REGULATION OF INTRACELLULAR ENERGY SUPPLIES BY CELL CYCLE KINETICS USING A SINGLE CELL APPROACH.

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ABSTRACT

Adenosine triphosphate (ATP) is the main energy provider for intracellular processes, through its cleavage into adenosine diphosphate (ADP) and hydrogen phosphate. The ATP/ADP ratio reflects the energy status of a cell. It is generally assumed that a high intracellular ATP/ADP ratio (i.e. a high energy potential) constitutes a signal for proliferation. Conversely, energy depletion leads to cell death. However, the consequences of cell cycle progression on cell energy consumption are unknown. Here, we quantified the ATP/ADP ratio in live cells using a single cell approach. We thus characterize a reproducible pattern of variation of the energy level throughout the cell cycle, notably with a post-mitotic decrease in the ATP/ADP ratio. In addition, the cellular energy status can be controlled by inhibiting or stimulating cell proliferation. Finally, we show that proliferating cells are more sensitive to an aggression than non-proliferating ones. Our results establish that the proliferation rate critically impacts the cellular energy state, with major implications for the pathophysiology of organ injury.

Adenosine triphosphate (ATP) is the main energy provider for intracellular processes, through its cleavage into adenosine diphosphate (ADP) and hydrogen phosphate [1,2]. The ATP/ADP ratio reflects the energy status of a cell [3–5]. It is generally assumed that a high intracellular ATP/ADP ratio (i.e. a high energy potential) constitutes a signal for proliferation [6–9]. Conversely, energy depletion leads to cell death [10,11]. However, the consequences of cell cycle progression on cell energy consumption are unknown. Here, we quantified the ATP/ADP ratio in live cells using a single cell approach. We thus characterize a reproducible pattern of variation of the energy level throughout the cell cycle, notably with a post-mitotic decrease in the ATP/ADP ratio. In addition, the cellular energy status can be controlled by inhibiting or stimulating cell proliferation. Finally, we show that proliferating cells are more sensitive to an aggression than non-proliferating ones. Our results establish that the proliferation rate critically impacts the cellular energy state, with major implications for the pathophysiology of organ injury.

Adenosine triphosphate (ATP) is the cell's main cellular energy provider. In tumors, its levels are correlated with proliferation. Thus, increased cellular ATP is generally considered to be a signal for cell proliferation [12,13]. Multiple molecules act as ATP sensors, either intracellular (adenosine monophosphate-activated protein kinase, APMPK) or extracellular (PY2 purinergic receptor), and foster proliferation in response to elevated ATP levels [6,14,15]. Conversely, a decrease in the level of intracellular ATP is associated with cell death, both in tumor and non-tumor cells [11,16].

Thus, intracellular ATP is a major determinant of cell fate. In addition, however, several studies suggest that intracellular ATP levels can be affected by the rate of proliferation through increased energy consumption. Such an effect of proliferation on ATP could explain why an increase in cell proliferation aggravates the course of acute kidney injury caused by an ischemic episode [17]. The kidney has a very high energy turnover and is thus very sensitive to injury (and

especially ischemia), which causes an immediate energy depletion and epithelial cell death [18]. After an injury, cell proliferation is necessary for epithelial repair [19]. Surprisingly, 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 [20–22]. Similarly, 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 [23]. 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 [24]. Taken together, these studies suggest that an increase in cell proliferation in conditions of stress might cause critical energy depletion, and result in cell death.

In order to follow the cellular energy level during the course of a cell cycle, we measured the ATP/ADP ratio in proliferating human renal epithelial cells (HK2 cell line). Indeed, if the ATP concentration is positively correlated with the number of cells *in vitro* [25], the ATP/ADP ratio is a better indicator of the cellular energy status, with a low ATP/ADP indicating a decreased energy state [26]. PercevalHR is a genetically encoded fluorescent marker enabling the live monitoring of the ATP/ADP ratio [26,27]. To monitor the single cell-ATP/ADP ratio over time in proliferating epithelial cells, we generated human renal epithelial HK2 cells stably expressing PercevalHR (Figure 1a). We first validated the quantification of the ATP/ADP ratio by PercevalHR (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] ($r=0.96$, $p=0.0001$). We also 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) (Figure 1b).

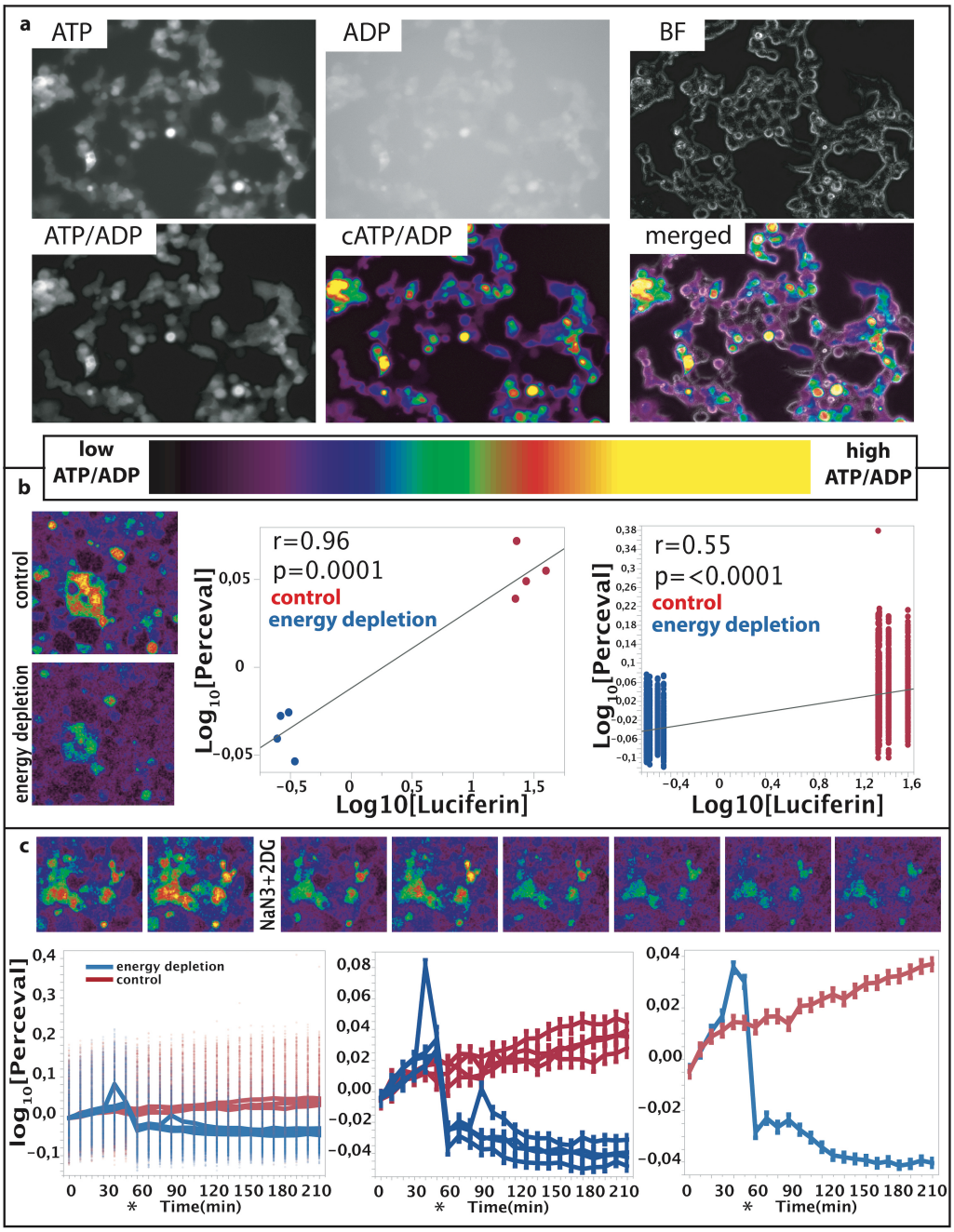

Figure 1: Monitoring of intracellular ATP/ADP ratio.

A. Fluorescence imaging of ATP-bound PercevalHR (ATP), ADP-bound PercevalHR (ADP), brightfield image (BF), 2D-representation of the ATP/ADP ratio in black and white (ATP/ADP) and using artificial colors (cATP/ADP), the cATP/ADP image can then be merged with the brightfield image (merged). **B**. Comparison of PercevalHR and Luciferin based assay for ATP/ADP quantification. **C**. Real time single cell ATP/ADP monitoring with PercevalHR. Left: ATP/ADP values of individual cells represented by dots. Middle: each line represents the mean ATP/ADP value of the cells from a single well. Right: each line represents the mean ATP/ADP value of cells from each condition (control vs ischemia). The «*» sign indicates the time when the cells were treated with 2DG and NaN_3 for energy depletion.

We then performed a single cell quantification of ATP/ADP[Perceval] in real-time. Although changes in mean ATP/ADP values over time with or without energy depletion were reproducible, this ratio was highly variable among proliferating cells in the same well and at the same time (Figure 1c). 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 to the cell cycle stage. To test this hypothesis, we plated PercevalHR-expressing epithelial cells at various densities to achieve different levels of contact-inhibition of proliferation (Figure 2a). We found that the intracellular ATP/ADP ratio increased when the cell density was higher. To rule out a direct effect of the confluence, we cultured the cells at the same densities in the presence of rigosertib, a potent cell cycle inhibitor inducing G2/M arrest. Non-proliferating cells showed a strong increase in the ATP/ADP ratio, regardless of their degree of confluence. Conversely, performing a scratch assay in confluent cells induced the proliferation and migration of the cells, which then displayed a low ATP/ADP ratio. Again, rigosertib increased the cellular ATP/ADP ratio while inhibiting wound closure (Figure 2a).

A panel of pharmaceutical compounds was then used to study the changes in the ATP/ADP ratio over time when the cell cycle is perturbed (Figure 2b and 2c). Tenovin-1 (a p53 agonist) and Rigosertib (a PLK1 inhibitor) were used to inhibit the cell cycle during the G1/S or G2/M transition, respectively. Conversely, pifithrin-α (a p53 antagonist promoting G1/S and G2/M transition) and KU-55933 (an ATM inhibitor promoting G2/M transition) were used to stimulate cell proliferation. Single-cell monitoring demonstrated that cell cycle inhibition increased ATP/ADP progressively over time whereas cell proliferation decreased it (Figure 2c).

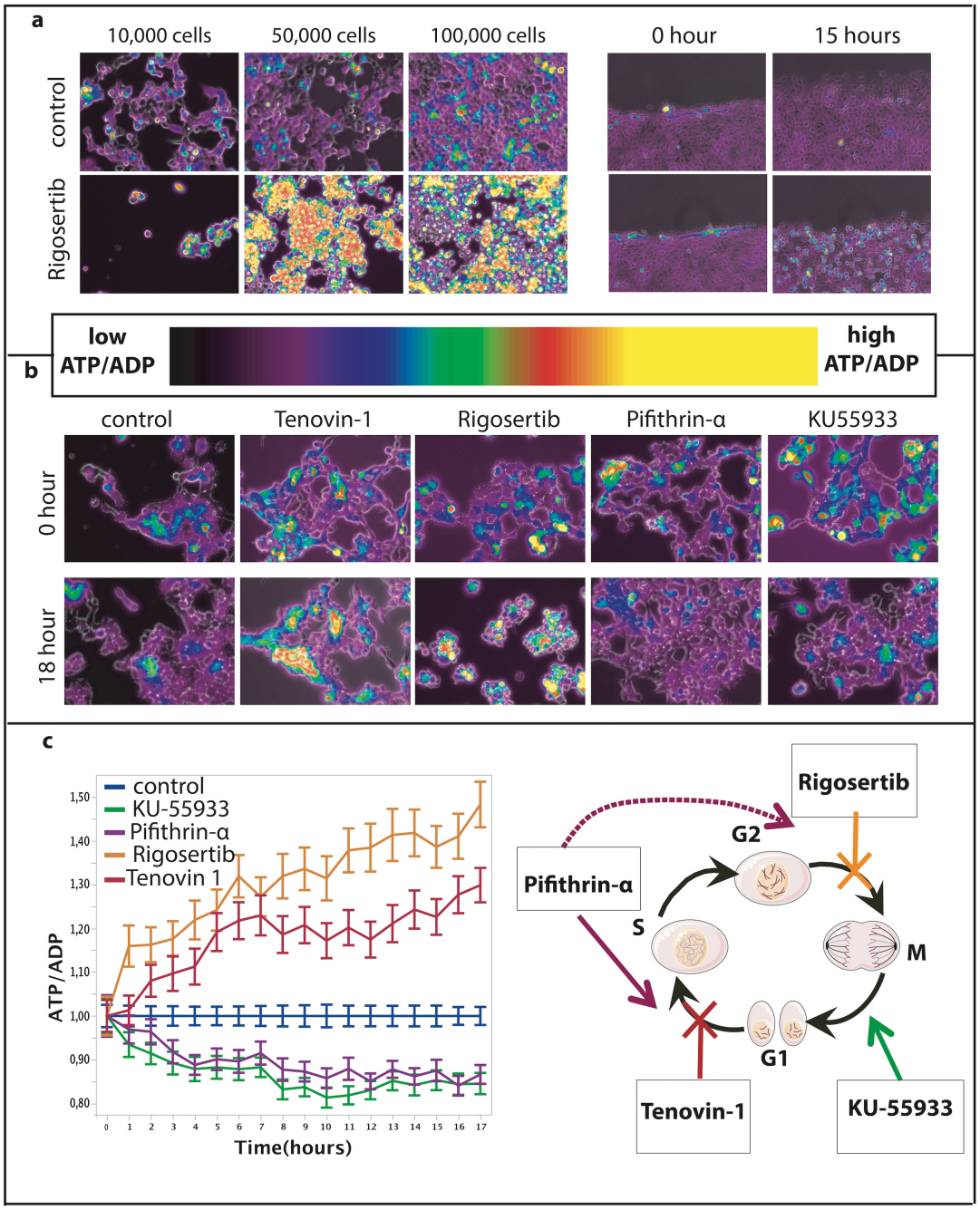

Figure 2: Interventions on cell cycle and their effect on ATP/ADP ratio.
A. Wells plated with increasing numbers of PercevalHR expressing cells to obtain various levels of confluence in control condition or with 1µM rigosertib for cell cycle inhibition. Scratch assay performed in control condition or with 1µM rigosertib to inhibit wound closure, shown 0 and 15 hours after the scratch. **B**. Pharmaceutical interventions on the cell cycle using various compounds on PercevalHR expressing cells over an 18-hr time course: KU55933 (10µM), pifithrin-α (50µM), rigosertib (10µM), tenovin-1 (10µM). **C**. Quantification of changes in single cell intracellular ATP/ADP ratio over an 18-hours exposure to various cell cycle modifiers.

We observed that the single-cell ATP/ADP[Perceval] ratio was maximal around cytokinesis in non-synchronized cells (Figure 3a). Therefore, we quantified the single-cell ATP/ADP ratio over time of the cell cycle in cells undergoing division. We found that the variations of the ATP/ADP ratio before and after cytokinesis follow a systematic pattern (Figure 3a): 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 (Figure 3b). We concluded that the intracellular energy level, evaluated by the ATP/ADP ratio, is directly influenced by the cell cycle.

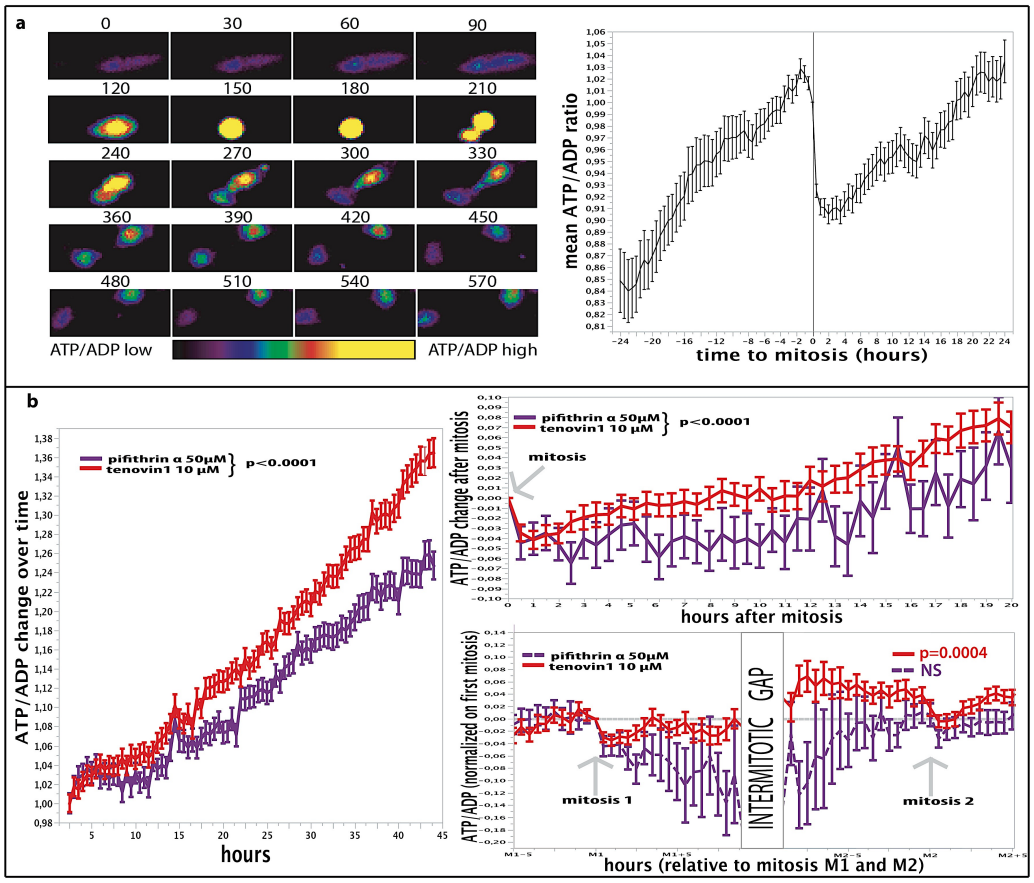

Figure 3: ATP/ADP variations throughout the cell cycle.
A. Live imaging on a single PercevalHR expressing cell undergoing mitosis. Quantification of intracellular ATP/ADP ratio in single cells during the peri-mitotic period (*in silico* synchronization on mitosis time). **B**. ATP/ADP changes in PercevalHR expressing cells incubated with the cell cycle facilitator pifithrin-α or with the cell cycle inhibitor tenovin-1, over the whole experiment, after the first mitosis, and between 2 successive mitoses.

In order to study potential variations in the single cell ATP/ADP ratio in successive cell cycles within the same cell, we then selected the ATP/ADP ratio trajectories of cells undergoing division twice in the same live-imaging experiment. After a first mitosis, cells treated with tenovin-1 increased their ATP/ADP ratio significantly more than cells treated with pifithrin-α (Figure 3b, upper right). The cells had the same pattern of ATP/ADP variations for each of the two successive mitoses. In cells incubated with the cell cycle inhibitor tenovin-1, we observed an increment in the mitotic maxima of the ATP/ADP ratio from one mitosis to the next, whereas we did not find such an increment with the cell cycle facilitator pifithrin-α (Figure 3b, bottom right). We conclude that cell cycle inhibition increases ATP/ADP ratio in cells independently of the cell cycle stage, in a time dependent manner (Figure 3b). **This suggests that the slower the cell cycles, the more positive is the cellular energy balance.**

We then tested if the variations of the intracellular energy level during the cell cycle could impact the cell survival after a toxin. HK2 cells were incubated with puromycin, in the presence of tenovin-1 or pifithrin-α (Figure 4a and 4b). We observed that the stimulation of cell proliferation by pifithrin-α promoted the death of the cells exposed to puromycin (a well characterized toxicant acting through inhibition of protein synthesis, i.e. not directly targeting energy metabolism), whereas inhibition of cell proliferation by tenovin-1 was protective. Pifithrin-α or tenovin-1 alone were not toxic by themselves as they didn't cause cell death in the absence of puromycin (Figure 4a). The same result was obtained when cells were cultured under conditions of chemical hypoxia + no glucose versus control conditions (normoxia + glucose), showing that pifithrin-α caused cell death whereas tenovin-1 was protective under energy depleted conditions (Figure 4b). These data show that highly proliferating cells are more sensitive to an insult than non-proliferative ones.

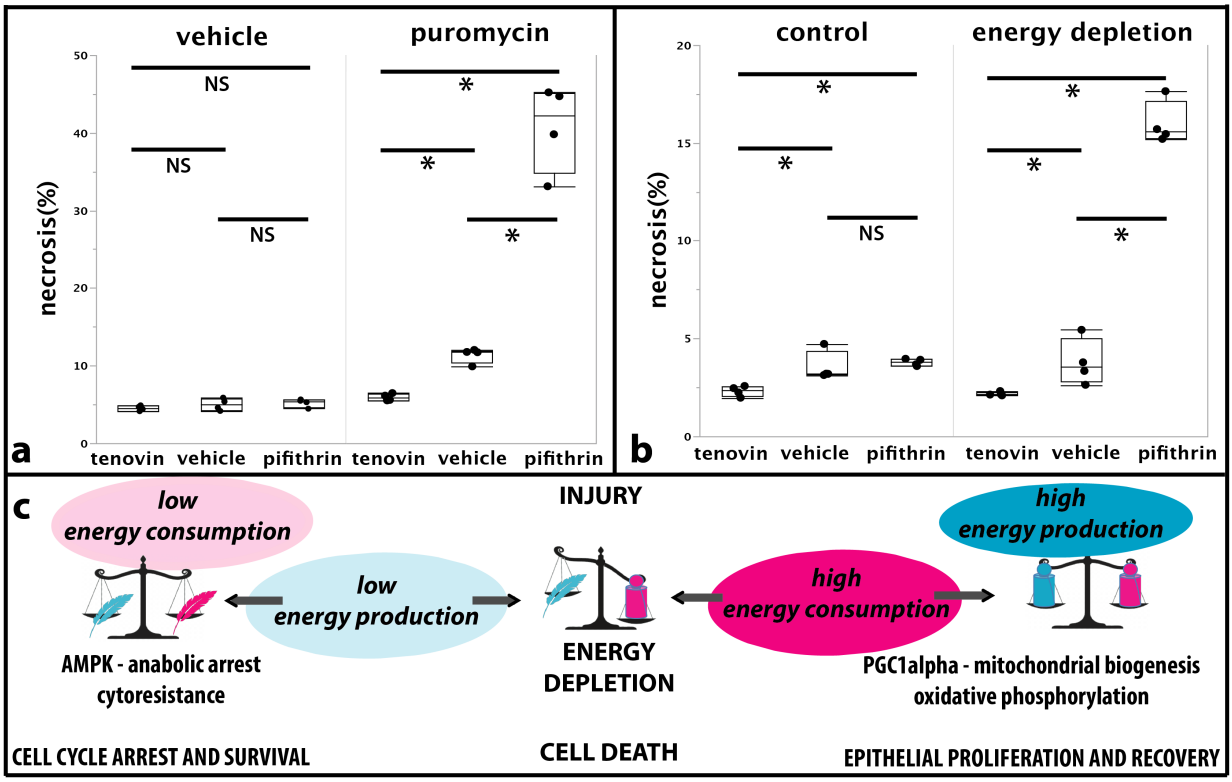

Figure 4: consequences of cell proliferation on susceptibility to necrosis. a. Effect of cell cycle inhibition by tenovin-1 or cell cycle facilitation by pifithrin-α on cellular necrosis caused by puromycin. **b**. Effect of cell cycle inhibition by tenovin-1 or cell cycle facilitation by pifithrin-α on cellular necrosis caused by energy depletion (oxygen and glucose deprivation). **c.** Implications of the proliferation/energy balance in the cellular fate. ATP depletion can be prevented through either decreased energy consumption or increased energy production. Here we show mechanisms participating in energy homeostasis during development, organ injury and tumorigenesis, and their consequences on cell survival, cell death and repair. *: p-value <0.05 ; NS: statistically non-significant.

Our observations provide important insight for the understanding of the consequences of a variation in the cell energy status in physiology and pathology (Figure 4c). We demonstrate that the ATP/ADP ratio oscillates throughout the cell cycle, a pattern reminiscent to cyclic variations of the TCA cycle flux described by Ahn and colleagues [28]. We also show here that the intracellular energy level not only varies during the cell cycle but also depends on the proliferation rate. We provide experimental evidence that the association of high intracellular energetic levels with cell proliferation, although usually observed, is not absolute. This finding is

supported by the work of Mendelsohn et al, who found in a high throughput CRISPRi screen that cell growth and ATP can follow dissociated patterns [29]. In addition, the cell survival in adverse conditions depends on proliferation inhibition, as cells in which proliferation is stimulated are more stress-sensitive than non-proliferating ones, likely related to accelerated energy consumption due to the requirements for protein synthesis and other metabolic processes necessary for genetic amplification prior to cell division.

This hypothesis is supported by studies performed in various types of tumors. Indeed, less proliferative tumors are often more resistant to cell death whereas highly proliferative tumors frequently undergo spontaneous necrosis. In addition, it is known that cancer cells with a high intracellular ATP level are more resistant to cytotoxic chemotherapy [30]. This could be due to the fact that these cells do not proliferate. It could also explain why these chemotherapies are more efficient in proliferating tumors [8,31,32].

Cell proliferation is also observed during organ repair, where restoring the balance between energy production and energy consumption could also increase cell survival after an injury. For example, recent studies have described the changes in energy metabolism occurring during acute kidney injury (AKI) [33–35]. They showed that fatty acid oxidative phosphorylation is defective in proximal tubular cells after injury. Restoring oxidative metabolism not only reverses established ischemic AKI but also prevents AKI. On the other hand, stabilization of the Hypoxia Inducible Factor (HIF) shifts the energy metabolism to anaerobic glycolysis, inhibits cell proliferation[36–38] and protects against ischemic acute kidney injury [39–41]. Activation of AMPK signaling reduces the cellular energy consumption and protects the kidneys from ischemia-reperfusion injury [42], while suppressing cell proliferation [43,44]. Our data shows that as a complement to metabolic reprogramming, inhibiting proliferation might be a useful strategy to prevent lethal energy deprivation in epithelial cells. This protective mechanism might be

responsible for the protective effect of the extensively studied phenomenon of ischemic preconditioning [45–47], where a mild and transient ischemia triggers cytoprotective pathways concomitant with transient cell cycle arrest [48,49], offering an endpoint to select a time frame for the difficult clinical implementation of a promising preclinical therapeutic approach [50,51]. With severe injury to an organ the increase in ATP/ADP ratio with cell cycle arrest may have adaptive effects to allow the cell more energy to repair damaged DNA. This may prevent malignant transformation and have a adaptive or maladaptive consequences with respect to long term sequelae of kidney injury depending upon whether the cell cycle arrest is at G1/S or G2/M due to a senescence associated secretory program resulting in production of pro-fibrotic cytokines[52,53].

ONLINE METHODS

Cell cultures and pharmaceutical inhibitors

HK2 cells are immortalized human renal proximal tubular cells cultured in DMEM with 10% fetal bovine serum. The experiments were conducted in Leibovitz's L-15 medium with no phenol red (Fisher, #21083027). L-15 was specifically designed for renal primary cell cultures. It provides a stable physiological pH without CO_2 supplementation, as it is buffered with non-bicarbonate ions. Pharmaceutical inhibitors were purchased from Selleckchem: tenovin-1 (#S8000), pifithrin-α (#S2929), Rigosertib (#S1362) and KU-55933 (#S1092).

Viability experiments

Puromycin toxicity was tested on cells grown in DMEM with 10% fetal bovine serum with or without 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% atmosphere

(ref 26700 from Air products) in a airtight chamber (ref 27310 and 27311 from Stemcell) during 24h, according to the manufacturer's instructions. The control cells were grown in Leibovitz's L-15 medium with 4,5 g/L glucose supplementation under ambient atmosphere. Flow cytometry analysis for assessment of dead cells was performed with the fixable viability dye Viobility 405/452 Fixable Dye (Miltenyi Biotec®). Viobility dyes react with amine groups of proteins and are non-toxic. In dead cells, intracellular proteins are stained, resulting in an increase in fluorescence.

ATP/ADP bioluminescence assay

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

ATP/ADP ratio live imaging

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 [27]. 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 [54]. PercevalHR (ATP/ADP ratio) was further corrected for pH by normalization on pHRed [27], using the Ratio Plus plugin.

Bioinformatic analysis of 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 [55]. 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.

Statistical analysis

Statistical analyses were conducted using JMP 11 (SAS). Log-transformation was performed to obtain normal distribution, when necessary. Correlations were evaluated using Pearson's test. P-values were considered significant when <0.05. Comparison between groups were considered significant when the p-value was <0.05 using a Wilcoxon rank-test. Differences in ATP/ADP changes between treatment groups over time were assessed by analysis of variance using a standard least square model.

Supplemental material:

Fiji Jython script for single cell ratiometric analysis

Live PercevalHR videos

ACKOWLEGMENTS

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