Energy depletion by cell proliferation sensitizes the kidney epithelial cells to injury

Pierre Galichon, Morgane Lannoy, Li Li, Justine Serre, Sophie Vandermeersch, David Legouis, M Todd Valerius, Juliette Hadchouel, Joseph V Bonventre

RESEARCH ARTICLE

RUNNING HEAD: Viability of proliferating cells

Energy depletion by cell proliferation sensitizes the kidney epithelial cells to injury.

Authors: Pierre Galichon[1,2,3*], Morgane Lannoy[2], Li Li [1], Justine Serre[2], Sophie Vandermeersch[2], David Legouis[4], M Todd Valerius[1], Juliette Hadchouel[2] and Joseph V Bonventre[1]

[1] Department of Medicine, Harvard Medical School, and Renal Division, Department of Medicine, Brigham and Women's Hospital; Boston, MA, USA,

[2] INSERM UMR_S1155, "Common and Rare and kidney diseases: from Molecular events to Precision Medicine"; Paris, France,

[3] Sorbonne Université, APHP, AP-HP. Sorbonne Université; F-75020 Paris, France,

[4] Laboratory of Nephrology, Department of Medicine and Cell Physiology, Division of Intensive Care, University Hospital of Geneva; Geneva, Switzerland,

Author contribution:

PG: conceptualization, data acquisition, data analysis, drafting and finalization of the manuscript

ML: data acquisition, data analysis, critical revision of the manuscript

JSB: data acquisition, data analysis, critical revision of the manuscript

LL: data acquisition, data analysis, critical revision of the manuscript

SV: data acquisition, data analysis, critical revision of the manuscript

DL: data analysis, critical revision of the manuscript

MTV: data analysis, critical revision of the manuscript

JH: data analysis, drafting and critical revision of the manuscript

JVB: conceptualization, data analysis, critical revision of the manuscript

*Correspondence: Prof Pierre Galichon
Inserm UMR_S1155 ; Tenon Hospital - Research Building
4 rue de la Chine
75020 Paris FRANCE
Phone : +33 1 56 01 76 27 ; email: pierre.galichon@aphp.fr

Word count

abstract: 196 ; text: 4422

ABSTRACT

Acute kidney injury activates both proliferative and anti-proliferative pathways, the consequences of which are not fully elucidated. If an initial proliferation of the renal epithelium is necessary for the successful repair, the persistence of proliferation markers is associated with the occurrence of chronic kidney disease. We hypothesized that proliferation in stress conditions impacts cell viability and renal outcomes. We found that proliferation is associated with cell death after various stresses in kidney cells. In vitro, the ATP/ADP ratio oscillates reproducibly throughout the cell cycle, and cell proliferation is associated with a decreased intracellular ATP/ADP ratio. In vivo, transcriptomic data from transplanted kidneys revealed that proliferation was strongly associated with a decrease in the expression of the mitochondria-encoded genes of the oxidative phosphorylation pathway, but not of the nuclear-encoded ones. These observations suggest that mitochondrial function is a limiting factor for energy production in proliferative kidney cells after injury. The association of increased proliferation and decreased mitochondrial function was indeed associated with poor renal outcomes. In summary, proliferation is an energy demanding process impairing the cellular ability to cope with an injury, highlighting proliferative repair and metabolic recovery as indispensable and interdependent features for successful kidney repair.

NEW & NOTEWORTHY

ATP depletion is a hallmark of acute kidney injury. Proliferation is instrumental to kidney repair. We show that ATP levels vary during the cell-cycle and that proliferation sensitizes renal epithelial cells to superimposed injuries *in vitro*. More proliferation and less energy production by the mitochondria are associated with adverse outcomes in injured kidney allografts. This suggests that controlling the timing of kidney repair might be beneficial to mitigate the extend of acute kidney injury.

Keywords: acute kidney injury ; cell proliferation ; energy metabolism

INTRODUCTION

Cell proliferation is essential for any life form. As the multiplication of organized structures, proliferation uses energy to decrease entropy. In his book "whant is Life?", Erwin Schrodinger coined the term "negentropy" to define Life.(44). Thus, proliferation requires energy. It is the cornerstone not only of development in multicellular organisms but also of several processes of organ repair. In the presence of various stresses, however, anti-proliferative pathways are triggered, suggesting that uncontrolled proliferation can be deleterious, even in non-cancerous diseases (12, 34). This is the case, for example, in the context of acute kidney injury (AKI). If it is known that cell proliferation is indispensable for the organ repair (37), it was also shown that alleviating cell cycle blockade by P21 or P53 aggravates the course of toxic or ischemic AKI (9, 27, 32, 33, 37, 49). These observations demonstrate the essential role of cell proliferation control in pathological situations.

A high cellular energy level is generally accepted as a marker of viability (6) and is considered a prerequisite for cell proliferation (10, 60). Conversely, a persistent decrease in the level of intracellular ATP is associated with cell injury and ultimately death, both in tumor and non-tumor cells (26, 57). It is generally assumed that the ATP level is regulated prior to changes in proliferation (15, 31, 45, 47). Indeed, a low ATP level stimulates AMPK, which induces cell cycle arrest through P27, P53 and P21 activation in various organs (16). By contrast, in cancer, a low ATP level is considered to allow cell proliferation by stimulating aerobic glycolysis and the Warburg effect (30). Although the effect of ATP variations on proliferation has been studied, the reverse mechanism, i.e. the effect of cell cycle progression on the cellular energy status, is yet unexplored. Since energy depletion leads to cell death (19, 57), the effect of proliferation on the energy level of the cell might critically impact cell viability. In particular, we hypothesized that cell proliferation in the context of energy depletion, such as an ischemic AKI, could be detrimental. We investigated the relationship between proliferation and viability in human renal epithelial cells. By quantifying the ATP/ADP ratio in live cells using a single cell approach, we studied the effect of proliferation on the cellular viability and characterized the pattern of variation of the intracellular energy level throughout the cell cycle. Lastly, we investigated how the balance between cell proliferation and energy production influences the outcomes of kidney allografts. We thus identified the downregulation of mitochondrial-encoded genes of the oxidative phosphorylation pathway in proliferating epithelial cells as a limiting factor for successful recovery after an episode of acute kidney injury.

MATERIALS AND METHODS

Reagents are referenced in Supplemental Table S4.

Cell cultures

HK2 (ATCC® CRL-2190™) cells are immortalized male adult human renal proximal tubular cells cultured at 37°C in DMEM with 10% fetal bovine serum. The live microscopy experiments were conducted in Leibovitz's L-15 medium with no phenol red (Fisher, #21083027). Pharmaceutical inhibitors were purchased from Selleckchem: tenovin-1 (#S8000), pifithrin-alpha (#S2929), Rigosertib (#S1362) and KU-55933 (#S1092). Cell count was performed on live microscopy data using the Fiji Trackmate plugin (53).

Western Blot

Proteins were extracted from HK2 cells with RIPA buffer containing protease and phosphatase inhibitor cocktails. Total protein concentration was measured using the BCA assay (Pierce). Samples were fractionated by SDS-PAGE under reducing conditions and then transferred to a nitrocellulose membrane. Membranes were incubated in TBST (TBS 1X with 0.1% Tween) with the appropriate primary antibodies: anti-ATP5a (Abcam ab14748), anti-MTCO1 (Abcam ab203912) and anti-HIF1a (Novus NB100-449). HSC70 (Abcam, ab51052, 1:500) was used as loading control. ImageJ software (National Institutes of Health) was used for quantification.

Viability experiments

Cells were grown in DMEM with 10% fetal bovine serum in the presence or absence of puromycin (2µg/mL). Energy depletion was achieved in cells grown in Leibovitz's L-15 medium without glucose supplementation and hypoxia obtained by applying a 100% N2 atmosphere (ref 26700 from Air products) in an airtight chamber (ref 27310 and 27311 from Stemcell) during 24h. The control cells were grown in L-15 medium with 4.5 g/dL glucose supplementation under ambient atmosphere. The expression of mitochondrial proteins ATP5A and MTCO1 and the induction of Hypoxia inducible factor 1 in hypoxic conditions were verified by western blot (Fig S1). Flow cytometry analysis for assessment of dead cells was performed with the fixable viability dye Viobility 405/452 Fixable Dye (Miltenyi Biotec®).

Mitochondrial potential assay

Cells were grown for 24 hours in L-15 medium with 1µmol/L JC-1 (ref 65-0851-38 from Thermo Fisher) under control conditions or energy depleting conditions (as described above). Mitochondria were identified by the presence of JC-1 aggregates fluorescing in red, and we assessed the mitochondrial potential by quantifying the red/green fluorescence ratio in mitochondria (reflecting aggregates/monomers), using Fiji(42).

ATP/ADP ratio measurements

ATP and ADP measurements were performed with the ApoSENSOR ADP/ATP ratio assay (Enzo Life Sciences), in accordance with the manufacturer's instructions.

PercevalHR was used to study the ATP/ADP ratio in live individual cells in real-time experiments, spanning multiple cell cycles. Using lentiviral transformation, we generated proximal tubular cells with stable expression of PercevalHR as well as pHRed, in order to adjust the PercevalHR signal on pH variations(51). ATP/ADP ratio and pH were assessed by dual signal acquisition for PercevalHR (ex 436/495; em 540) and pHRed (ex 436/550; em 640), followed by ratiometric normalization using the Ratio Plus plugin in Fiji(42). PercevalHR (ATP/ADP ratio) was further corrected for pH by normalization on pHRed (51), using the Ratio Plus plugin.

Bioinformatic analysis

ATP/ADP ratio. The Fiji Trackmate plugin was used to monitor single-cell ATP/ADP ratio over time, and to determine the time of cytokinesis for each cell(53). Briefly, every cell was assigned to a single trajectory at a specific timepoint, with a corresponding ATP/ADP value. The times of cytokinesis were noted for each cell trajectory. ATP/ADP values were represented on the y-axis as mean+/-standard error, and time was represented on the x-axis in hours from either the start of the experiment or from the time of cytokinesis. The full script is available as Supplemental data.

Computation of proliferation index, overall OXPHOS index, nuclear and mitochondrial OXPHOS index in bulk RNAseq data. We then used available RNA-seq data from kidney allograft biopsies in order to test the relationship between proliferation and energy metabolism *in vivo* in 163 kidney allograft biopsies from 42 patients (7). The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the 'Declaration of Istanbul on Organ Trafficking and Transplant Tourism'. We applied a previously published and validated method to compute a proliferation index as the median value of a list of proliferation associated genes (56). Similarly, we computed the overall OXPHOS index as the median value of the genes listed in the human OXPHOS pathway of the (Kyoto Encyclopedia of Genes and Genomes database(22). We performed the same computation on the subset of mitochondria-encoded genes to obtain the mitochondrial OXPHOS index. We used median value by analogy to the previously determined proliferation index, and because it has the theoretical advantage over mean values of mitigating the effect of extreme values in only a small subset of genes within the predefined list. However mean values correlated closely ($r \geq 0.8$) with median values suggesting non-skewed expression data for the studied genes.

Computation of proliferation index, overall OXPHOS index, nuclear and mitochondrial OXPHOS index in single-cell RNAseq data. We used the human rejecting kidney data set (61) and the Seurat package from R (14, 40) to identify the different cell types (Fig. 5). We computed the proliferation index, mitochondrial encoded OXPHOS and nuclear encoded OXPHOS in the epithelial cells as described for bulk RNA-seq in the previous paragraph. Because the single-cell depth of sequencing is much lower than for bulk RNA-seq, we used the sum of copies within the lists of genes instead of the median value.

Pathway analysis of proliferation associated genes. We computed Kendall's tau rank correlation coefficient to evaluate the correlation of every gene expression value with the proliferation index. The correlations' p-values and coefficient were then processed with the LrPath web-based software allowing a logistic regression pathway analysis to identify the gene sets enriched in genes with proliferation-correlated expression levels. Three prespecified genesets for energy-producing pathways were analyzed: "OXidative PHOSphorylation", "Tricarboxylic Acid Cycle", and "glycolysis". P-values and Odds Ratios were computed as indicators of the significance and the magnitude of the enrichment of these gene sets in genes with proliferation-correlated expression values.

Statistical analysis

Statistical analyses were conducted using JMP 11 (SAS). Log-transformation was performed to obtain normal distribution, when necessary. Proportions, 95% confidence interval and p-values were computed using the exact binomial test. Correlations were evaluated using Kendall's tau rank correlation coefficient. P-values were considered significant when <0.05. Comparison between groups were considered significant when the p-value was <0.05 using a Kruskall-Wallis non-parametrical test, and secondary comparisons between pairs were considered significant when the p-value was <0.05 by Wilcoxon signed-rank test. Differences in ATP/ADP changes between treatment groups over time were assessed by analysis of variance using a standard least square model.

RESULTS

Proliferating cells are more sensitive to an injury than non-proliferating ones

We subjected human renal epithelial cells (HK2 cell line) to a metabolic stress and compared their consequences in conditions of stimulated or inhibited proliferation. HK2 cells express markers of mitochondrial ATP production but are also known to be glycolytic. Thus we caused energy depletion by oxygen and glucose deprivation. Oxygen deprivation was confirmed by HIF1a induction. Although the expression of OXPHOS proteins remained stable in this model, it caused a decrease in mitochondrial potential (Figure 1a-b and S1A), mimicking the mitochondrial dysfunction observed in the injured renal tubules *in vivo* (20, 50). Proliferation was either enhanced by pifithrin-□ (a P53 antagonist promoting G1/S and G2/M transition) or inhibited by tenovin-1 (a P53 agonist inhibiting the G1/S transition) (Fig. 1c-d and S1B). . Pifithrin-□□alone or tenovin-1 alone were not toxic, as they did not cause cell death in the absence of puromycin. We observed that the stimulation of cell proliferation promoted the death of energy-depleted cells, whereas inhibition of cell proliferation was protective (Fig. 1c). Similar results were obtained when cells were exposed to puromycin, a well-characterized toxicant acting through inhibition of protein synthesis (Fig1 d).

Taken together, these data show that proliferation enhances susceptibility to noxious stimuli and/or impairs the ability of cells to survive an injury. Given that proliferation requires energy and that a decreased cellular energy level results in cell death, we hypothesized that the depletion of cellular energy in proliferating cells could be responsible for this enhanced susceptibility to toxic influences.

The ATP/ADP ratio decreases when cells proliferate

Adenosine triphosphate (ATP) is the main energy source for intracellular processes, through its cleavage into adenosine diphosphate (ADP) and hydrogen phosphate (4, 55). The intracellular

ATP/ADP or ATP/AMP ratio is a more reliable marker of the cellular energy status than the absolute ATP concentration (8, 30). PercevalHR is a genetically encoded markers of the ATP/ADP ratio, providing a reliable indicator of the intracellular energy status (2, 51). To monitor the single cell-ATP/ADP ratio over time in proliferating epithelial cells, we generated human renal tubular cells (HK2 cell line) stably expressing PercevalHR (Fig. S2A). We first validated the quantification of the ATP/ADP ratio (ATP/ADP[Perceval]) by an independent technique based on the measurement of luciferin/luciferase bioluminescence (ATP/ADP[Luciferin]). The total ATP/ADP[Perceval] signal correlated well with the ATP/ADP[Luciferin] ($r=0.96$, $p=0.0001$) with an intraclass correlation of 0.87. We observed the expected decrease of ATP/ADP[Perceval] after chemical energy depletion with blockage of glycolysis and oxidative metabolism using 2-deoxyglucose (2 DG) and sodium azide (NaN_3) (Fig. S2B-C).

We then quantitated the single cell ATP/ADP[Perceval]. The mean ATP/ADP values over time were reproducible between wells under basal conditions or during energy depletion (Fig. S2C, middle panel). However, this ratio was highly variable among proliferating cells in the same well and at the same time (Fig. S2C, right panel). This observation suggests that the ATP/ADP ratio is a dynamic parameter, undergoing variations that are at least partly independent of the extracellular environment. We hypothesized that the ATP/ADP ratio could be correlated with the cell cycle stage. To test this hypothesis, PercevalHR-expressing epithelial cells were plated at various densities to achieve different levels of contact inhibition of proliferation (Fig. 2A, left panel). The intracellular ATP/ADP ratio increased when the cells were more confluent. To rule out a direct effect of the confluency, we cultured the cells at the same densities in the presence of rigosertib, a potent polo-like-kinase 1 (PLK1) inhibitor inducing G2/M arrest. Non-proliferating cells showed a significant increase in the ATP/ADP ratio, regardless of their degree of confluence. Conversely, performing a scratch assay on confluent cells

induced the proliferation and migration of the cells, which then displayed a low ATP/ADP ratio (Fig. 2A, right panel). Rigosertib increased the cellular ATP/ADP ratio while inhibiting wound closure.

To exclude a possible cell cycle-independent effect of rigosertib, a panel of pharmaceutical compounds was used to study the changes in the ATP/ADP ratio over time when the cell cycle is perturbed (Fig. 2B-C). Tenovin-1 and Rigosertib were used to inhibit the cell cycle during the G1/S or G2/M transition, respectively. Conversely, pifithrin-α and KU-55933 (an ATM inhibitor promoting G2/M transition) were used to stimulate cell proliferation. Single-cell monitoring demonstrated that the ATP/ADP ratio increased progressively over time when cell proliferation was inhibited whereas it decreased when cell proliferation was stimulated (Fig. 2C).

Cell cycle and proliferation-dependent variations in the intracellular ATP/ADP ratio

We then followed the variation of single-cell ATP/ADP ratio throughout the cell cycle. We thus observed that the ratio was maximal around cytokinesis (Fig. 3A, left panel). This analysis was performed using single cell tracking in non-synchronized cells, ruling out an effect of time or an effect of the extracellular environment and suggesting a direct association with the cell cycle stage. Variations of the ATP/ADP ratio before and after cytokinesis follow a systematic pattern (Fig. 3A, right panel): after a gradual increase, the ratio peaks at mitosis before dropping dramatically. This pattern was highly reproducible, including in cells exposed to pharmaceutical inhibition or stimulation of the cell cycle (Fig. 3B). The cell cycle inhibitor tenovin-1 increased the mean ATP/ADP ratio over time compared to the cell cycle facilitator pifithrin-α (Fig. 3B). Thus, the intracellular energy level, evaluated by the ATP/ADP ratio, is directly influenced by the cell cycle.

In order to study the variations of the ATP/ADP ratio during successive cell cycles within the same cell, the trajectories of this ratio were evaluated in cells undergoing division twice in the same live-imaging experiment. The cells had the same pattern of ATP/ADP variations for each of the two

253 successive mitoses. After a first mitosis, cells treated with tenovin-1 reached the second mitosis with a
254 significantly higher ATP/ADP ratio than the first mitosis, compared to cells treated with pifithrin-α, in
255 which such an increment did not occur (Fig. 3B). We conclude that cell cycle inhibition increases the
256 ATP/ADP ratio in cells independently of the cell cycle stage, in a time-dependent manner: the slower
257 the cell cycles, the more positive the cellular energy balance.

258 ## Association of a mitochondrial defect with proliferation in injured kidney allografts

259 The proliferation of tubular cells is indispensable for the repair of the kidney after an acute
260 injury. However, the inhibition of anti-proliferative factors such as P21 and P53 (35, 38, 49, 52) leads
261 to the aggravation of the kidney lesions and loss of function. Therefore, to test the relevance of our
262 findings in proliferating cells *in vivo*, we performed an unbiased gene enrichment analysis to identify
263 pathways associated with cell proliferation in injured kidneys by analyzing the RNA-sequencing data
264 from kidney allografts before and after transplantation by Cippa and colleagues (7). Indeed, all
265 allografts undergo an episode of acute ischemic injury between the organ procurement and the
266 transplantation into the recipient.

267 The level of proliferation was estimated using a previously published and validated proliferation
268 index, which corresponds to the median value of a list of proliferation associated genes (56). We found
269 that the KEGG items that are the most significantly associated with cell proliferation are 'Metabolic
270 pathways' (FDR: 2.87e-39) and 'Oxidative phosphorylation (OXPHOS)' (FDR: 2.94e-20). Proliferation
271 and the expression level of OXPHOS genes of the pathway were negatively correlated in kidney
272 allografts immediately after transplantation (Fig. 4A and Tables S1 and S2). A further analysis showed
273 that this decrease in OXPHOS genes expression with proliferation was in fact due to a profound
274 downregulation of the expression of mitochondria-encoded genes. In contrast, the transcription of the
275 nucleus-encoded OXPHOS genes was moderately upregulated with proliferation (Fig. 4B-C,Fig. S3

Table S3), suggesting that mitochondrial failure is characteristic of proliferating epithelial cells in the acutely injured kidney.

In order to verify this hypothesis at the single-cell level, we used single-cell RNA-seq data from rejecting human kidneys (61) to analyze the relationship between proliferation and OXPHOS related genes specifically in renal epithelial cells, identified by unsupervised analysis and verified by the expression of tubular-specific markers (Fig. 5A and S4). Our analysis confirmed that proliferation was negatively correlated with mitochondria-encoded OXPHOS genes, but not with nuclear-encoded OXPHOS genes (Fig. 5B).

Association of proliferation, mitochondrial status and outcomes.

The study reported by Cippà and colleagues was performed on biopsies performed on the same kidney allografts before, just after, 3 and 12 months after transplantation (7). The first two series of samples constituted the 'early' group, while the last two constituted the 'late' group. Furthermore, three types of evolution were distinguished within the late biopsies: recovery, transition towards chronic kidney disease and established chronic kidney disease (CKD) (7). As shown in Fig. S5A, the computed proliferation index was higher in the kidney biopsies performed 3 or 12 months after transplantation than in biopsies performed immediately before or after transplantation. Moreover, a higher proliferation index was associated with the progression towards CKD (Fig. S5B). OXPHOS transcripts were found to be downregulated in the nucleus only during the acute phase of injury (reperfusion), whereas they were downregulated in the mitochondria during the transition towards chronic kidney disease (Fig. S5D, E, G,H).

We then studied the relationship between proliferation, the regulation of OXPHOS genes and the long-term clinical outcomes, i.e. the glomerular filtration rate (GFR) and incidence of fibrosis (Fig. S5C, F, I and Fig. S6). We found that the timing dramatically modified the relationship between proliferation, the OXPHOS level and the incidence of fibrosis in the kidney. Although there was no

correlation between the proliferation index and fibrosis in the early biopsies, a low mitochondrial OXPHOS index in the early biopsies (immediately before and after transplantation) was associated with a lesser incidence of fibrosis in the kidney between 3 and 12 months after transplantation (Fig. S5I). In contrast, a decrease in the mitochondrial OXPHOS index and an increase in the proliferation index in the late biopsies (3 and 12 months after transplantation) were associated with an increased fibrosis and a decreased GFR at 12 months (Fig. S5I and Fig. S6).

Taken together, these transcriptomic analyses suggest that the decrease of mitochondria-encoded genes represents a limiting factor for energy production by OXPHOS required for the adaptive proliferative repair of the kidney allografts after injury.

DISCUSSION

Here we show that proliferating cells are more sensitive to injury than non-proliferating ones, and that the inhibition of proliferation is protective. Proliferation causes a decrease in intracellular energy with superimposed energy oscillations depending on the cell-cycle stage. *In vivo*, we found that mitochondria-encoded genes were profoundly downregulated in the proliferating kidney after injury, indicating mitochondrial shutdown. This is in contrast with the upregulation of nucleus-encoded genes of the OXPHOS gene set (Fig. 4c). This dissociated transcriptomic pattern between mitochondrial and nuclear-encoded genes suggests that the cellular energy production is limited by mitochondrial damage. Finally, we found that the persistence of proliferation associated with the downregulation of mitochondrial OXPHOS genes is a pattern associated with an unfavorable evolution towards chronic kidney disease.

Proliferation and energy metabolism are interdependent

The fact that a low intracellular ATP induces molecular pathways downstream of AMPK to control proliferation suggests the presence of a regulatory feedback to mitigate the decrease in intracellular ATP levels caused by increased energy consumption. Such an effect of proliferation on ATP could explain why alleviating cell cycle blockade by P21 or P53 aggravates the course of acute kidney injury caused by an ischemic episode (37), and why highly proliferative tumors frequently undergo spontaneous necrosis (57). The kidney energy turnover is very high. It is thus very sensitive to injury (and especially ischemia), which causes an immediate decrease in energy production, overall energy depletion and epithelial cell death (58). After an injury, cell proliferation is necessary to replace lost cells (18). However, an increase in cell proliferation caused by the inactivation of the anti-proliferative factors P53 and P21 was shown to worsen the lesions caused by an episode of ischemia-reperfusion in the kidney (27, 32, 49). The p53 pathway is important to arrest the cell cycle in case of DNA damage, delaying proliferation until DNA is repaired (24). Short-term blockage of p53 performed after the acute phase of ischemia reperfusion may have beneficial effects on subsequent fibrosis due to a decrease in

the senescence-associated secretory phenotype (63) whereas earlier or continued blockade actually enhances fibrosis (9)

DNA repair is a highly energy-demanding process fueled by mitochondrial ATP generation (39). Thus, proliferation arrest might be an energy-saving mechanism, especially in an injured state. In agreement with this hypothesis, we found an association between increased proliferation, ATP depletion and increased cell death in renal epithelial cells in which *Nupr1* (Nuclear Protein 1) was inactivated (11). Nupr1 is a downstream effector of ATF4, a master regulator of endoplasmic reticulum stress, the activation of which leads to excessive protein synthesis, causing energy depletion and cell death (13). Taken together, these studies suggest that an increase in cell proliferation in conditions of stress might cause critical energy depletion due to enhanced energy needs for both cell maintenance and biosynthetic processes, thus resulting in cell death if a critical energy threshold is not maintained (29). Ultimately if the cells do not die, they may enhance the population of DNA-damaged, cell cycle arrested senescent cells and lead to fibrosis.

Intercellular and intracellular variations in energy metabolism

Our observations change the understanding of energy variations in proliferating cells (Fig. 7). Previous experiments were limited by the fact that timed bulk cell analysis did not allow the study of the ATP/ADP trajectories of single cells within the same extracellular environment. We show by single-cell analysis that a progressive increase in the ATP/ADP ratio in proliferating cells can be observed when confluence and contact inhibition increase, whereas proliferation itself (at the single-cell level) is associated with a decreased ATP/ADP ratio. In addition, we show that within proliferating cells, the ATP/ADP ratio oscillates with the cell cycle. These oscillations in ATP/ADP ratio are reminiscent of cyclic variations of the TCA cycle flux described by Ahn and colleagues (1). This is also in keeping with our *in vivo* transcriptomic data showing that nuclear OXPHOS transcripts are overexpressed in proliferating cells. The intracellular energy level not only varies during the cell cycle but also depends

on the proliferation rate. The live monitoring in single cells demonstrates a transient physiological decrease in ATP/ADP in proliferating cells.

Effect of proliferation on cell viability upon injury

In certain conditions, inhibiting proliferation might be a protective mechanism to prevent lethal energy depletion in epithelial cells or prevent propagation of cells with damaged DNA. This mechanism might be responsible for the protective effect of the extensively studied phenomenon of ischemic preconditioning (3, 5, 28), where a mild and transient ischemia triggers cytoprotective pathways concomitant with transient cell cycle arrest (35, 41). Among these pathways, p53, p21 and AMPK were shown to protect the kidneys from the early lesions induced by ischemia-reperfusion injury while suppressing cell proliferation (21, 34).

However, cell proliferation is absolutely necessary for vital processes. Following a renal epithelial injury, epithelial proliferation is known to be instrumental to organ repair (18). Proliferation demands a high energetic input, and oxidative phosphorylation is the most efficient pathway for energy production. In keeping with that, we found that proliferation is associated with increased expression of nucleus-encoded OXPHOS genes in injured kidneys. Kidneys transitioning to fibrotic chronic kidney disease display a dissociated transcriptomic pattern: high proliferation index with maintained expression of nucleus-encoded OXPHOS genes but decreased expression of mitochondria-encoded OXPHOS genes. This mitochondrial imbalance is known to be deleterious (48). In vitro, hypoxia and glucose deprivation did decrease mitochondrial potential, and caused cell death in proliferating cells, without causing mitonuclear imbalance, suggesting proliferating cells are sensitive to mitochondrial dysfunction in general (Figure S1). This is in keeping with recent studies showing that maintaining oxidative phosphorylation during and after acute kidney injury (AKI) is protective (23, 36, 54). Other studies also suggested that energy-parsimonious approaches might mitigate renal damage at the early stage of acute kidney injury. For example, the stabilization of the hypoxia inducible factor (HIF) shifts

383 the energy metabolism to anaerobic glycolysis, inhibits cell proliferation (12, 17, 25) and protects
384 against ischemic acute kidney injury (43, 46, 59).

385 ### Proliferation as a therapeutic target in kidney diseases

386 We identified kidney epithelial proliferation as a state of energy crisis with increased sensitivity to cell
387 death. However, epithelial cell proliferation is necessary for kidney repair. Thus, an interesting strategy
388 to mitigate necrosis would be to delay proliferative repair in kidneys with ongoing injury, and allow
389 proliferation after cessation of the injury. However, identifying the optimal time frame for the
390 inhibition of proliferation after kidney injury in individual patients will necessitate the implementation
391 of dynamic biomarkers of acute kidney injury. In this perspective, the cell cycle arrest markers IGFB7
392 and TIMP2 are clinically available urinary markers that might help to refine the different stages of
393 injury and repair after acute kidney injury (62). Urinary quinolinate/tryptophan ratio is another
394 emerging non-invasive marker allowing to pinpoint the mitochondrial defect during acute kidney injury
395 (36).

396 Targeting the cell cycle to orchestrate kidney repair is an attractive option, but raises the concern of
397 extrarenal side effects, as proliferation is both necessary for the self-renewal of certain tissues like gut
398 or blood cells, and also instrumental in the development of cancers. Thus, targeting proliferation in a
399 time and tissue specific manner would be an advantageous strategy to improve renal outcomes while
400 minimizing the risk of side effects.

401

402 ## PERSPECTIVES AND SIGNIFICANCE

403 Proliferation is an energy demanding process impairing the cellular ability to cope with a toxic or
404 ischemic injury. We postulate that interventions to mitigate proliferation and restore energy production
405 can enhance cell survival and organ recovery (Fig. 7).

406

SUPPLEMENTAL MATERIAL:
https://doi.org/10.6084/m9.figshare.24504028

Fig. S1. A. Expression of HIF1alpha, ATP5A and MTCO1. **B.** Proliferation in cells incubated with vehicle, Pifithrin alpha or tenovin-1, represented as relative cell count per field.

Fig. S2. Monitoring of intracellular ATP/ADP ratio.

Fig. S3. Transcriptomic signature associated with proliferation at different time points of kidney transplantation.

Fig. S4. Proliferation index and renal epithelial cell differentiation markers in single cells from a rejecting human kidney.

Fig. S5. Association of proliferation index and OXPHOS with chronic kidney disease progression.

Fig. S6. Association of the proliferation index and the OXPHOS indexes with long term outcomes.

Table S1. Association of proliferation-associated genes with energy metabolism pathways from the KEGG encyclopedia.

Table S2. Metabolic pathways and genes associated with proliferation in injured kidneys (reperfusion).

Table S4. Key resources.

Data File S1. Fiji Jython script for single cell ratiometric analysis

Movie S1. Live PercevalHR video: scratch assay.

Movie S2. Live PercevalHR video: effect of various cell-cycle acting molecules.

DISCLOSURE:

Authors declare that they have no competing interests.

GRANTS:

Institut National de la Santé et de la Recherche Médicale ATIP Avenir program (PG)
Monahan Foundation (PG)
Fondation pour la Recherche Médicale (PG)
Groupe Pasteur Mutualité (PG)
Société Francophone de Transplantation (PG)
Société Francophone de Néphrologie, Dialyse et Transplantation (PG)
Arthur Sachs fellowship (PG)
Philippe Foundation (PG)
Fulbright Scholarship (PG)
Fondation de l'Avenir (PG)
National Institute of Health/National Institute of Diabetes and Digestive and Kidney Diseases 2R01DK072381 (JVB)
National Institute of Health/ National Institute of Diabetes and Digestive and Kidney Diseases R37DK039773(JVB)
National Institute of Health/ National Institute of Diabetes and Digestive and Kidney Diseases UH3 TR002155 (JVB)

REFERENCES

1. **Ahn E, Kumar P, Mukha D, Tzur A, Shlomi T.** Temporal fluxomics reveals oscillations in TCA cycle flux throughout the mammalian cell cycle. *Mol Syst Biol* 13:953,2017. doi: 10.15252/msb.20177763
2. **Berg J, Hung YP, Yellen G.** A genetically encoded fluorescent reporter of ATP:ADP ratio. *Nat Methods* 6:161-166,2009. doi: 10.1038/nmeth.1288
3. **Bon D, Chatauret N, Giraud S, Thuillier R, Favreau F, Hauet T.** New strategies to optimize kidney recovery and preservation in transplantation. *Nat Rev Nephrol* 8:339-347,2012. doi: 10.1038/nrneph.2012.83
4. **Bonora M, Patergnani S, Rimessi A, De Marchi E, Suski JM, Bononi A, Giorgi C, Marchi S, Missiroli S, Poletti F, Wieckowski MR, Pinton P.** ATP synthesis and storage. *Purinergic Signal* 8:343-357,2012. doi: 10.1007/s11302-012-9305-8
5. **Bonventre JV.** Kidney ischemic preconditioning. *Curr Opin Nephrol Hypertens* 11:43-48,2002.
6. **Chan GK, Kleinheinz TL, Peterson D, Moffat JG.** A simple high-content cell cycle assay reveals frequent discrepancies between cell number and ATP and MTS proliferation assays. *PLoS One* 8:e63583,2013. doi: 10.1371/journal.pone.0063583
7. **Cippà PE, Sun B, Liu J, Chen L, Naesens M, McMahon AP.** Transcriptional trajectories of human kidney injury progression. *JCI Insight* 32018. doi: 10.1172/jci.insight.123151
8. **Crouch SP, Kozlowski R, Slater KJ, Fletcher J.** The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J Immunol Methods* 160:81-88,1993. doi: 10.1016/0022-1759(93)90011-u
9. **Dagher PC, Mai EM, Hato T, Lee SY, Anderson MD, Karozos SC, Mang HE, Knipe NL, Plotkin Z, Sutton TA.** The p53 inhibitor pifithrin-α can stimulate fibrosis in a rat model of ischemic acute kidney injury. *Am J Physiol Renal Physiol* 302:F284-91,2012. doi: 10.1152/ajprenal.00317.2011
10. **Fu XF, Yao K, Du X, Li Y, Yang XY, Yu M, Li MZ, Cui QH.** PGC-1α regulates the cell cycle through ATP and ROS in CH1 cells. *J Zhejiang Univ Sci B* 17:136-146,2016. doi: 10.1631/jzus.B1500158
11. **Galichon P, Bataille A, Vandermeersch S, Wetzstein M, Xu-Dubois YC, Legouis D, Hertig A, Buob D, Placier S, Bigé N, Lefevre G, Jouanneau C, Martin C, Iovanna JL, Rondeau E.** Stress Response Gene Nupr1 Alleviates Cyclosporin A Nephrotoxicity In Vivo. *J Am Soc Nephrol* 28:545-556,2017. doi: 10.1681/ASN.2015080936
12. **Gordan JD, Thompson CB, Simon MC.** HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. *Cancer Cell* 12:108-113,2007. doi: 10.1016/j.ccr.2007.07.006
13. **Han J, Back SH, Hur J, Lin YH, Gildersleeve R, Shan J, Yuan CL, Krokowski D, Wang S, Hatzoglou M, Kilberg MS, Sartor MA, Kaufman RJ.** ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat Cell Biol* 15:481-490,2013. doi: 10.1038/ncb2738
14. **Hao Y, Hao S, Andersen-Nissen E, Mauck WM, Zheng S, Butler A, Lee MJ, Wilk AJ, Darby C, Zagar M, Hoffman P, Stoeckius M, Papalexi E, Mimitou EP, Jain J, Srivastava A, Stuart T, Fleming LB, Yeung B, Rogers AJ, McElrath JM, Blish CA, Gottardo R, Smibert P, Satija R.** Integrated analysis of multimodal single-cell data. *bioRxiv* 2020.10.12.335331,2020. doi: 10.1101/2020.10.12.335331
15. **Hardie DG.** AMP-activated protein kinase--an energy sensor that regulates all aspects of cell function. *Genes & Development* 25:1895-1908,2011. doi: 10.1101/gad.17420111
16. **Hardie DG, Ross FA, Hawley SA.** AMP-activated protein kinase: a target for drugs both ancient

and modern. *Chem Biol* 19:1222-1236,2012. doi: 10.1016/j.chembiol.2012.08.019
17. **Hubbi ME, Semenza GL.** Regulation of cell proliferation by hypoxia-inducible factors. *Am J Physiol Cell Physiol* 309:C775-82,2015. doi: 10.1152/ajpcell.00279.2015
18. **Humphreys BD, Valerius MT, Kobayashi A, Mugford JW, Soeung S, Duffield JS, McMahon AP, Bonventre JV.** Intrinsic Epithelial Cells Repair the Kidney after Injury. *Cell Stem Cell* 2:284-291,2008. doi: 10.1016/j.stem.2008.01.014
19. **Izyumov DS, Avetisyan AV, Pletjushkina OY, Sakharov DV, Wirtz KW, Chernyak BV, Skulachev VP.** Wages of fear": transient threefold decrease in intracellular ATP level imposes apoptosis. *Biochim Biophys Acta* 1658:141-147,2004. doi: 10.1016/j.bbabio.2004.05.007
20. **Jiang M, Bai M, Lei J, Xie Y, Xu S, Jia Z, Zhang A.** Mitochondrial dysfunction and the AKI-to-CKD transition. *Am J Physiol Renal Physiol* 319:F1105-F1116,2020. doi: 10.1152/ajprenal.00285.2020
21. **Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, Birnbaum MJ, Thompson CB.** AMP-Activated Protein Kinase Induces a p53-Dependent Metabolic Checkpoint. *Molecular Cell* 18:283-293,2005. doi: 10.1016/j.molcel.2005.03.027
22. **Kanehisa M, Goto S.** KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28:27-30,2000. doi: 10.1093/nar/28.1.27
23. **Kang HM, Ahn SH, Choi P, Ko YA, Han SH, Chinga F, Park AS, Tao J, Sharma K, Pullman J, Bottinger EP, Goldberg IJ, Susztak K.** Defective fatty acid oxidation in renal tubular epithelial cells has a key role in kidney fibrosis development. *Nat Med* 21:37-46,2015. doi: 10.1038/nm.3762
24. **Kishi S, Brooks CR, Taguchi K, Ichimura T, Mori Y, Akinfolarin A, Gupta N, Galichon P, Elias BC, Suzuki T, Wang Q, Gewin L, Morizane R, Bonventre JV.** Proximal tubule ATR regulates DNA repair to prevent maladaptive renal injury responses. *J Clin Invest* 129:4797-4816,2019. doi: 10.1172/JCI122313
25. **Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, Huang LE.** HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. *EMBO J* 23:1949-1956,2004. doi: 10.1038/sj.emboj.7600196
26. **Lelli JL, Becks LL, Dabrowska MI, Hinshaw DB.** ATP converts necrosis to apoptosis in oxidant-injured endothelial cells. *Free Radic Biol Med* 25:694-702,1998. doi: 10.1016/s0891-5849(98)00107-5
27. **Li Y, Liu J, Li W, Brown A, Baddoo M, Li M, Carroll T, Oxburgh L, Feng Y, Saifudeen Z.** p53 enables metabolic fitness and self-renewal of nephron progenitor cells. *Development* 142:1228-1241,2015. doi: 10.1242/dev.111617
28. **Lieberthal W, Tang M, Lusco M, Abate M, Levine JS.** Preconditioning mice with activators of AMPK ameliorates ischemic acute kidney injury in vivo. *Am J Physiol Renal Physiol* 311:F731-F739,2016. doi: 10.1152/ajprenal.00541.2015
29. **Lunt SY, Vander Heiden MG.** Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol* 27:441-464,2011. doi: 10.1146/annurev-cellbio-092910-154237
30. **Maldonado EN, Lemasters JJ.** ATP/ADP ratio, the missed connection between mitochondria and the Warburg effect. *Mitochondrion* 19:78-84,2014. doi: 10.1016/j.mito.2014.09.002
31. **Marcussen M, Larsen PJ.** Cell cycle-dependent regulation of cellular ATP concentration, and depolymerization of the interphase microtubular network induced by elevated cellular ATP concentration in whole fibroblasts. *Cell Motil Cytoskeleton* 35:94-99,1996. doi: 10.1002/(SICI)1097-0169(1996)35:2<94::AID-CM2>3.0.CO;2-I
32. **Megyesi J, Andrade L, Vieira JM, Safirstein RL, Price PM.** Positive effect of the induction of p21WAF1/CIP1 on the course of ischemic acute renal failure. *Kidney Int* 60:2164-2172,2001. doi: 10.1046/j.1523-1755.2001.00044.x

33. **Megyesi J, Price PM, Tamayo E, Safirstein RL.** The lack of a functional p21(WAF1/CIP1) gene ameliorates progression to chronic renal failure. *Proc Natl Acad Sci U S A* 96:10830-10835,1999.
34. **Motoshima H, Goldstein BJ, Igata M, Araki E.** AMPK and cell proliferation--AMPK as a therapeutic target for atherosclerosis and cancer. *J Physiol* 574:63-71,2006. doi: 10.1113/jphysiol.2006.108324
35. **Nishioka S, Nakano D, Kitada K, Sofue T, Ohsaki H, Moriwaki K, Hara T, Ohmori K, Kohno M, Nishiyama A.** The cyclin-dependent kinase inhibitor p21 is essential for the beneficial effects of renal ischemic preconditioning on renal ischemia/reperfusion injury in mice. *Kidney Int* 85:871-879,2014. doi: 10.1038/ki.2013.496
36. **Poyan Mehr A, Tran MT, Ralto KM, Leaf DE, Washco V, Messmer J, Lerner A, Kher A, Kim SH, Khoury CC, Herzig SJ, Trovato ME, Simon-Tillaux N, Lynch MR, Thadhani RI, Clish CB, Khabbaz KR, Rhee EP, Waikar SS, Berg AH, Parikh SM.** De novo NAD+ biosynthetic impairment in acute kidney injury in humans. *Nat Med* 24:1351-1359,2018. doi: 10.1038/s41591-018-0138-z
37. **Price PM, Megyesi J, Saf Irstein RL.** Cell cycle regulation: repair and regeneration in acute renal failure. *Kidney Int* 66:509-514,2004. doi: 10.1111/j.1523-1755.2004.761_8.x
38. **Price PM, Safirstein RL, Megyesi J.** Protection of renal cells from cisplatin toxicity by cell cycle inhibitors. *Am J Physiol Renal Physiol* 286:F378-84,2004. doi: 10.1152/ajprenal.00192.2003
39. **Qin L, Fan M, Candas D, Jiang G, Papadopoulos S, Tian L, Woloschak G, Grdina DJ, Li JJ.** CDK1 Enhances Mitochondrial Bioenergetics for Radiation-Induced DNA Repair. *Cell Rep* 13:2056-2063,2015. doi: 10.1016/j.celrep.2015.11.015
40. R CT, *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2013.
41. **Sano K, Fujigaki Y, Miyaji T, Ikegaya N, Ohishi K, Yonemura K, Hishida A.** Role of apoptosis in uranyl acetate-induced acute renal failure and acquired resistance to uranyl acetate. *Kidney Int* 57:1560-1570,2000. doi: 10.1046/j.1523-1755.2000.00777.x
42. **Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A.** Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9:676-682,2012. doi: 10.1038/nmeth.2019
43. **Schley G, Klanke B, Schodel J, Forstreuter F, Shukla D, Kurtz A, Amann K, Wiesener MS, Rosen S, Eckardt KU, Maxwell PH, Willam C.** Hypoxia-inducible transcription factors stabilization in the thick ascending limb protects against ischemic acute kidney injury. *J Am Soc Nephrol* 22:2004-2015,2011. doi: 10.1681/ASN.2010121249
44. Schrodinger E. What is Life? In: edited by London: Cambridge University Press, 1944,
45. **Sholl-Franco A, Fragel-Madeira L, Macama AC, Linden R, Ventura AL.** ATP controls cell cycle and induces proliferation in the mouse developing retina. *Int J Dev Neurosci* 28:63-73,2010. doi: 10.1016/j.ijdevneu.2009.09.004
46. **Shu S, Wang Y, Zheng M, Liu Z, Cai J, Tang C, Dong Z.** Hypoxia and Hypoxia-Inducible Factors in Kidney Injury and Repair. *Cells* 82019. doi: 10.3390/cells8030207
47. **Song D, Liu X, Liu R, Yang L, Zuo J, Liu W.** Connexin 43 hemichannel regulates H9c2 cell proliferation by modulating intracellular ATP and [Ca2+]. *Acta Biochimica et Biophysica Sinica* 42:472-482,2010. doi: 10.1093/abbs/gmq047
48. **Soto I, Couvillion M, Hansen KG, McShane E, Moran JC, Barrientos A, Churchman LS.** Balanced mitochondrial and cytosolic translatomes underlie the biogenesis of human respiratory complexes. *Genome Biol* 23:170,2022. doi: 10.1186/s13059-022-02732-9
49. **Sutton TA, Hato T, Mai E, Yoshimoto M, Kuehl S, Anderson M, Mang H, Plotkin Z, Chan**

RJ, Dagher PC. p53 is renoprotective after ischemic kidney injury by reducing inflammation. *J Am Soc Nephrol* 24:113-124,2013. doi: 10.1681/ASN.2012050469

50. **Tang C, Dong Z.** Mitochondria in Kidney Injury: When the Power Plant Fails. *J Am Soc Nephrol* 27:1869-1872,2016. doi: 10.1681/ASN.2015111277
51. **Tantama M, Martínez-François JR, Mongeon R, Yellen G.** Imaging energy status in live cells with a fluorescent biosensor of the intracellular ATP-to-ADP ratio. *Nat Commun* 4:2550,2013. doi: 10.1038/ncomms3550
52. **Thoreen CC, Sabatini DM.** AMPK and p53 help cells through lean times. *Cell Metab* 1:287-288,2005. doi: 10.1016/j.cmet.2005.04.009
53. **Tinevez JY, Perry N, Schindelin J, Hoopes GM, Reynolds GD, Laplantine E, Bednarek SY, Shorte SL, Eliceiri KW.** TrackMate: An open and extensible platform for single-particle tracking. *Methods* 115:80-90,2017. doi: 10.1016/j.ymeth.2016.09.016
54. **Tran MT, Zsengeller ZK, Berg AH, Khankin EV, Bhasin MK, Kim W, Clish CB, Stillman IE, Karumanchi SA, Rhee EP, Parikh SM.** PGC1α drives NAD biosynthesis linking oxidative metabolism to renal protection. *Nature* 531:528-532,2016. doi: 10.1038/nature17184
55. **Veech RL, Lawson JW, Cornell NW, Krebs HA.** Cytosolic phosphorylation potential. *J Biol Chem* 254:6538-6547,1979.
56. **Venet D, Dumont JE, Detours V.** Most random gene expression signatures are significantly associated with breast cancer outcome. *PLoS Comput Biol* 7:e1002240,2011. doi: 10.1371/journal.pcbi.1002240
57. **Verrax J, Dejeans N, Sid B, Glorieux C, Calderon PB.** Intracellular ATP levels determine cell death fate of cancer cells exposed to both standard and redox chemotherapeutic agents. *Biochem Pharmacol* 82:1540-1548,2011. doi: 10.1016/j.bcp.2011.07.102
58. **Vogt MT, Farber E.** On the molecular pathology of ischemic renal cell death. Reversible and irreversible cellular and mitochondrial metabolic alterations. *Am J Pathol* 53:1-26,1968.
59. **Wang J, Biju MP, Wang MH, Haase VH, Dong Z.** Cytoprotective effects of hypoxia against cisplatin-induced tubular cell apoptosis: involvement of mitochondrial inhibition and p53 suppression. *J Am Soc Nephrol* 17:1875-1885,2006. doi: 10.1681/ASN.2005121371
60. **Wang X, Li L, Guan R, Zhu D, Song N, Shen L.** Emodin Inhibits ATP-Induced Proliferation and Migration by Suppressing P2Y Receptors in Human Lung Adenocarcinoma Cells. *Cell Physiol Biochem* 44:1337-1351,2017. doi: 10.1159/000485495
61. **Wu H, Malone AF, Donnelly EL, Kirita Y, Uchimura K, Ramakrishnan SM, Gaut JP, Humphreys BD.** Single-Cell Transcriptomics of a Human Kidney Allograft Biopsy Specimen Defines a Diverse Inflammatory Response. *J Am Soc Nephrol* 29:2069-2080,2018. doi: 10.1681/ASN.2018020125
62. **Xie Y, Ankawi G, Yang B, Garzotto F, Passannante A, Breglia A, Digvijay K, Ferrari F, Brendolan A, Raffaele B, Giavarina D, Gregori D, Ronco C.** Tissue inhibitor metalloproteinase-2 (TIMP-2) • IGF-binding protein-7 (IGFBP7) levels are associated with adverse outcomes in patients in the intensive care unit with acute kidney injury. *Kidney Int* 95:1486-1493,2019. doi: 10.1016/j.kint.2019.01.020
63. **Yang L, Besschetnova TY, Brooks CR, Shah JV, Bonventre JV.** Epithelial cell cycle arrest in G2/M mediates kidney fibrosis after injury. *Nat Med* 16:535-43, 1p following 143,2010. doi: 10.1038/nm.2144

FIGURE LEGENDS

Figure 1. Consequences of cell proliferation on susceptibility to cell death. (a) Representative images of JC-1 staining in control conditions (CTL) or under energy depletion (ED). The red fluorescence indicates aggregates forming in high potential mitochondria. The green fluorescence indicates JC-1 monomers in low potential mitochondria. The composite image shows the increase in JC-1 monomers under energy depletion. (b) Quantification of mitochondrial potential by JC-1 in control condition, and energy depletion, showing the ratio of red/green fluorescence intensity in arbitrary units. (c) Effect of cell cycle inhibition by tenovin-1 or cell cycle facilitation by pifithrin-α on cellular death caused by puromycin (pre-incubation for 4 hours before puromycin exposure). (d) Effect of cell cycle inhibition by tenovin-1 or cell cycle facilitation by pifithrin-α on cellular necrosis caused by energy depletion (preincubaiton for 4 hours before oxygen and glucose deprivation). *: p-value <0.05 ; NS: statistically non-significant.

Figure 2. Cell cycle interventions and their effects on ATP/ADP ratio. (A). Left panel: Wells were plated with increasing numbers of PercevalHR expressing cells to obtain various levels of confluence in control condition or with 1µM rigosertib to inhibit the cell cycle. Right panel: Representative pictures obtained 0 and 15 hours after a scratch performed under control condition or with exposure to 1µM rigosertib to inhibit wound closure. (B) Pharmaceutical interventions on the cell cycle using various compounds on PercevalHR expressing cells over an 18-hr time course (total= 7345 events): KU55933 (10 µM), pifithrin-alpha (50 µM), rigosertib (10 µM), tenovin-1 (10 µM). C Quantification of changes in single cell intracellular ATP/ADP ratio over an 18-hr exposure to various cell cycle modifiers.

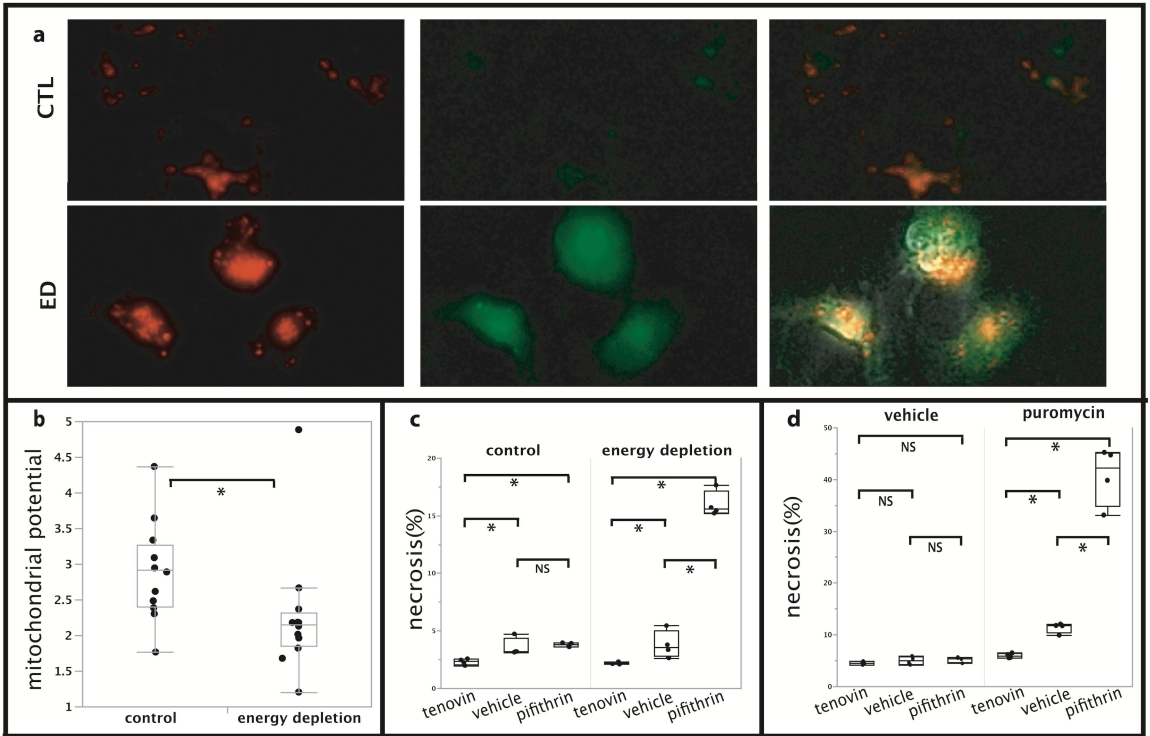
Figure 3. ATP/ADP variations throughout the cell cycle. (A, left panel): Live imaging on a single PercevalHR expressing cell undergoing mitosis. (A, right panel): Quantification of the intracellular ATP/ADP ratio in single cells during the perimitotic period with in silico synchronization on time of mitosis (n= 117 mitoses). (B) ATP/ADP changes in PercevalHR expressing cells incubated with the cell cycle facilitator pifithrin-alpha or with the cell cycle inhibitor tenovin-1 between 2 successive mitoses (n= 42 mitoses). ATP/ADP ratio values are represented as means and whiskers indicate the standard error. P-values represent the significance of the comparison between the first and second mitosis within each treatment group, using a Wilcoxon signed-rank test.

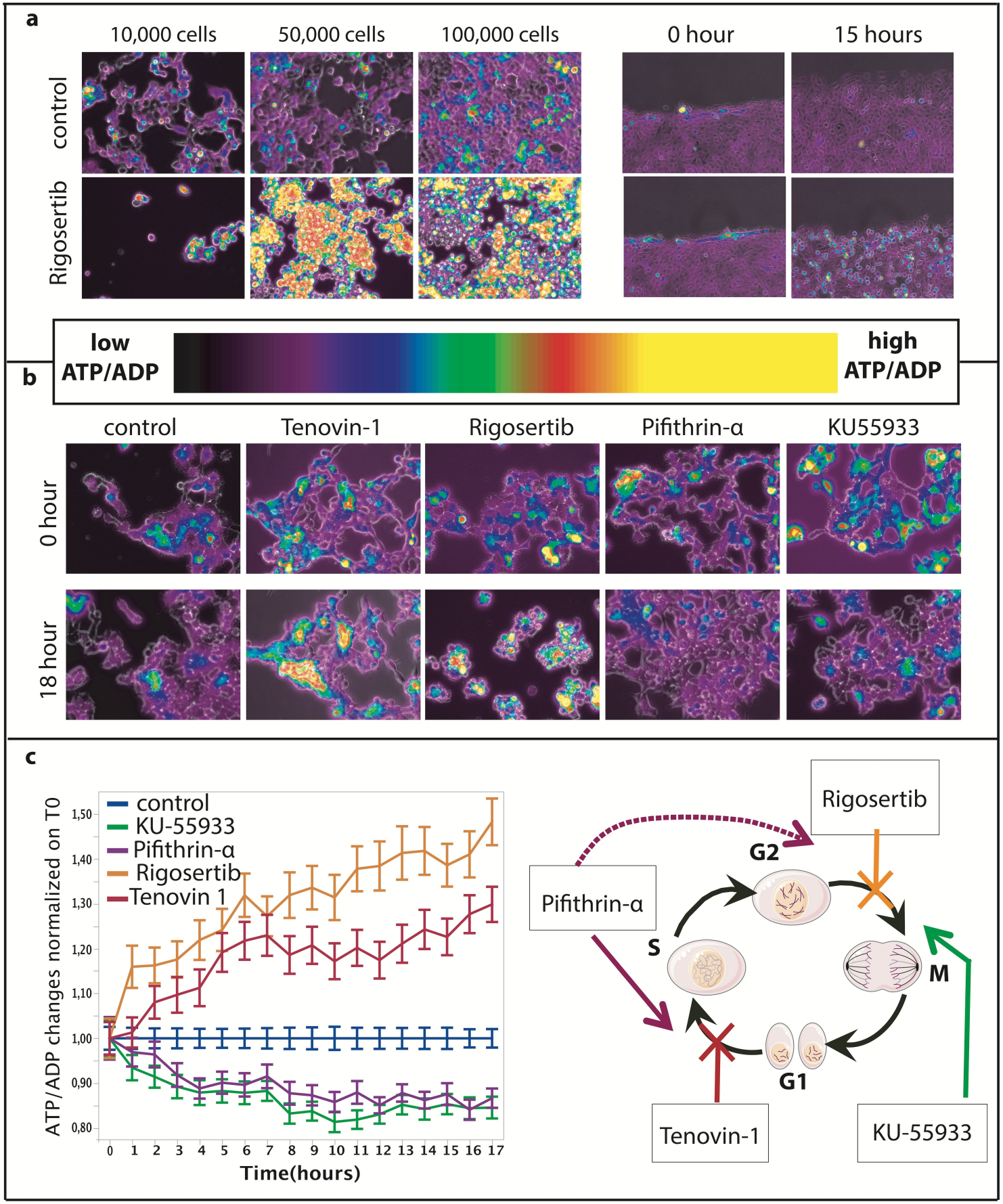
Figure 4. Transcriptomic signature associated with proliferation. (A). KEGG pathways enrichment associated with proliferation in kidney allografts immediately after reperfusion. The 20 most significant pathways are labeled. (B). Vulcano plot showing the correlation between OXPHOS gene expression and the computed
proliferation index in kidney allografts (right panel). x-axis: Kendall's k correlation coefficient; y-axis: significance represented by the -log(p-value) of the correlation (Kendall); blue: mitochondria-encoded genes; red: nuclear-encoded genes. (C). Differential correlations of nuclei and mitochondria-encoded OXPHOS with the proliferation index in kidney allografts.

Figure 5. Oxidative phosphorylation in proliferating epithelial cells. (A) Single-cell RNA-seq analysis of human rejecting kidney showing clusters corresponding to different cell types within the kidney. The subset of epithelial cells is circled in red. (B) In the subset of renal epithelial cells (PT, LOH and CD), an increased proliferation correlated with a decreased expression of mitochondria-encoded OXPHOS genes (mean values of oxphos gene expression with standard-error whiskers, by proliferation index values rounded to the nearest 10^{-5}).

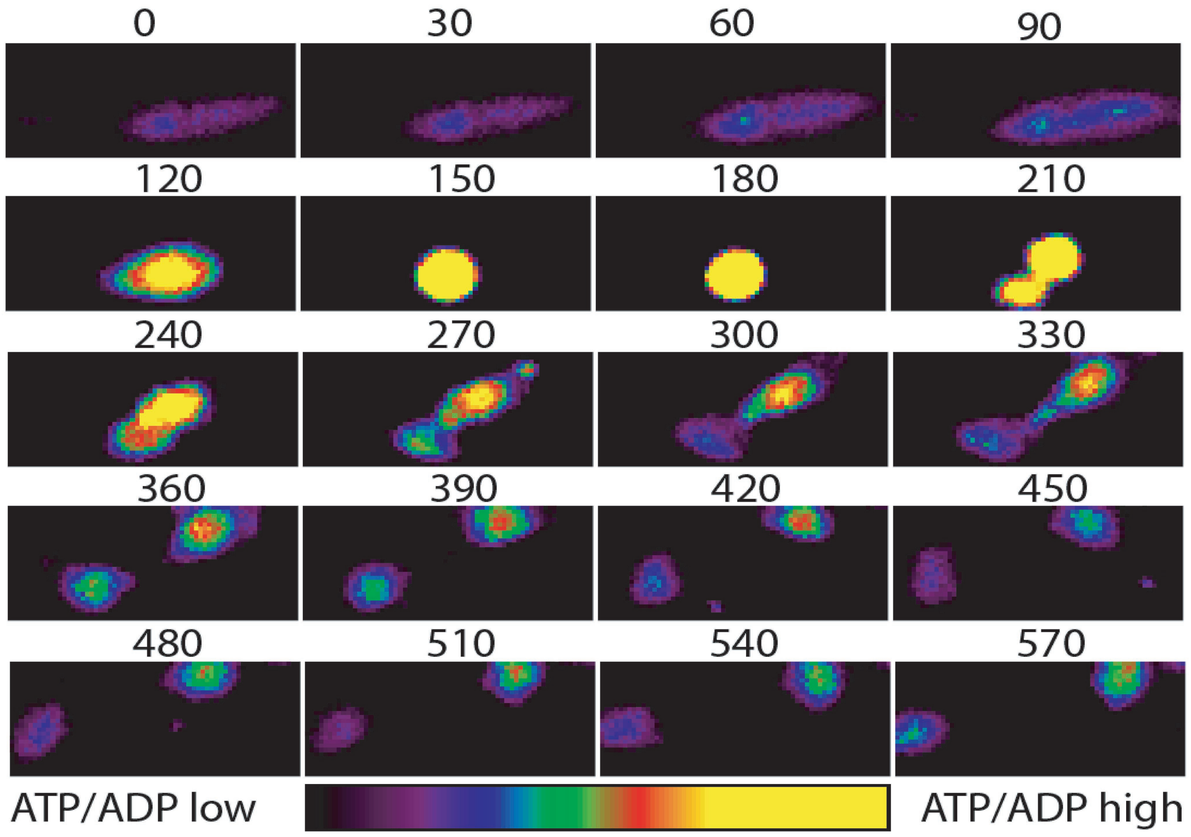
Figure 6. Association of proliferation index and OXPHOS mitochondrial genes expression with chronic kidney disease progression. (A) Expression of nucleus-encoded OXPHOS genes was not correlated with proliferation index or evolution towards chronic kidney disease. (B) Expression of mitochondria-encoded OXPHOS genes was strongly correlated with proliferation index and evolution towards chronic kidney disease.

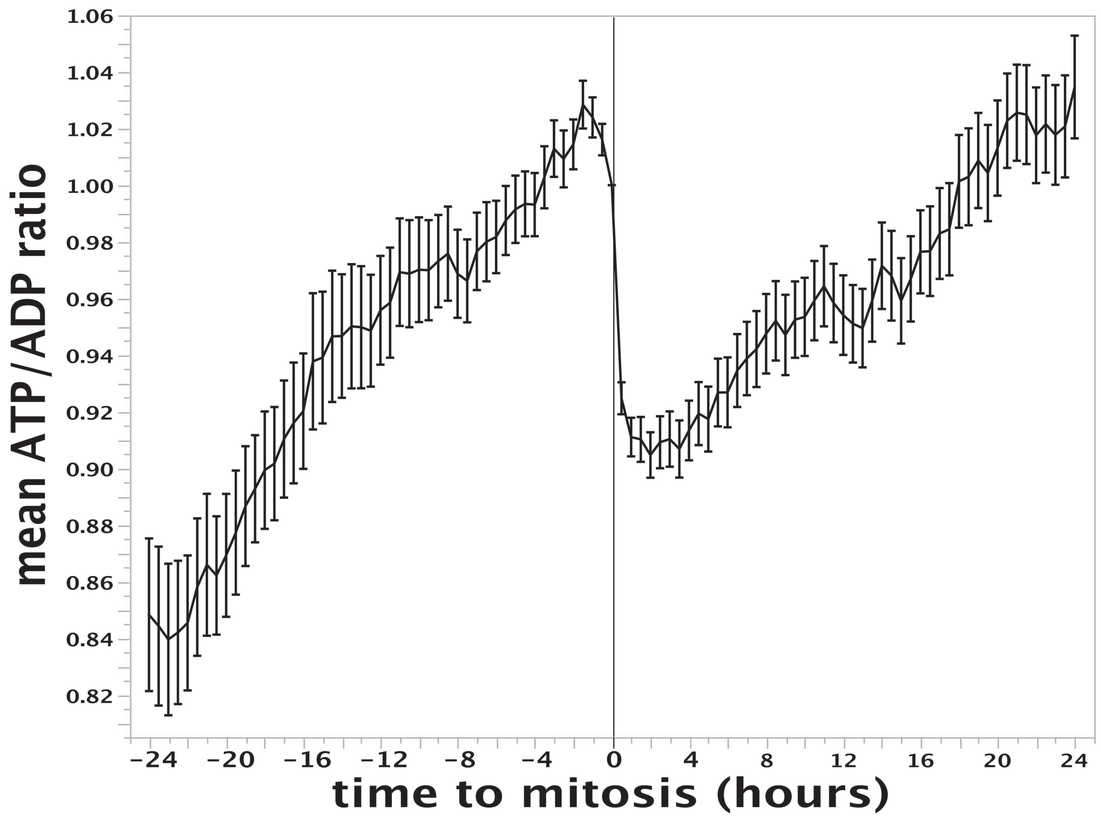
Figure 7. Impact of energy metabolism changes leading to cellular injury and proliferation in injured kidneys. Top: the lines show the physiological changes in oxidative phosphorylation (pink) and proliferation (blue) leading to full recovery. Bottom: the lines show pathological processes with excessive proliferation leading to cell death by energy depletion (hatched purple area) and persistent oxidative phosphorylation defect leading to chronic kidney disease and fibrosis (hatched green area).


a
CTL
ED
b
mitochondrial potential
5
4.5
4
3.5
3
2.5
2
1.5
1
*
control
energy depletion
c
control
energy depletion
necrosis(%)
20
15
10
5
0
*
NS
tenovin
vehicle
pifithrin
d
vehicle
puromycin
necrosis(%)
50
40
30
20
10
0
NS
*
tenovin
vehicle
pifithrin

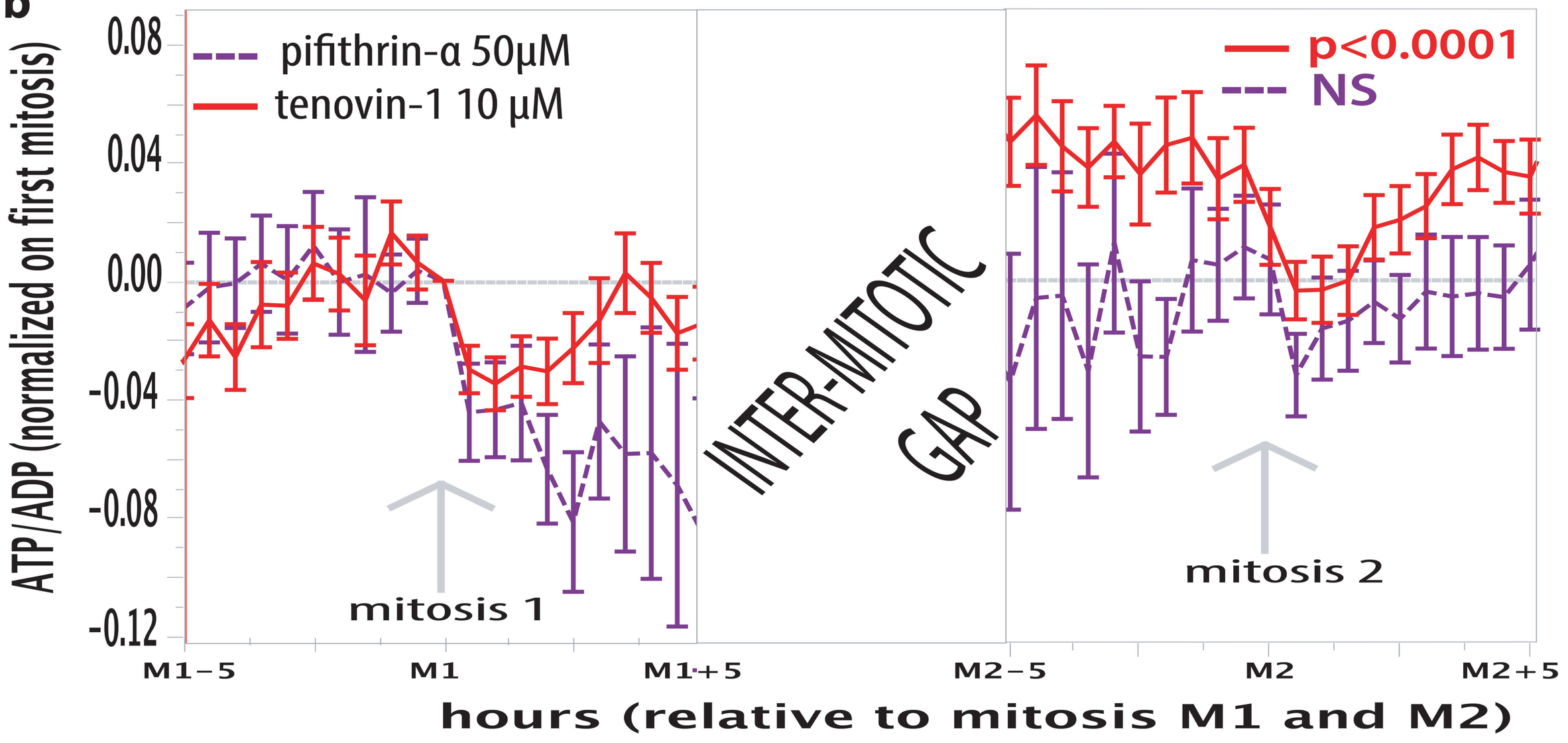

a
10,000 cells
50,000 cells
100,000 cells
0 hour
15 hours
control
Rigosertib
low ATP/ADP
high ATP/ADP
b
control
Tenovin-1
Rigosertib
Pifithrin-α
KU55933
0 hour
18 hour
c
control
KU-55933
Pifithrin-α
Rigosertib
Tenovin 1
ATP/ADP changes normalized on T0
1,50
1,40
1,30
1,20
1,10
1,00
0,90
0,80
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Time(hours)
Rigosertib
G2
Pifithrin-α
S
M
G1
Tenovin-1
KU-55933

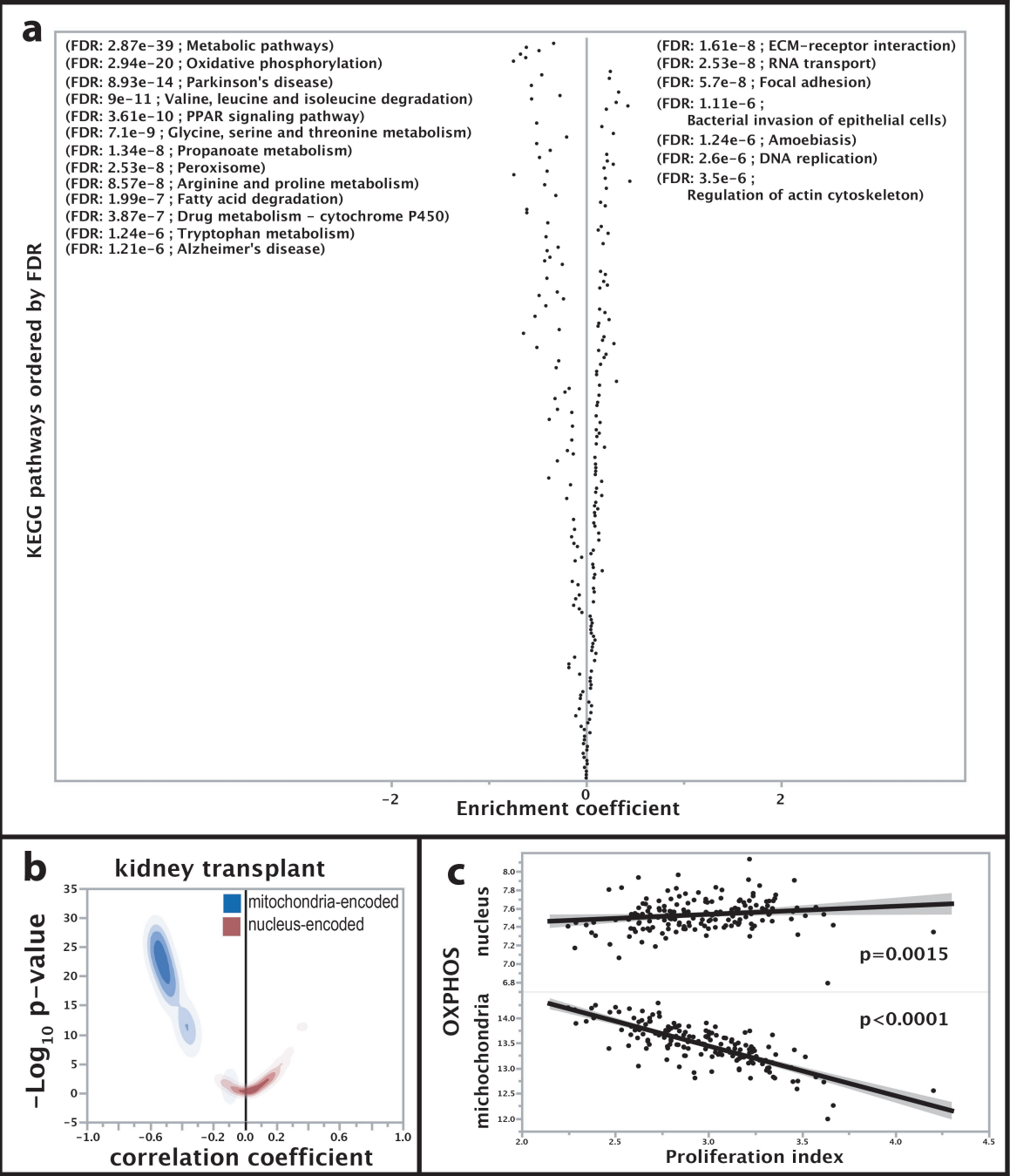
a

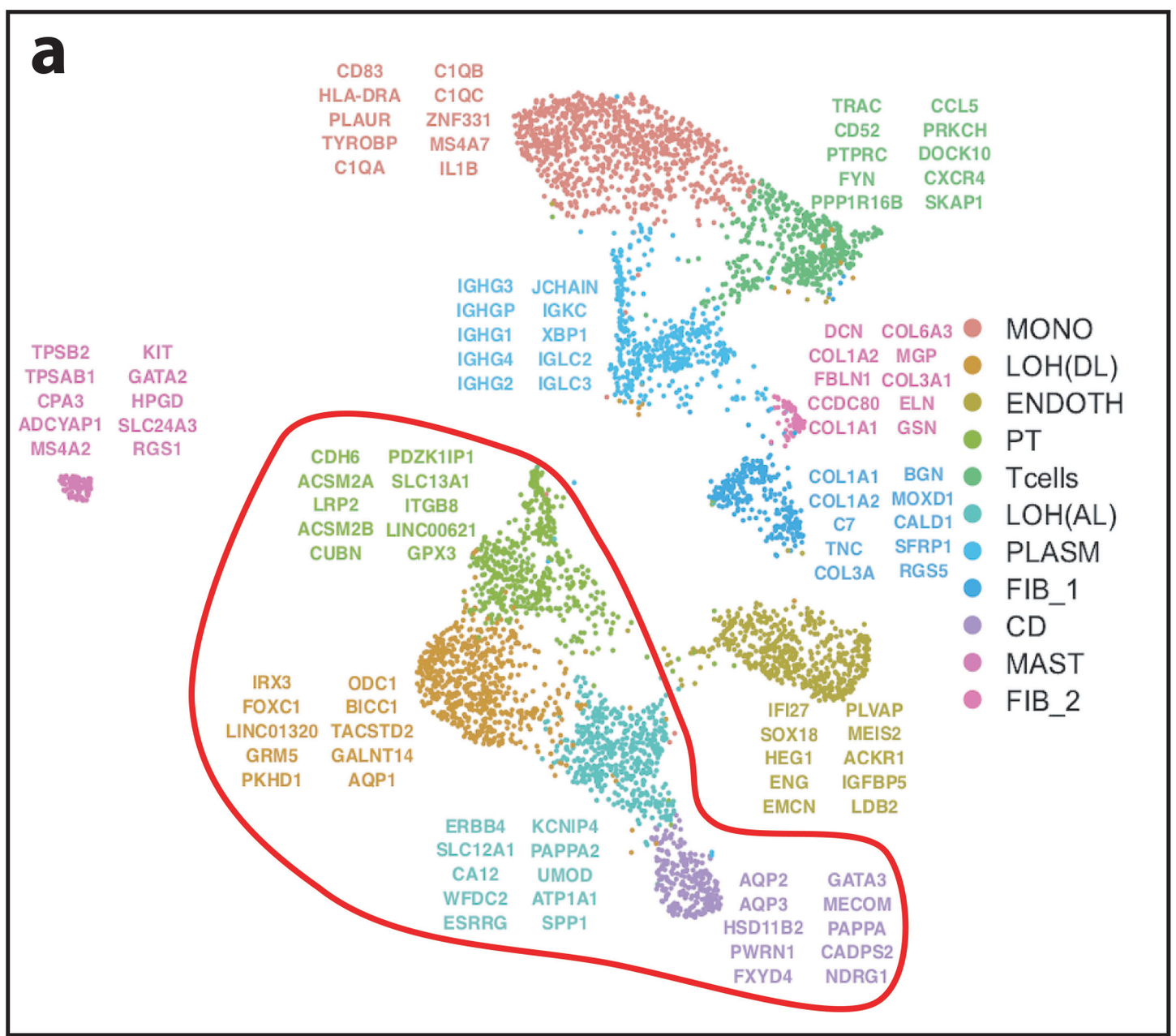

0
30
60
90
120
150
180
210
240
270
300
330
360
390
420
450
480
510
540
570
ATP/ADP low
ATP/ADP high

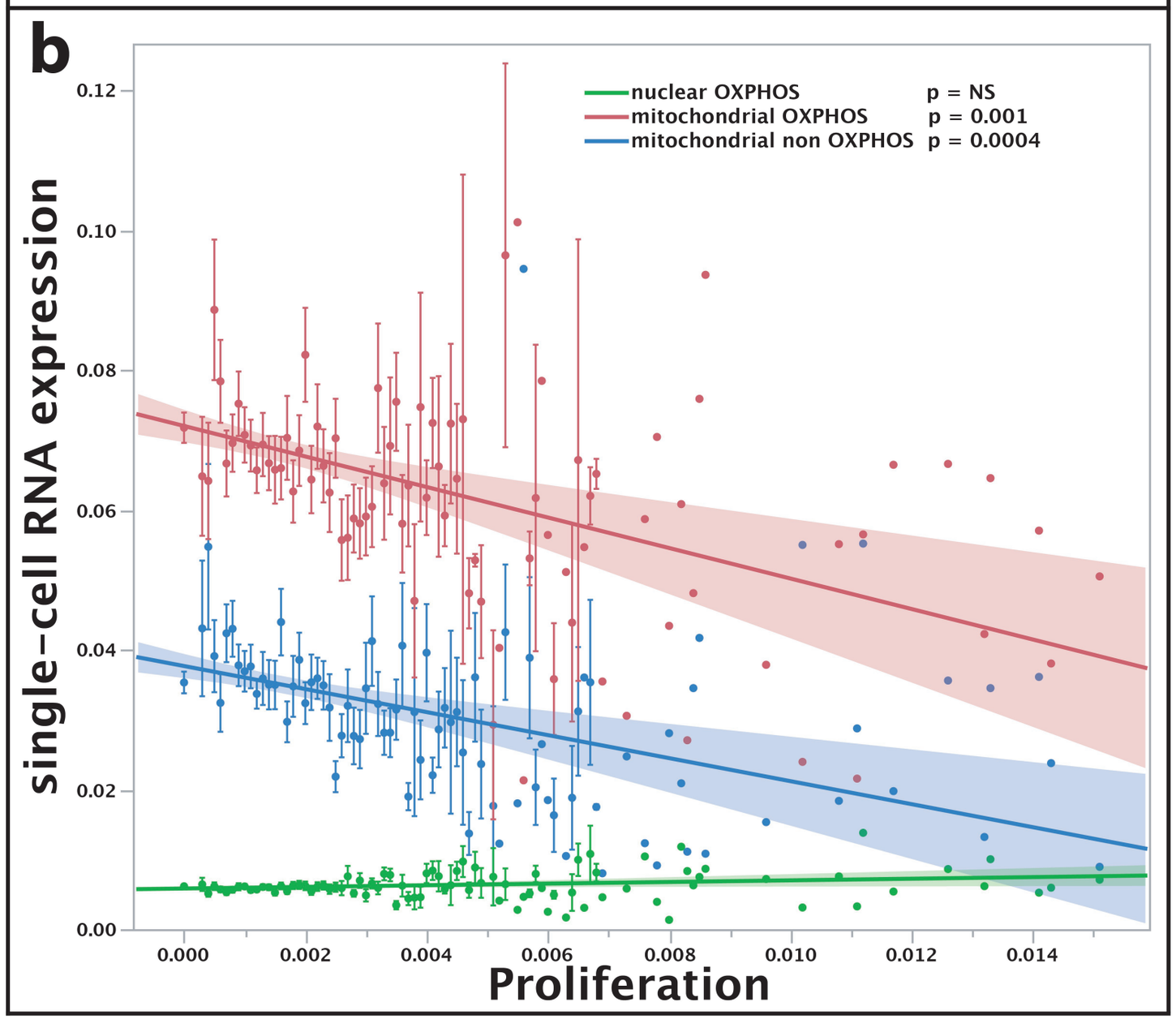

mean ATP/ADP ratio
1.06
1.04
1.02
1.00
0.98
0.96
0.94
0.92
0.90
0.88
0.86
0.84
0.82
−24
−20
−16
−12
−8
−4
0
4
8
12
16
20
24
time to mitosis (hours)

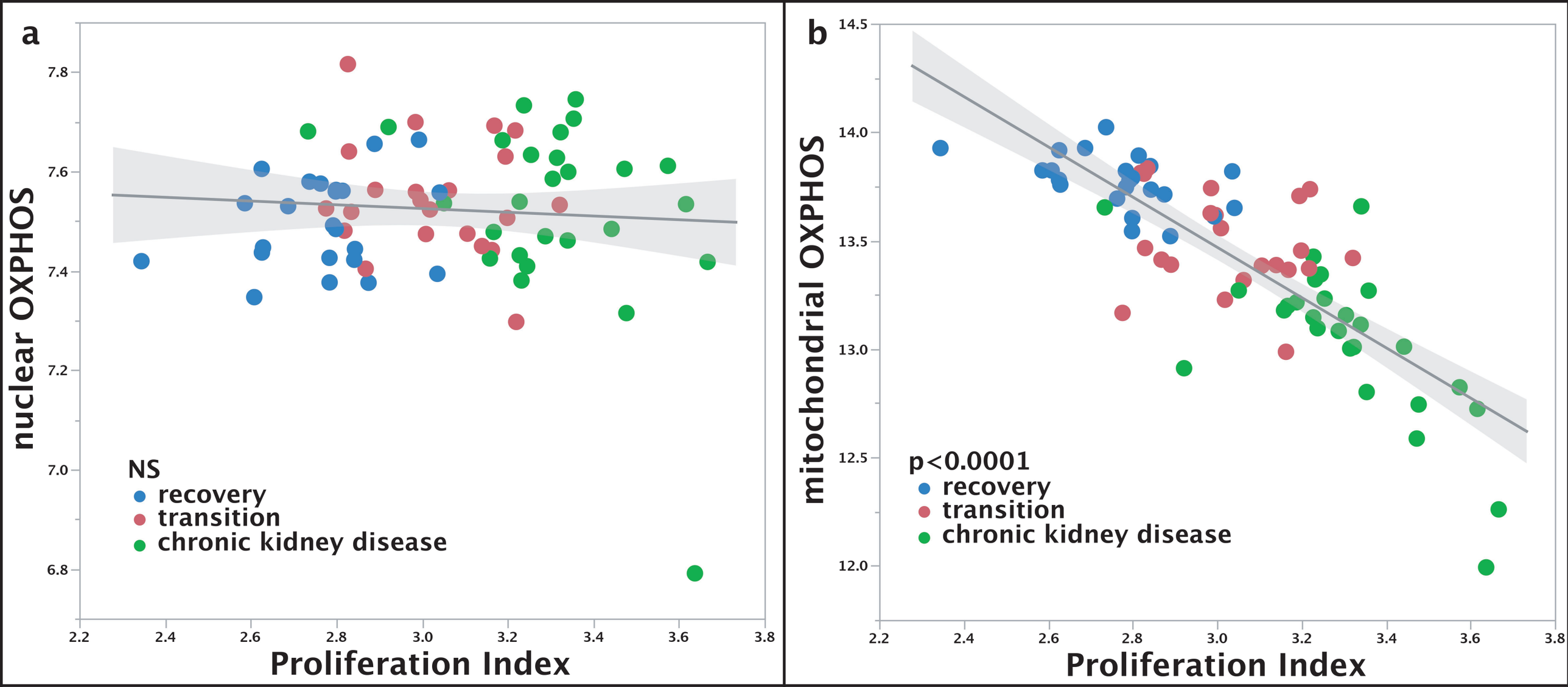
b

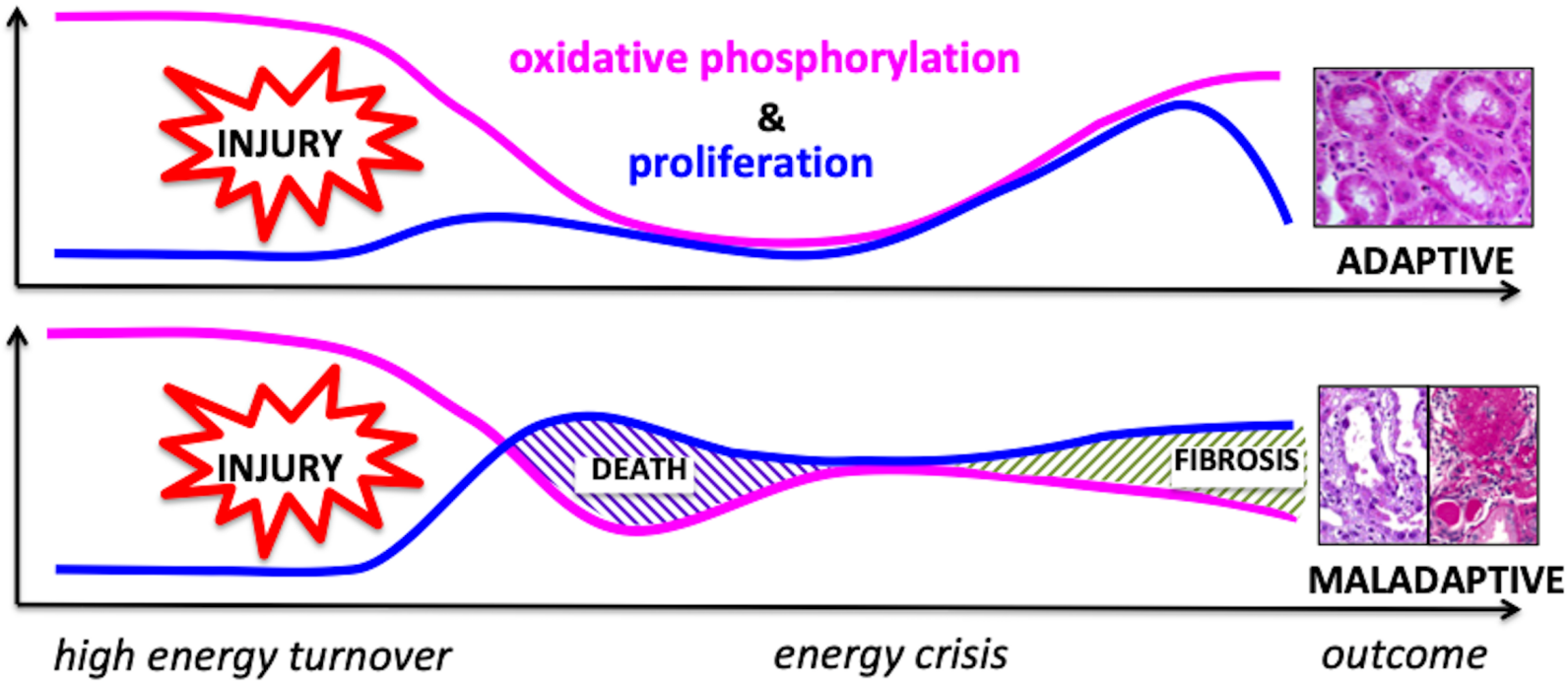

ATP/ADP (normalized on first mitosis)
0.08
0.04
0.00
-0.04
-0.08
-0.12
pifithrin-α 50μM
tenovin-1 10 μM
p<0.0001
NS
INTER-MITOTIC GAP
mitosis 1
mitosis 2
M1−5
M1
M1+5
M2−5
M2
M2+5
hours (relative to mitosis M1 and M2)


a
(FDR: 2.87e-39 ; Metabolic pathways)
(FDR: 2.94e-20 ; Oxidative phosphorylation)
(FDR: 8.93e-14 ; Parkinson's disease)
(FDR: 9e-11 ; Valine, leucine and isoleucine degradation)
(FDR: 3.61e-10 ; PPAR signaling pathway)
(FDR: 7.1e-9 ; Glycine, serine and threonine metabolism)
(FDR: 1.34e-8 ; Propanoate metabolism)
(FDR: 2.53e-8 ; Peroxisome)
(FDR: 8.57e-8 ; Arginine and proline metabolism)
(FDR: 1.99e-7 ; Fatty acid degradation)
(FDR: 3.87e-7 ; Drug metabolism - cytochrome P450)
(FDR: 1.24e-6 ; Tryptophan metabolism)
(FDR: 1.21e-6 ; Alzheimer's disease)
(FDR: 1.61e-8 ; ECM-receptor interaction)
(FDR: 2.53e-8 ; RNA transport)
(FDR: 5.7e-8 ; Focal adhesion)
(FDR: 1.11e-6 ; Bacterial invasion of epithelial cells)
(FDR: 1.24e-6 ; Amoebiasis)
(FDR: 2.6e-6 ; DNA replication)
(FDR: 3.5e-6 ; Regulation of actin cytoskeleton)
KEGG pathways ordered by FDR
-2
0
2
Enrichment coefficient
b
kidney transplant
mitochondria-encoded
nucleus-encoded
-Log10 p-value
35
30
25
20
15
10
5
0
-5
-1.0
-0.6
-0.2
0.0
0.2
0.6
1.0
correlation coefficient
c
OXPHOS
nucleus
mitochondria
8.0
7.8
7.6
7.4
7.2
7.0
6.8
p=0.0015
14.0
13.5
13.0
12.5
12.0
p<0.0001
2.0
2.5
3.0
3.5
4.0
4.5
Proliferation index


a
CD83
HLA-DRA
PLAUR
TYROBP
C1QA
C1QB
C1QC
ZNF331
MS4A7
IL1B
TRAC
CD52
PTPRC
FYN
PPP1R16B
CCL5
PRKCH
DOCK10
CXCR4
SKAP1
IGHG3
IGHGP
IGHG1
IGHG4
IGHG2
JCHAIN
IGKC
XBP1
IGLC2
IGLC3
TPSB2
TPSAB1
CPA3
ADCYAP1
MS4A2
KIT
GATA2
HPGD
SLC24A3
RGS1
DCN
COL1A2
FBLN1
CCDC80
COL1A1
COL6A3
MGP
COL3A1
ELN
GSN
CDH6
ACSM2A
LRP2
ACSM2B
CUBN
PDZK1IP1
SLC13A1
ITGB8
LINC00621
GPX3
COL1A1
COL1A2
C7
TNC
COL3A
BGN
MOXD1
CALD1
SFRP1
RGS5
IRX3
FOXC1
LINC01320
GRM5
PKHD1
ODC1
BICC1
TACSTD2
GALNT14
AQP1
IFI27
SOX18
HEG1
ENG
EMCN
PLVAP
MEIS2
ACKR1
IGFBP5
LDB2
ERBB4
SLC12A1
CA12
WFDC2
ESRRG
KCNIP4
PAPPA2
UMOD
ATP1A1
SPP1
AQP2
AQP3
HSD11B2
PWRN1
FXYD4
GATA3
MECOM
PAPPA
CADPS2
NDRG1
MONO
LOH(DL)
ENDOTH
PT
Tcells
LOH(AL)
PLASM
FIB_1
CD
MAST
FIB_2

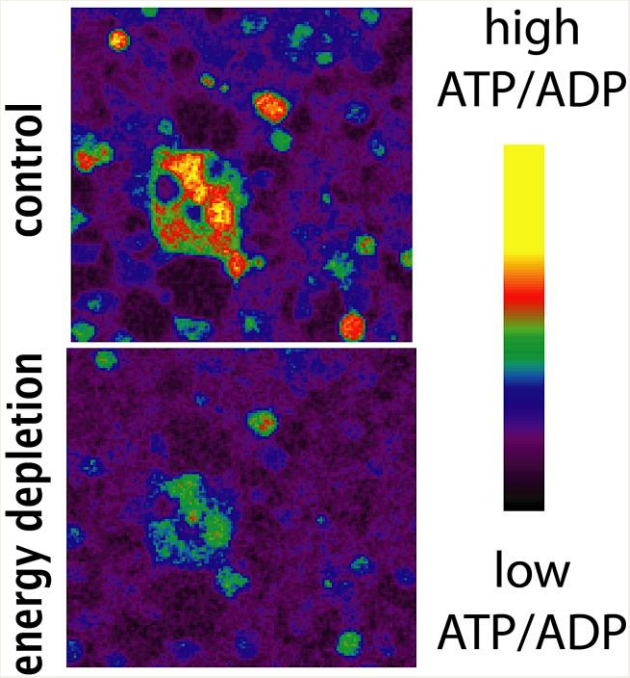

b
nuclear OXPHOS p = NS
mitochondrial OXPHOS p = 0.001
mitochondrial non OXPHOS p = 0.0004
single-cell RNA expression
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.000
0.002
0.004
0.006
0.008
0.010
0.012
0.014
Proliferation


a
nuclear OXPHOS
7.8
7.6
7.4
7.2
7.0
6.8
NS
recovery
transition
chronic kidney disease
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
Proliferation Index
b
mitochondrial OXPHOS
14.5
14.0
13.5
13.0
12.5
12.0
p<0.0001
recovery
transition
chronic kidney disease
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
Proliferation Index

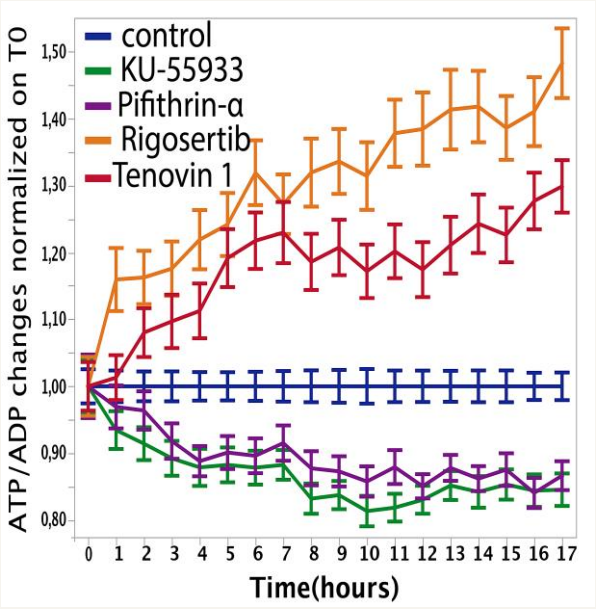

oxidative phosphorylation
&
proliferation
INJURY
ADAPTIVE
INJURY
DEATH
FIBROSIS
MALADAPTIVE
high energy turnover
energy crisis
outcome

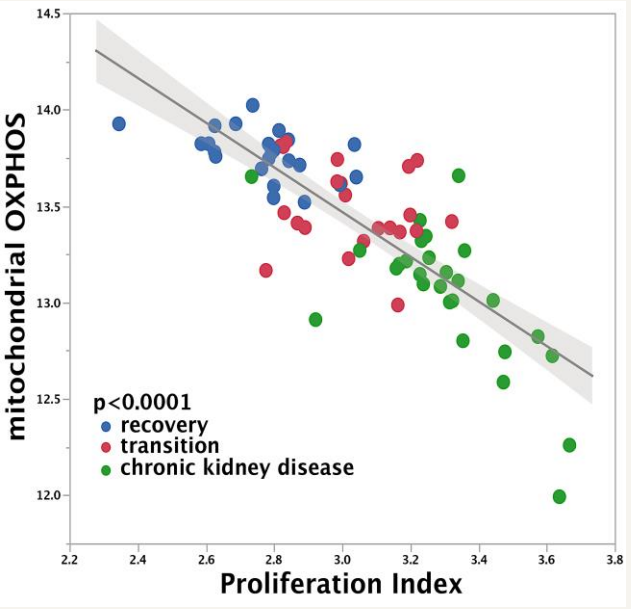
Viability of proliferating kidney cells

METHODS



PROLIFERATION IMPAIRS ENERGETIC STATUS





Proliferation is an energy crisis sensitizing renal epithelial cells to maladaptive repair.