Optogenetic control of lysosome function

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Abbreviations:

HEK293T: Human Embryonic Kidney 293 with expression of mutant version of the SV40 large T antigen cell line for immortalization through p53 inhibition; HeLa: Henrietta Lacks's cancerous cervical tumor cell line; SH-SY5Y: human neuroblastoma cell line; DMEM: Dulbecco's modified Eagle's medium; pCMV: expression vector with Cytomegalovirus promoter; CD63: Cluster of Differentiation 63; βHK: β-subunit of H+,K+-ATPase; *Ns*XeR: xenorhodopsin from the nanohalosarchaeon Nanosalina; Arch3: archaerhodopsin from *Halorubrum sodomense*; ATR: all-trans retinal; lyso-: lysosomal; GFP: green fluorescent protein; se-pHluorin: super-ecliptic pH-sensitive GFP mutant pHluorin; RFP: red fluorescent protein; CLSM – confocal light scanning microscope; BafA1: Bafilomycin A1; CTSB: cathepsin B; HCQ: hydroxychloroquine; FCCP: Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; CCCP: Carbonyl cyanide 3-chlorophenylhydrazone; ATP: adenosine triphosphate; HBSS: Hank's Balanced Salt Solution; LMP: lysosomal membrane permeabilization; LSD: lysosomal storage disease; GTPase: hydrolase enzymes that bind to the nucleotide guanosine triphosphate (GTP) and hydrolyze it to guanosine diphosphate (GDP); ARL8B: ADP-ribosylation factor-like protein 8B; mTORC1: mechanistic target of rapamycin complex 1; TFEB: transcription factor EB; vATPase: Vacuolar-type H^+-ATPase.

Abstract

Lysosome protective, metabolic, signaling functions are highly dependent on their pH. A lack of tools of high spatial and temporal resolution for pH control is a bottleneck of lysosome related cell research. Light-driven inward proton pump *Ns*XeR, targeted to the lysosomes of mammalian cells, produces lysosome alkalization simply by light. Complementary use of outward proton pumping Arch3 rhodopsins in lysosomes offers an approach to vary pH in a range from around 5 to 6.5 in both directions (alkalization and acidification). Lyso-*Ns*XeR optogenetics efficiency was demonstrated, in particular, by its ability to inhibit lysosome proteolytic enzymes. Unprecedented time resolution of the optogenetic approach allowed direct *in situ* monitoring of vATPase activity. Thus, optogenetic monitoring and regulation of the lysosome function, through pH control over a wide range, could serve as an approach to studying fundamental cell processes, and rational drug design.

Introduction

Lysosomes are indispensable organelles of the cells serving as a waste disposal/food digesting system cleaving used cellular components, macromolecules, and viruses through autophagy and endocytosis. They are involved not only in material and energy metabolism but also in other organelles regulation, plasma membrane repair, programmed cell death, secretion and signaling (Ballabio and Bonifacino, 2020). In general, pH of cellular compartments is a vital physiological parameter of homeostasis virtually in all cellular compartments, and pH alterations are associated with severe diseases (Weisz, 2003). Acidic environment is especially crucial for endocytic/phagocytic and autophagic pathways organelles, lysosomes in particular. Highly acidic environment (pH 4.5 – 5.0) in lysosomal lumen is required for various purposes like the efficient operation of hydrolytic enzymes that degrade damaged biomolecules or nutrients, iron uptake, receptors recycling, lysosome positioning and release the viral genome. Low pH is also necessary for some amino acid transporters participating in the export of digested nutrients and metabolic signaling.

The ion balance in lysosomes is maintained by chloride, calcium, sodium, other ion transporters and by a proton pumping vATPase (Xu and Ren, 2015), which plays a major role in the pH regulation. Inhibition of vATPase, lysosome membrane permeabilization, garbage accumulation lead to deacidification of lysosomes, which, in its turn, has severe consequences for human health. Dysregulations of lysosomes lead to impairment of energetics, proteostasis, and some other cell functions causing severe diseases, including cancer, neurodegeneration, age-related macular degeneration, infectious diseases, and metabolic disorders (Bonam et al., 2019). On the contrary, stabilization of lysosome activity, first of all acidic pH, is important for longevity (Adam Bohnert and Kenyon, 2017; Leeman et al., 2018; Sun et al., 2020).

On the other hand, controlled lysosome alkalization could be used as simulation of diseases for the studies of their pathogenesis process. Many physiological cellular responses induced by lysosome alkalization (like lysosomal-mitochondrial calcium exchange, lysosome-to-nucleus signaling via TFEB, ER remodeling, metabolic adaptation and rewiring, and virus egress) should be re-investigated under spatio-temporally precise lysosome pH control. Lysosome alkalization could prevent viruses infectivity, cancer invasiveness and chemotherapy resistance, systemic autoimmune diseases. The analysis of lysosome pH influence on cellular processes carried out in the Supplementary Note 1.

Thus, manipulation of lysosomal pH is a key for the studies of their function and also therapeutic intervention. Optogenetic deacidification of lysosomes would be a missing, problem-solving approach to the studies of lysosome functions and dysfunctions. Optogenetics is already widely applied for the multimodal remote control of neurons by use of microbial rhodopsins (Gordeliy, 2022), ion pumps, and ion channels selectively expressed in the plasma membrane of the cells (Adamantidis et al., 2015). Though the rhodopsin optogenetic approach in cell organelles is a minimally invasive treatment, few, comparing to neuroscience applications, examples of intracellular optogenetic tools exist. There are rhodopsin-assisted studies of synaptic vesicles and lysosomes (Rost et al., 2015), endosomes (Kakegawa et al., 2018), mitochondria (Berry and Wojtovich, 2020), and endoplasmatic reticulum (Asano et al., 2018). For example,

functional incorporation of rhodopsins to the cell organelles allows optogenetic control of the electrochemical gradient by channelrhodopsin in mitochondria (Tkatch et al., 2017). Also, application of the outwardly directed proton pump Arch3 in synaptic vesicles enables light control of vesicular acidification and neurotransmitter accumulation (Rost et al., 2015). Recently optogenetic approaches to cytosol alkalization and acidification were developed (Donahue et al., 2021; Nakao et al., 2022; Vlasova et al., 2023). The reason for limited representation of optogenetics in the control of different cellular compartments is unresolved difficulties of specific targeted expression of optogenetic tools (Garita-Hernandez et al., 2018). Thus, specific organelle optogenetics is a promising emerging area.

In our work, we expand the optogenetics toolbox to control lysosome functions not only through their acidification but also through their alkalization via expression of outward and inward light driven pumps, correspondingly, in lysosomes of mammalian cells. We demonstrate the efficiency of lysosome optogenetics with an inward proton pump, xenorhodopsin from the nanohalosarchaeon *Nanosalina* (*Ns*XeR) (Shevchenko et al., 2017), enabling alkalization of the organelle up to 1 pH unit. It makes it possible to control lysosome functions through optogenetic regulation of pH in wide physiological and pathological ranges (Leung et al., 2019), confirmed here by controlled inhibition of lysosomal pH-dependent enzyme Cathepsin B.

The introduced here inward proton pump based optogenetics revealed diversity of lysosomes within individual cells and functional consequences of this phenomenon. vATPase activity, revealing by post-interventional re-acidification kinetics, was different in cells periphery and juxtanuclear location, as it was reported previously by lysosome pH measurements (Johnson et al., 2016).

Passive lysosome alkalization via proton leakage under vATPase inhibition (Xu and Ren, 2015) or active alkalization (lysosome membrane permeabilization or proton sequestration by garbage during lysosome storage diseases) reveal themselves by difference in amplitude of optogenetical alkalization and kinetics of vATPase-assisted re-acidification.

Thus, we suggest that the lyso-*Ns*XeR system could reveal non-functional lysosomes even when acidic pH masks problems. Lysosome optogenetics could be used for the study and early stage diagnosis of human diseases associated with the dysfunction of lysosomes, such as vATPase inhibition, lysosome storage, and membrane permeabilization diseases (Bonam et al., 2019).

Optogenetic control of pH in a wide range (from acidic to alkaline) opens a new way to the studies of key processes in which lysosomes are involved, particularly those driven by alkalization of lysosomes. Since acidification is required for the function of different cellular organelles (Weisz, 2003), the optogenetics introduced here with light-driven inward proton pumps might have applications beyond lysosomes, for the studies of cellular functions and dysfunctions dependent on the pH state of other organelles.

Results

Lyso-*Ns*XeR: new optogenetic tool for the pH increase of lysosomes

The first goal of our work was to develop an optogenetic tool for reversible increase of pH (hereafter "alkalization") of lysosomes. For this end, we expressed an inward proton pump *Ns*XeR (Shevchenko et al., 2017) in a fusion construct CD63N-se-pHluorin-*Ns*XeR-mKate2-βHK-CD63C (hereafter lyso-*Ns*XeR, Fig. 1a) with the pH sensitive green fluorescent protein super-ecliptic (se-) pHluorin (hereafter pHluorin, in lysosome lumen), and the pH insensitive red fluorescent protein mKate2 (facing the cytosol). This approach has been used with an outward proton pump archaerhodopsin from Halorubrum sodomense Arch3 (in so-called lyso-pHoenix construct, named here as lyso-Arch3), which was shown to localize in the lysosome membrane and could induce transient lysosome acidification in Bafilomycin A1 (BafA1) treated cells (Rost et al., 2015).

The lyso-*Ns*XeR lysosomal localization and the ability to alkalize lysosomes are presented in Fig. 1. Fluorescence confocal microscopy in the HEK293T cells transfected with lyso-*Ns*XeR shows that mKate2 (red fluorescence) from the lyso-*Ns*XeR construct is co-localized with LysoTracker Red (orange fluorescence), staining acidic lysosomes (Fig. 1b). An increase of pHluorin fluorescence in the untreated HEK293T cells is observed under illumination with 590nm light (Fig. 1c, green curves). The undesirable effects linked to the lysosomal movement during imaging are not present since mKate2 red fluorescence does not change significantly (Fig. 1c, red curves); whereas pHluorin fluorescence is noticeably and synchronously increased under illumination and decreased without it. Therefore, the pHluorin fluorescence changes reflect pH alteration rather than the lysosome movement.

Notably, the pH-sensing fluorescent protein pHluorin is located in the vicinity of *Ns*XeR, but collected fluorescence change curves reflect pH of the bulk phase of lysosomal lumen due to fast H^+ diffusion in water volume (diffusion coefficient is ~10^4 μm^2/s (Agmon, 1995)). Having in mind that lysosome diameter is ~0.5μm (de Araujo et al., 2020), so its volume is ~10^{-17} l, under pH 4.5, 5.5, 6.5 proton amount is ~400, 40, 4 protons, correspondingly. Thus, proton pumping by *Ns*XeR cause firstly fast local 'surface' juxta-membrane effect of alkalization and pHluorin fluorescence increase, following fast proton diffusion out of lumen and pumping out, but, under used experimental setup, we assume that these steps that could not be separated within monitoring with the period of ~4s.

Amplitude and kinetics of light-induced lysosome alkalization depends, on the one hand, on the rhodopsin expression level and, on the other hand, on alkalization compensation by vATPase, which also has heterogeneity by the cellular state and the localization of lysosomes in the cells (Johnson et al., 2016). Additionally, endogenous proton leaks also occur (Xu and Ren, 2015). Our experiments were performed without inhibition of vATPase so expressed lyso-*Ns*XeRs can pump protons more effectively than endogenous lysosomal vATPase allowing increase of lysosomal pH in the untreated cells.

The proposed here approach allows time-resolved monitoring of re-acidification (relaxation of intraluminal acidification) kinetics just after turning off the illumination of the cells (Fig. 1c, green curves, time segment

marked with blue boundaries). Since *Ns*XeR proton pumping terminates within 30 ms (the length of photocycle) (Shevchenko et al., 2017) after the light is switched off, post-illumination relaxation shows pure endogeneous pH adaptation that could be mainly attributed to vATPase working capacity (Riederer et al., 2023), with some impact of other acidifiers (Fujii et al., 2023). The origin of fast but weak and slow but high re-acidification effects in two regions with lysosomes in different cells (Fig. 1c, green curves 1 and 2) could arise from a different balance of the vATPase acidification and proton leaks alkalization (outside the lysosome or inside the garbage "proton sponges", accumulated in the lumen), maintaining the equilibrium pH of lysosomes (Casey et al., 2010). Lyso-*Ns*XeR throws the system out of balance for a while, so stability of equilibrium could be shown. Slow relaxation with a high decrease of pH after lyso-*Ns*XeR extra-alkalization for lysosomes with curve 2 demonstrates working vATPase acidification with weak leaks. So more acidic pH for lysosomal cluster 2 could be reached. Fast relaxation with a low decrease of pH for lysosomal cluster 1 could reveal that normal acidification/alkalization balance is disrupted due to weaker vATPase or stronger leaks. Further experiments should be done for clarification. Altogether, coupled pH modulation and detection is a unique *in situ* approach to vATPase probing in terms of simplicity and pithiness.

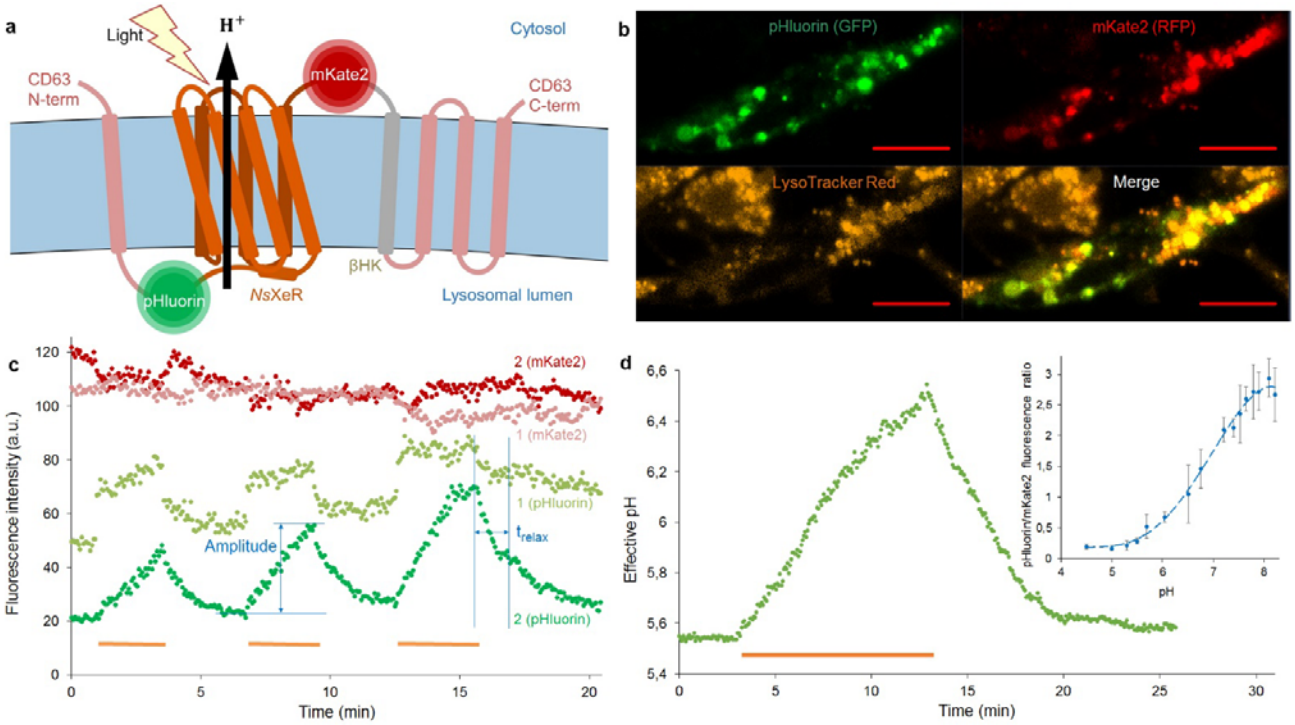

Figure 1 | Lyso-*Ns*XeR optogenetical alkalization of lysosomes. (**a**) Schematic representation of the lyso-*Ns*XeR (CD63N-se-pHluorin-*Ns*XeR-mKate2-βHK-CD63C) construct consisting of CD63 alpha-helixes flanking *Ns*XeR, the fluorophores (pHluorin and mKate2), and the βHK transmembrane domain. Term: terminus. (**b**) Lyso-*Ns*XeR construct expressed in the HEK293T cells and colocalized with LysoTracker Red DND-99. mKate2 (red), pHluorin (green), and LysoTracker Red (orange) reveal all, alkaline and acidic lysosomes, correspondingly. Scale bars 10µm. (**c**) Demonstration of characteristic kinetics of lysosomal alkalization under illumination on the example of two different lysosomal regions (1 and 2). The illumination time intervals (with 590 nm light) are highlighted by orange lines. The absence of significant mKate2 fluorescence change (red curves) indicates that lysosomes are not moving in the course of the experiment. Lysosomal regions 1 and 2 demonstrate different alkalization and re-acidification kinetics due

to the differences in their vATPase/leaks equilibrium (see the main text). The fluorescence signals were corrected on background to remove backlight from LED. See also Supplementary Movie 1 for lysosomal cluster 2. (**d**) Demonstration of maximum lysosomal alkalization under prolonged illumination, pHluorin fluorescence converted to pH by calibration plot (inset). The pH change about 1 unit reveals high potency of the approach.

We measured in absolute units the pH increase triggered by light activated lyso-*Ns*XeR. The pHluorin fluorescent changes were normalized on the protein expression by measuring the ratio of pHluorin to mKate2 fluorescence. This ratio determined at different lysosomal pH allows calibrating the pH-sensor and calculating ΔpH in absolute units from pHluorin rises after light activation of the rhodopsins. The calibration plot (Fig. 1d, inset), which agrees well with the literature data (Sankaranarayanan et al., 2000), shows that lyso-*Ns*XeR increases pH up to 1 unit under illumination (Fig. 1d).

Our control experiments with the construct lacking rhodopsin (lyso-pHluorin) and lyso-*Ns*XeR with protonophore FCCP show that the main reason for the observed pHluorin fluorescence change is *Ns*XeR light sensitivity. The protonophore diminishes the lyso-*Ns*XeR induced pHluorin fluorescence increase (pH change) equilibrating proton concentrations inside and outside lysosome (Fig. 2a). As expected, the emerging pH change produced by lyso-*Ns*XeR depends on the light intensity, increasing up to saturation under higher LED power values (Fig. 2b).

Post-illumination re-acidification takes minutes; therefore one could analyze lysosomes alkalization also by flow cytometry to obtain data from many cells. The flow cytometry data confirmed the pronounced effect of lyso-*Ns*XeR on the intralysosomal pH (Fig. 2c). It is shown that *Ns*XeR-dependent alkalization under illumination could be similar to the alkalization effect of vATPase inhibition by BafA1, *i.e.*, up to pH ~6.5 (Yoshimori et al., 1991), which is consistent with microscopy pH change evaluation (Fig. 1d).

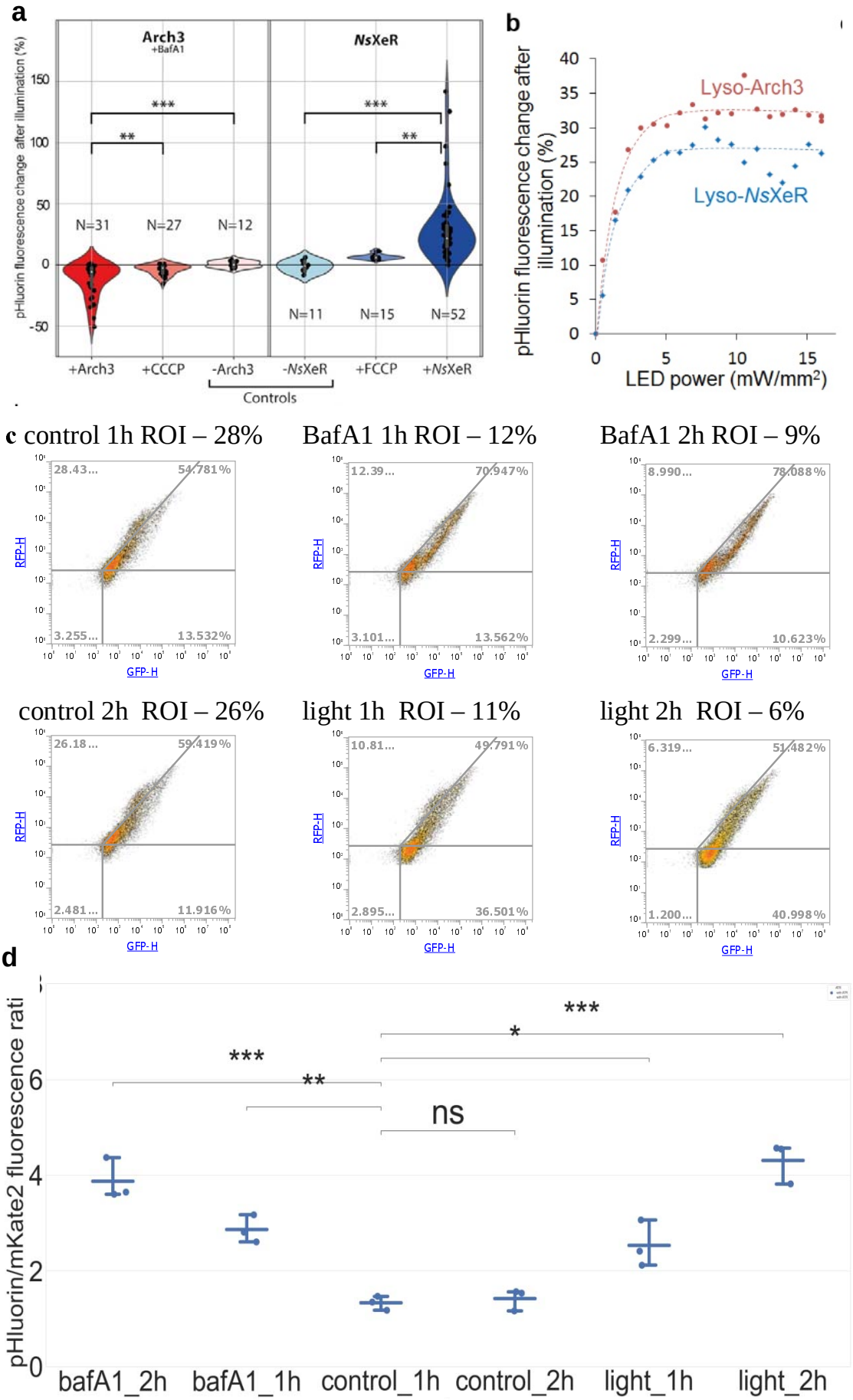

Figure 2 | Control experiments with lyso-*Ns*XeR and lyso-Arch3 constructs in HEK293T cells. (**a**) The absence of significant effects on pHluorin fluorescence under illumination of the lyso-pHluorin construct

(without rhodopsins, Controls), under illumination of the lyso-*Ns*XeR, lyso-Arch3 constructs in the presence of protonophores in contrast with the lyso-*Ns*XeR, lyso-Arch3 constructs. N corresponds to the number of the light shots in all experiments. lyso-Arch3 (with overnight 50nM BafA1) - CCCP: 50uM 1h, lyso-*Ns*XeR - FCCP: 50uM 1h. Choose of FCCP as stronger protonophore for lyso-*Ns*XeR was only effective in the case of untreated cells. (**b**) The dependence of the lyso-*Ns*XeR and lyso-Arch3 light-induced pHluorin fluorescence change on illumination intensity (LED power). The effect plateaued after exponential growth. Characteristic curves are shown. (**c**) The representative flow cytometry data comparing BafA1 and lyso-*Ns*XeR effects on lysosomal pH in the lyso-*Ns*XeR-expressing cells, control – lyso-*Ns*XeR expressing cells without treatments. Cells were treated with light or BafA1 and analyzed for a shift in pHluorin/mKate2 ratio, revealing average lysosomes pH per cell, at different time points of treatments. Scatter plots of pHluorin/mKate2 ratio are shown with Demarcation line made such as in the control sample almost all transfected cells with high mKate2 signal were to the left of the straight line (in the ROI – region of interest).
Number of cells in ROI (representing normal acidification of lysosomes) written under scatter plots. (**d**) pHluorin/mKate2 ratio, revealing average lysosomes pH per cell, in transfected cells with high mKate2 signal. Mean ± s.d. from three independent experiments. One-way t-test. ns: 0.05 < p <= 1, *: 0.01 < p <= 0.05, **: 0.001 < p <= 0.01, ***: 0.0001 < p <= 0.001.

Lyso-Arch3, a complementary optogenetic tool for lysosomal acidification: determination of the corresponding pH change in absolute units

The combined lyso-*Ns*XeR and lyso-Arch3 optogenetic system would allow controlling lysosomal pH in the widest possible pH range. To understand the real performance of the approach and be able to rationally plan future optogenetic studies of lysosome and lysosome-related processes in the cells, one should also know the pH shift of lyso-Arch3 in absolute pH units. We expressed the outward rhodopsin proton pump Arch3 in lysosomes of the HEK293T cells (Fig. 3a), as described by Rost *et al.* (Rost et al., 2015). First, as for lyso-*Ns*XeR, we proved for lyso-Arch3 that the pHluorin fluorescence change is due to the change of pH rather than the lysosomal movement (Fig. 3b, Supplementary Movie 2). Second, the experiments showed that the emerging ΔpH produced by lyso-Arch3 disappears under the protonophore treatment (Fig. 2a and Fig. 3d) and depends on the light intensity (Fig. 2b).

Lyso-Arch3 can decrease pH in BafA1-treated HEK293T by about 0.5 units (Fig. 3c). Precise determination of the maximum drop of pH is limited since super-ecliptic pHluorin loses linearity of the pH-response below pH ~5.5 and is insensitive below pH ~5.0 (Fig. 1d, inset).

For higher accuracy, it should be pointed out that the control experiments with the lyso-pHluorin (without rhodopsin) construct in BafA1-treated and untreated cells showed a slight change of the amplitude of pHluorin fluorescence upon the maximum power of 590 nm illumination (Fig. 2a). However, this effect is significantly lower than the pH change triggered by lyso-*Ns*XeR and lyso-Arch3 and should not complicate interpretation of the data obtained in the optogenetic experiments.

Thus, a complementary use of lyso-*Ns*XeR and lyso-Arch3 already can modify the pH of lysosomes within two units. We expect that further optimization of the proteins and their expression in lysosomes may further increase the range. Considering high sensitivity of the cell and, in particular, lysosome functions to pH changes, we also expect that lyso-*Ns*XeR itself and in combination with lyso-Arch3 provides powerful optogenetic approach to the studies of the pH dependent functions and dysfunctions of lysosomes.

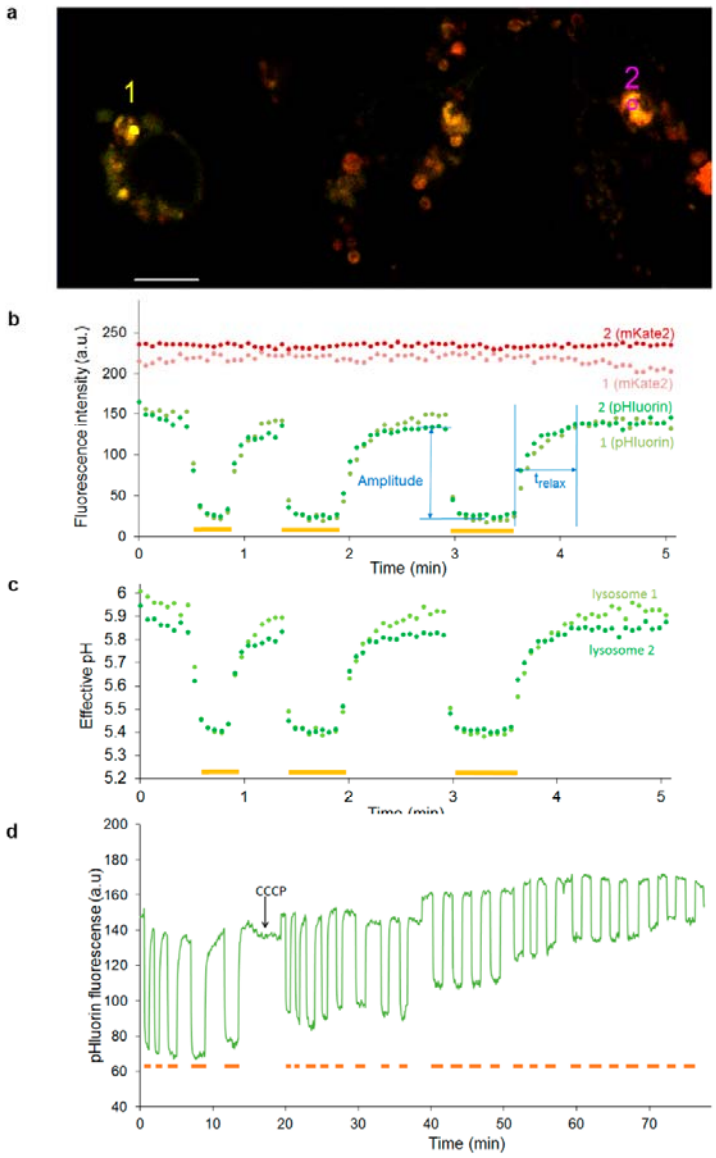

Figure 3 | Lyso-Arch3 in HEK293T cells treated with vATPase inhibitor BafA1. (**a**) Image of confocal fluorescence microscopy showing co-expression of pH sensitive green fluorescent protein pHluorin and pH insensitive red fluorescent protein mKate2. Scale bar 10µm. (**b**) Demonstration of lysosomal acidification (in the vicinity of pHluorin) under illumination. Fluorescence is measured at regions 1 and 2 highlighted on figure a. Time-intervals of illumination by 590nm light are highlighted by orange lines. The absence of mKate2 fluorescence change proves that lysosomes are not moving under illumination. (**c**) The changes in effective lysosomal pH obtained by calibration. **(d)** The level of lysosomal acidification by lyso-Arch3 decreases during the incubation with the protonophore 50uM CCCP. All experiments with lyso-Arch3 were done after overnight treatment with 50nM of BafA1.

Justification of the optogenetic control of bulk pH and functional properties of lysosomes: decreased LysoTracker accumulation and lysosome proteases inactivation by means of lyso-*Ns*XeR

Our optogenetic approach is indispensable for fundamental studies of lysosome functioning and those cell functions and dysfunctions that are coupled with lysosomes. We used LysoTracker Red accumulation and Magic Red CTSB cleavage assays in the corresponding experiments (Fig. 4). To demonstrate that

optogenetics can control proteases, which are key lysosome functional components, we first show that the lyso-*Ns*XeR changes pH quickly not only in the vicinity of a lysosome membrane where pHluorin is placed but also in lysosome lumen. LysoTracker Red is a fluorophore containing a weak basic amine that selectively accumulates in acidic compartments and exhibits red fluorescence. Therefore high pHluorin fluorescence accompanied by low LysoTracker Red staining of lyso-*Ns*XeR expressing cells in comparison to non-transfected cells (Fig. 4a) indicates optogenetically induced alkalization of lysosome bulk.

To show directly that lyso-*Ns*XeR induced alkalization influences the activity of lysosome proteases, we performed an experiment with Magic Red CTSB substrate. The Magic Red CTSB is a reagent exhibiting red fluoresce upon cleavage by Cathepsin B enzyme. Lysosomal cysteine proteases, a subgroup of the Cathepsin family, are critical for normal cellular functions such as general protein turnover, antigen processing, and bone remodeling. Cathepsin B functions in lysosomes for protein degradation and maintenance of cellular homeostasis (Brix et al., 2008). Cathepsin B normally has highest activity at acidic pH in lysosomes. It also cleaves small molecule substrates like Magic Red CTSB at the carboxyl side of Arg-Arg bonds (Cui et al., 2019). In our experiments, Magic Red CTSB was cleaved within 5 min, as shown by the fluorescence increase (Fig. 4b). Light illumination of the lyso-*Ns*XeR expressing cells significantly decreased the Magic Red CTSB cleavage. Thus, lyso-*Ns*XeR optogenetics enables unprecedented time resolution control of the activity of lysosomal enzymes.

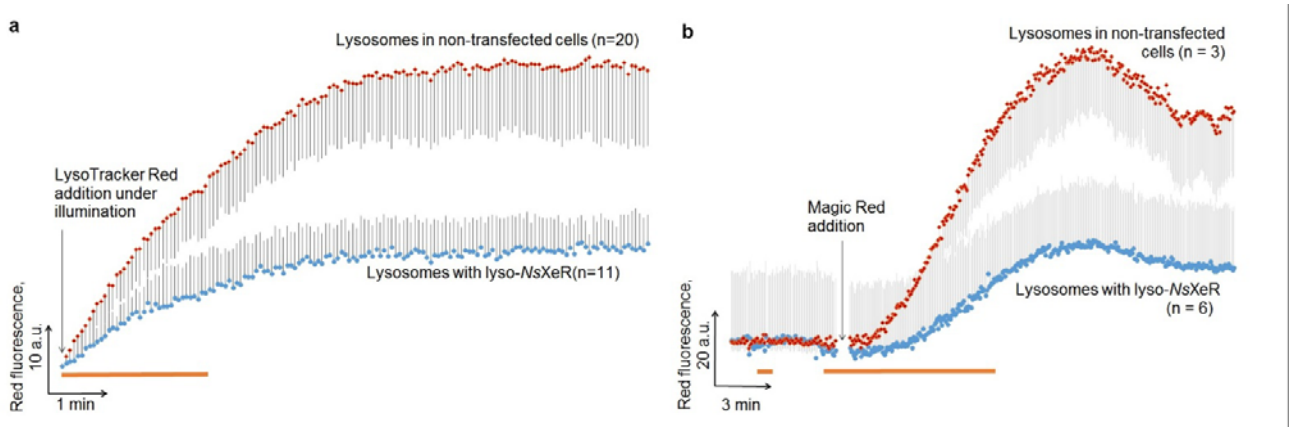

Figure 4 | Activation of lyso-*Ns*XeR increases pH of lysosomal lumen and inhibits proteases. (**a**) Lyso-*Ns*XeR induced pH change. Weaker accumulation of pH-sensitive fluorescent dye LysoTracker Red in lysosomes of the transfected cells under illumination shows that lyso-*Ns*XeR increases the lumenal pH of lysosomes. (**b**) Lyso-*Ns*XeR-mediated optogenetically induced pH increase inhibits lysosomal proteases. Fluorescence of Magic Red CTSB, which becomes fluorescent only after cleavage by Cathepsin B, is significantly inhibited by light activation of lyso-*Ns*XeR compared to the control (non-transfected) cells. Constant background of red fluorescence from mKate2, which is independent on light, was subtracted. The error bars reflect the error of the mean value and are shown on one side only for clarity.

Lysosomal optogenetically-induced pH change and pH relaxation kinetics are sensitive to vATPase activity

Lysosomal optogenetics allows probing vATPase activity by measuring the kinetics of pHluorin fluorescence after light-activation of rhodopsins. The approach allows efficient detection of the difference between lysosomes placed on the periphery of the cell and near the nucleus in absence of nutrients. A small

GTPase ARL8B drives lysosomal transport to the cell periphery (Johnson et al., 2016; Ponsford et al., 2021). Previously, the differences of pH of perinuclear and peripheral lysosomes were observed only upon overexpression of ARL8B GTPase (Ponsford et al., 2021).

Optogenetics reveals a noticeable difference of the re-acidification kinetics for these two subpopulations of lysosomes (Fig. 5) even without ARL8B overexpression. Slower lysosomes re-acidification on the cell periphery could be explained by inhibition of the vATPase activity by ARL8B in peripheral lysosomes (Johnson et al., 2016). Thus, the introduced here optogenetic approach is highly informative due to its outstanding time resolution.

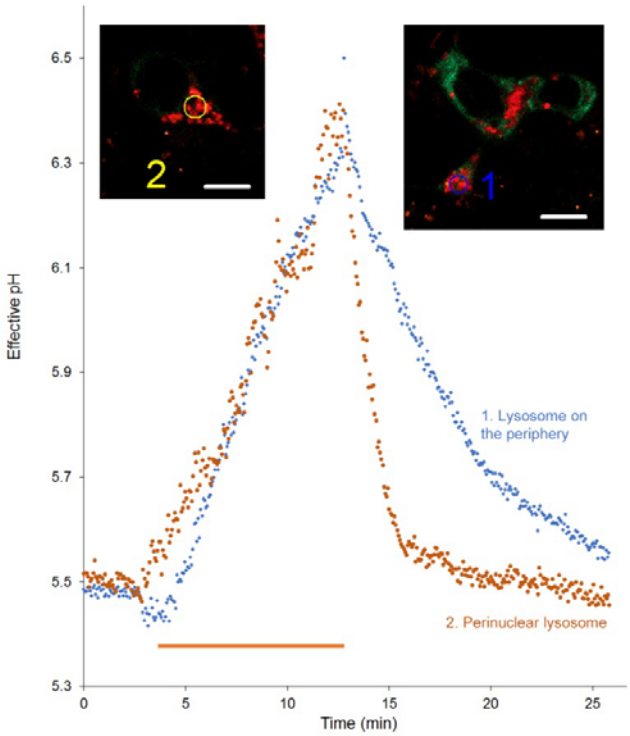

Figure 5 | Lyso-*Ns*XeR senses vATPase activity. Two sub-populations of lysosomes under physiological conditions, in absence of nutrients. Lysosomes on the cell periphery (1) have a significantly longer time of re-acidification (relaxation) in comparison with perinuclear lysosomes (2).

Lysosomes are heterogeneous in a given cell. The ratio of acidic and alkaline lysosomes can be a hallmark of 'health'. Around 23% of lysosomes are acidic, and 68% are alkaline in normal cells (Leung et al., 2019). However, for instance, around 80% are acidic, and only the rest of lysosomes are alkaline in cancer cells (Webb et al., 2021). Optogenetic probing of vATPase activity can be used for the analysis of cellular lysosome state ('health'), for instance, for the studies of vATPase dysfunctions, accumulation of molecular 'garbage' in lysosomes in lysosome storage diseases, state of permeability of lysosome membrane. It is important to emphasize that re-acidification kinetics can be significantly different among lysosomes within the same cell. A search for approaches to vATPase activity restoration might be greatly facilitated by lyso-*Ns*XeR optogenetics. The proposed here lyso-*Ns*XeR is the first and only known tool allowing for high time-resolution and controlled alkalization of lysosomes. In general, lyso-*Ns*XeRs can be a valuable tool in a number of studies of functions and dysfunctions of lysosomes and cells on the whole.

Discussion

In the present work we introduced a new approach and demonstrated that important cell functions can be controlled simply by light through modulation of lysosomal pH with unprecedented spatial and time resolution. It is shown that it is a highly efficient approach to the studies of lysosome associated functions and dysfunctions of cells. Expression of inward light driven proton pump (*Ns*XeR) in lysosomes is highly selective, and this provides outstanding spatial resolution of the approach. Complementary use of lyso-*Ns*XeR and lyso-Arch3 can vary lysosome proton concentration by about two orders in both directions (alkalization (Fig. 1) and acidification (Fig. 3)).

Lysosome alkalization by lyso-*Ns*XeR is unique by its selectivity of action in contrast with other vATPases inhibiting drugs. For example, BafA1 blocks vATPases in lysosome and cellular membranes (Forgac, 2007) and furthermore inhibiting Ca^{2+} pump SERCA (P-ATPase) in endoplasmatic reticulum (Mauvezin et al., 2015). Concanamycins also binds with v and P- ATPases (Dröse and Altendorf, 1997).

Moreover, simultaneous compensation or exacerbation of the possible cytosolic acidification, produced by lyso-*Ns*XeR, could be done by usage of rhodopsin proton pumps, targeted to plasma membrane. Thus, pure consequences of lysosomal alkalization could be investigated by lyso-*Ns*XeR system.

To make clear whether lysosome optogenetics is indeed able to control the key lysosomal functions, we demonstrated the lyso-*Ns*XeR optogenetic regulation of Cathepsin B activity, which provides protein degradation and supports cellular proteostasis.

We demonstrated that an unprecedented time resolution, a great advantage of optogenetics, allows determining even small deviations in lysosomal functions and dysfunctions that are not manifested in increased luminal pH and thus cannot be detected by classical detection-only techniques. This conclusion was made on the basis of a significant difference of pH relaxation kinetics between the control experiments and the experiments with vATPase inhibition (at cell periphery (Fig. 5); whereas the corresponding pH value differences were negligible. Hence, our work proves that reversible lysosome alkalization could serve as an in situ monitoring tool for the lysosomal health and pathogenesis progression.

The fact that proton concentration can be controlled via lyso-rhodopsin proton pump optogenetics in a range of at least two orders of magnitude is encouraging for the extension of the proposed approach to other rhodopsin ion pumps (chloride, sodium, etc.). Indeed, rhodopsin ion pumps have a comparable length of photocycle and therefore similar ion pumping efficiency. Passive permeability of biological membranes to protons is usually considerably higher than to other ions. It opens new perspectives in lysosome and cell studies. Lysosomes are significantly enriched with chloride, and a decrease of chloride concentration correlates directly with a loss in the degradative function of the lysosome (Chakraborty et al., 2017; Graves et al., 2008). A decrease of lysosomal chloride influences Ca^{2+} release from the lysosome and impedes the activity of specific lysosomal enzymes, indicating a broad role for chloride in lysosomal function (Chakraborty et al., 2017).

Considerable variation of ion concentration is possible due to a high ratio of the membrane surface to the volume of lysosomes determined by a small size of the organelle. The size of other cell organelles is also

small in comparison to the size of the cells. It means that organelle optogenetics based on ion transporters can control many cell processes through light modulation of different ions concentrations. In future, lysosome optogenetics could be translated to lysosome-related organelles (Saftig and Klumperman, 2009) and other organelles for the studies of cellular functions and dysfunctions dependent on ions concentration (Thattai, 2017; Weisz, 2003). It encourages extension of the organelle optogenetic toolkit with other ion pumps and channels.

Finally, the number of slowly re-acidifying lysosomes could be used as a health biomarker for a normal cell. It could be a more powerful approach than just a pH measurement (Leung et al., 2019) because it might reveal an early onset of pathogenesis linked to lysosome abnormities.

Optogenetic control of the lysosome function through pH regulation over a wide range allows studying a wide range of fundamental lysosome depending cell functions, including lysosomal-mitochondrial axis, lysosome-to-nucleus signaling, ER remodeling, metabolic adaptation and rewiring, and virus egress. The introduced lysosome optogenetics may find a range of applications in the studies of the diseases associated with the dysfunction of lysosomes, like lysosomal storage diseases (Parenti et al., 2021; Platt et al., 2018). For instance, abnormally fast re-acidification kinetics could be a sign for malignization progression (Liu et al., 2018), process linked to reversed extra-/intra-cellular pH gradient (Webb et al., 2011), highly acidic lysosomes (Webb et al., 2021) of cancer cells. The analysis of possible applications of lysosome pH control was carried out in the Supplementary Note 1.

One might think that lysosomal alkalization has only pathogenic impact. It is tempting to apply lyso-*Ns*XeR tool to kill diseased cell, for example overactivated autoimmune cells in autoimmune disorders (Cao et al., 2021), influencing specific tumor features (Bonam et al., 2019), relying on lysosome over-acidification (autophagy addiction (Levy et al., 2017), invasion (Hämälistö and Jäättelä, 2016), cytosolic alkalization(Webb et al., 2011)). Alternative resembling approaches for light-controlled cellular toxicity like photodynamic cancer therapy using chemical photosensitizers is known (Li et al., 2020). Feasible advantage of optogenetical impact on cancer is the possibility of using selective expression (e.g. by hypoxia-specific promoters (Brown and Wilson, 2004), or exploiting of transcriptional addiction (Vervoort et al., 2022)).

Lysosome acidification is a mainstream among anti-aging interventions (Aman et al., 2021). However, there is possible advantage of transient lysosome alkalization over acidification. Senescent cells have alkali lysosomes due to their membrane permeabilization (Johmura et al., 2021), so acidification is senseless at this stage. On the contrary, transient alkalization on pre-senescent cells could initiate lysosome biogenesis by TFEB activation (Lie and Nixon, 2019), delaying process of autophagy decay.

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Methods

Reagents

Bafilomycin A1 (BafA1) (B1793), Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (C2759), Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (C2920), hydroxychloroquine (HCQ) (H0915) were from Sigma (Merck). Cathepsin B Assay Kit (Magic Red CTSB) (ab270772) was from Abcam. LysoTracker Red DND-99 (L7528), pHrodo Red AM (P35372), Phusion™ High-Fidelity DNA Polymerase (F530), FastAP Thermosensitive Alkaline Phosphatase (E0651), FastDigest NotI (FD0594), FastDigest KpnI (FD0524) were from ThermoFisher Scientific. Polynucleotidekinase T4 (E311), DNA ligase T4 (E319) were from SibEnzyme. Plasmid MiniPrep (BC021S) and CleanUp Standart (BC022) were from Evrogen, Russia.

Genetic constructs creation

pCMvlyso-pHoenix and pCMvlyso-pHluorin were a gift from Christian Rosenmund (Addgene plasmid # 70112, 70113). The lyso-*Ns*XeR constructs were made by followed procedure. In the pCMvlyso-pHoenix, plasmid BsiWI was replaced with the NotI restriction site by PCR. Afterwards, the *Ns*XeR gene (Shevchenko et al., 2017) was cloned by restriction-ligation with the KpnI & NotI sites. Cloning was approved by DNA sequencing.

Cell cultivation, transfections and treatments

Human cells HEK293T (ECACC 12022001) were cultivated in DMEM (Gibco, Cat.No: 41966) in CO2-incubators (Binder C150 E2) at 37’C and high humidity. For microscopy experiments, cells were seeded on the ibitreated 35 mm dishes (ibidi, Cat.No: 81156). Transient transfections were done on these dishes using 1ug plasmid and 6ul X-tremeGENE HP DNA Transfection Reagent (Roche, Cat.No:XTGHP-RO) two days before imaging. All-trans retinal was added to the final 10uM concentration to the cells growth medium simultaneously with transfection. For the lyso-Arch3 experiments, the HEK293T cells were treated with 50 nM BafA1 overnight for full lysosome alkalization. For the lyso-*Ns*XeR experiments, untreated HEK293T cells were used.

Optogenetic experiments

Live-cell imaging was done, as previously described (Bogorodskiy et al., 2021). We used an inverted confocal LSM780 microscope (Carl Zeiss, Germany), 63x (NA = 1.4, oil immersive) objective. Optical fiber (400 um diameter) guided through a microinjection needle holder was placed just above the cells of interest by means of a microinjection micromanipulator InjectMan NI2 (Eppendorf). LED590 (ThorLabs M590F2, 590nm emission maximum) was used with a power of 16 mW/mm2 (ThorLabs Thermal Power Sensor Head S302C measured) in all experiments, excluding the ones with LED power variation (ThorLabs 4-Channel LED Driver, DC4104). During the experiments, the cells were placed in a Tokai Hit CO2-incubator (model INUBG2H-ELY), HEPES (pH 7.4) for the final concentration 25 mM was added. For time series with light illumination, two approaches were used: a channel mode and a λ-mode.

Channel mode. First, for the control experiments, excitation at 488, 561 nm was used respectively for the simultaneous detection of mKate2 and pHluorin fluorescence for lysosome movement accounting. The

emission was measured in CLSM Channel-mode using a 2-channel detector (Carl Zeiss, Germany) set to a 488–523 nm (Channel 1) and 635–712 nm ranges (Channel 2) to avoid detection of maximum intensity of LED590. Slight LED590 emission was passed to Channel 1 and Channel 2 and was used to indicate time when LED590 worked. Time series experiments were done with time for one image obtaining 3.88s with no interval between the images, so pHluorin intensities were collected with 3.88s intervals. Time curves were obtained using ZEN software by Zeiss.

LED590 emission, which added intensity to Channel 1 or 2, was obtained from background ROI (near with lysosomes ROI) and then was subtracted from the time curves of lysosomes ROI to produce pure pHluorin and mKate2 fluorescence intensity time curves, correspondingly.

λ-mode. pHluorin fluorescence intensity only was excited by a 488 nm laser. The emission was measured in CLSM λ-mode using a 34-channel QUASAR detector (Carl Zeiss, Germany) set to a 488–535 nm range to avoid LED590 maximum intensity detection. Time curves were obtained using ZEN software by Zeiss after performing spectral Linear unmixing (in ZEN software by Zeiss) processing option (GFP spectrum assignment for spectrum of each pixel was done).

For Figure 2A, pHluorin fluorescence relative change were automatically obtained from the time series for the each illumination. Each pHluorin fluorescence relative change value is calculated for the full field of view (image) with many cells and lysosomes in each. Video files obtained were converted to png images by ZEN software by Zeiss. Python code (Supplement Notebook) did data array for all images of each experiment. The masks for cells, lysosomes and extracellular background for the each image were found using min max filter (Verbeek et al., 1988). Average intensity in pixels of the lysosome mask was found for each image and average intensity of the extracellular background (reflecting LED illumination) was subtracted. Curves of fluorescence changes in time for lysosomes were generated. Optogenetic effect was calculated as (Ff-Fi)/ (Ff+Fi)*100%, where Ff and Fi correspond to pHluorin fluorescence intensity right after finishing the illumination and before illumination, respectively. Tukey's post hoc tests were done to calculate a P value.

For Figure 2C pHluorin fluorescence change calculated as |(Ff-Fi)/Fi|*100%.

LED590 emission, which added intensity to the GFP spectrum, was obtained from background ROI and then was subtracted manually from the time curves of lysosomes ROI to produce pure pHluorin fluorescence intensity time curves. Finally, the images were aligned and positioned using Adobe Photoshop (Adobe Systems, USA).

pH measurements in lysosomes

HEK293T cells were incubated with different pH buffer solutions (based on HBSS, Gibco, cat No 14170) in the presence of Digitonin (10 μM) and Bafilomycin A1 (100 nM). For calibration by the GFP/RFP ratio (pH from 5.5 to 8.5) images from five cells in three different dishes were imaged in λ-mode (488–700 nm range), and Unmixing option of ZEN software by Zeiss was used for spectrum generation. For quantification of the lysosome acidity, the GFP/RFP fluorescence ratio was measured after excitation by a

488 nm laser - 0.4%, 561nm - 0.1%, because of 6-fold lower power of 488nm laser but 1,5-fold higher quantum yield of pHluorin (GFP) compared to mKate2 (RFP). Then the ratios of fluorescence intensities of pHluorin at 510nm (GFP value) and mKate2 at 620nm (RFP value) were calculated from the spectrum generated for the Region of Interest by Unmixing option in ZEN software by Zeiss.

Cytosolic pH measurements by pHrodo Red AM

To stain HEK293T cell, transiently transfected with lyso-*Ns*XeR, pHrodo Red AM (1000x stock) was mixed with Power Load Concentrate (100x stock). This mixture was added directly to cell medium for 20 min with subsequent exchange to the new full medium. Intracellular pH changes by lyso-*Ns*XeR was measured on confocal LSM780 microscope (Carl Zeiss, Germany) like in part Optogenetic experiments, λ-mode. First, effect of lyso-*Ns*XeR has been checked using reduced LED590 power (5mW/mm2) to avoid pHrodo bleaching with 490-535nm detection, 488nm excitation. Second, time series experiment was repeated with 635-700nm detection, 561nm excitation for pHrodo changes measurements. LED590 (5mW/mm2) was turned on and fluorescence signal increase after correction to background was used to assess cytosolic pH changes by manufacturer calibration.

Cytometry

Cell analysis on stable alkalization was done on an Attune NxT flow-cytometer (Applied Biosystems, Thermo Fisher) using BL1 (488nm laser excitation, 512/25nm detection), YL2 (561nm laser excitation, 620/15nm detection) channels for pHluorin and mKate2 fluorescence detection, correspondingly. Light illumination of the detached cells in a 1,5ml tube was done using Olympus KL1500-LCD (250W) with a 605/70nm optical filter.

Live imaging of LysoTracker Red and Magic Red CTSB staining

LysoTracker Red DND-99 (L7528, ThermoFisher), dye for the acidic cell compartments, was added to the final concentration 50uM to HEK293 cells during the Time Series experiment directly to 2ml medium. The addition was done without LED590 illumination to demonstrate equal staining rate for the transfected and not-transfected cells. LysoTracker addition was done during LED590 illumination to demonstrate that the activation of lyso-*Ns*XeR causes a different staining rate of the transfected and not-transfected cells, revealing bulk alkalization of *Ns*XeR-containing lysosomes with light by slower LysoTracker staining.

Magic Red (Arg-Arg-Cresyl Violet-Arg-Arg) (Abcam), fluoregenic substrate, emitting red fluorescence after Cathepsin B cleavage, was added to the HEK293T cells during the Time Series experiment directly to 2 ml medium. Vial with dry substrate for 50 reactions was dissolved in 50ul DMSO. This stock solution was diluted 1:10 with UltraPure Water (10977-035, Invitrogen) and added to the cells in the amount of 5-10ul depending on the cells quantity on the imaging dish. The Rate of Magic Red CTSB cleavage, revealed by the appearance of red fluorescence, is slower in alkali lysosomes than in acidic ones, which should be another proof of possibility of lyso-*Ns*XeR to alkalize lysosomes.

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Author contributions

FT, SB, AV, AEM, OM did work on the genetic constructs creation; NI, SB, did cell cultivation, transfections, and treatments; VG, NI, SB, SN planned the experiments design; NI supervised most of the experiments; NI, SB, performed the optogenetic experiments, including the study of different responses under various treatments, NI, SB, studied the optogenetic impact on the effectiveness of lysosomal enzymes and acidotrophic dye accumulation; SB, NI, AB, VIB revealed artifacts in the microscopy data; AA did automatization of image processing; VG, AA, EB supervised the optogenetic experiments; SN, VG, NI, EB, AR made manuscript conceptualization; NI, DB did the cytometry experiments; VG initiated, provided general supervision of the project and together with NI, SN, and the contribution from all other co-authors who prepared the manuscript.

Additional information

Supplementary Information accompanies this paper at link

Competing interest

Authors declare that they have no competing interests.