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Spatiotemporally controlled initiation of Parkin-mediated mitophagy within single cells

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Mitophagy, the selective removal of mitochondria through the autophagic pathway, is involved in cellular mitochondria quality control. Dysfunctional mitochondria can be selectively eliminated through Parkin-mediated mitophagy. Parkin is a ubiquitin E3 ligase that selectively translocates onto impaired mitochondria to initiate mitophagy, and mutations in Parkin have been identified in autosomal recessive forms of Parkinson disease. Here with the use of a genetically encoded, mitochondria-matrix targeting photosensitizer, we established a robust strategy that allow for spatiotemporally controlled initiation of Parkin-mediated mitophagy in single cells with light. The method can specifically target varying numbers of mitochondria into the Parkin-mediated mitophagy pathway for clearance. Combined with live cell imaging, we demonstrated that mitochondria can be cleared by Parkin-mediated mitophagy without juxtanuclear mitoaggresome formation. Autophagy proceeded with the asynchronous appearance of small LC3B-coated structures on Parkin-labeled mitochondria subsections in a nucleation-expansion manner. Our method allows for quantitative measurement on the Parkin-mediated mitophagy process, and can be multiplexed in imaging for higher throughput studies.

Key words: Parkin, mitochondria, autophagy, Parkinson disease, mitophagy, ROS, KillerRed, photoactivation

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Introduction

Mitochondria are key cellular energy production centers, and their proper maintenance is critical to cell health and function. With high levels of potentially damaging reactive oxygen species (ROS) present within mitochondrial matrices, cells have developed a multitude of schemes to prevent the accumulation of dysfunctional mitochondria.[1] Recent evidence shows that autophagy plays a key role in mitochondria maintenance utilizing a process termed mitophagy.[2] In mitophagy, dysfunctional mitochondria are selectively sequestered into phagophores and delivered to lysosomes for degradation. It is therefore thought that mitophagy is involved in the quality control of mitochondria.

Alterations to mitophagy has been implicated in neurodegenerative disorders such as Parkinson disease.[3] Two proteins found mutated in autosomal recessive forms of Parkinson disease, Parkin and PTEN-induced putative kinase protein 1 (PINK1),[4,5] have recently been described as playing central roles in mitophagy. In this form of mitophagy (herein referred to as Parkin-mediated mitophagy), PINK1 responds to mitochondria impairment by recruiting Parkin, an ubiquitin E3 ligase, from the cytoplasm onto dysfunctional mitochondria.[6-11] This Parkin translocation appears to signal the onset of selective mitochondrial removal through Parkin-mediated ubiquitination of mitochondria outer-membrane proteins,[6,10,12-18] leading to the eventual recruitment of autophagic machineries. Many disease-associated Parkin mutants have recently been demonstrated to alter cellular mitophagy activities, further connecting Parkin-mediated mitophagy to Parkinsonism.[16]

A robust way to investigate Parkin-mediated mitophagy is through the use of carbonyl cyanide

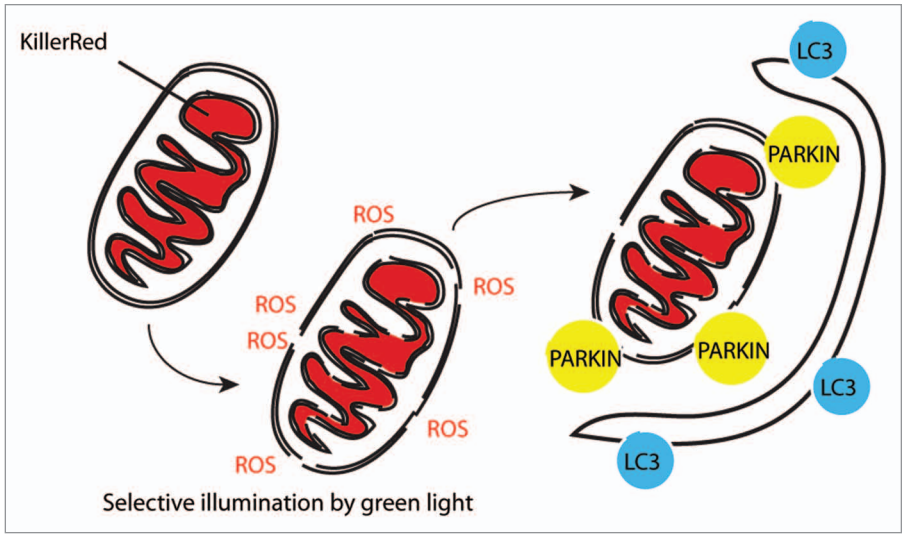

Figure 1. Schematic for light-assisted autophagic clearing of mitochondria. Specific mitochondria expressing matrix-targeted KillerRed protein in a Parkin-carrying cell (top left) is illuminated with 559 nm light, resulting in local display of ROS at the impaired mitochondria surface (middle). This leads to clearing of specific mitochondria by the autophagic machinery (LC3-coated autophagic structures) through Parkin-mediated actions (right).

3-chlorophenylhydrazone (CCCP), which can globally uncouple and impair cellular mitochondria. Under CCCP treatments, all cellular mitochondria eventually become targets of Parkin, making Parkin-mediated mitophagy events simple to visualize. However, the delivery of CCCP through direct feeding results in highly asynchronous and spatially uncorrelated initiation of Parkin-mediated mitophagy in cells. CCCP-treatment is therefore not the ideal tool for investigating the dynamics and the potential spatial coordination between different molecular components in Parkin-mediated mitophagy. The treatment also cannot be used to investigate Parkin-mediated mitophagy in specific cells within intact organisms.

We sought to improve upon existing methods for specific initiation of Parkin-mediated mitophagy by using light signals. Precise control and perturbation over cellular fate and activities such as neuronal firing,[19,20] activation of specific signaling cascades,[21-23] gene transcription,[24] protein production,[25] and modulation of protein-protein interactions[26] in living cells and organisms have been achieved using light signals. These light-based methods can modulate individual cell behaviors at the subcellular level with high time resolution, allowing for quantitative dynamic information on cellular processes to be obtained. It has previously been demonstrated that illuminating living hepatocytes with intense 488 nm light can lead to cellular damage that triggers mitophagy.[2] Herein with the use of a genetically encoded, mitochondria-matrix targeting photosensitizer, we show that low intensity green light can be specifically used to signal varying numbers of mitochondria for Parkin-mediated mitophagic turnover in a spatially- and temporally-controlled manner. Combined with live cell imaging, we demonstrated that mitochondria can be cleared by Parkin-mediated mitophagy without the formation of juxtanuclear mitochondrial aggregates (mito-aggresomes). In addition, we found that LC3-coated autophagic structures asynchronously appeared on small subsections of Parkin-labeled mitochondria in a nucleation-expansion manner. This method can be easily coupled with live cell imaging for following Parkin-mediated mitophagy events, and can be robustly multiplexed for higher throughput studies.

Results

Light-signaled parkin-mediated mitophagy within single cells. It has recently been reported that overall photodamage (through the use of intense 488 nm light illumination) to portions of a hepatocyte can lead to local occurrence of mitophagy events.[2] To specifically initiate mitophagy within single cells through the Parkin-mediated pathway in a spatially and temporally controlled fashion, and to avoid damaging other cellular components in the process, we designed a scheme based on the findings made in CCCP-treated cells. Studies using CCCP-treated cells revealed molecular factors that regulate Parkin-mediated mitochondria turnover through autophagy. With CCCP treatment, mammalian cells expressing high levels of ubiquitin E3-ligase Parkin (i.e., muscle and neuronal cells) demonstrated enhanced mitochondria clearing abilities. Parkin can specifically translocate onto impaired mitochondria to ubiquitinate mitochondria outer membrane proteins, resulting in the efficient recruitment of the autophagic machinery.[6,8] Moreover, reactive oxygen species are generated upon CCCP treatment, and this augments cellular autophagic activities for rapid progression of Parkin-mediated mitochondria turnover.[27]

In our design, single cells to be manipulated need to express two auxiliary proteins, *KillerRed-dMito* and *Parkin*, as illustrated in **Figure 1**.

Protein 1. *KillerRed-dMito.* Our HeLa cell model expressed a mitochondria-matrix targeted KillerRed protein (containing 2X mitochondria targeting sequence SVL TPL LLR GLT GSA RRL PVP RAK HIS LGD,[28] **Fig. 1** top left). KillerRed is a genetically encoded photosensitizer, and can effectively produce ROS in response to low levels of 559 nm light.[29] By illuminating desired area(s) within a cell with 559 nm light, mitochondria containing KillerRed-dMito in these defined region(s) will become specifically impaired, and display ROS at their surface (**Fig. 1** and middle), generating an effect similar to that of CCCP treatment. The use of a genetically encoded photosensitizer can allow for simple application of the scheme to cultured cells and entire organisms.

Protein 2. *Parkin.* Parkin can efficiently tag impaired mitochondria for autophagic clearing. Many cells do not normally carry high amounts of Parkin (i.e., HeLa cells). In our model system, we supplemented HeLa cells with YFP-Parkin. Having high levels of Parkin

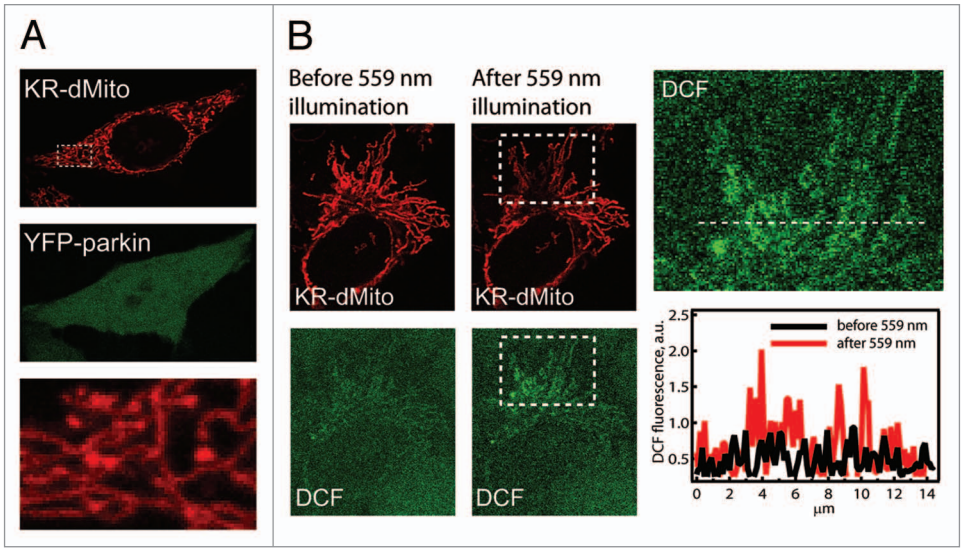

Figure 2. KillerRed for spatio-temporal controlled ROS generation. (A) Mitochondrial network remained intact in HeLa cells coexpressing mitochondria-matrix targeted KillerRed protein (KR-dMito) and YFP-Parkin. YFP-Parkin distributed normally (evenly in the cytoplasm) in these cells. Bottom: Zoomed-in view of the dotted region within the top image. (B) HeLa cell expressing KR-dMito and loaded with DCF showed homogenous green fluorescence (from DCF; left parts). Spatially encoded ROS generation in specific mitochondria can be achieved in a desired cell (in this case the region outlined with white dotted lines) through 559 nm light stimulation. This is indicated by the increase of DCF fluorescence in specific mitochondria (and the loss of KR-dMito fluorescence) upon illumination (middle parts). Top right: Zoomed-in view of the 559 nm illuminated region, with DCF fluorescence specifically highlighting mitochondria. Bottom right: DCF fluorescence intensity along the white-dotted line in the top right image. Mitochondria DCF fluorescence in this region dramatically increased after 559 nm illumination.

allows the 559 nm light-impaired mitochondria to be efficiently tagged, and this is followed by engulfment into LC3-coated autophagic membranes for removal (**Fig. 1** right). Parkin-mediated mitophagy can therefore be easily triggered in this manner with subcellular resolution in a single (or a group of) living cell(s). The use of a mitochondria-matrix targeted photosensitizer (making light stimulation specific) together with low intensity green light (559 nm, low phototoxicity) significantly reduces the potential side effects from illumination.

KillerRed in spatiotemporal generation of ROS within single cells. We first confirmed that the presence of KillerRed-dMito did not apparently affect the cells. Co-expressing YFP-Parkin and KillerRed-dMito in HeLa cells maintained the normal tubular appearance of their mitochondrial network as visualized through KillerRed-dMito (**Fig. 2A**). It also did not affect the mitochondrial fusion/fission dynamics and cell survival (**Sup. Vid. 1**). The KillerRed-dMito fluorescence highly localized to the mitochondrial networks (**Fig. 2A** and bottom). This minimizes potential ROS production directly within the cell cytoplasm.

Knowing that the co-expression of YFP-Parkin and KillerRed-dMito is well tolerated by cells, we then tested the use of KillerRed protein for spatially-encoded ROS generation in living cells using a well-established ROS sensor: 2,7-Dichlorodihydrofluorescein diacetate (DCF). DCF is a ROS sensor that, when oxidized, become highly green-fluorescent. As can be seen in **Figure 2B**, untreated HeLa cells show apparent homogenous DCF fluorescence (left). Upon 559 nm illumination, the cellular region that received light stimulation showed marked increase in its mitochondrial DCF fluorescence (tubular shaped; middle and right parts), confirming spatially selective ROS production.

Parkin-translocation triggered by light. The co-expression of YFP-Parkin and KillerRed-dMito did not alter live HeLa cells' native ability to efficiently initiate Parkin-mediated mitophagy in response to CCCP treatments.[8] The hallmark in the CCCP-induced effect is the translocation of Parkin onto mitochondria after treatment. In cells not treated with CCCP, YFP-Parkin remained diffused throughout the cytoplasm (**Fig. 2A**). When CCCP was added to the culture medium, YFP-Parkin began to translocate onto mitochondria (**Fig. 3A** and left), as evidenced by the colocalization between the YFP-Parkin and the KillerRed-dMito fluorescence in cells. The fraction of cells that showed colocalization increased in a time-dependent manner (**Fig. 3A** and right); in addition, a global change in mitochondria morphology (clustered and swollen, see **Fig. 3A**) and a decrease in total cellular mitochondria were observed.

With the use of HeLa cells co-expressing YFP-Parkin and KillerRed-dMito, we next demonstrated spatiotemporal-controlled initiation of Parkin-mediated mitophagy within single cells utilizing low intensity 559 nm illumination. An arbitrary region within a live HeLa cell was designated (**Fig. 3B** and bottom left, white dotted circle) as the area for initiating Parkin-mediated mitophagy. Immediately after 559 nm illumination, a decrease in local KillerRed-dMito fluorescence (due to KillerRed photobleaching), but not the YFP-Parkin signals, was observed (**Fig. 3B** and 2nd column). Afterwards, YFP-Parkin fluorescence translocated into the 559 nm-illuminated region with an apparent tubular

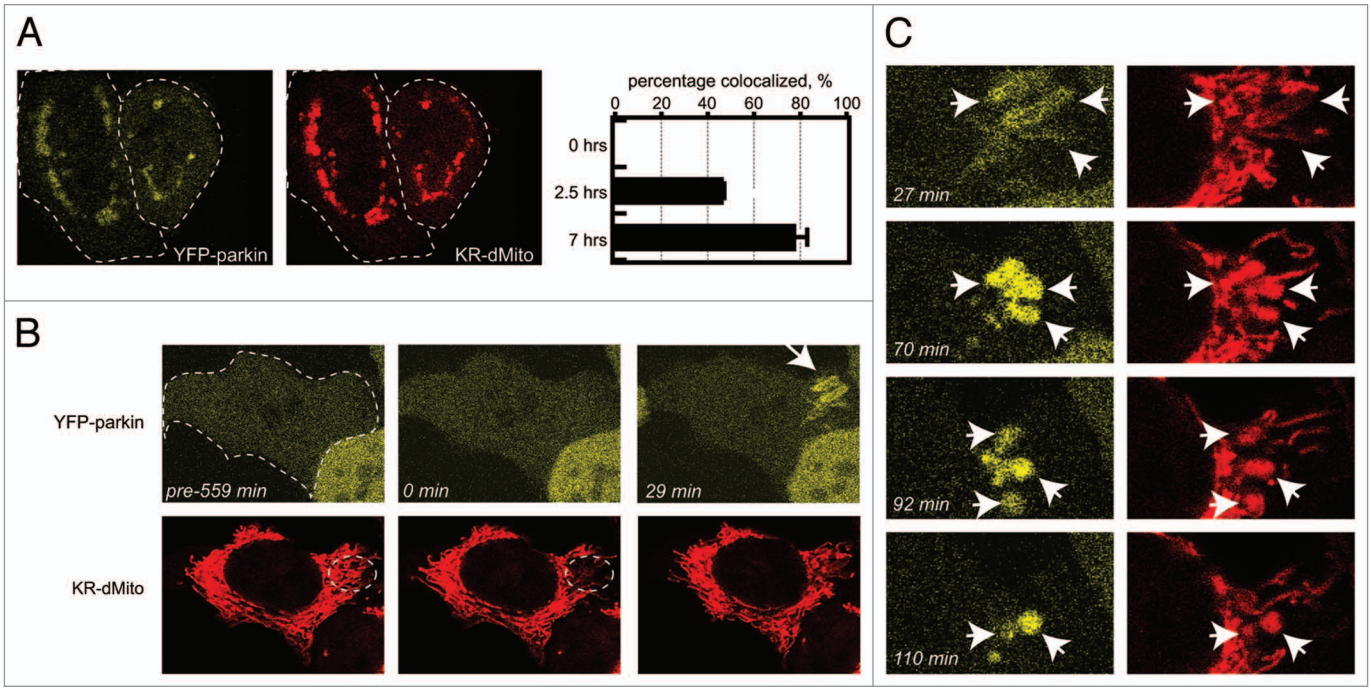

Figure 3. Spatiotemporally controlled initiation of Parkin-mediated mitophagy within single cells. (A) CCCP-induced YFP-Parkin translocation onto KillerRed-dMito labeled mitochondria (white dotted lined outline two individual cells): HeLa cells expressing KillerRed-dMito and YFP-Parkin displayed normal CCCP-induced mitochondria clearing. Percentage of cells showing YFP-Parkin/KillerRed-dMito colocalization after 0, 2.5 and 7 h of 10 μM CCCP treatments is plotted on the graph to the right. (B) Light-induced YFP-Parkin translocation: a HeLa cell expressing YFP-Parkin and KillerRed-dMito was illuminated with 559 nm light at the dotted-circle region, resulting in YFP-Parkin translocation to this specific site. Left column: the HeLa cell before illumination; 2nd column: the HeLa cell immediately after 559 nm light illumination, resulting in the loss of KillerRed-dMito fluorescence; 3rd column: the HeLa cell 29 min after 559 nm illumination, displaying YFP-Parkin tranloaction to the site of light exposure. (C) Magnified image of the cell at the site of 559 nm illumination. Top row: YFP-Parkin initially localized onto tubular-shaped mitochondria. White arrows indicate the YFP-Parkin labeled mitochondria. 2nd row: YFP-Parkin labeled mitochondria nucleated and compacted in the process of being removed. 3rd row: Compacted YFP-Parkin coated mitochondria separated into multiple smaller round structures. Last row: Many small round YFP-Parkin coated structures become degraded.

distribution (~30 min, not observed in nonilluminated areas), indicating the tagging of local mitochondria for autophagic clearing (**Fig. 3B** and 3rd column, and **Sup. Vid. 2**). The tagging of only the 559 nm illuminated mitochondria indicated the exquisite selectivity afforded by both the light-based signal as well as the PINK1/Parkin molecular machinery. The selective recruitment of YFP-Parkin to the 559 nm-illuminated regions did not occur in cells lacking KillerRed-dMito expression.

Clearance of parkin-tagged mitochondria without juxtanuclear mito-aggresome formation. The mitochondria fate upon mitophagy initiation can be directly monitored within the model HeLa cell system used. YFP-Parkin was first seen to highlight the original tubular-shaped mitochondria (**Fig. 3C** and top row). After YFP-Parkin tagging, mitochondria began to compact and cluster, leading to a swollen morphology as indicated by both KillerRed-dMito and YFP-Parkin (**Fig. 3C** and 2nd row). This apparent mitochondria assembly then separated into multiple smaller round-shaped clusters for further removal/turnover (**Fig. 3C** and 3rd–4th row). The direct observation that mitochondria apparently "aggregates" only after tagging is consistent with Parkin's ability in polyubiquinating mitochondrial outer-membrane proteins. Polyubiquitinated proteins can attract p62, a protein that possess the self-oligomerizating PB1 domain,[30] and therefore have been proposed to help cluster mitochondria.[8,18]

In contrast to CCCP-induced mitophagy, Parkin-tagged mitochondrial clusters observed here can be entirely cleared off without the formation of a juxtanuclear aggregate. While CCCP treatment targets the entire mitochondria population within a cell, our scheme can limit Parkin-mediated mitophagy to small portions of the cytoplasm. This suggests that observing juxtanuclear mitochondria aggregates represent a case of substrate overload in Parkin-mediated mitophagy, and that the degradation-delayed mitophagy substrates accumulate to perinuclear regions. The observation that Parkin-mediated mitophagy can proceed independent of mito-aggresome formation is in accordance with the observation that in p62 knockout MEF cells, Parkin-mediated mitophagy can still occur without mitochondrial juxtanuclear clustering.[17]

Efficiencies for KillerRed-dMito and TMRE based schemes for initiating parkin-mediated mitophagy. We next benchmarked the efficiency of our scheme in successfully initiating Parkin-mediated mitophagy within single cells. As shown in **Figure 4A**, three cells carrying both YFP-Parkin and KillerRed-dMito were sequentially illuminated with 559 nm light at portions of their cytoplasm. The proper appearance of the Parkin-labeled mitochondria at respective areas in all three cells was observed (**Fig. 4A** and

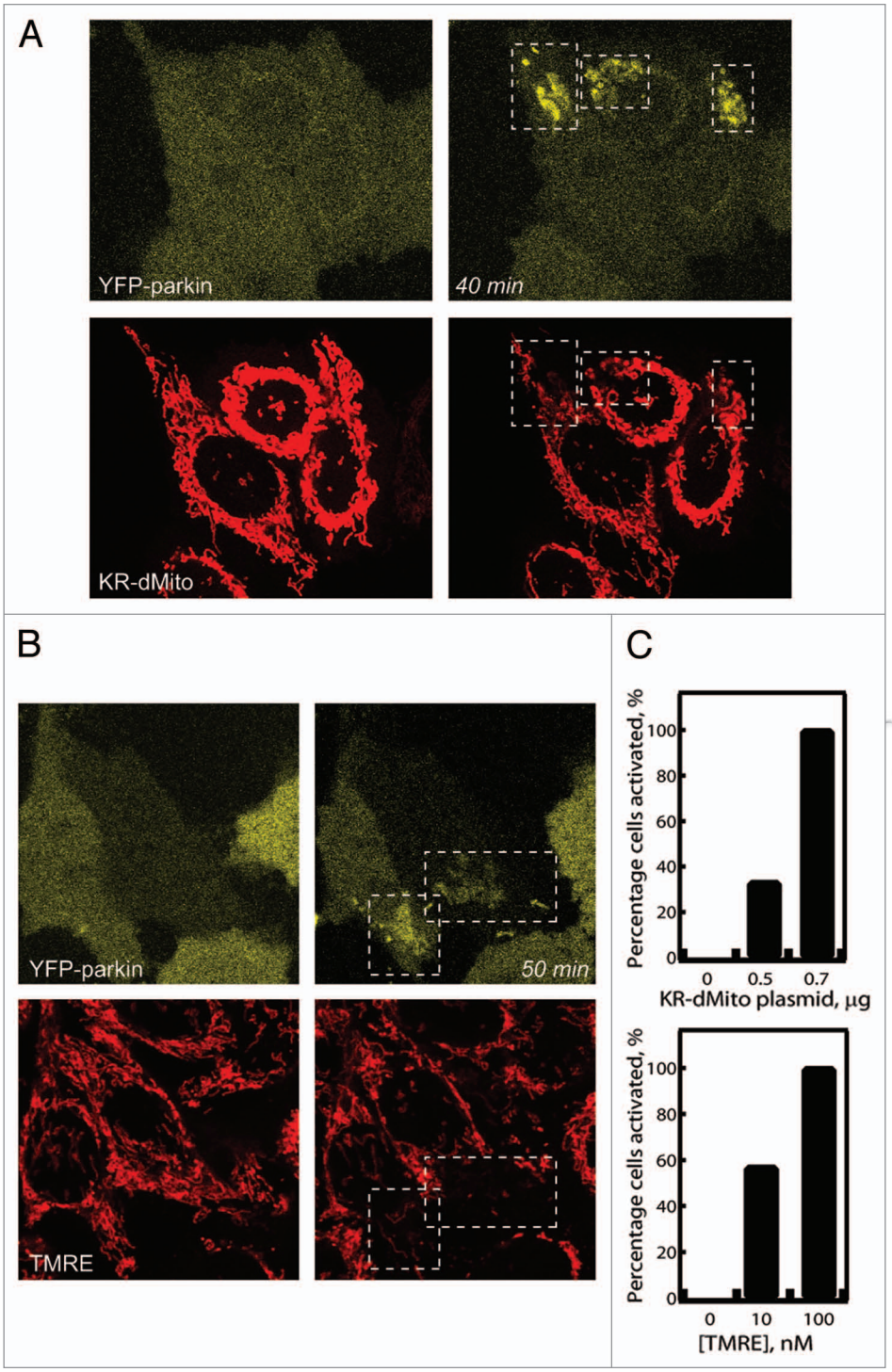

Figure 4. KR-dMito/TMRE for efficient initiation of Parkin-mediated mitophagy. (A) Specific regions within three HeLa cells carrying both YFP-Parkin and KillerRed-dMito were sequentially illuminated with 559 nm light (left column), resulting in the appearance of YFP-Parkin puncta (marked with white dotted line squares) within individual cells (right column). (B) Specific regions within two HeLa cells carrying both YFP-Parkin and TMRE were sequentially illuminated with 559 nm light (left column), resulting in the appearance of YFP-Parkin puncta (marked with white dotted line squares) within individual cells (right column). (C) Efficiency for initiating Parkin-mediated mitophagy using either KillerRed-dMito or TMRE. Top: Percentage of KillerRed-dMito transfected cells that show Parkin-mediated mitophagy (YFP-Parkin translocation) after 559 nm illumination (μg of plasmid per 5 x 10^4 cells for transfection). Bottom: Percentage of TMRE loaded cells (37°C, 20 min) that show Parkin-mediated mitophagy after 559 nm illumination.

right), demonstrating the robustness of the established method. The efficiency for the KillerRed-based method was dependent on the amount of KR-dMito overexpressed in cells (**Fig. 4C** and top), suggesting that sufficient ROS generation within mitochondria was required for the proper initiation of Parkin-mediated mitophagy. High throughput imaging of the molecular processes in Parkin-mediated mitophagy can be achieved through multiplexing the KillerRed-dMito based methodology, allowing for multiple autophagy events to be simultaneously tracked. When only slower dynamic information is needed (therefore lower time resolution movies suffice), the use of automatic microscope stages to allow the simultaneous monitoring of multiple imaging fields will further increase throughput.

For applications not requiring genetically encoded photosensitizers, tetramethylrhodamine (TMRE) can be used in place of KillerRed-dMito in our established method. TMRE is a potent photosensitizer that accumulates in active mitochondria. As shown in **Figure 4B**, two cells carrying YFP-Parkin and loaded with TMRE were sequentially illuminated with 559 nm light at portions of their cytoplasm. The proper appearance of the Parkin-labeled mitochondria at respective areas in both cells was again observed (**Fig. 4B** and right), demonstrating the applicability of TMRE. Similar to the KR-dMito based method, the efficiency for proper initiation of Parkin-mediated mitophagy was dependent on the TMRE amount loaded into cells (**Fig. 4C** and bottom). Unlike KR-dMito, however, TMRE does not effectively label light-impaired mitochondria.

LC3 structures nucleate and expand around subsections of parkin-labeled mitochondria at a time. LC3B is a ubiquitous mammalian autophagic structure marker (autophagosome and phagophores),[31] that enables visualization of substrate capturing by the autophagic machinery. We followed the progression of Parkin-mediated mitophagy using a HeLa cell model co-expressing EBFP2-LC3B, YFP-Parkin and KillerRed-dMito (**Fig. 5A** and top). Exposure of a selected region to 559 nm light in single cells (i.e., **Fig. 5A**, top left part, white dotted rectangle) first led to the appearance of YFP-Parkin tagged mitochondria in the illuminated area (**Fig. 5A** and lower part, showing the illuminated area 25 min after light exposure), followed by asynchronous appearance of numerous small LC3B-labeled autophagic structures around clustered mitochondria subsections (**Fig. 5A** and lower part, showing the illuminated area 41–55 min after light exposure, and **Sup. Vid. 3**. The illuminated area is rotated 90 degrees counter-clockwise in the **Fig. 5**). Quantification of Parkin/LC3B levels within the 559 nm illuminated area showed that the appearance of LC3B was accompanied by a concomitant decrease in Parkin-tagged mitochondria signal within the region (**Fig. 5B**), indicating LC3B-facilitated mitochondria removal. The presence of Parkin enhanced the recruitment of LC3B into the 559 nm illuminated region; cells transfected with only KillerRed-dMito and EBFP2-LC3B did not demonstrate the same efficacy in 559 nm induced LC3B recruitment (**Fig. 5C**).

We found that the appearance of LC3B-structures on Parkin-labeled mitochondria proceeded in parallel to Parkin-mediated mitochondria clustering, suggesting that Parkin-mediated mitochondria clustering is not a prerequisite for turnover (**Sup. Vid. 3**). Together with the observation that Parkin-labeled mitochondria can be cleared off before the formation of any juxtanuclear aggregates (as those observed in CCCP treated cells), our results imply that when the amount of mitochondria damage remain within the immediate capacity of Parkin-mediated mitophagy, impaired mitochondria can be tagged and engulfed at the site of damage for autophagic clearance.

A close-up view of the LC3B actions revealed how LC3B-structures act on Parkin-labeled mitochondria. The LC3B-structures first nucleated at single sites of Parkin-labeled mitochondria (**Fig. 5D** and left). LC3B-structures then elongated/expanded into cup-shaped membranes, wrapping around Parkin-labeled structures for turnover (**Fig. 5D** and right). These actions took on the order of minutes to complete.

Discussion

With a mitochondria-matrix targeted KillerRed protein, we can now robustly initiate Parkin-mediated mitophagy in a spatially- and temporally-controlled fashion within single cells using light signals. This new method presents numerous opportunities for studies on Parkin-mediated mitophagy. The method allows triggering of Parkin-mediated mitophagy with defined magnitude, which will permit one to probe cellular responses to varying degrees of mitophagy activation. The possibility to initiate

Figure 5 (See opposite page). Engulfment of YFP-Parkin coated mitochondria by autophagic membranes. (A) A HeLa cell co-expressing EBFP2-LC3B (pseudo-colored green), YFP-Parkin (pseudo-colored red) and KillerRed-dMito (pseudo-colored cyan) was illuminated with 559 nm at the white-dotted rectangle area, resulting in YFP-Parkin translocation and mitochondria engulfment by EBFP2-LC3B coated autophagic membranes. Top row: the HeLa cell before illumination; 2nd row (left): the illuminated area (rotated by 90 degrees counter-clockwise) at different times after 559 nm exposure (merged image of YFP-Parkin and EBFP2-LC3B). No YFP-Parkin translocation was visible right after 559 nm exposure. At 25 min after illumination clear YFP-Parkin translocation was seen; the images between 41–55 min clearly showed EBFP2-LC3B on YFP-Parkin coated mitochondria (circular yellow signals). (B) Single cell LC3/Parkin kinetics within the 559 nm illuminated area in (A) (quantifying % of area with EBFP2-LC3B or YFP-Parkin signals). Parkin signal appeared first, followed by the recruitment of LC3B signal into the area. A decrease in total Parkin level accompanied the LC3B appearance. (C) Parkin-dependent recruitment of LC3B into the 559 nm illuminated area. Cells co-expressing EBFP2-LC3B/KillerRed-dMito with or without YFP-Parkin were quantified for their LC3B containing area after 559 nm illumination (average of 5 cells each). Cells with Parkin showed enhanced recruitment of LC3B into the 559 nm illuminated area within 60 min, in contrast to cells without YFP-Parkin. (D) Detailed view on the engulfment process of YFP-Parkin labeled mitochondria by EBFP2-LC3B coated membrane [magnification of the white solid square region in (A)]. Left to right: An EBFP2-LC3B coated autophagic structure first nucleated at one site on the periphery of YFP-Parkin labeled mitochondria. The autophagic membrane then elongated, ending up wrapping around the entire YFP-Parkin labeled mitochondria.

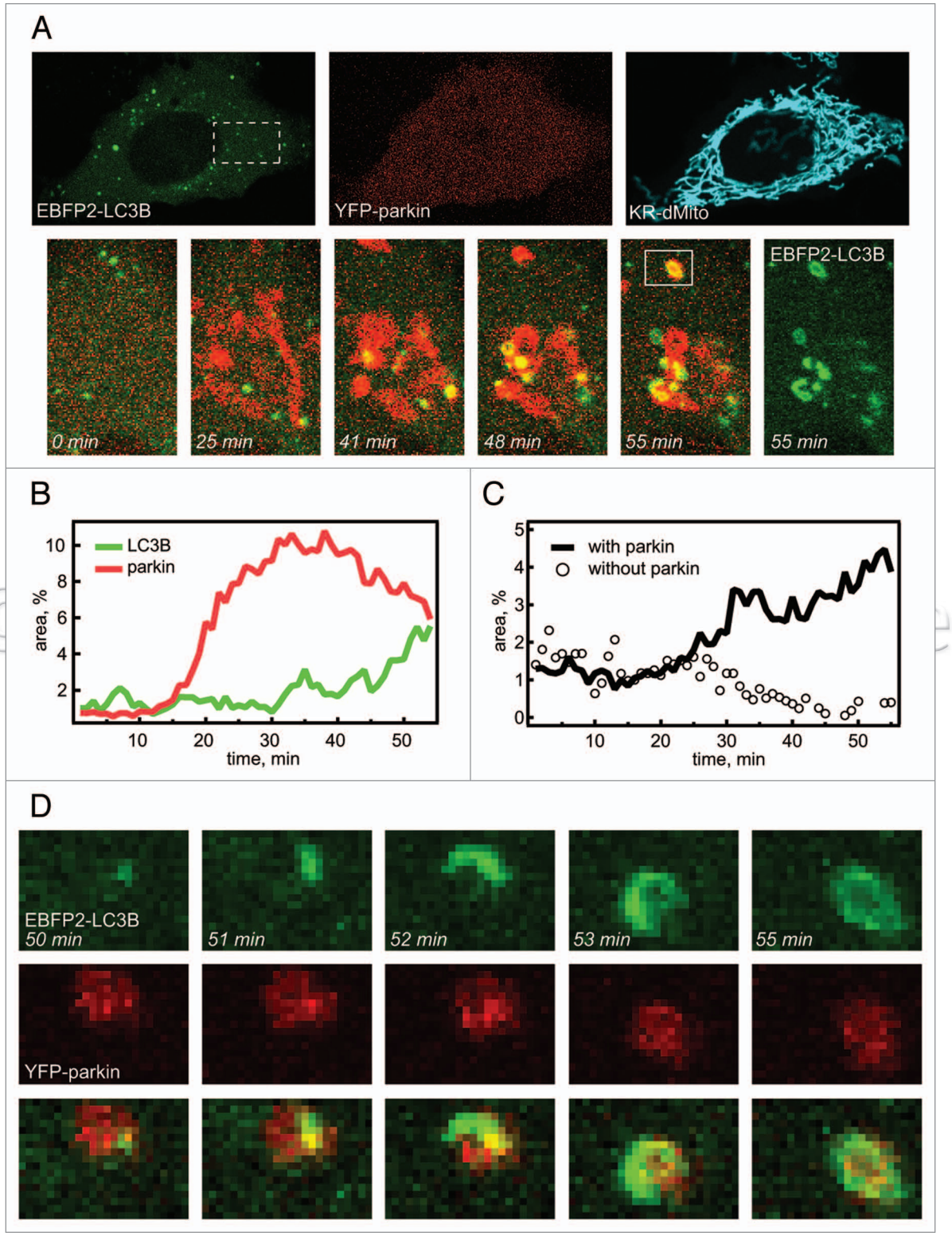

Figure 5. For figure legend, see page 6.

Parkin-mediated mitophagy with spatiotemporal precision will be useful for unveiling the molecular dynamics in Parkin-mediated mitophagy. In addition, activating Parkin-mediated mitophagy in a selected cell (or a group of selected cells) within intact organisms will now be possible, permitting the examination of this process in physiological settings. The method should be applicable to multiple cell types; for example, during the revision of this manuscript, it was reported

elsewhere that mitochondria-targeted KillerRed can also trigger Parkin translocation in PC12 cells.[32]

CCCP-based studies on Parkin-mediated mitophagy have led to the model that mitochondria destined for turnover need to be first concentrated to the juxtanuclear position of a cell.[33] Furthermore, it has been proposed that many disease-causing Parkin mutants leads to deficiencies in cellular Parkin-mediated mitophagy by preventing proper formation of juxtanuclear mitochondrial aggregates.[16] In contrast, previous data showed that in p62 knockout MEF cells, Parkin-mediated mitophagy can still occur without mitochondrial juxtanuclear clustering.[17] Using our method, we found that formation of such juxtanuclear aggregate(s) is not a prerequisite for Parkin-mediated mitochondria turnover. It is therefore possible that the observation of juxtanuclear mitochondrial aggregate(s) in CCCP-treated cells reflects that fact that when large numbers of mitochondria become impaired at once, the capacity of cellular autophagic machinery becomes overwhelmed. Excess mitochondria destined for turnover are then gradually transported to the juxtanuclear position, segregated from other cellular structures, and slowly degraded. In support of this model, we observed that the appearance of LC3-structures on Parkin-labeled mitochondria paralleled Parkin-mediated mitochondria compaction. Furthermore, we directly observed that LC3-structures nucleates at sites on Parkin-labeled mitochondria, and expands around small portions of these mitochondria at a time for eventual degradation.

The recently discovered role of Parkin-mediated mitophagy in maintaining mitochondria and potentially in causing Parkinson disease underlies the importance for developing new methods to decipher this pathway. With our current method, it will be possible to detail how various disease-causing Parkin mutants affect cellular Parkin-mediated mitophagy. Moreover, as Parkin-mediated mitophagy requires a multitude of cellular components, including the autophagy proteins and the cytoskeleton,[16,33] to converge spatially and temporally to the site of impaired mitochondria, it will be interesting to use our method to probe for how coordination between these molecular species occur.

Materials and Methods

Plasmids. YFP-Parkin was obtained from Addgene (plasmid 23955).[7] KillerRed (cytosolic) and KillerRed-dMito were purchased from Evrogen (FP961 and FP964). EBFP2-LC3B was constructed through PCR amplification of human LC3B from HeLa cell total cDNA, followed by its insertion into the EBFP2 vector obtained from Addgene (plasmid 14893).[34]

Cell culture conditions and transfections. HeLa cells (ATCC, CCL-2) were cultured in DMEM medium (Invitrogen, 11965) supplemented with 10% FBS (Gibco, 10437) and 1% penicillin/streptomycin (Gibco, 15140), and maintained at 37°C and 5% CO_2. For transfection into HeLa cells, Lipofectamine 2000 (Invitrogen, 11668) was used based on the manufacturer's protocol. CCCP (Sigma, C2759) was used at 10 μM. DCF (Cayman Chemicals, 85155) was supplemented at 20 μM into the cell medium for 20 min, washed three times by PBS, followed by imaging with confocal microscopy. TMRE (Invitrogen, T-669) was supplemented into cells at either 10 or 100 nM in cell medium for 20 min at 37°C for TMRE-based mitophagy induction.

Live-cell manipulation and imaging. Light-assisted autophagy was performed on an Olympus FV1000 confocal microscope (60x N.A. = 1.2 water objective) equipped with a SIM scanner. Before observation, the HeLa cells were transferred into phenol-red free medium for observation (Gibco, 31053, containing FBS and P/S). Live HeLa cells were maintained under 37°C and 5% CO_2 on the microscope for manipulation and observation (Tokai Hit, MIU-IBC).

To achieve light-assisted autophagy, we point-scanned a 50 μW 559 nm laser light through a ~10 μm x 10 μm region in a HeLa cell for a total of 30 sec using Olympus FV1000s tornado scanning. The YFP-Parkin (detected using 488 nm excitation), EBFP2-LC3B (405 nm excitation), and KillerRed-dMito (559 nm excitation) dynamics were imaged at 1 frame/min, and a zero drift compensator module (ZDC from Olympus) was used to eliminate sample drift in the z-direction. The images were not processed after acquisition.

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Note

Supplemental materials can be found at: www.landesbioscience.com/journals/autophagy/article/16626

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