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A FRET biosensor for necroptosis uncovers two different modes of the release of DAMPs

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Necroptosis is a regulated form of necrosis that depends on receptor-interacting protein kinase (RIPK)3 and mixed lineage kinase domain-like (MLKL). While danger-associated molecular pattern (DAMPs) are involved in various pathological conditions and released from dead cells, the underlying mechanisms are not fully understood. Here we develop a fluorescence resonance energy transfer (FRET) biosensor, termed SMART (a sensor for MLKL activation by RIPK3 based on FRET). SMART is composed of a fragment of MLKL and monitors necroptosis, but not apoptosis or necrosis. Mechanistically, SMART monitors plasma membrane translocation of oligomerized MLKL, which is induced by RIPK3 or mutational activation. SMART in combination with imaging of the release of nuclear DAMPs and Live-Cell Imaging for Secretion activity (LCI-S) reveals two different modes of the release of High Mobility Group Box 1 from necroptotic cells. Thus, SMART and LCI-S uncover novel regulation of the release of DAMPs during necroptosis.

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While apoptosis has been considered to be a typical form of programmed or regulated cell death, recent studies have revealed novel types of regulated or programmed cell death, including necroptosis, pyroptosis, and ferroptosis. Necroptosis is morphologically similar to necrosis and can be induced by virus infection, or death ligands including tumor necrosis factor (TNF), Fas ligand, and TRAIL when caspase activation is blocked. Upon stimulation with these death ligands, sequential phosphorylation and activation of receptor-interacting protein kinase (RIPK)1 and RIPK3 are induced. Activated RIPK3 phosphorylates mixed lineage kinase domain-like (MLKL), resulting in a conformational change of MLKL. Phosphorylated MLKL forms oligomers and translocates to biological membranes, resulting in the execution of necroptosis through pore formation and membrane rupture.

Danger-associated molecular pattern (DAMP)/s are host-derived molecules released from dead cells through ruptured nuclear and cytoplasmic membranes. DAMPs include nuclear proteins such as High-Mobility Group Box (HMGB)1 and histones but no heat shock proteins, IL-1 family members, and ATP. A previous study reported that HMGB1 is passively released from necrotic cells, but actively secreted from macrophages upon lipopolysaccharide (LPS)-stimulation. Released HMGB1 mediates inflammation and also plays a crucial role in the development of septic shock through binding to Toll-like receptor (TLR)2 and 4, and the receptor for advanced glycated endproducts (RAGE). IL-33, a cytokine belonging to the IL-1 family, is released from the nucleus of necrotic epithelial cells and promotes Th2 helper (Th2)-type immune responses. Intriguingly, IL-33 is cleaved by caspase 3 in apoptotic cells, resulting in the inactivation of its biological activity. Moreover, we recently reported that large amounts of histone H3 are released from apoptotic hepatocytes and potentially involved in the development of endothelial cell injury in the livers and lungs. Together, these results suggest that DAMPs are critically involved in inflammation, immune responses, and tissue injury. Thus, blockade of the release of DAMPs or neutralization of their biological activities might be novel strategies to treat various pathological conditions. However, it is not fully understood whether these DAMPs are actively secreted from dead cells or passively released from the nucleus or the cytosol to the extracellular space through the ruptured membrane. To investigate the mechanism(s) underlying the release of DAMPs from necrotic cells, it is crucial to monitor the activation of MLKL and subsequent releases of DAMPs in a single cell. We previously developed a Live-Cell Imaging for Secretion activity (LCI-S) platform. LCI-S enables us to immediately capture a cytokine secreted from a single cell, and a secreted cytokine is visualized by total internal reflection fluorescence microscopy-based fluorescence immunoassay (TIRFM-FIA).

Fluorescence resonance energy transfer (FRET) is a technique to measure the distance between two molecules based on energy transfer from a fluorescent donor dye to a fluorescent acceptor dye within a narrow distance. FRET biosensors enable us to monitor dynamic changes of interaction, phosphorylation, and conformational changes of various signaling molecules in living cells. We previously developed a FRET biosensor termed SCAT (a Sensor for Caspase Activation based on FRET). SCAT1 and SCAT3 comprise peptides containing consensus caspase 1 and caspase 3 cleavage sequences between Venus and cyan fluorescent protein (CFP), enabling us to monitor pyroptosis and apoptosis, respectively. Imaging of macrophages derived from mice stably expressing SCAT1 uncovered an intimate crosstalk between interleukin (IL)-1β release and membrane rupture along with caspase 1 activation. On the other hand, analysis of SCAT3 transgenic mice revealed that the removal of apoptotic cells plays a crucial role in neural tube closure during the development of murine embryos. Together, these results indicate that live cell imaging of caspase activation in vitro and in vivo increases our understanding of pyroptosis and apoptosis under physiological and pathological conditions. In contrast to apoptosis or pyroptosis, it is unclear how signaling molecules crucial for necroptosis are regulated within a single cell during the execution of cell death.

To investigate the mechanisms underlying the release of DAMPs from necrotic cells, we developed a FRET biosensor termed SMART (a Sensor for MLKL Activation by RIPK3 based on FRET). SMART monitored necroptosis, but not apoptosis or necrosis. An increase in the FRET/CFP ratio of SMART depended on endogenous RIPK3 and MLKL and correlated with phosphorylation of MLKL and RIPK3. More importantly, SMART monitored plasma membrane translocation of oligomerized MLKL. SMART in combination with imaging of the release of nuclear HMGB1 and LCI-S revealed that the release of HMGB1 was tightly correlated with the increase in the FRET/CFP ratio of SMART. We also found that there were two different modes of the release of HMGB1 from necrotic cells. Thus, SMART and LCI-S uncover novel regulation of the release of DAMPs during necroptosis.

Results

Development of a FRET biosensor that monitors necroptosis.

To develop a FRET biosensor that monitors necroptosis, we focused on the conformational change of MLKL. Murine MLKL (mMLKL) is composed of an N-terminal four-helix bundle domain comprising 4 α helices and a C-terminal kinase-like (KL) domain. The KL domain of mMLKL is composed of 9 α helices and 7 β strands. Crystal structures of the KL domain of MLKL show that it adopts an inactive conformation through a hydrogen bond between K219 and Q343 thereby preventing spontaneous oligomer formation. RIPK3-dependent phosphorylation of serines and threonine in α helix promotes MLKL plasma membrane translocation and oligomerization, resulting in membrane permeabilization. Since the conformational change in the KL domain of mMLKL might increase the FRET efficiency, we designed intramolecular FRET biosensors comprising fragments of the KL domain between enhanced cyan fluorescent protein (ECFP) and modified yellow fluorescent protein (YPet) serving as FRET donor and acceptor, respectively. We inserted α1 to α9 (α19), α1 to α4 (α14), and α5 to α9 (α59) helices of the KL domain into a FRET backbone vector. A murine fibrosarcoma cell line, I929 cells undergo necroptosis when treated with TNF and a caspase inhibitor, z-VAD-fmk (TZ). To test our FRET biosensors, we transfected I929 cells with them and stimulated the cells with TZ. Transfection of α19 blocked TZ-induced necroptosis, and α19 showed a marginal increase in the FRET/CFP ratio in I929 cells. Maximum changes of the FRET/CFP ratio of α14 were significantly higher than those of α59 (Fig. 1b). Cotransfection experiments revealed that three biosensors interacted with RIPK3, and MLKL in the absence or presence of RIPK3 and without induction of necroptosis (Fig. 1c–e), suggesting that the interaction of FRET biosensors with RIPK3 or MLKL is not sufficient for FRET induction. Notably, Flag-RIPK3 was efficiently coimmunoprecipitated with Myc-MLKL in the presence of α19 (Fig. 1e), indicating that α19 did not block the binding of MLKL to RIPK3. Thus, we surmised that α19 might block the oligomerization of endogenous MLKL, thereby inhibiting TZ-induced necroptosis.
Substitution of serines and threonine in the α4 helix of mMLKL (S345, S347, T349, and S352) with alanine (3ST4A for short) blocks TNF-induced necroptosis by preventing MLKL phosphorylation and oligomerization formation. Unexpectedly, TZ stimulation increased the FRET/CFP ratio in cells expressing α14 mutants 3ST4A, S228A, or S248A (Fig. 1f, g, Supplementary Fig. 1d), and these mutants bound to RIPK3 (Fig. 1h). Thus, while phosphorylation of MLKL is required for oligomerization of MLKL, phosphorylation of α14 is not necessary for FRET induction. Nevertheless, a RIPK1 inhibitor, Necrostatin-1 (Nec-1) or a RIPK3 inhibitor, GSK 872 blocked necroptosis and abolished an increase in the FRET/CFP ratio in cells expressing α14 upon TZ stimulation (Fig. 1i, j). Taken together these data paradoxically indicate that phosphorylation of α14 is not required for FRET activity yet kinase activities of RIPK1 and RIPK3 are nevertheless required for α14 FRET activation.

Fig. 1 Development of a FRET biosensor that monitors necroptosis. a Domain structures of murine MLKL and designed FRET biosensors. 4HBD and KL indicate four-helical bundle and kinase-like domains, respectively. Red boxes indicate each α helix. b α14 monitors necroptosis. L929 cells were transiently transfected with α14 or α59, and then treated with TZ. The FRET/CFP ratio was calculated and analyzed as in Methods, and maximum changes of the FRET/CFP ratio are shown. Each dot indicates individual cell (n = 12 cells). Statistical significance was determined using the unpaired two-tailed Student’s t test. ***p < 0.001. c-e HEK293T cells were transiently transfected with α9, α14, and α59 along with FLAG-RIPK3 (c), Myc-MLKL (d), or Myc-MLKL plus FLAG-RIPK3 (e). Otherwise indicated, murine RIPK3 and murine MLKL were used for experiments. At 24 h after transfection, cell lysates were immunoprecipitated with control, anti-FLAG (c) or anti-Myc antibodies (d, e), and then immunoprecipitates were analyzed by immunoblotting with the anti-GFP antibody. Expression of transfected constructs was verified by immunoblotting using total cell lysates. C, F, and M indicate control, anti-FLAG, and anti-Myc antibodies, respectively. f Diagrams of α14 mutants. 3ST4A indicates the quadruple mutant where S345, S347, T349, and S352 are replaced with alanines. g L929 cells were transiently transfected with the indicated mutants and then stimulated with TZ, and analyzed as in b. Maximum changes of the FRET/CFP ratio. Each dot indicates individual cell (n = 10–14 cells). Statistical significance was determined using the one-way ANOVA test. ns, not significant. h HEK293T cells were transiently transfected with the indicated mutants along with FLAG-RIPK3. Cell lysates were immunoprecipitated and analyzed as in c. i, j L929 cells were transiently transfected with α14 and then stimulated with TZ + Nec-1 (TZN) (i) or TZ + GSK872 (j), and the FRET/CFP ratio was analyzed as in b. Kinetics of average of ΔFRET/CFP ratio is shown (n = 9 cells). Results are representative of two or three independent experiments, or pooled results of two independent experiments (g). Error bars indicate s.e.m.
Further refinement of α14 to generate SMART. Although α14 monitored necroptosis, transient transfection of α14 blocked cell proliferation. To investigate the mechanisms how α14 monitors necroptosis and circumvent this drawback, we generated a further series of α14 mutants. To test whether binding of α14 to RIPK3 is required for monitoring necroptosis, we first mutated phenylalanine at position 234 of MLKL, that is critical for RIPK3 binding in vitro, to glutamic acid (F234E) (Fig. 2a). Unexpectedly, an F234E mutant of α14 still interacted with RIPK3 and showed the increase in the FRET/CFP ratio upon TZ stimulation (Fig. 2b, c). We next replaced amino acids at the indicated fragments with a set of four flexible Ser-Ala-Gly-Gly (SAGG) repeats to maintain the same spacing between Ypet and ECFP (Fig. 2a, Supplementary Fig. 2). The TZ-induced increase in the FRET/CFP ratio was partially or completely abolished in cells expressing Δα2α3, Δα3, or Δαbc. In sharp contrast, TZ increased the FRET/CFP ratio of Δα and Δαb comparable to that of α14 (Fig. 2b, Supplementary Fig. 3a, b). Notably, Δα2α3 did not interact with RIPK3 or show the increase in the FRET/CFP ratio (Fig. 2b, c). Three biosensors including α19, α59, and Δαbc bound to RIPK3, but did not show the increase in the FRET/CFP ratio (Fig. 2c), suggesting that the interaction of RIPK3 with the FRET biosensors is prerequisite, but not sufficient for monitoring necroptosis. Among FRET biosensors showing the increase in the FRET/CFP ratio upon TZ stimulation, we were only able to obtain cells stably expressing Δαb. Thus, this construct is henceforth referred to as SMART (a Sensor for MLKL activation by RIPK3 based on FRET).

Cotransfection experiments revealed that SMART and a mutant of SMART where all putatively phosphorylated serines and threonine were replaced with alanine (4ST5A), still interacted with RIPK3 in the absence or presence of GSK872 (Fig. 2d). Thus, neither the kinase activity of RIPK3 nor the phosphorylation of SMART is required for their binding.

SMART does not monitor apoptosis or necrosis. To characterize the specificity of SMART further, we stably expressed SMART in L929 cells. Consistent with transient transfection assays, the FRET/CFP ratio rapidly increased in L929-SMART cells treated with TZ, but not TZ and GSK872 (TZG) (Fig. 3a–c, Supplementary Movie 1, 2). We next treated L929-SMART cells with TG to induce apoptosis visualized by the rapid appearance of the cleaved form caspase 3 (Fig. 3d). Under these experimental conditions, the FRET/CFP ratio was not increased in TG-stimulated L929-SMART cells (Fig. 3c, e, Supplementary Movie 3). To test whether SMART monitors RIPK3-independent necrosis, we treated cells with an uncoupler, carbonyl cyanide m-chlorophenyl hydrazone (CCCP). As expected, CCCP-induced cell death was not blocked by either zVAD or GSK872 (Fig. 3f), suggesting that these cells did not die by apoptosis or necroptosis. The FRET/CFP ratio was not increased in CCCP-stimulated L929-SMART cells and, on the contrary, rapidly decreased over time even prior to SYTOX positive staining indicating membrane permeabilization (Fig. 3c, g, Supplementary Movie 4). Together, SMART is a FRET biosensor that monitors necroptosis, but not apoptosis or necrosis.

SMART monitors necroptosis in MEFs and aMoC1 cells. To test whether SMART might be used generically to monitor necroptosis, we stably expressed SMART in murine embryonic fibroblast (MEFs) and a murine colonic epithelial cell line, aMoC1 cells. When these cells were treated with the combination of TNF, a SMAC mimetic, BV6, and zVAD (TBZ), they underwent necroptosis (Supplementary Fig. 4a). As expected, TBZ increased the FRET/CFP ratio of SMART in both MEFs and aMoC1 cells (Supplementary Fig. 4b, c, Supplementary Movie 5, 6).

In sharp contrast, TG induced apoptosis, but did not increase the FRET/CFP ratio of SMART in both MEFs and aMoC1 cells (Supplementary Fig. 4d, e). Thus, SMART monitors necroptosis, but not apoptosis in MEFs and aMoC1 cells as well as L929 cells.

![Image](https://example.com/image1.png)
Monitoring of necroptosis by SMART depends on RIPK3 and MLKL. To further investigate the mechanisms how SMART monitors necroptosis, we knocked down the expression of Ripk3 or Mlkl in L929-SMART cells. Treatment of cells with Ripk3 or Mlkl, but not control siRNA blocked TZ-induced LDH release (Fig. 4a, b). As expected, knockdown of Ripk3 abolished TZ-induced increase in the FRET/CFP ratio of SMART (Fig. 4c, Supplementary Fig. 5). TZ- and TBZ-induced increase in the
we transfected the oligomerization of endogenous MLKL. To test this possibility, induced necroptosis24. We veri
mers, resulting in necroptosis, whereas MLKL L280P blocks TBZ-
SMART (Figs. 3b and 4a SMART monitors oligomerization of MLKL and/or sub-
location of oligomerized MLKL, but not oligomerization of MLKL itself (Fig. 6f). Although hSMART does not possess C86, NSA treatment nevertheless abolished the increase in the FRET/ CFP ratio following TBZ stimulation (Fig. 6d, e). Since hSMART did not translocate to membrane fraction under these experimental conditions (Fig. 6f), direct interaction of hSMART with oligomerized MLKL is not required for the increase in the FRET/ CFP ratio of hSMART.
SMART monitors poly(I:C)-induced necroptosis. A previous study reported that poly(I:C) + BZ (PBZ) induces necroptosis in a human keratinocyte cell line, HaCaT cells27. We found that hSMART also monitored PBZ-induced necroptosis in HaCaT cells, but not PBG-induced apoptosis (Fig. 7a–d). As poly (I:C) induction stimulates necroptosis through TLR327,28, SMART monitors both TLR3-induced and TNFR1-induced necroptosis.

Two sequential steps of HMGB1 release during necroptosis. While DAMPs, such as HMGB1 and histones, are considered to be released from dead cells due to the collapse of both the nuclear and cytoplasmic membrane integrity9,10, the underlying mechanisms are not fully understood. To address this issue, we investigated the kinetics of the release of various DAMPs along with the progression of cell death. To visualize nuclear DAMPs, we fused mCherry to the C-terminal portion of HMGB1 and histone H3, resulting in the generation of HMGB1-mCherry and histone H3-mCherry, respectively. We first confirmed that the kinetics of the release of HMGB1-mCherry was comparable to that of endogenous HMGB1 from L929 cells stably expressing HMGB1-mCherry cells stimulated with TZ (Supplementary Fig. 7a). We next transfected L929-SMART cells with HMGB1- mCherry or histone H3-mCherry. As expected, HMGB1- mCherry and histone H3-mCherry were localized in the nucleus (Fig. 8a, b). HMGB1-mCherry abruptly disappeared between 2 and 3 h after TZ stimulation and this happened before the nucleus became positive for SYTOX (Fig. 8a, c). However, in the same time-frame, the histone H3-mCherry remained constant in the nucleus even after the nucleus became positive for SYTOX (Fig. 8b). The FRET/CFP ratio of SMART gradually increased before the signals of HMGB1-mCherry disappeared (Fig. 8c).

Loss of HMGB1 from the nucleus occurred quickly and corresponded with a precipitate rise in cytosolic HMGB1 levels (Fig. 8c, d). However, this increase in cytosolic HMGB1 was short-lived reflecting rapid release of HMGB1 into the extracellular space (Fig. 8c, d, Supplementary Movie 7). These results suggest that the integrity of the nuclear membrane was already disrupted before the cytoplasmic membrane became ruptured. Western blotting analysis showed that the release of endogenous
HMGB1 in the culture supernatant started approximately 2 h after a necroptotic stimulus, and as previously reported, was almost undetectable in the culture supernatant of cells undergoing apoptosis (Supplementary Fig. 7a, b). Similar findings were obtained by MEFs and aMoC1 cells (Supplementary Fig. 7c, d). The release of HMGB1 was relatively slow in HT29 and HaCaT cells compared to murine cell lines (Supplementary Fig. 7e, f). On the other hand, histone H3 was not, or marginally released into the extracellular spaces from cells died by either apoptosis or necroptosis at least under our experimental conditions (Supplementary Fig. 7b-f).

LCI-S uncovers two different modes of HMGB1 release. To further investigate the kinetics of the release of HMGB1 and execution of necroptosis, we visualized the extracellular release of HMGB1 by LCI-S in combination with SMART at the single cell level. Our preliminary experiments revealed that commercially available antibodies against HMGB1 were not suitable for sandwich fluoro-immunoassay under physiological conditions. We plated a single cell of L929 co-expressing SMART and HMGB1-mCherry on a microfabricated-well array chip that was precoated with the anti-mCherry antibody. Simultaneous live imaging of SMART, intracellular HMGB1-mCherry, and extracellularly
released HMGB1-mCherry by LCI-S further substantiated that the FRET/CFP ratio started to increase before the signals of HMGB1-mCherry disappeared in the cell (Fig. 9a, b, Supplementary Movie 8). Given that there was a time lag between the disappearance of the signals of HMGB1-mCherry in the nucleus and detection of extracellularly released HMGB1-mCherry by LCI-S (Fig. 9a, b), these results further substantiated that HMGB1-mCherry was released from the nucleus before plasma membrane ruptured. Average time from an increase in the FRET/CFP ratio to the extracellular release of HMGB1-mCherry was approximately 43.8 min (Fig. 9c).

Intriguingly, we found that in some cells, HMGB1-mCherry release was stopped within 10 min, whereas in other cells, HMGB1-mCherry release lasted more than 100 min (Fig. 9d–f, Supplementary Movie 9, 10). Thus, we surmised the release mode of HMGB1-mCherry could be divided into two groups: one is a burst-mode and the other is a sustained-mode. To compare these two modes quantitatively, we estimated the duration of the HMGB1-mCherry release in individual cells and classified them into two groups by k-means clustering. The representative duration of extracellular HMGB1 release of the burst-mode and the sustained-mode are 7.1 and 109 min, respectively (Fig. 9f). To investigate the kinetics of the extracellular release of HMGB1-mCherry in more detail, we quantified the relative intensities of intracellular (total), extracellular (TIRF), and nuclear HMGB1-mCherry in a single cell, respectively. Then, we plotted representative kinetics of signal intensities of a single cell at the indicated times before and after the extracellular release of HMGB1-mCherry (Fig. 9g). The intracellular and nuclear signals of HMGB1-mCherry drastically diminished in burst-mode cells, but remained relatively high in sustained-mode cells after the cytoplasmic membrane rupture (Fig. 9g, left vs right). Consistently, some portions of nuclear HMGB1-mCherry still existed in the nucleus of a sustained-mode cell, whereas most nuclear HMGB1-mCherry disappeared in a burst-mode cell (Epi, Fig. 9d).

Together, the extent of both nuclear and cytoplasmic membrane damage induced by MLKL might critically determine whether cells release HMGB1 in a burst-mode or a sustained-mode.

**CHMP4B is involved in a sustained-mode release of HMGB1.**

The endosomal sorting complex required for transport (ESCRT) is involved in mediating receptor sorting, membrane remodeling, and membrane scission. A recent study has shown that MLKL binds to the ESCRT proteins and generates extracellular vesicles. Moreover, CHMP2A and CHMP4B, components of the ESCRT-III complex, have been shown to delay or prevent TNF-induced necroptosis through shedding MLKL-containing vesicles. Taken that the knockout of *Chmp2a* or *Chmp4b* enhances TNF-induced necroptosis, we surmised that the ESCRT-III proteins maintained a sustained-mode release of HMGB1 by promoting membrane repair. To test this possibility, we knocked down *Chmp4b* in L929-SMART/HMGB1-mCherry cells by siRNA (Fig. 10a). After TZ stimulation, we monitored HMGB1-mCherry release by LCI-S and estimated the duration of the release of HMGB1 of individual cell. Intriguingly, knockdown of *Chmp4b* substantially reduced the duration of the HMGB1-mCherry release compared to control siRNA-treated cells (Fig. 10b). Moreover, when we classified the assembly from both of these siRNA-treated cells into two groups based on the duration of the HMGB1-mCherry release by k-means clustering, cells that released HMGB1-mCherry via the sustained-mode were abolished in *Chmp4b*, but not in control siRNA-treated cell populations (Fig. 10b, Supplementary Fig. 8).

As expected, the time between the start of the release of HMGB1 and the burst of cells was shortened, and ΔFRET/CFP ratio was more rapidly increased in cells treated with *Chmp4b* siRNA than those with control siRNA (Fig. 10c, d). Together, these results suggest that CHMP4B contributes to maintain a sustained-mode of HMGB1 release, possibly by promoting plasma membrane repair.

**Discussion**

In the present study, we developed a FRET biosensor that detected necroptosis in living cells. The increase in the FRET/CFP ratio of SMART depended on RIPK3 and MLKL, and was correlated with phosphorylation of RIPK3 and MLKL, hallmarks of necroptosis. Moreover, SMART monitored plasma membrane translocation of oligomerized MLKL even in the absence of TNF stimulation. SMART monitored necroptosis, but not apoptosis or necrosis. Simultaneous live imaging of SMART and the release of nuclear DAMPs by LCI-S uncovered two different modes of the release of HMGB1 from cells undergoing necroptosis. Moreover, CHMP4B, a component of the ESCRT-III complex might determine whether a cell exhibits a burst-mode or a sustained-mode of HMGB1 release.

Many groups including us developed FRET biosensors to monitor apoptosis in living cells. Imaging of necroptosis is rather difficult, since there has been no specific polypeptide(s) that are cleaved by protease(s) activated during necroptosis. Taken that the phosphorylation of MLKL is prerequisite for necroptosis, we assumed that a short fragment containing a MLKL-phosphorylation site (~15 amino acids) and a phosphopeptide recognition domain, such as the Forkhead-associated (FHA) domain fused by a linker peptide might be suitable for generating a FRET biosensor to monitor necroptosis. However, such fragment could not monitor necroptosis. Thus, we next detected necroptosis by SMART depends on RIPK3 and MLKL. Knockdown of *Ripk3* or *Mlkl* abolishes the TZ-induced increase in the FRET/CFP ratio of SMART. L929-SMART cells were transfected with control, *Ripk3*, or *Mlkl* siRNAs. Expression of *Ripk3* or *Mlkl* was analyzed by immunoblotting with the indicated antibodies. After transfection, cells were unstimulated or stimulated with TZ for 8 h. Cell viability was determined by LDH release assay. Results are mean ± s.d. of triplicate samples. Statistical significance was determined using the one-way ANOVA test. ***P < 0.001. C L929-SMART cells were treated with the indicated siRNAs and then stimulated with TZ. The FRET/CFP ratio was analyzed as in Fig. 1b. Kinetics of average ΔFRET/CFP ratio of cells (n = 10 cells). Time 0 for control siRNA indicates the time when cells underwent rupture, whereas the time for *Ripk3* or *Mlkl* siRNAs indicates the time after stimulation. D, E The TZ-induced increase in the FRET/CFP ratio of SMART is abolished in *Mlkl*−/− MEFS. Expression of MLKL in *Mlkl*−/− MEFS was analyzed by immunoblotting (D). Wild-type and *Mlkl*−/− MEFS stably expressing SMART were stimulated with TBZ, and the FRET/CFP ratio was analyzed as in Fig. 1b. Maximum changes of the FRET/CFP ratio (E). Each dot indicates individual cell (n = 10 cells). Statistical significance was determined using the unpaired two-tailed Student’s t test. ***P < 0.001. F, H L929-SMART cells and MEFS-SMART were stimulated with TBZ and the FRET/CFP ratio was analyzed as in Fig. 1b. Representative images of the ratio of a single cell (left) (n = 10 cells). Ratio and DIC + SYTOX indicate the FRET/CFP ratio, and merged images of DIC and SYTOX Orange, respectively. Red arrowheads indicate SYTOX-positive cells. Scale bars, 20 μm. Color scales indicate pseudocolor images of the FRET/CFP ratio. G, I L929-SMART cells and MEFS-SMART were stimulated with TBZ for the indicated times and cell lysates were analyzed by immunoblotting with the indicated antibodies. All results are representative of two independent experiments. Error bars indicate s.e.m. (C, E)
focused on conformational changes of MLKL induced by RIPK3 binding\(^{19,24}\). Consistent with our hypothesis, one of the FRET probes containing the \(\alpha_{14}\) fragments of MLKL allowed us to monitor necroptosis. Unexpectedly, the quadruple mutant 3ST4A of \(\alpha_{14}\) and SMART 4ST5A did monitor necroptosis after TZ stimulation. In sharp contrast, GSK872 suppressed TZ-induced necroptosis and the increase in the FRET/CFP ratio of SMART. Moreover, TZ- or TBZ-induced increase in the FRET/CFP ratio of SMART was abolished in L929 cells treated with Ripk3 or \(\text{Mlkl}^{-/-}\) MEFs. Together, these results suggest that the phosphorylation of endogenous MLKL, but not the phosphorylation of SMART probe itself is indispensable for the increase in the FRET/CFP ratio of SMART. Expression of a constitutive active mutant, MLKL Q343A that spontaneously forms oligomers and induced necroptosis, resulted in the increase in the FRET/CFP ratio of SMART even in the absence of TNF stimulation. NSA suppressed TBZ-induced increase in the FRET/CFP ratio of hSMART in HT29 cells, suggesting that SMART monitors plasma membrane translocation of oligomerized MLKL. At this moment, the detailed molecular mechanisms how SMART

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**Fig. 5** SMART monitors oligomerization of MLKL. **a** \(\text{Mlkl}^{-/-}\) MEFs-SMART were transfected with Dox-inducible lentiviral vectors for WT MLKL, MLKL L280P, or MLKL Q343A. Cells were treated with Dox for the indicated times. Expression of MLKL mutants was analyzed by immunoblotting with the anti-MLKL antibody. **b** \(\text{Mlkl}^{-/-}\) MEFs-SMART/WT MLKL and MLKL L280P were treated with Dox as in (a) and then untreated or further stimulated with TBZ for 12 h. \(\text{Mlkl}^{-/-}\) MEFs-SMART/MLKL Q343A were treated with Dox alone for 12 h. Cell viability was determined by LDH release assay. Results are mean ± s.d. of triplicate samples. Statistical significance was determined using the unpaired two-tailed Student-t test. ****\(P < 0.001\), ns, not significant. **c** \(\text{Mlkl}^{-/-}\) MEFs-SMART/MLKL Q343A were treated with Dox for the indicated times and cell lysates were subjected to SDS-PAGE under non-reducing conditions. Oligomers of MLKL were analyzed by immunoblotting with the anti-MLKL antibody. Asterisk indicates oligomers. **d** Cells were treated as in (b) and the FRET/CFP ratio was analyzed as in Fig. 1b. Maximum changes of the FRET/CFP ratio. Each dot indicates individual cell (\(n = 10\) cells). Statistical significance was determined using the unpaired two-tailed Student-t test. ****\(P < 0.001\), ns, not significant. Error bars indicate s.e.m. **e** Cells were treated and analyzed as in (b). Representative images of the ratio of a single cell expressing SMART (\(n = 10\) cells). Ratio and DIC + SYTOX indicate the FRET/CFP ratio and merged images of DIC and SYTOX Orange, respectively. Red and white arrowheads indicate SYTOX-positive cells and cells undergoing necroptosis, respectively. Scale bars, 20 \(\mu\)m. Color scales indicate pseudocolor images of the FRET/CFP ratio. All results are representative of two independent experiments.
senses plasma membrane translocation of oligomerized MLKL are currently unknown. Since SMART did not translocate to the plasma membrane in cells undergoing necroptosis, the increase in the FRET/CFP ratio of SMART does not appear to be mediated by direct interaction with oligomerized MLKL on the plasma membrane. One of the plausible explanations would be that SMART might monitor drastic changes of cellular conditions induced by plasma membrane translocation of oligomerized MLKL, such as relative ratios of RIPK3 and MLKL in the cytosol. Further study will be required to address this issue.

Time-lapse imaging of the release of nuclear DAMPs and SMART uncovered differential regulation of the release of HMGB1 and histone H3 from cells undergoing necroptosis or apoptosis. HMGB1 was rapidly released from necroptotic cells just after cells became positive for SYTOX. It is currently unknown why HMGB1 bound to chromatin was rapidly released.
from the nucleus into the extracellular spaces. Disulfide-HMGB1, but not the reduced form of HMGB1, is released from cells during necroptosis. Thus, we surmise that conformation change of but not the reduced form of HMGB1, is released from cells during necroptosis.

Currently, the exact biological significances of these two different modes of the releases of DAMPs remain unclear. It is reasonable to surmise that a burst-mode and a sustained-mode cell might elicit qualitatively or quantitatively different biological responses of neighboring cells that sense DAMPs, such as macrophages and dendritic cells (DCs). Thus, it would be interesting to test whether amounts of production of inflammatory cytokines by macrophages or DCs might be different when cells were cocultured with a burst-mode cell or a sustained-mode cell. Alternatively, taken that HMGB1 also has a chemoattractant activity, a burst-mode cell might recruit cells such as DCs or neutrophils from a distant site to inflammation sites where cells undergo necroptosis by a concentration gradient of HMGB1 like a chemokine.

RIPK3-dependent oligomerization and subsequent translocation of MLKL to the cytoplasmic and nuclear membrane are reminiscent of caspase 1- or 11-dependent cleavage of Gasdermin (GSDM) D and its subsequent translocation to the cytoplasmic membrane. Very recently, two groups have reported that caspase 3-dependent cleavage of GSDM/DFNA5 mediates secondary necrosis. So far, it is unclear whether GSDM or GSDME translocate to the nuclear membrane and participate in the release of nuclear DAMPs. In this respect, simultaneous live imaging of nuclear DAMPs, LCI-S, and FRET biosensors that monitor cleavages of the GSDM family will address whether GSDM or GSDME are also involved in the collapse of the nuclear membrane and the release of nuclear DAMPs.

Consistent with the results of HMGB1, IL-1α and IL-33 are nuclear proteins that do not possess signal peptides and released from damaged cells. Our preliminary experiments showed that kinetics of the release of IL-33 from cells undergoing necroptosis appeared to be different from that of HMGB1. Thus, SMART in combination with LCI-S might further increase the understanding of mechanisms of necroptosis and the release of DAMPs.
Fig. 8 Sequential two-step release of HMGB1 from cells undergoing necroptosis. **a, b** L929 cells stably expressing HMGB1-mCherry or histone H3-mCherry were stimulated with TZ. The signals of HMGB1- or histone H3-mCherry were analyzed every 2 min. Representative images of HMGB1-mCherry (a) or histone H3-mCherry (b) (upper), SYTOX Green (middle), and merged images of DIC and Hoechst (lower) (left) (n = 5 cells). Scale bars, 20 μm. Intensities of mCherry (magenta) and SYTOX Green (green) were quantified and relative intensities were plotted at the indicated times (right). **c, d** L929-SMART cells were transiently transfected with HMGB1-mCherry and stimulated with TZ. The FRET/CFP ratio was analyzed as in Fig. 1b. Representative images of the FRET/CFP ratio (upper), HMGB1-mCherry (middle), and merged images of DIC and HMGB1-mCherry (lower) (n = 5 cells) (c). White arrowheads indicate released HMGB1-mCherry in the cytoplasm. Scale bars, 20 μm. ΔFRET/CFP ratio (left), and relative intensity of HMGB1-mCherry in the nucleus and the cytoplasm (right) were plotted at the indicated times after stimulation (d). Intensities of HMGB1-mCherry in the cytoplasm were determined by subtracting nuclear intensities of HMGB1-mCherry from intracellular (total) intensities of HMGB1-mCherry. Two vertical dotted lines indicate the times when nuclear efflux of HMGB1-mCherry started and extracellular release of HMGB1-mCherry was terminated, respectively. All results are representative of at least three independent experiments.
Methods

Reagents. Murine TNF (34-8321, eBioscience), human TNF (BMS301, eBioscience), poly(I:C) (ALX-746-021, Enzo Life Sciences), Birinapant (CT-BIRI, Tetralogic Pharmaceuticals), BV6 (B4653, ApexBio), CCCP (CAS 555-60-2, Calbiochem), GSK872 (530389, Merck), Hoechst 33342 (H3570, ThermoFisher Scientific), Nec-1 (N9037, Sigma-Aldrich), Necrosulfonamide (NSA) (ab143839, Abcam), SYTOX Green (S34860, ThermoFisher Scientific), SYTOX Orange (S34861, ThermoFisher Scientific), and zVAD (3188-v, Peptide Institute) were purchased from the indicated sources. The following antibodies used in this study were obtained from the indicated sources: anti-phospho-RIPK1 (31122, Cell Signaling, 1:1000), anti-RIPK1 (610459, BD Biosciences, 1:1000), anti-phospho-RIPK3 (57220, Cell Signaling, 1:1000), anti-RIPK3 (IMG-5523-2, Immugenex, 1:3000),...
stimulated with TZ. The FRET/CFP ratio was analyzed as in Fig. 1b. Representative images of the FRET/CFP ratio (Ratio), intracellular (Epi), and extracellularly released (TIRF) HMGB1-mCherry (a) (n = 19 cells). Epi shows merged images of bright field and intracellular HMGB1-mCherry. Scale bar, 25 μm. Representative temporal relationship between MLKL activation and extracellular release of HMGB1-mCherry (b). The FRET/CFP ratio and the signals of extracellularly released HMGB1-mCherry were plotted at the indicated times after stimulation. Two vertical dotted lines at 94 and 142 min indicate initiation of an increase in the FRET/CFP ratio and extracellular release of HMGB1-mCherry, respectively. Average intervals between the initiation of the increase in the FRET/CFP ratio and extracellular release of HMGB1 (c) (n = 19 cells). Two different modes of extracellular release of HMGB1 during necrosis. L929-HMGB1-mCherry cells were stimulated with TZ, and extracellularly released and intracellular HMGB1-mCherry were analyzed every 2 min. Representative images of extracellularly released (TIRF) and intracellular (Epi) HMGB1-mCherry at the indicated times (d). Epi indicates merged images of intracellular HMGB1-mCherry and bright field. Time 0 indicates the time when the extracellular release of HMGB1-mCherry started. Scale bar, 25 μm. Representative plots of the change in intensities of extracellularly released HMGB1-mCherry in a burst-mode cell and a sustained-mode cell (e). The duration of the HMGB1-mCherry release in individual cell (f). The duration of the HMGB1-mCherry release of a single cell is plotted and clustered into two groups. Centers of each group are 109 and 7.1 min, respectively. Temporal relationship of a decrease in intensities of total and nuclear HMGB1-mCherry and an increase in extracellularly released HMGB1-mCherry (g). Intensities of each fraction of HMGB1-mCherry were calculated by epifluorescence (left vertical axis) microscopy and TIRF (right vertical axis) and plotted at the indicated times. Representative plots of a burst-mode cell (left, n = 10 cells) and a sustained-mode cell (right, n = 8 cells) in the same experiment. All results are representative of at least two independent experiments.

 anti-phospho-MLKL (62233, Cell Signaling, 1:1000), anti-MLKL (3H1, made in house, 1:1000), anti-human MLKL (ab184718, Abcam, 1:1000), anti-cleaved caspase-3 (9661, Cell Signaling, 1:1000), anti-caspase-3 (9662, Cell Signaling, 1:1000), anti-actin (A2066, Sigma-Aldrich, 1:1000), anti-cytchrome c oxidase subunit (COX) IV (Ab16056, Abcam, 1:1000), anti-tubulin (Sigma, T-5168, 1:40,000), anti-FLAG (M2, Sigma-Aldrich, 1:1000), anti-Myc (9E10, Sigma-Aldrich, 1:1000), anti-GFP (sc-8334, Santa Cruz, 1:5000), anti-GFP (66002-1-H, Proteintech, 1:5000), anti-mCherry (600-401-379, Rockland Immunochemicals, 1:1000), anti-HMGB1 (ab18256, Abcam, 1:1000), anti-histone H3 (ab1791, Abcam, 1:1000), and HRP-conjugated donkey anti-rabbit IgG (712-035-153, Jackson ImmunoResearch, 1:10,000) antibodies. HRP-conjugated sheep anti-mouse IgG (NA934, 1:10,000) and HRP-conjugated donkey anti-rabbit IgG (NA934, 1:10,000) antibodies were from GE Healthcare.

**Generation of murine and human SMART.** A backbone vector for a FRET biosensor, 3536NES was kindly provided by M. Matsuda (http://www.fret.lif.kyoto-u.ac.jp/). To generate FRET biosensors for necroptosis, fragments of murine MLKL cDNAs were amplified by PCR using the following primers. α1: 5′-GGCCTGAGGAGGCGGAAAGTTGTTGGAATAG-3′ and 5′-TAGGCGGCGACCACCTCTTCTGCGTGTGACATT-3′; α2: 5′-GGCCTGAGGAGGCGGAAAGTTGTTGGAATAG-3′ and 5′-TAGGGCCGCGCCTTCTGCTGTCGGCGCT-3′ (1056R); α3: 5′-GGCTTCTAGGAACTATGATTGAATGAGG-3′ and 5′-ACCATGGCGGCCCTTCTGTCGTAATCC-3′. A linker sequence between Ypet and ECFP of 3536NES was replaced with respective cDNA frag ments. Primers used in the study were purchased from Eurofin Genomics.

Mutations of S228A, S248A, F234E, and 3ST4A were generated by introducing respective mutations into α1 by PCR-based mutagenesis using the following primers as described previously46. S228A: 5′-GGGAGCGTCAGGAGGCGCAGCTTTCTGCTGTCGGCGCT-3′ (1056R) and 5′-ACATATGATGTTGTCTAGGAACTATGATTGAATGAGG-3′ and 5′-ACCATGGCGGCCCTTCTGTCGTAATCC-3′. Amino acids 235–335 and 293–329 of α1 were replaced with 29 and 9 repeats of SAGG, respectively, resulting in the generation of Δα2α3 (Figure S2). Briefly, fragments corresponding to the α1 and α4 helices of murine MLKL were amplified by PCR using the following primers, respectively: α1: 5′-GGCCTGAGGAGGCGGAAAGTTGTTGGAATAG-3′ and 5′-ACCATGGCGGCCCTTCTGTCGTAATCC-3′ and 5′-ACCATGGCGGCCCTTCTGTCGTAATCC-3′; and 5′-ACCATGGCGGCCCTTCTGTCGTAATCC-3′. Two different modes of extracellular release of HMGB1 release. α2: L929-SMART/HMGB1-mCherry cells were transfected with control or Chmp4b siRNA, and knockdown efficiency was determined by qPCR at 24 h after transfection. Results are means ± s.d. of triplicate samples and representative of two independent experiments. Statistical significance was determined using the unpaired two-tailed Student’s t test. *P < 0.001. b Duration of the HMGB1-mCherry release of a single cell. Cells were treated as in (a) and then stimulated with TZ. The release of HMGB1 was analyzed as in Fig. 8 and the duration of the release was determined (n = 29 cells for control siRNA and n = 26 cells for Chmp4b siRNA). Centers of each group of cells treated with control siRNA are 144 and 4.4 min, whereas that of Chmp4b siRNA is 2.9 min. Each red dot indicates individual cell showing a sustained-mode of HMGB1 release. Results are representative of two independent experiments.

Statistical significance was determined using the Mann–Whitney test. **P < 0.001. c, d Cells were treated with siRNAs and stimulated with TZ. The times between the start of an increase in ΔFRET/CFP ratio and the burst of cells were calculated and plotted (n = 10 cells for each treatment) (c). Pooled results of two independent experiments. Statistical significance was determined using the Mann–Whitney test. **P < 0.001. Kinetics of averages of ΔFRET/CFP ratio of a single cell was plotted (n = 10 cells for control or Chmp4b siRNA) (d). Time 0 indicates the start of an increase in ΔFRET/CFP ratio. Error bars indicate s.e.m.
hal5. Then, oligonucleotides where the a and b regions corresponding to murine MLKL were replaced with SAGG repeats were synthesized and ligated into Xhol and Stul sites of hal5, resulting in the generation of hSMART (Supplementary Table 1c and Supplementary Fig. 2c).

Cell culture and transfection. L929, HT29, HEK293T, and HaCaT cells were obtained from ATCC. aMoC1, murine colonic epithelial cells, were previously described42. Wild-type and Mlkl−/− MEFs were prepared from mice of the indicated genotypes at E14.5 after coitus using as a standard method. MEFs were cated genotype at E14.5 after coitus using as a standard method. MEFs were

 Knockdown by siRNAs. L929-SMART/HMGB1-mcherry cells were transiently transfected with D-00810-10-05, Rfpk3 (L-049919-00-0005), Mki (LQ-061420-00-0002, pools of J-061420-05 and -07, Chmp6b (L-041531-01-0020) siRNAs by lipofection 2000 (Invitrogen). siRNAs were purchased from Dharmacon. Knockdown of the expression of RIPK3 and MLKL was analyzed by immuno- blotting with the indicated antibodies at 24 h after transfection. After transfection, cells were stimulated with TNF and subjected to LDH release assay or FRET analysis. Since anti-CHMP4B antibody did not work well in L929 cells, knockdown of the expression of Chmp6b was determined by quantitative polymerase chain reaction (qPCR) using the following primers. Chmp6b-F: 5′-GGAGAGAAGGTCGACAGAG- GAT-3′ and Chmp6b-R: 5′-TGTAGAGGCGGCTCCCGGACG-3′. qPCR analysis was performed with the 7500 Real-Time PCR detection system with SYBR green fluorinate, the 1% digitonin soluble membrane fraction and cytosolic fraction. The resulting pellet was resuspended with the 1% digitonin soluble membrane fraction used for the final concentration of digitonin was adjusted to 1% w/v and kept on ice for 20 min. After centrifugation, the supernatant was collected as a cytosolic fraction. The resulting pellet was resuspended with the 1% digitonin soluble membrane fraction used for the final concentration of digitonin was adjusted to 1% w/v and kept on ice for 20 min. After centrifugation, the 1% digitonin soluble membrane fraction and cytosolic fraction were subjected to 4–16% Bis-Tris PAGE (BN10002B, ThermoFisher) and stained with Coomassie brilliant blue. In parallel, the membrane and cytosolic fractions were subjected to reducing SDS-PAGE and then transferred onto PVDF membrane. The membrane was analyzed as described above.

Inducible expression of MLK mutants by lentiviral vectors. Dox-inducible lentiviral expression vectors, pF-TRE3G-PGK-puro encoding MLK1 Q343A and MLK2 L280P were previously described. To produce lentiviruses encoding MLK mutants, we transfected HEK293T cells with pF-TRE3G-PGK-puro encoding wild-type or the indicated MLK mutants along with packaging plasmids including pCAG-HVg and pCMV-VSV-G-RSV-Rev (provided by H. Miyoshi) as described previously. After the infection of cells with culture supernatants containing viruses, cells were selected in the presence of 5 µg/ml of puromycin, resulting in the generation of Mkl−/− MEFs-SMART/MLK Q343A or MLK L280P. To induce the expression of transfected genes, Mkl−/− MEFs-SMART expressing the indicated mutants were incubated with 100 ng/ml of Dox for 12 h (for MLK Q343A) or 24 h (for MLK L280P). After confirming the expression of inducible genes, cells expressing MLK L280P were stimulated with TBZ and subjected to the FRET analysis. Since the expression of MLK1 Q343A resulted in cell death, Mkl−/− MEFS-SMART/MLK Q343A were subjected to the FRET analysis just after the addition of Dox.

Immunoprecipitation and Western blotting. HEK293T cells (1 x 10⁶) were plated on 60 mm dishes, and then transfected with an expression vector for each FRET probe along with an expression vector for FLAG-mRIPK3 by PEI MAX (Molecular Devices). 440AF21 excitation (Ex) filter set for TRITC was used. Two emission (Em) filters (480/30 for ECFP and 536/38 for YPet) were used for imaging. The FRET emission ratio (ECFP/YPet) was calculated by dividing Ex440/Em480 (CFP) using MetaMorph. For the detection of SYTOX Orange, cells were incubated in growth medium and placed in a heated chamber. Imaging analysis was carried out using a fluorescence microscope (IX-81; Olympus) with a CCD camera (ORCA-R2, Hamamatsu) controlled by MetaMorph 7.0 Software (Molecular Devices). 440AF21 excitation (Ex) filter, 455DLPC dichroic mirror, and two emission (Em) filters (480/30 for ECFP and 536/38 for YPet) were used for imaging. The FRET emission ratio (ECFP/YPet) was calculated by dividing Ex440/Em536 (FRET) by Ex440/Em480 (CFP) using MetaMorph. For the detection of SYTOX Orange uptake, an additional filter set for TRITC was used.

Imaging analysis. Initial experiments, L929 cells were transiently transfected with the indicated FRET biosensors with Lipofectamine 2000. Otherwise indicated, L929, MEFs, aMoC1, HT29, and HaCaT cells stably expressing mSMART or hSMART were used for imaging. Cells were seeded on gelatin-coated glass bottom dishes for 18 h and then stimulated with the indicated agents. To confirm the concentrations of agents to stimulate cells are as follows: murine TNF (10 ng/ml), human TNF (30 ng/ml), poly(I:C) (20 µg/ml), zVAD (20 µM), Nec-1 (20 µM), BV6 (1 µM), GSK872 (5 µM), and NSA (5 µM).

In the former experiments (Figs. 1, 2, Supplementary Fig. 1, 3, 5), cells were incubated in growth medium and placed in a heated chamber. Imaging analysis was carried out using a fluorescence microscope (IX-81; Olympus) with a CCD camera (ORCA-R2, Hamamatsu) controlled by MetaMorph 7.0 Software (Molecular Devices). 440AF21 excitation (Ex) filter, 455DLPC dichroic mirror, and two emission (Em) filters (480/30 for ECFP and 536/38 for YPet) were used for imaging. The FRET emission ratio (ECFP/YPet) was calculated by dividing Ex440/Em536 (FRET) by Ex440/Em480 (CFP) using MetaMorph. For the detection of SYTOX Orange uptake, an additional filter set for TRITC was used.

In the latter experiments (Figs. 3–8, 10, Supplementary Fig. 4, 5), imaging of FRET was collected using a DeltaVision microscopy system (GE Healthcare) built on an Olympus IX-71 inverted microscope base equipped with Photometrics CoolSnap HQ2 CCD camera, using 60×/NA.516 PlanApo oil immersion lens (Olympus). For live cell imaging with FRET sensors, cells were seeded on
HMGB1 release was estimated by purchasing from Semrock (Rochester, NY). Eight to fifteen hours before observation, the culture supernatant was replaced with a fresh to 456 nm was used to detect SYTOX Orange or mCherry, mCherry filter (Ex 575 nm/Em 625 nm) was used for data collection.

Simultaneous LCI-S, intracellular HMGB1-mCherry, and SMART. Imaging of the release of HMGB1-mCherry by LCI-S was performed as previously described (16). It was used to detect SYTOX Orange or mCherry, mCherry filter (Ex 575 nm/Em 625 nm) was used for data collection.

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