellular features

To parse these massive sections into smaller components and explore whether further trends could be identified in the kidneys, we used a very high solidity threshold to select nuclei and compare nuclear size distribution - in the left versus right kidneys. Interestingly, the nuclei appeared to cluster into distinct peaks or populations, which refined further as solidity was increased. When the solidity score of >0.99 was chosen, all the kidneys showed 3 distinct clusters of nuclei, designated small, medium, and large nuclei, and the size of these populations decreased with increasing nuclear area (**figure 10a**). In many cases, these peaks were nearly identical in the left versus right kidneys (**figure 10a**, red and blue lines follow a near identical path), indicating that both kidneys showed similar populations of nuclei. In others, there were more distinct shifts in the relative sizes of these populations (suggesting that they are distributed slightly differently), but they still followed a trimodal pattern.

Visualizing these nuclei as a function of their distance from the kidney section center (**figure 10b**) showed that they are evenly distributed across the tissue. Mapping these populations back to their locations in the kidney section (based on the nuclear X and Y coordinates) did not reveal any specific localization of these 3 populations (**figure 10c and 10d**). Given the length and complexity of the tubules, many different cell types are present in all areas of the kidney cross-section and it is not surprising that these nuclear sizes are distributed throughout the kidney cross-section. However, further experiments staining different cell population markers or tubule sections will shed light on whether these size peaks represent particular cell types. Mapping nuclei of different solidity scores or intensities in a similar manner will enable me to generate more details of the nuclear landscape of kidneys pre- and post-UNx.

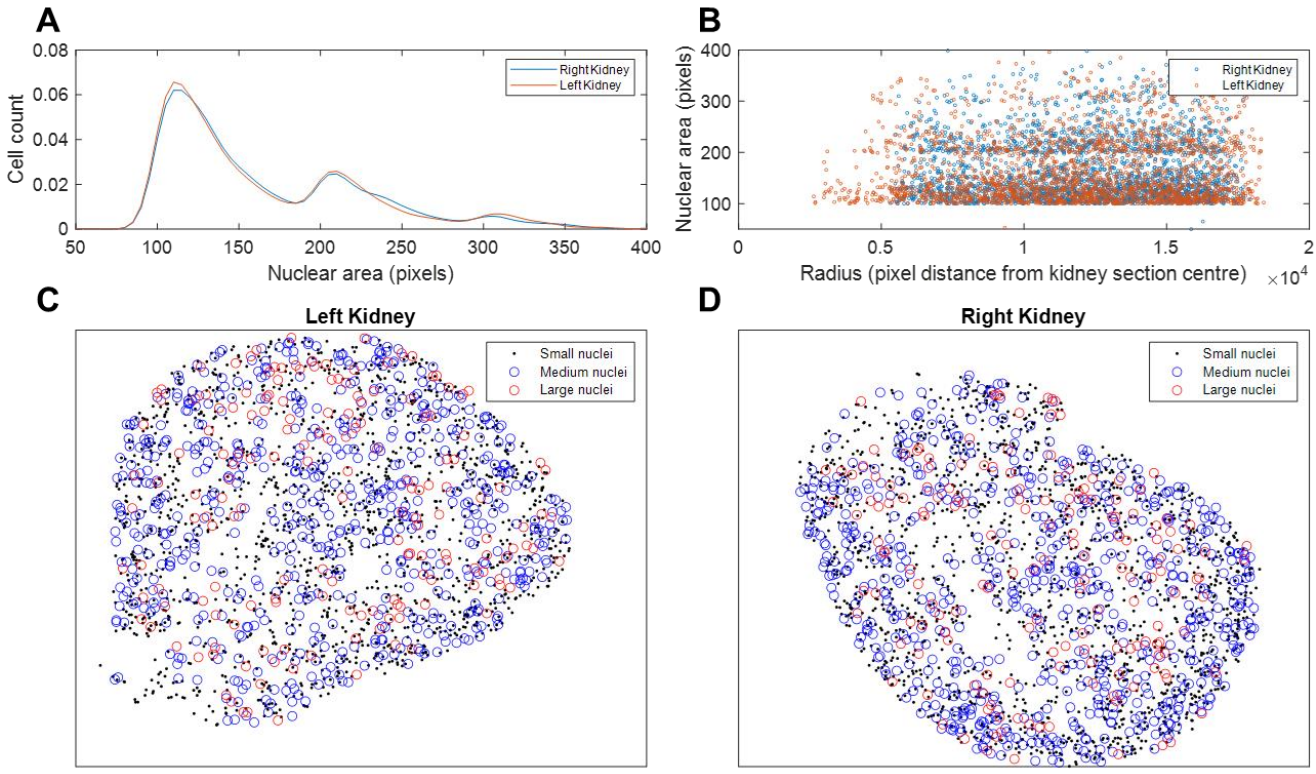

Figure 10. Representative plots of nuclei with high solidity (>0.99) within kidney sections: A) Nuclei cluster into three main size ranges in both left (red) and right (blue) kidneys. B) All the nuclei are spread across the kidney at varying distances from the centre of the kidney section. C) The nuclei can be mapped back to their location within the renal cross-section using the nuclear X and Y coordinates obtained from image processing.

Chapter 4
Discussion, conclusion, future directions

4 Discussion, conclusion, future directions

4.1 Animal experiment results and gross metrics

In order to restore filtration capacity to ensure homeostasis, there are several options following loss of renal mass. The first is the classically assumed phenomenon of CRH, whereby the remnant kidney increases its mass, and can sustainably increase its function due to a greater mass and surface area. The second option is simply the upregulation of function within the remnant (existing) kidney mass. While temporarily effective, this may result in hyperfiltration-overworking of the kidney-which can eventually lead to kidney injury or disease.[9,57,58] Alternatively, if the animal is unable to upregulate its renal function (with or without an accompanying size increase), it may progress to disease due to insufficient renal function.

Outside of cell/renal structure size quantitation, several metrics are used to quantify the size changes in the remnant kidney following UNx. The first readout is usually wet kidney weights, whereby the weight of the remnant kidney is compared to that of a sham-operated animal, or to the weight of the nephrectomized (removed) kidney from that animal. The kidney is typically decapsulated to remove any blood or tissue which could interfere with a clean weight.[29,35,36,40,67–69] To control for the organ's water content as well as animal weight gain throughout the duration of the experiments, the kidney weight is often presented as a ratio of kidney-to-body weight, similar to the weight correction factor used here. Studies in various strains of adult male mice have shown changes in this ratio ranging from ~20-40% within 7 days post-UNx.[27,48,80] An approach which excludes water weight is using the dry weight of the kidney by desiccation[25], although this is less common as it is established that CRH induces increases in cellular protein, and not just water weight.

4.1.1 Variability in CRH

The removal of a single kidney is not a minor procedure, and CRH has been shown to occur in nearly all species with the exception of some cats and baboons.[81] Animal studies typically show consistent and robust growth in response to the removal of a single kidney; however, in every

biological phenomenon there will be a varied response to a given stimulus and this is certainly reflected in the clinical data. For example, Shehab *et al*. measured CRH in kidney donors at 3 months post-op by ultrasound, and found that kidney volume increased in all subjects but ranged from 7 to 45%, whereas other studies showed that approximately 20% of live kidney donors do not show a compensatory response.[3,82] Within different donor populations (eg older, obese, hypertensive, and standard) it is common for volume changes to have standard deviations of 15-20% or more.[83] Some studies report that the initial post-op enlargement later decreases by either a small percentage or a dramatic loss of renal volume.[84,85] In contrast, other studies show that the increases in kidney volume are sustained.

Given the tremendous variability in clinical data, it is surprising that rates of non-response to UNx have not been reported in animal studies, raising the question of whether these animals are ruled to be outliers and excluded from studies, or if the response is simply highly consistent, unlike the human response. Factors such as species, strain, sex, and age will affect the outcomes of such studies, but in comparing studies with similar conditions from different groups, there is variability in the responses.[27,28,35] Based on the literature around human kidney donors and consultation with experienced experimenters, we suspected that up to 20% of the animals would not show the hypertrophic response; however, this subgroup is rarely, if ever, addressed in existing literature, with most studies showing successful and consistent hypertrophy exclusively. In my results, both males and females showed CRH, although males to a much larger extent, consistent with animal studies.[29,38–41] My analysis of the growth data in male animals also showed considerably more variability in the compensatory response than seen in existing literature. This variability could be due to lower sample sizes in my experiments versus existing studies, as well as due to different cut-off values for inclusion in previous studies (these animals may have been considered outliers and therefore removed). Due to the lack of studies exploring CRH in female mice, it is unclear whether the variability in my results reflects the variability in this biological process in females.

These results also reflect the sex variability that is seen in clinical studies of kidney donors or those who underwent nephrectomy for renal cancer. For example, one study showed that males who underwent a donor UNx showed greater CRH, based on kidney length changes (approximately 7% increase in males and 1.6% in females)[86], in contrast to another study of patients who had undergone UNx for renal cancer and showed no difference between males and females in the extent of CRH based on CT volume (approximately 20% increase in volume over 2 years in both).[87]

Assuming that the renal cancer for which these patients underwent nephrectomy for was localized to the nephrectomized kidney, and the remnant kidney was normal, these clinical studies show similar sex-specific responses to UNx.

One of the central drawbacks of the nephrectomy model is the variability in kidney sizes both between and within animals. The assumption that the right and left kidneys are the exact same size by wet weight is often not the case, and therefore it is impossible to rule out that the apparent changes in weight triggered by UNx were reduced or magnified by initial inequalities. I aimed to accompany some of these studies by MRI or other non-invasive imaging, but unfortunately was unable to complete them due to an equipment issue. Future experiments including this type of imaging would shed light on these organ size inequalities and the true values of growth.

4.1.2 Disproportional relationship between restoration of renal function versus increase in renal mass

Within the group of animals that showed significant increases in remnant kidney size, many showed a functional increase that was not proportional to the magnitude of the size increase. If one operates on the assumption that renal function is directly proportional to size, then restoration of pre-operative levels of renal function would require a doubling of the remnant renal mass. Even in animals who showed increased renal mass, none showed a doubling of renal mass. However, it would be surprising that a single kidney, even enlarged, would be able to double its filtration rate to produce a clearance identical to the pre-operative levels. High levels of kidney function can lead to overwork and atrophy of remnant nephrons, a renal "burnout".[9,57,58] Studies of single-kidney GFR in nephrectomized adult male white rats plateaued at 30-40% higher than sham animals at 5-7 days post-UNx, and was sustained at this level at 6 weeks post-UNx.[17] Similarly, by 30 days post-UNx, adult male Long-Evans rats showed a 40% increase in GFR which correlated with 40% increase in remnant kidney weight.[88] Human kidney donors showed ~30% increase in sCr within the first 18 days post-UNx, and overall recover 60-90% of their pre-operative (2-kidney) function.[4–6,89,90]

These GFR increases appear relatively modest in comparison to mouse studies which compare blood urea nitrogen (BUN) levels before and after nephrectomy, which are nearly identical, suggesting complete-as opposed to partial-recovery of renal function.[27,28] Unchanging levels of BUN before and after UNx suggest that renal function is not affected or decreased by UNx. This

response is more consistent with my results, which show that animals recover near pre-operative levels of function by 14-28 days post-UNx, implying that their creatinine clearance with a single kidney is largely as efficient as with two kidneys. Because renal mass did not increase to such a degree, this result suggests that both increases in size as well as functional upregulation may contribute to the restoration of kidney function. Additionally, they demonstrate that some animals, particularly females, can functionally compensate for loss a single kidney with very minimal upregulation of kidney size, at least for the short term. Exploring renal function and histology at later time points would provide clarification of how the kidney fares longer term, and if these ideal sCr ratios can be maintained.

The apparent difference in recovery of pre-op function in rats and humans as compared to mice may be due to the method by which renal function was assessed. sCr or BUN levels are static measurements collected before and after UNx and subject to natural fluctuation. GFR can provide higher control against this stochasticity as it involves the injection of a known concentration of an exogenous substance that is filtered by the kidneys, but neither secreted nor absorbed by the tubules. The levels of these substances can be measured after a very defined time point and provide a more detailed readout of the *rate* at which they are filtered. While more complex and invasive, it provides a more dynamic readout of kidney function. These differences in functional recovery may also be representative of a species difference between mice and rats/humans.

A concept which may be linked to this upregulated function is known as “renal functional reserve” (RFR) or “renal reserve capacity” (RRC). RRC is the capacity of kidneys to upregulate their filtration and use “reserve” or excess, should there be an increased demand above the usual demands. This phenomenon suggests that at rest, kidneys do not function at their maximal capacity. RRC is measured by introducing a heavier renal load, or a vasodilator such as dopamine or amino acids, and measuring GFR in response.[91] In this vein, it would follow that if an animal failed to upregulate its remnant kidney mass in response to nephrectomy, depending on its RRC, it may still be able to accommodate the burden of increased filtration without any additional renal growth. It is unclear whether this would be pathological if sustained because of the effects of hyperfiltration.

Clinical studies show that RRC can still be stimulated by dopamine or hydration and protein loading, but not by amino acid infusion, in individuals who have undergone UNx. Even though

RRC is dampened in those with a single kidney as opposed to two, this is a remarkable display of the kidney's ability to continuously upregulate function when needed.[92,93] Few studies explore sex-specific differences in RRC following UNx, but two smaller studies found that RRC stimulated by protein loading is higher in females than males.[94–96] Together with my data, perhaps these results represent that females have a greater ability to upregulate kidney function, even without concomitant increases in kidney size. Further studies are needed to clarify these sex-specific differences in RRC in both non-nephrectomized and nephrectomized individuals.

4.1.3 Through the lens of size-sensing

The negative correlation we found between initial kidney-to-body weight is interesting when considering whether mechanisms of size sensing are at play in CRH. While this correlation is certainly not direct evidence for such mechanisms, there is some evidence in clinical literature which may be indicative of similar trends in clinical studies of UNx. For example, a small study reported that nephrectomized patients with smaller initial kidney volume showed greater remnant kidney volume 2-7 years post-UNx, (although this finding was not statistically significant, possibly due to the small sample size).[87] Another study examining kidney volume changes in donors after 3 and 7 days post-UNx found that smaller kidneys tended to show a larger increase in volume.[6] An additional study showed that the extent of remnant kidney hypertrophy correlated with the amount of kidney mass removed, in a comparison of partial versus unilateral nephrectomy.[90] This again suggests a connection between removed and remnant kidney mass, potentially through a mechanism which senses and adjusts for necessary renal mass.

This negative correlation between renal size and relative growth echoes the relationship between cell size and growth duration. It is reminiscent of the negative correlation between cell size and G1 proportion noted by Liu *et al*., suggesting that cells can sense their size and regulate growth accordingly.[51] Similarly, the negative correlation seen here may potentially reflect a sensing mechanism that detects if there is enough renal mass in proportion to body weight, to activate or regulate growth accordingly. The concept of a necessary ratio of kidney-to-body weight could explain the discrepancy sometimes seen between left and right kidney weights: rather than an identical division of renal mass and labor, there may simply be a requirement of *total* renal mass-to-body mass which is not perfectly divided between the right and left kidneys. This too would be

in line with a potential size sensing mechanism, employed to ensure that renal mass is sufficient for function even when right and left kidney sizes are not equal.

Under the assumption that renal size correlates with nephron number and kidney function, when considering a population of similarly sized animals, there will be some variability in their kidney sizes relative to their body sizes. Intuitively, the animals with larger kidneys relative to their body size at baseline (pre-operatively) can afford less compensatory growth following a decrease to their renal mass. This is because their initially larger kidneys will ensure that they retain sufficient filtration function. In my analysis, females showed a strong correlation between nephrectomized kidney weight and increase in remnant kidney weight at the end of the experimental period (**figure 7b**). Given that females show overall less growth following UNx than males (**figure 6a and 6b**), it is possible that these regulatory mechanisms are stronger in the cohort, representing a more tightly controlled growth process. Further experiments expanding on the relationship between renal size and function are warranted. Overall, these findings show different patterns of remnant kidney growth in males versus females. Both time post-UNx and initial kidney-to-body weight ratios appear to play a role in the extent of CRH, and the different sexes show different trends with regards to these two main variables. However, these two factors are not independent of each other and it is likely that both play a role in CRH in both sexes. Further experiments with larger sample sizes are required to tease apart these two important contributing factors.

Despite many years of research, the factors that trigger CRH or signal its termination remain elusive. The phenomenon of CRH in itself--an organ increasing its size in response to increased demand--is suggestive of a link between size and function. There is mixed evidence for increased renal work being the trigger for CRH, as some studies have shown that increases in size/growth related biological activities begin even before renal work/transport is upregulated.[5,97] This has led many groups to suggest that CRH is activated by a circulating or humoral factor, but this factor has not been pinpointed. While it is possible that the organ receives a general growth signal, there is evidence which would point to this phenomenon being regulated at the cellular level. In order to avoid disruption of renal structures and function, the growth process would need to be tightly controlled--for a process driven by cell hypertrophy and some hyperplasia to be coordinated, there must be regulation at the individual cell level, and between cells. Additionally, it has been shown that hypertrophic growth takes place primarily in the PT, which means there is preferential growth in certain cell populations. Unless this selective growth was related to the nature of the renotrophic

signal, it implies that there is some control over which cells hypertrophy. Conversely, if the nature of the signal was specific to a cell type, this would negate it being a general signal to the whole organ. These points together suggest that there is some regulation of CRH at the cellular level, as opposed to blind activation of growth, which either promotes hypertrophy of specific cell populations, or overall, proportional growth of all renal structures and cells.

4.2 Cell size quantitation

To explore changes in cell size, many groups quantify the change in the ratio of protein-to-DNA. A higher ratio in response to UNx suggests that CRH occurs via hypertrophy rather than hyperplasia, since such changes must be due to increased cellular protein levels or decreased DNA One of the caveats of this method is that it gives a global picture of the changes in protein, rather than detailing cellular or morphological changes, and thus the localization of growth is not well-identified through this method. An additional consideration is that while increases in organ size via cell hyperplasia would contribute to increased DNA levels, they would also contribute to increased protein levels due to increasing cytoplasmic mass. Therefore, to use this method to quantify increases in individual cell size or relative changes before and after UNx, one would have to quantify cell number, perhaps through DNA content[29], or by organ dissociation and flow cytometry. Antibody-based cell sorting would be an alternate way to obtain specific populations of cells for protein:DNA ratio quantification.

There are clearly many limitations of my analysis, particularly of using nuclear size or area as a proxy for cell size.[73,74] Small changes in area can have large impacts on sizes or volumes of a 3D structure. Additionally, the processing procedures may induce changes in the tissues which are reflected in the image processing. Image processing iterations are continuously improving, and further analysis will continue to shed light on new features of the images, and reveal further layers of complexity.

4.2.1 Nuclear area and density changes

Our analyses of nuclear size changes showed that relative mean nuclear size of the right vs left kidneys decreased over time in the male cohort. This was highly surprising, as the existing literature points to a largely hypertrophic method of growth and as such we expected to find increasing nuclear sizes. Much like the changes in wet weight, there was variability in the nuclear

size and density changes within particular time points. Relative nuclear sizes were larger at the early time points, but by day 28 were largely equal. The decreasing nuclear sizes correlated negatively with increases in nuclear density in the male cohort. These initial increases in nuclear size followed by a decrease at 14 days post-UNx and then restoration to roughly equal to the left kidney may suggest an early hypertrophic response where cells are enlarging, followed by a wave of cell division which reduces relative nuclear sizes. Alternatively, they may represent the early increases in nuclear size which precede cell division, followed by cell division and subsequent growth. Similar results have not been raised in the literature, although few studies have explored this proliferation in C57Bl/6 mice, and even less so in female mice. My work also extends past the typical endpoint (animals are usually sacrificed at 7-10 days post-UNx), and thus may be revealing a proliferative wave which occurs later in the compensatory response, particularly at 14 days post-UNx in males where the relative nuclear size was smallest (**figure 9a**). These results may be consistent with Liu and Preisig's model of cell cycle-dependent hypertrophy, whereby cells initiate the cell cycle but stop before entering into the S phase, resulting in enlarged cells. However, their work does not extend past 7 days post-UNx, and as such may not capture a later wave of proliferation, or a release into the S phase and progress into the cell cycle.[26]

4.2.2 Changes in lumina and non-cellular space

The relationship between increasing nuclear density and increased lumen sizes/non-cellular compartment size may reflect dilation of the lumens or peritubular capillaries, which could push cells closer together. The enlarged lumina could also contribute to significant increases in kidney wet weight, as larger lumina can hold more filtrate or blood, thus contributing to a larger water weight, particularly in the male animals. Increases in tubular lumen diameter are noted by Hayslett et al at 2-4 weeks post-UNx in male Sprague-Dawley rats.[54] Unfortunately I am currently unable to resolve the differences between lumina and non-cell area based on the image processing data, but further improvements to the pipeline will enable better resolution of these different renal spaces.

4.2.3 Nuclear size distribution

A final feature which was obtained from image processing was the clustering of nuclei with high solidity scores into three distinct populations. These nuclei were not localized to distinct areas in the kidney cross-section, but rather were spread all over. This is consistent with the complex

structure of kidney tubules which are weave throughout the different layers of depth and portions of the kidney. Using similar techniques to map other nuclei back to their locations in the tissue will continue to shed light on the localization of different nuclei within the renal tissue.

4.3 Conclusion

Overall, my work has contributed to the development of a more detailed picture of the process of compensatory renal hypertrophy in adult male and female C57Bl/6 mice up to 28 days post-UNx. It has followed the trajectory of kidney weight increases and functional recovery of sCr levels in a longitudinal manner not previously reported. I have shown that mice display near-perfect restoration of kidney function which is not proportional to the magnitude of renal growth by weight increase, suggesting that size increase is not the exclusive determinant of functional recovery after a loss of renal mass. This data is consistent with several clinical studies.[3,82–85] I identified sex-specific differences in the compensatory growth response and notably the relationship between nephrectomized kidney-to-body weight and extent of growth post-UNx, which may serve as a potential predictor of the extent of CRH. This negative correlation is evocative of the negative correlation between cell size at birth and G1 duration and may hint at similar mechanisms of regulation via size sensing. Future work exploring whether identified cell size regulators are at play in this process is a highly exciting prospect.

Further, we developed an image processing pipeline to automate analysis of whole kidneys sections to extract cellular features. Using this pipeline, I identified unexpected changes in nuclear size and density which were not previously reported, with evident sex-specific differences as well. Males show larger nuclear sizes in the nephrectomized kidney at early time points, which continue to decrease until 14 days post-UNx, and then restore to sizes similar to the nephrectomized kidney by 28 days-post UNx. These decreases correlated with increased nuclear density, potentially suggesting a hyperplastic component to CRH. Conversely, females did not show a specific trend in nuclear size changes over time, but showed relatively larger nuclei in the remnant kidney at 14 days post-UNx. These also restored to sizes similar to the nephrectomized kidney by 28 days-post UNx. The relative nuclear sizes in females correlated positively with changes in nuclear density. Together, these data suggest that both hypertrophy and hyperplasia play into compensatory renal growth, and these cellular changes may take place later on in the growth process than previously considered. Both males and females showed increased lumen sizes/non-cellular space with

increased time post-UNx, suggesting that water weight may contribute considerably to the increase in wet weight following UNx. Finally, I identified distinct populations of differently sized nuclei which are randomly distributed throughout the kidney and may represent specific cell populations.

Together, these findings represent novel sex-specific differences present in the functional and cellular changes following UNx. They suggest that the process of CRH is more variable than previously reported, and illuminate the process of CRH at time points not previously explored. Finally, they hint at a potential size-sensing mechanism whereby initial kidney mass may act to regulate remnant kidney growth following UNx.

4.4 Future directions and experiments

My work has uncovered sex-specific differences in the organ weights, renal function, and nuclear size and density following unilateral nephrectomy. However, the small sample sizes preclude deeper analysis of some of the factors which promote organ growth. Increasing the sample sizes at all time points (as well as the number of sham-operated animals) would provide a larger sample population which is amenable to deeper analysis such as partial correlation analysis. This would enable us to better understand the contributions to the wet weight increases following UNx of time post-UNx, versus initial kidney-to-body-weight. Additionally, carrying out these studies and including MRI or other non-invasive imaging prior to UNx will provide better data on the initial kidney size discrepancies. This will help determine the true increases in renal mass or volume that UNx induces, rather than relying on the assumption that kidney sizes are equal to begin with.

To explore whether the functional changes in the single kidney are sustainable over time, the experimental timeline should be extended past 28 days post-UNx. Collecting sCre samples consistently over a long time period will allow us to establish whether the single kidney can maintain the level of filtration it shows within the first month following UNx. Additionally, assessing renal histology for symptoms of pathology may also shed light on the renal health of remnant kidneys at different time points following UNx.

In order to directly assess changes in the renal cell populations, combining staining of particular renal cell markers with image processing will allow us to select and analyze specific cell populations. Similarly, staining for known cell size regulators (such as those identified by the Kafri lab) may provide insight as to whether these genes or proteins are activated or inhibited in the

process of CRH. Additionally, using markers for proliferation such as ki67 or PCNA at various time points (particularly the later ones) will help pinpoint if, when, and where proliferation is taking place in the process of CRH. More direct methods of assessing proliferation such as BrdU or EdU injection combined with surgery will shed light on dividing cells as well. Studies which focus on the details of renal cell morphology and high-powered microscopy will provide more insight in the morphological changes that renal cells undergo.

Finally, continued optimization of the image processing pipeline will enable us to extract more features from the rich datasets that are the kidney cross section images. Better identification of tubule lumens versus peritubular capillaries, obtaining tubule numbers and their sizes will add layers to our understanding of the changes in these structures. Optimizing the image processing to map more features back to the kidney cross section images (such as **figure 10**) will provide a more detailed picture of the individual cells and changes they undergo throughout the process of UNx, and detect trends within different cell populations.

Together, the gross-organ, functional, and cellular data gained from these studies has established a fascinating mode through which to explore cell size changes in a clinically-relevant model. A better understanding of the cellular and molecular basis of CRH will not only provide insight into the clinical phenomenon of CRH and the factors which affect it, but also into the process of cell size regulation and growth in a whole organ.

5 References

1. Lote, C. J. *Principles of renal physiology*. *Principles of Renal Physiology* (2013). doi:10.1007/978-1-4614-3785-7.

2. Taner, T., Iqbal, C. W., Textor, S. C., Stegall, M. D. & Ishitani, M. B. Compensatory hypertrophy of the remaining kidney in medically complex living kidney donors over the long term. *Transplantation* **99**, 555–559 (2015).

3. Chen, K. W. *et al.* Compensatory Hypertrophy after Living Donor Nephrectomy. *Transplant. Proc.* **48**, 716–719 (2016).

4. Rojas-Canales, D. M., Li, J. Y., Makuei, L. & Gleadle, J. M. Compensatory renal hypertrophy following nephrectomy: When and how? *Nephrology* **24**, 1225–1232 (2019).

5. Fine, L. The biology of renal hypertrophy. *Kidney Int.* **29**, 619–634 (1986).

6. Song, T. *et al.* Change in renal parenchymal volume in living kidney transplant donors. *Int. Urol. Nephrol.* **46**, 743–747 (2014).

7. Donati-Bourne, J., Roberts, H. W. & Coleman, R. A. Donor-Recipient Size Mismatch in Paediatric Renal Transplantation. *J. Transplant.* **2014**, 1–5 (2014).

8. Salvatierra, O. *et al.* Successful transplantation of adult-sized kidneys into infants requires maintenance of high aortic blood flow. *Transplantation* **66**, 819–823 (1998).

9. Brenner, B. M. Nephron adaptation to renal injury or ablation. *Am. J. Physiol. Ren. Physiol.* **249**, F324–F337 (1985).

10. Cheung, K. L. & Lafayette, R. A. Renal Physiology of Pregnancy. *Adv. Chronic Kidney Dis.* **20**, 209–214 (2013).

11. Christensen, T., Klebe, J. G., Bertelsen, V. & Hansen, H. E. Changes In Renal Volume During Normal Pregnancy. *Acta Obstet. Gynecol. Scand.* **68**, 541–543 (1989).

12. Beydoun, S. N. Morphologic changes in the renal tract in pregnancy. *Clinical Obstetrics*

and Gynecology vol. 28 249–256 (1985).

13. Davison, J. M. Changes in Renal Function in Early Pregnancy in Women with One Kidney. *YALE J. Biol. Med.* **51**, 347–349 (1978).

14. Modai, D. *et al.* Uninephrectomy and repetitive pregnancies in mice: A combined stimulus for renal hypertrophy. *Nephrol. Dial. Transplant.* **4**, 701–706 (1989).

15. Hostetter, T. H., Meyer, T. W., Rennke, H. G. & Brenner, B. M. Chronic effects of dietary protein in the rat with intact and reduced renal mass. *Kidney Int.* **30**, 509–517 (1986).

16. Martin, W. F., Armstrong, L. E. & Rodriguez, N. R. Dietary protein intake and renal function. (2005) doi:10.1186/1743-7075-2-25.

17. Dicker, S. E. & Shirley, D. G. Mechanism of compensatory renal hypertrophy. *J. Physiol.* **219**, 507–523 (1971).

18. Kanzaki, G., Tsuboi, N., Shimizu, A. & Yokoo, T. Human nephron number, hypertension, and renal pathology. *Anat. Rec.* (2019) doi:10.1002/ar.24302.

19. Briggs, J. P., Kriz, W. & Schnermann, J. B. Overview of kidney function and structure. in *National Kidney Foundation's Primer on Kidney Diseases, Sixth Edition* 2–18 (Elsevier Health Sciences, 2013). doi:10.1016/B978-1-4557-4617-0.00001-7.

20. Fromm, M., Piontek, J., Rosenthal, R., Günzel, D. & Krug, S. M. Tight junctions of the proximal tubule and their channel proteins. *Pflugers Arch. Eur. J. Physiol.* **469**, 877–887 (2017).

21. Ullrich, K. J. Sugar, Amino Acid, and NA+ Cotransport in the Proximal Tubule. *Annu. Rev. Physiol.* **41**, 181–195 (1979).

22. Curthoys, N. P. & Moe, O. W. Renal Physiology Proximal Tubule Function and Response to Acidosis. *Clin J Am Soc Nephrol* **9**, 1627–1638 (2014).

23. Zimmerhackl, B. L., Robertson, C. R. & Jamison, R. L. The medullary microcirculation. *Kidney Int.* **31**, 641–647 (1987).

24. Nephron - Servier Medical Art. https://smart.servier.com/smart_image/nephron-2/.

25. Johnson, H. A. & Roman, J. M. V. Compensatory Renal Enlargement: Hypertrophy versus Hyperplasia. *Am. J. Pathol.* **49**, 1–13 (1966).

26. Liu, B. & Preisig, P. A. Compensatory renal hypertrophy is mediated by a cell cycle-dependent mechanism. *Kidney Int.* **62**, 1650–1658 (2002).

27. Liu, T. *et al.* Cre/loxP approach-mediated downregulation of Pik3c3 inhibits the hypertrophic growth of renal proximal tubule cells. *J. Cell. Physiol.* **1**, (2020).

28. Ting, L., Dai, C., Xu, J., Li, S. & Chen, J.-K. The expression level of class III phosphatidylinositol-3 kinase controls the degree of compensatory nephron hypertrophy. *Am J Physiol Ren. Physiol* **318**, 628–638 (2020).

29. Azurmendi, P. J. *et al.* Sexual hormones modulate compensatory renal growth and function. *Medicina (B. Aires).* **73**, 513–9 (2013).

30. Lim, S. & Kaldis, P. Cdks, cyclins and CKIs: Roles beyond cell cycle regulation. *Dev.* **140**, 3079–3093 (2013).

31. Saxton, R. A. & Sabatini, D. M. mTOR Signaling in Growth, Metabolism, and Disease. *Cell* **168**, 960–976 (2017).

32. Chen, J.-K., Chen, J., Neilson, E. G. & Harris, R. C. Role of mammalian target of rapamycin signaling in compensatory renal hypertrophy. *J. Am. Soc. Nephrol.* **16**, 1384–1391 (2005).

33. Schmelzle, T. & Hall, M. N. TOR, a Central Controller Review of Cell Growth. *Cell* **103**, 253–262 (2000).

34. Jefferies, H. B. J. *et al.* Rapamycin suppresses 5’TOP mRNA translation through inhibition of p70 s6k represent up to 20% of the total mRNA in the cell. *EMBO J.* **16**, 3693–3704 (1997).

35. Chen, J.-K., Chen, J., Thomas, G., Kozma, S. C. & Harris, R. C. S6 kinase 1 knockout

inhibits uninephrectomy- or diabetes-induced renal hypertrophy. *Am. J. Physiol. Physiol.* **297**, F585–F593 (2009).

36. Xu, J., Chen, J., Dong, Z., Meyuhas, O. & Chen, J.-K. Phosphorylation of ribosomal protein S6 mediates compensatory renal hypertrophy. *Kidney Int* **87**, 543–556 (2015).

37. Garovic, V. D. & August, P. Sex Differences and Renal Protection: Keeping in Touch with Your Feminine Side. *Am J Physiol Ren. Physiol* **133**, 1–9 (2006).

38. Mulroney, S. E. *et al.* Temporal Changes in Insulin-Like Growth Factor I, c-fos, and c-jun Gene Expression during Hyperplastic Kidney Growth in Weanling Rats. *Endocrinology* **137**, 839–845 (1996).

39. Mulroney, S. E. & Pesce, C. Early hyperplastic renal growth after uninephrectomy in adult female rats. *Endocrinology* **141**, 932–937 (2000).

40. Mulroney, S. E., Woda, C., Johnson, M. & Pesce, C. Gender differences in renal growth and function after uninephrectomy in adult rats. *Kidney Int.* **56**, 944–953 (1999).

41. Gafter, U., Ben-Bassat, M. & Levi, J. Castration inhibits glomerular hypertrophy and proteinuria in uninephrectomized male rats. *Eur. J. Clin. Invest.* **20**, 360–365 (1990).

42. Baylis, C. Age-dependent glomerular damage in the rat. Dissociation between glomerular injury and both glomerular hypertension and hypertrophy. Male gender as a primary risk factor. *J. Clin. Invest.* **94**, 1823–1829 (1994).

43. Fine, L. The biology of renal hypertrophy. *Kidney Int.* **29**, 619–634 (1986).

44. Preisig, P. A. What Makes Cells Grow Larger and How Do They Do It? Renal Hypertrophy Revisited. *Exp Nephrol* **7**, 273–283 (1999).

45. Wolf, G. & Neilson, E. G. Angiotensin II as a Renal Growth Factor. *J. Am. Soc. Nephrol* **3**, 1531–1540 (1993).

46. Haramati, A., Lumpkin, M. D. & Mulroney, S. E. Early increase in pulsatile growth hormone release after unilateral nephrectomy in adult rats. *Am. J. Physiol. - Ren. Fluid*

Electrolyte Physiol. **266**, (1994).

47. Domínguez-Calderón, A. *et al.* ZO-2 silencing induces renal hypertrophy through a cell cycle mechanism and the activation of YAP and the mTOR pathway. *Mol. Biol. Cell* **27**, 1581–1595 (2016).

48. Chen, J.-K. *et al.* Phosphatidylinositol 3-kinase signaling determines kidney size. *J. Clin. Invest.* **125**, 2429–2444 (2015).

49. Yoon, M.-S. Vps34 and PLD1 take center stage in nutrient signaling: their dual roles in regulating autophagy. (2015) doi:10.1186/s12964-015-0122-x.

50. Byfield, M. P., Murray, J. T. & Backer, J. M. hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. *J. Biol. Chem.* **280**, 33076–33082 (2005).

51. Liu, S. *et al.* Size uniformity of animal cells is actively maintained by a p38 MAPK-dependent regulation of G1-length. *Elife* **7:e26947**, (2018).

52. Ginzberg, M. B. *et al.* Cell size sensing in animal cells coordinates anabolic growth rates and cell cycle progression to maintain cell size uniformity. *Elife* **7**, e26957 (2018).

53. Clark, J. Z. *et al.* Representation and relative abundance of cell-type selective markers in whole-kidney RNA-Seq data. doi:10.1016/j.kint.2018.11.028.

54. Hayslett, J. P., Kashgarian, M. & Epstein, F. H. Functional correlates of compensatory renal hypertrophy. *J. Clin. Invest.* **47**, 774–799 (1968).

55. Information, C. I. for H. *Annual Statistics on Organ Replacement in Canada: Dialysis, Transplantation and Donation, 2009 to 2018*. (2019).

56. Lentine, K. L. *et al.* KDIGO Clinical Practice Guideline on the Evaluation and Care of Living Kidney Donors. *Transplantation* **101**, S1–S109 (2017).

57. Fattah, H., Layton, A. & Vallon, V. How do Kidneys Adapt to a Deficit or Loss in Nephron Number? *Physiology* **34**, 189–197 (2019).

58. Elsherbiny, H. E. *et al.* Nephron Hypertrophy and Glomerulosclerosis and Their

Association with Kidney Function and Risk Factors among Living Kidney Donors. *Clin J Am Soc Nephrol* **9**, 1892–1902 (2014).

59. Hinchliffe, S. A., Sargent, P. H., Howard, C. V., Chan, Y. F. & Van Velzen, D. Human intrauterine renal growth expressed in absolute number of glomeruli assessed by the disector method and cavalieri principle. *Lab. Investig.* **64**, 777–784 (1991).

60. Fine, L. G. & Norman, J. Cellular Events In Renal Hypertrophy. *Rev. Physiol* **51**, 19–32 (1989).

61. Hartman, H. A., Lai, H. L. & Patterson, L. T. Cessation of renal morphogenesis in mice. *Dev. Biol.* **310**, 379–387 (2007).

62. Douglas-Denton, R., Moritz, K. M., Bertram, J. F. & Marelyn Wintour, E. *Compensatory Renal Growth after Unilateral Nephrectomy in the Ovine Fetus*. https://jasn.asnjournals.org/content/jnephrol/13/2/406.full.pdf (2002).

63. Hartshorne, N., Shepard, T. & Barr, M. Compensatory renal growth in human fetuses with unilateral renal agenesis. *Teratology* **44**, 7–10 (1991).

64. Westland, R., Schreuder, M. F., van Goudoever, J. B., Sanna-Cherchi, S. & van Wijk, J. A. E. Clinical implications of the solitary functioning kidney. *Clin. J. Am. Soc. Nephrol.* **0**, 1–8 (2013).

65. Kajiwara, M. & Masuda, S. Role of mTOR inhibitors in kidney disease. *Int. J. Mol. Sci.* **17**, 1–12 (2016).

66. Foss, A., Line, P. D., Brabrand, K., Midtvedt, K. & Hartmann, A. A prospective study on size and function of paediatric kidneys (<10 years) transplanted to adults. *Nephrol. Dial. Transplant.* **22**, 1738–1742 (2007).

67. Sands, J., Dobbing, J. & Gratrix, C. A. Cell Number And Cell Size: Organ Growth And Development And The Control Of Catch-Up Growth In Rats. *Lancet* **314**, 503–505 (1979).

68. Sakaguchi, M. *et al.* Inhibition of mTOR signaling with rapamycin attenuates renal

hypertrophy in the early diabetic mice. (2005) doi:10.1016/j.bbrc.2005.12.012.

69. Haylor, J. *et al.* JB3, an IGF-I receptor antagonist, inhibits early renal growth in diabetic and uninephrectomized rats. *J. Am. Soc. Nephrol.* **11**, 2027–2035 (2000).

70. Schnell, S. A., Staines, W. A. & Wessendorf, M. W. Reduction of lipofuscin-like autofluorescence in fluorescently labeled tissue. *J. Histochem. Cytochem.* **47**, 719–730 (1999).

71. Sun, Y. *et al.* Sudan black B reduces autofluorescence in murine renal tissue. *Arch. Pathol. Lab. Med.* **135**, 1335–1342 (2011).

72. Baschong, W., Suetterlin, R., Hubert, R. & Muller, L. M. E. Control of Autofluorescence of Archival Formaldehyde-fixed, Paraffin-embedded Tissue in Confocal Laser Scanning Microscopy (CLSM). *J. Histochem. Cytochem.* **49**, 1565–1571 (2001).

73. Jorgensen, P. *et al.* The Size of the Nucleus Increases as Yeast Cells Grow. *Mol. Biol. Cell* **18**, 3523–3532 (2007).

74. Levy, D. L. & Heald, R. Nuclear Size Is Regulated by Importin α and Ntf2 in Xenopus. *Cell* **143**, 288–298 (2010).

75. Dai, Q. Y. & Yu, Y. L. Advances of mathematical morphology in image processing. *Kongzhi Lilun Yu Yingyong/Control Theory Appl.* **18**, 478 (2001).

76. Jagannathan, M., Cummings, R. & Yamashita, Y. M. A conserved function for pericentromeric satellite DNA. (2018) doi:10.7554/eLife.34122.001.

77. Kidney - Servier Medical Art. https://smart.servier.com/smart_image/kidney-2/.

78. Mouse - Servier Medical Art. https://smart.servier.com/smart_image/mouse/.

79. Thongprayoon, C., Cheungpasitporn, W. & Kashani, K. Serum creatinine level, a surrogate of muscle mass, predicts mortality in critically ill patients. *J. Thorac. Dis.* **8**, E305–E311 (2016).

80. Ting Liu, X., Dai, C., Xu, J., Li, S. & Chen, J.-K. The expression level of class III

phosphatidylinositol-3 kinase controls the degree of compensatory nephron hypertrophy. *Am J Physiol Ren. Physiol* **318**, 628–638 (2020).

81. Dicker, S. E. & Morris, C. A. Absence of compensatory renal hypertrophy in baboons. *J. Physiol.* **223**, 365–373 (1972).

82. Shehab, A. B. *et al.* Early Changes in Volume and Function of the Remaining Kidney After Unilateral Donor Nephrectomy. *Saudi J Kidney Dis Transpl.* **5**, 474–478 (1994).

83. Taner, T., Iqbal, C. W., Textor, S. C., Stegall, M. D. & Ishitani, M. B. Compensatory hypertrophy of the remaining kidney in medically complex living kidney donors over the long term. *Transplantation* **99**, 555–559 (2015).

84. Funahashi, Y. *et al.* Change in Contralateral Renal Parenchymal Volume 1 Week After Unilateral Nephrectomy. *Urology* **74**, 708–712 (2009).

85. Prassopoulos, P., Gourtsoyiannis, N., Cavouras, D. & Pantelidis, N. CT Evaluation of Compensatory Renal Growth in Relation to Postnephrectomy Time. *Acta radiol.* **33**, 566–568 (1992).

86. Tapson, J. S. *et al.* Compensatory Renal Hypertrophy After Donor Nephrectomy. *Clin. Radiol.* **36**, 307–310 (1985).

87. Sugaya, K. *et al.* Compensatory renal hypertrophy and changes of renal function following nephrectomy. *Hinyokika Kiyo.* **46**, 235–240 (2000).

88. Shirley, D. G. & Walter, S. J. Acute and chronic changes in renal function following unilateral nephrectomy. *Kidney Int.* **40**, 62–68 (1991).

89. Krohn, A. G., Ogden, D. A. & Holmes, J. H. Renal Function in 29 Healthy Adults Before and After Nephrectomy. *JAMA J. Am. Med. Assoc.* **196**, 322–324 (1966).

90. Takagi, T. *et al.* Compensatory Hypertrophy after Partial and Radical Nephrectomy in Adults. *J. Urol.* **192**, 1612–1619 (2014).

91. Marco Van Londen, X. *et al.* Renal functional reserve capacity before and after living

kidney donation. *Am J Physiol Ren. Physiol* **315**, 1550–1554 (2018).

92. Ter Wee, P. M., Tegzess, A. M. & Donker, A. J. M. Renal reserve filtration capacity before and after kidney donation. *J. Intern. Med.* **228**, 393–399 (1990).

93. Spinelli, A. *et al.* Rationale for the Evaluation of Renal Functional Reserve in Living Kidney Donors and Recipients: A Pilot Study. *Nephron* **135**, 268–276 (2017).

94. Figurek, A., Luyckx, V. A. & Mueller, T. F. A Systematic Review of Renal Functional Reserve in Adult Living Kidney Donors. *Kidney Int. Reports* **5**, 448–458 (2020).

95. Cassidy, M. J. D. & Beck, R. M. Renal Functional Reserve in Live Related Kidney Donors. *Am. J. Kidney Dis.* **11**, 468–472 (1988).

96. Tapson, J. S., Mansy, H., Marshall, S. M., Tisdall, S. R. & Wilkinson, R. Renal Functional Reserve in Kidney Donors. *Q. J. Med.* **60**, 725–732 (1986).

97. Katz, A. I., Gary Toback, F. & Lindheimer, M. The Role of Renal 'Work' in Compensatory Kidney Growth. *YALE J. Biol. Med.* **51**, 331–337 (1978).

Appendices

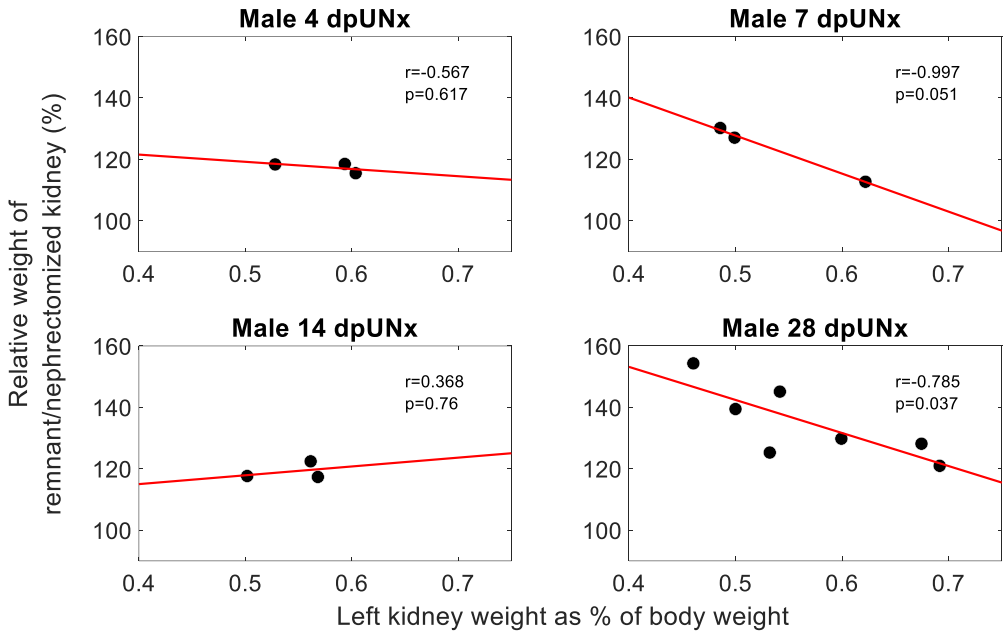

Figure A1. Correlation between initial kidney-to-body weight of removed (left) kidney, and extent of remnant (right) kidney growth, at specific time points within the male cohort.

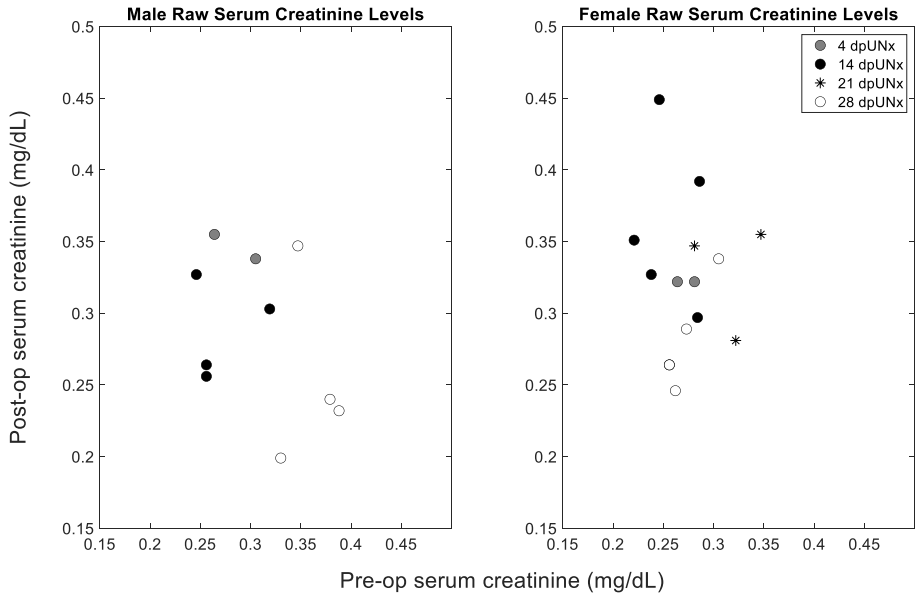

Figure A2. Raw serum creatinine levels in males and females (mg/dL)