Secreted autotransporter toxin (Sat) triggers autophagy in epithelial cells that relies on cell detachment

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Summary

The secreted autotransporter toxin, Sat, which belongs to the subfamily of serine protease autotransporters of Enterobacteriaceae, acts as a virulence factor in extraintestinal and intestinal pathogenic strains of Escherichia coli. We observed that HeLa cells exposed to the cell-free culture supernatant of recombinant strain AAEC185pSat-IH1128 producing the Sat toxin (CFCSSat), displayed dramatic disorganization of the F-actin cytoskeleton before loosening cell-to-cell junctions and detachment. Examination of the effect of Sat on GFP-microtubule-associated protein light chain 3 (LC3) HeLa cells revealed that CFCSSat-induced autophagy follows CFCSSat-induced F-actin cytoskeleton rearrangement. The induced autophagy shows an acceleration of the autophagy flux soon after Sat treatment, followed later by a blockade of the flux leading to the accumulation of large GFP-LC3-positive vacuoles in the cell cytoplasm. CFCSSat did not induce cell detachment in autophagy-deficient mouse embryonic fibroblasts in contrast with wild-type mouse embryonic fibroblasts. The CFCSSat-induced large GFP-LC3 dots do not display the characteristics of autophagolysosomes including expression of cathepsin D and Lamp-1 and 2 proteins, and Lysotracker Red- and DQ-BSA-positive labelling. We provide evidences that CFCSSat-induced autophagy is not a cell response intended to get rid of the intracellular toxin. By a pharmacological blockers approach, we found that the blockade of Erk1/2 and p38 MAPKs, but not JNK, inhibited the CFCSSat-induced autophagy and cell detachment whereas phosphatidylinositol-3 kinase blockers inhibiting canonical autophagy were inactive. When attached CFCSSat-treated cells start to detach they showed caspase-independent cell death and rearrangements of the focal adhesion-associated vinculin and paxillin. Collectively, our results support that Sat triggers autophagy in epithelial cells that relies on its cell-detachment effect.

Introduction

Secreted autotransporter toxin (Sat) belongs to the subfamily of serine protease autotransporters of Enterobacteriaceae (SPATEs) toxins (Henderson and Nataro, 2001) that are secreted through the type V secretion pathway (Henderson et al., 2004). Mature SPATEs display an N-terminal serine protease catalytic domain; followed by a highly conserved beta-helix motif (Henderson et al., 2004). SPATEs have been divided phylogenetically into classes I and II on the basis of the structures and activities of their toxins (Dutta et al., 2002). Class I includes the plasmid-encoded toxin, Pet (Eslava et al., 1998) of enteroaggregative Escherichia coli, the extracellular serine protease, plasmid-encoded, the EspP of enterohemorrhagic E. coli (Brunder et al., 1997), the EspC of enteropathogenic E. coli (Stein et al., 1996), the SigA of Shigella flexneri 2a (Al-Hasani et al., 2000) and the Sat of uropathogenic E. coli (UPEC) (Guyer et al., 2000) and diffusely adhering Afa/Dr E. coli (Afa/Dr DAEC) (Guignot et al., 2007). The sat gene has been previously identified in the strain CFT073 (Guyer et al., 2000), a prototype strain of UPEC (Mobley et al., 1990), where it resides within pathogenicity island II (PAI-II[CFT073]) (Guyer et al., 2000; 2001). The sat gene has been found to be prevalent in UPEC strains (Guyer et al., 2002; Ruiz et al., 2002a; Vila et al., 2002), resident intestinal microbiota, and pathogenic strains of E. coli including enteroaggregative...
Engulfment by autophagosomes of intracellular Gram-positive and Gram-negative bacterial pathogens, leading to their destruction (Levine, 2005; Deretic and Levine, 2009). Moreover, autophagy has been observed occurring in cells intoxicated by the vacuolating VacA cytotoxin of Helicobacter pylori (Terebiznik et al., 2009), Vibrio cholerae cytolsyn (Gutierrez et al., 2007), Shiga toxin (Sandvig and van Deurs, 1992) and cytotoxic necrotizing factor 1 (Fiorentini and Malorni, 2006) of E. coli, α-haemolysin of Staphylococcus aureus (Mestre et al., 2010), anthrax lethal toxin of Bacillus anthracis (Tan et al., 2009) and neurotoxin MPP (Zhu et al., 2007).

Results

Sat triggers a cell-detaching effect in HeLa epithelial cells

The CFCS

\( \text{Sat}\)-induced cell-detaching effect was investigated using human uterine cervical carcinoma HeLa cells at sub-confluency. Cells forming a confluent cell layer with well-organized, cell-to-cell contact detached from the glass substratum as a function of time and of the CFCS

\( \text{Sat}\) concentration (Fig. 1). Observation of CFCS

\( \text{Sat}\)-treated cells by differential interferential contrast microscopy revealed that the cells exposed to CFCS

\( \text{Sat}\) for 18 h formed a confluent cell layer with well-organized cell-to-cell contacts (Fig. 1A). In contrast, the cells exposed to CFCS

\( \text{Sat}\) for 18 h detached from the glass substratum and the cells that remained attached displayed dramatic elongation (Fig. 1A). Development of CFCS

\( \text{Sat}\)-induced cell detachment was concentration- and time-dependent (Fig. 1B). Sat contains a serine protease motif that plays a pivotal role in the activities of SPATES (Dutta et al., 2002). We used the CFCS of strain AAECS

\( \text{Sat}\)S256I (CFCS

\( \text{Sat}\)S256I) containing the mutated toxin in which an isoleucine replaces the serine at residue 256 of the serine protease motif (S256I) (Guignot et al., 2007). As seen in Fig. 1C and F, the cells exposed to CFCS

\( \text{Sat}\)S256I for 18 h showed no cell detachment. To confirm this, when HeLa cells were exposed to CFCS

\( \text{Sat}\) pretreated with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF), they showed no cell detachment in contrast to those exposed to untreated CFCS

\( \text{Sat}\) (Fig. 1C and F).

We further examined the presence of Sat within HeLa cells exposed to CFCS

\( \text{Sat}\). As Sat displays 75% amino acid sequence similarity with Pet (Eslava et al., 1998; Guyer et al., 2000), we took advantage of the anti-Pet antibody (Navarro-Garcia et al., 2001). Indirect immunofluorescence labelling of CFCS

\( \text{Sat}\)exposed cells with the anti-Pet antibody revealed the punctuate presence of positive immunofluorescence throughout the cytoplasm (Fig. 1D). As a control, there was no positive immunofluorescence in cells exposed to CFCS

\( \text{Sat}\).
the anti-Pet antibody (Fig. 1D). As Pet internalization and effects of Pet on epithelial cells were inhibited by brefeldin A (Navarro-Garcia et al., 2001), when HeLa cells were pretreated with brefeldin A, a blocker of membrane traffic, the intracellular presence of Sat was dramatically decreased and all the cells remained attached to the glass substratum, indicating that the intracellular presence Sat was needed for the toxin to trigger its cell-detaching effect (Fig. 1E and F).

Collectively, these findings demonstrate that the Sat toxin present in the CFCS of the recombinant strain AAEC185pSat-IH11128 is responsible for the cell detachment of epithelial cells after it has penetrated into the cells.

**CFCS Sat induces dramatic disorganization of the F-actin stress fibres before producing cell detachment**

The Class I SPATES, including Pet (Navarro-Garcia et al., 1999), EspC (Navarro-Garcia et al., 2004) and Sat (Guyer et al., 2000; Maroncle et al., 2006), produce their cytotoxic effects by disorganizing the host cell cytoskeleton in a manner characterized by the loss of F-actin stress fibres.
in epithelial cells. We observed marked disorganization of the F-actin cytoskeleton in the sub-confluent CFCSSat-treated HeLa cells compared with control CFCSAAEC185-treated cells (Fig. 2A). The well-organized F-actin stress fibres disappeared, and the F-actin was redistributed as ruffles. In contrast, F-actin cytoskeleton disorganization was not observed when the cells were exposed to CFCSSat-S256I or to CFCSSat pretreated with PMSF (Fig. 2B). A time kinetic shows that the CFCSSat-induced F-actin network disassembly in CFCSSat-treated cells increased time-dependently (Fig. 2C) and before the above observed cell detachment (Fig. 1B). Moreover, the effect of Sat on F-actin cytoskeleton was inhibited by brefeldin A treatment (not shown).

Sat cleaves fodrin in vitro and it has been postulated that Class I SPATES-induced disassembly of F-actin cytoskeleton follows a rearrangement of fodrin (Dutta et al., 2002). To find out whether Sat redistributes fodrin in HeLa cells, CFCSAEC185 and CFCSSat-treated cells were stained with an anti-spectrin α II antibody (Villaseca et al., 2000; Sui et al., 2003). Surprisingly, fodrin distribution is not modified in CFCSSat-treated HeLa cells compared with CFCSAEC185-treated cells (Fig. 2D).

CFCSSat rearranges focal adhesions (FAs)

Cell-to-cell contacts and the cell adhesome mediating cell attachment to the substratum consist of a complex network composed of a large number of linked components (Geiger et al., 2009). We chose to investigate the distribution of vinculin and paxillin, two representative actin-binding proteins that is associated with cell–cell contacts and cell–extracellular matrix junctions at FAs (Humphries et al., 2007; Maddugoda et al., 2007). Immunostaining of CFCSAAEC185-, CFCSSat- and CFCSSat-S256I-treated HeLa cells with anti-vinculin and anti-paxillin antibodies revealed a time-dependent rearrangement in vinculin- and paxillin-positive FAs in CFCSSat-treated cells compared with CFCSAAEC185-treated cells (Fig. 3A and B, respectively). We observed long and well-ordered paxillin-positive FAs in CFCSAAEC185-treated cells (Fig. 3A left panel and 3C). The observation that the CFCSSat-induced modification of paxillin-positive FAs did not occur in cells exposed to CFCSSat-S256I indicated that the serine protease activity of the toxin must be involved in the FAs proteins.
rearrangement (Fig. 3B and C). The effect of Sat on rearrangement of vinculin-positive FAs was inhibited by brefeldin A treatment (not shown). Moreover, observation of sub-confluent CFCS\textsubscript{Sat}-treated cells at 10 h post-treatment (p-t), i.e. before the cell detachment started, revealed a loss of cell-to-cell contacts, and cells that were rounded up compared with CFCSS\textsubscript{AEC185}-treated cells (Fig. 3D).

Sat promotes autophagy in epithelial HeLa cells before producing cell detachment

The loss of attachment of epithelial cells to the extracellular matrix promotes autophagy via three potential signalling pathways: the energy-sensing pathway, the growth factor- and nutrient-sensing pathway and the integrated stress response pathway (Lock and Debnath, 2008). We investigated whether autophagy occurred in sub-confluent CFCS\textsubscript{Sat}-treated HeLa cells (Fig. 4). Microtubule-associated protein light chain 3 (LC3), the mammalian equivalent of yeast Atg8, a marker of autophagy, is located in the cytosol (LC3-I) under normal conditions, but in response to the induction of autophagy, LC3-I is processed and conjugated to phosphatidylethanolamine, forming LC3-II that attaches to the autophagosomal membranes (Kabeya et al., 2000). LC3-II can therefore be used to estimate the abundance of autophagosomes using both biochemistry and microscopy (Klion-
sky et al., 2008). We took advantage of the fact that HeLa cells stably expressed the GFP-tagged phosphatidylethanolamide-conjugated LC3-II form (GFP-LC3) (Kabeya et al., 2000), which appeared under fluorescence microscopy as punctuate dots corresponding to the formation of autophagosomes during autophagy induction. GFP-LC3 cells exposed to CFCSAAEC185 displayed a few randomly distributed GFP-LC3 dots (Fig. 4A). In contrast, there was a dramatic increase in the number of GFP-LC3 dots in GFP-LC3 HeLa cells that had been exposed to CFCSSat for 4 h (Fig. 4A). GFP-LC3 HeLa cells cultured for 4 h in the presence of nutrient-free EBSS medium showed the same increase in GFP-LC3 dots, showing that autophagy was stimulated by amino acid deprivation (Fig. 4A). To test the role played by Sat serine protease activity in CFCSSat-induced autophagy, GFP-LC3 HeLa cells were exposed either to CFCSSat or to Sat mutated on the serine protease domain (CFCSSat-S256I) (50 µl). In C, the time- and concentration-dependent appearance of GFP-LC3 dots in CFCSSat-treated GFP-LC3 HeLa cells. The micrographs are representative of three separate experiments. Each value shown is the mean ± SD of three separate experiments. In A, *P < 0.01 compared with CFCSAAEC185. In B, *P < 0.01 at 4, 5, 6 and 10 h p-t for CFCSSat (10, 25, 50 and 75 µl) compared with CFCSAAEC185.
GFP-LC3 dots in CFCSsat-treated GFP-LC3 HeLa cells, as the phenomenon was inhibited by the blocker of membrane traffic, brefeldin A (Fig. 4B). Image analysis conducted by scanning laser microscopy of fixed cells and the quantification of cells with GFP-LC3 dots over time as a function of the concentration of CFCSsat showed that GFP-LC3 dots soon appeared and increased in number in a time- and concentration-dependent manner before cell detachment started (Fig. 4C).

During the time-course of CFCSsat treatment, two populations of GFP-LC3 dots were observed (Fig. 5). Dots varied in size; some were ~2–4 μm in diameter, which is relatively normal for vesicles in the autophagic pathway (Klionsky et al., 2008), while others were extremely large (approaching ~25 μm in diameter). Small GFP-LC3 dots appeared rapidly and their number per cell increased regularly as a function of the time p-t, peaked at 6 h p-t and remained stable thereafter (Fig. 5A). In contrast, only a slight increase in the number of small dots per cell was observed in CFCSAAEC185-treated cells (Fig. 5A). Video microscopy examination of live cells reveals the late appearance of large GFP-LC3 dots (Fig. 5B). Image...
analysis of large GFP-LC3 dots conducted by scanning laser microscopy of fixed cells (Fig. 5C left panel) and their quantification (Fig. 5C right panel) confirms that they appeared at 6 h p-t, peaked at 10 h p-t, and then remained stable. It was noted that the thinly scattered CFCS_{Sat}-treated GFP-LC3 HeLa cells that remained attached to the glass substratum at 18 h p-t displayed a large number of large GFP-LC3 dots (Fig. 5C left and right panels). Image analysis of CFCS_{Sat}-treated cells was conducted to measure the sizes and distribution of small and large GFP-LC3 dots at 4 h and 8 h p-t (Fig. 5D). At 4 h p-t, only small GFP-LC3 dots were present (Fig. 5D upper panel). At 8 h p-t, a decreased number of small dots and the presence of large dots of various different sizes were observed (Fig. 5D lower panel). It was noted that the CFCS_{AAEC185}-treated cells remained devoid of large GFP-LC3 dots at all times p-t examined (Fig. 5D). Collectively, these results demonstrate that Sat triggers the rapid production of small and large autophagic vacuoles in a time- and concentration-dependent manner before it triggers a cell-detaching effect.

**CFCS_{Sat} treatment soon induces acceleration of the autophagic flux that is followed by a blockade of the flux at later times p-t**

In response to the induction of autophagy, the LC3 protein is processed for lipid conjugation leading to the conversion of the 45 kDa LC3-I form to the 43 kDa LC3-II form (Klionsky et al., 2008). We see a time-dependent increase of the LC3-II form in CFCS_{Sat}-treated GFP-LC3 HeLa cells at early times p-t compared with control and CFCS_{AAEC185}^treated cells (Fig. 6A left panel). As expected, the control...
experiment shows that the GFP-LC3 HeLa cells exposed to amino acid deprivation displayed an increased level of the LC3-II form (Fig. 6A right panel). We assessed the autophagic flux by measuring LC3-II levels in the presence or absence of the proton ATPase inhibitor bafilomycin A1, which is known to inhibit lysosomal degradation without affecting autophagolysosome formation (Klionsky et al., 2008). We found a substantial increase in the LC3-II level at 2 h p-t in CFCSSat-treated GFP-LC3 HeLa cells in the presence of bafilomycin A1 compared with that in its absence (Fig. 6B left panel). As a positive control of an increase of autophagic flux, cells responding to nutrient starvation displayed a significant increase in LC3-II levels after the addition of bafilomycin A1 (Fig. 6B right panel). In contrast, at 8 h and 10 h p-t, the presence of bafilomycin A1 caused not increase in the LC3-II levels in CFCSSat-treated GFP-LC3 HeLa cells (Fig. 6C). Collectively, these results indicate that the cells responded to Sat intoxication by increasing the autophagic flux at early times p-t, resulting in the appearance of an increased number of small GFP-LC3 dots, and that this phenomenon is followed by a blockade of the autophagic flux at later times p-t.

**CFCSSat did not induce cell detachment in autophagy-deficient MEF**

Atg5 is essential for the initiation of autophagosome formation (Kuma et al., 2004). Autophagy-deficient Atg5-deficient mouse embryonic fibroblasts (MEF Atg5−/−) (Mizushima et al., 2001) were used at sub-confluency to demonstrate that the CFCSSat-induced cell detachment is related to the CFCSSat-induced autophagy. Figure 7A left panel and Fig. 7C show that the MEF Atg5−/− cells exposed to CFCSSat for 18 h showed a concentration-dependent detachment, and that at the higher concentrations more than 90% of the cells were detached. When exposed to CFCSSat-S256I containing the mutated toxin, MEF Atg5−/− showed no cell detachment (Fig. 7A, right micrograph). No cell detachment occurred in MEF Atg5+/− cells treated with 10–50 μl of CFCSSat (Fig. 7B and C). Fewer than 20% of the MEF Atg5−/− cells detached when exposed to a high concentration (100 μl) of CFCSSat (Fig. 7B and C). Vinculin-positive FAs examined by immunostaining and CLMS revealed long and well-organized vinculin-positive FAs in CFCSSat-treated MEF Atg5−/− cells and small, vinculin-positive dots in CFCSSat-treated MEF Atg5−/− cells (not shown). As expected, large GFP-LC3 dots appeared in GFP-LC3-MEF Atg5−/− exposed to CFCSSat (Fig. 7D). In contrast, the GFP-LC3-MEF Atg5−/− showed an absence of GFP-LC3 dots when exposed to CFCSSat (Fig. 7D). Taken together, this finding indicates that Atg5 is necessary for CFCSSat-induced autophagy to occur.

**Phosphatidylinositol-3 kinase (PI3K) blockers did not reverse CFCSSat-induced autophagy and cell detachment**

Inhibitors of Class III PI3K (PI3K/hVps34) activity were widely used to block canonical autophagy (Blommaart et al., 1997; Petiot et al., 2000). We conducted an inhibitory experiment with the PI3K inhibitors 3-methyladenine (3-MA) and wortmannin in order to determine whether CFCSSat induces canonical autophagy in sub-confluent GFP-LC3 HeLa cells. As 3-MA in full medium promotes autophagy in cells treated for a prolonged period (Wu et al., 2010), effect of 3-MA was measured at 4 h p-t. Neither 3-MA at 4 h CFCSSat p-t nor wortmannin at 8 h CFCSSat p-t inhibited the CFCSSat-induced appearance of GFP-LC3 dots in CFCSSat-treated GFP-LC3 HeLa cells (Fig. S1A). As expected, a control experiment with starved GFP-LC3 HeLa cells shows that both 3-MA and wortmannin inhibited the appearance of GFP-LC3 dots (Fig. S1B). Importantly, it was noted that wortmannin fails to inhibit CFCSSat-induced cell detachment (Fig. S1C).

**CFCSSat-induced autophagy not relies to a vacuolating effect and is not a cell response intended to get rid of the intracellular toxin**

Sat has been found forming vacuoles only in bladder CRL-1749 and kidney CRL-1573 cell lines compared with different epithelial cell lines (Guyer et al., 2000; 2002; Maronde et al., 2006). The formation of large vacuoles in cells exposed to the V. cholerae cytolsin has been shown to be autophagy-dependent (Gutierrez et al., 2007), and in cells exposed to the vacuole-forming VacA cytotoxin of H. pylori the autophagy induced plays no role in the formation of the large vacuoles, but does control their size (Terebiznik et al., 2009). Consequently, we investigated whether cytoplasmic vacuolization accompanied the appearance of autophagy in CFCSSat-treated HeLa cells or not. To do this, untreated and CFCSSat-treated cells were labelled with the CellTracker Red in order to detect the cytoplasmic vacuoles, which were visible as ‘holes’ not stained by CellTracker Red. There were no cytoplasmic vacuoles in CFCSSat-treated cells with large GFP-LC3 dots (Fig. S2).

In response to a subset of vacuolating and non-vacuolating bacterial toxins, the cells engaged autophagy in an attempt to eliminate the toxins present intracellularly (Gutierrez et al., 2007; Tan et al., 2009; Terebiznik et al., 2009). We investigated whether intracellular Sat colocalized with the GFP-LC3 dots in CFCSSat-treated GFP-LC3 HeLa cells, and found that no colocalization occurred between Sat immunolabelling and the small or large GFP-LC3 dots (Fig. 8). This indicated that the autophagy observed in CFCSSat-treated cells was not a protective
response induced by the cell for addressing the intracellular toxin into the autophagy degradation compartment.

**CFCS<sub>Sat</sub>-induced large GFP-LC3 dots do not display the hallmarks of autophagolysosomes**

During the autophagy process (Klionsky and Emr, 2000), the double-membraned vesicles known as autophagic vacuoles or autophagosomes can eventually ultimately fuse with lysosomes to form autophagolysosomes, which are also known as autolysosomes (Yoshimori and Noda, 2008). After the fusion of autophagosomes with lysosomes, the inner pool of LC3 is degraded, and the LC3 associated with the vesicular membrane is cleaved by Atg4, and recycled back into the cytosol (Kimura et al., 2007). Surprisingly, we observed that the LC3 signal decorating the CFCS<sub>Sat</sub>-induced large dots did not disappear in a time-dependent fashion, and was still observable in the few remaining attached cells at late times post-treatment. This prompted us to try to find out whether the CFCS<sub>Sat</sub>-induced large GFP-LC3 dots displayed the characteristics of autophagolysosomes. To assess the degree of maturation of these structures, we evaluated their acidifiability, whether a lysosomal enzyme was present, the degradative activity and the presence of lysosome-associated membrane-proteins. CFCS<sub>Sat</sub>-treated GFP-LC3 HeLa cells were treated with an acidophilic, lysosomotropic...
agent, LysoTracker Red (LTR) in order to make it possible to examine simultaneously the localizations of lysosomes and of GFP-LC3 dots. As depicted in Fig. 9A, LTR-positive vesicles and large GFP-LC3 dots could be observed separately in CFCS\textsubscript{Sat}-treated GFP-LC3 HeLa cells, and no colocalization between LTR and GFP-LC3 occurred. In addition, CFCS\textsubscript{Sat}-treated GFP-LC3 HeLa cells were immunolabelled with an antibody directed against cathepsin D, a marker of lysosomes that is present in autophagolysosomes. Cathepsin D-positive vesicles were identified in CFCS\textsubscript{Sat}-treated GFP-LC3 HeLa cells, without any colocalization of cathepsin D and large GFP-LC3 dots (Fig. 9B). As a control experiment, in starved GFP-LC3 HeLa cells LTR-positive and GFP-LC3-positive vesicles, and vesicles that were positive for both LTR and GFP-LC3 were detected separately (Fig. 9C). Knockout of lysosome-associated membrane-protein-2 (Lamp-2) in mice and siRNA silencing of Lamp-2 in starved epithelial cells led to the accumulation of autophagic vacuoles in various tissues and in the cytoplasm of cultured epithelial cells respectively (Tanaka et al., 2000; Eskelinen et al., 2002; Gonzalez-Polo et al., 2005). We completed our investigation by checking for the presence of Lamp-1 and/or Lamp-2 in CFCS\textsubscript{Sat}-induced large GFP-LC3 dots. As shown in Fig. 9D, small Lamp-1- and Lamp-2-positive vesicles were present in CFCS\textsubscript{Sat}-treated GFP-LC3 HeLa cells, but the large GFP-LC3 dots were devoid of Lamp-1- and Lamp-2-positive immunolabelling. Finally, the degradative character of CFCS\textsubscript{Sat}-induced large GFP-LC3 dots was evaluated with DQ-BSA (DeQuenched Bovine Serum Albumin) Red, which emits fluorescence when it is degraded, thus labelling intracellular degradative compartments. No colocalization between DQ-BSA Red and large GFP-LC3 dots was observed in CFCS\textsubscript{Sat}-treated GFP-LC3 HeLa cells (Fig. 9E). This accumulation of autophagosomes within the cell cytoplasm that we observed at late times p-t in CFCS\textsubscript{Sat}-treated cells is a hallmark of Type-II cell death, which is also known as autophagic cell death (Kroemer and Levine, 2008).

Autophagosomes move in an MT-dependent manner (Kimura et al., 2008), and MT disassembly in response to nocodazole treatment, which inhibits the autophagosome-lysosome fusion, leads to the appearance of large LC3-positive vesicles (Munafo and Colombo, 2001; Webb et al., 2004). This prompted us to investigate whether the CFCS\textsubscript{Sat}-induced appearance of large GFP-LC3 dots was correlated with CFCS\textsubscript{Sat}-induced MTs network disassociation. Examination of the MTs network in CFCS\textsubscript{Sat}-treated GFP-LC3 HeLa cells by immunolabelling of tubulin reveals that the organization remains the same as in CFCS\textsubscript{SEC185}-treated cells (Fig. S3A). Consistent with previous reports (Munafo and Colombo, 2001; Webb et al., 2004), a control experiment showed that the strong association of both LTR and Lamp-2 with a population of GFP-LC3 dots in starved HeLa cells was abolished when MTs were disassembled after nocodazole treatment (Fig. S3B). Overall, these results indicate that the Sat-
induced accumulation of large GFP-LC3 dots does not result from a Sat-induced dismantling of the MTs network.

**CFCS<sub>Sat</sub>-induced autophagy and cell detachment were unrelated to Type I cell death and necrosis**

In attached CFCS<sub>Sat</sub>, intoxicated cells, a decrease of cell viability, measured with the MTT assay, was observed starting between 6 h and 8 h p-t and increasing after before the cells to detach (Fig. 10). As revealed by the trypan blue exclusion assay, all the detached cells are dead (not shown). Importantly, the detached cells did not attach when they are cultivated (not shown).

In some cases, Type I cell death, also known as apoptosis, develops alongside autophagy (Kroemer and Levine, 2008). We investigated whether the effects of CFCS<sub>Sat</sub> observed above were related to apoptosis or not. Compared with CFCS<sub>SAAEC185</sub>-treated cells, the CFCS<sub>Sat</sub>-treated GFP-LC3 HeLa cells showed no change in distribution of mitochondria stained with the MitoTracker Red, no release of cytochrome c or apoptosis-inducing factor (AIF) (Fig. S4A), no cleavage of PARP (not shown) and no nuclear condensation (Fig. S4B). Moreover, CFCS<sub>Sat</sub>-treated GFP-LC3 HeLa cells when treated with an apoptosis suppressor, the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), showed no inhibition of the appearance of GFP-LC3 dots or cell detachment (Fig. S4C). After investigating whether the CFCS<sub>Sat</sub>-induced autophagy was related to necrosis (Golstein and Kroemer, 2007) or pyroptosis (Bergsbaken et al., 2009) cell death, we found that glycine, a non-specific cytoprotective agent providing...
The level of GFP-LC3 dots in CFCSSat-treated GFP-LC3-expressing HeLa cells was dose-dependently decreased by the p38 mitogen-activated protein kinase (p38) (Corcelle et al., 2007). We therefore next investigated whether the PI3K-independent, CFCSSat-induced autophagy was regulated by MAPKs or not. To do this, the sub-confluent GFP-LC3-expressing HeLa cells were exposed to CFCSSat with or without specific blockers of Erk1/2, of p38, or of stress-activated protein kinase/c-Jun N-terminal kinase (JNK) (JNK). Blockade of extracellular signal-regulated protein kinase (Erk1/2) and p38, but not c-Jun NH2-terminal kinase (JNK), inhibited the CFCSSat-induced autophagy and cell detachment (Fig. S4D). Agreeing with this result, during the 10 h period of treatment, attached CFCSSat-intoxicated cells did not release LDH (not shown). Taken together, these findings indicate that the above reported Sat-induced cell detachment and autophagy are not related to a Type-I cell death, necrosis or pyroptosis.

**Discussion**

We further investigated whether the Erk1/2 and p38 inhibitors modified the CFCSSat-induced cell detachment in sub-confluent HeLa cells. CFCSSat inducing rearrangements in FAs (Fig. 3), we investigated the distribution of vinculin-positive FAs in cells exposed to CFCSSat in the presence of Erk1/2, p38 or JNK inhibitor II inhibitors. Cells displayed normal expression of vinculin-positive FAs when exposed to CFCSSat in the presence of U0126 or of SB203580 but not in the presence of JNK inhibitor II (Fig. 11E). Consistent with this, results in Fig. 11F shows that SB203580 and U0126, but not JNK inhibitor II, inhibited the CFCSSat-induced cell detachment. In contrast and importantly, data showed that in cells exposed to CFCSSat in the presence of MAPK inhibitors U0126 or SB203580, the F-actin cytoskeleton remains disorganized (Fig. S6).

PI3K-independent autophagy in cells exposed to a toxin has been shown to be regulated by extracellular signal-regulated kinase 1/2 (Erk1/2) (Zhu et al., 2007). Moreover, depending on the cell line and stimuli involved, autophagy can be regulated by Erk1/2 or p38 mitogen-activated protein kinase (p38) (Corcelle et al., 2007). We therefore next investigated whether the PI3K-independent, CFCSSat-induced autophagy was regulated by MAPKs or not. To do this, the sub-confluent GFP-LC3-expressing HeLa cells were exposed to CFCSSat with or without specific blockers of Erk1/2, of p38, or of stress-activated protein kinase/c-Jun N-terminal kinase (JNK) (JNK). The level of GFP-LC3 dots in CFCSSat-treated GFP-LC3-expressing HeLa cells was dose-dependently decreased by the p38 inhibitor, SB203580, and to a lesser extent by the Erk1/2 inhibitor, UO126 (Fig. 11A and B). In contrast, treatment with JNK inhibitor II, which blocks both JNK-I and JNK-II, did not reduce the level of GFP-LC3 dots in cells exposed to CFCSSat treatment (Fig. 11A and B). Treatment with SB203580 reduced the size of the GFP-LC3 dots in CFCSSat-treated GFP-LC3-expressing HeLa cells (Fig. 11C). In addition, the conversion of LC3-I to LC3-II was lower in GFP-LC3-expressing HeLa cells exposed to CFCSSat in the presence of SB203580 than in untreated cells (Fig. 11D). We checked that CFCSSat-treated HeLa cells did indeed display increased phosphorylation of Erk1/2 and p38, but not of JNK, compared with CFCSSat-treated controls (Fig. S5).

We report here a newly discovered mechanism involving autophagy, by which the internalized Sat toxin exerts its cytotoxic effect in epithelial cells. At early times p-t, we observed a time- and concentration-dependent increase in the number of small GFP-LC3-positive vacuoles within the cell cytoplasm of CFCSSat-intoxicated cells (Fig. 12). Moreover, we found that the conversion of the LC3-I form into the LC3-II form was improved in the presence of a specific inhibitor of vacuolar-type H+-ATPase, indicating an acceleration of the autophagic flux. In addition, examination of the characteristics of the CFCSSat-induced autophagy revealed that it was insensitive to PI3K inhibitors. PI3K-independent autophagy has been previously observed in cells intoxicated with the neurotoxin 1-methyl-4-phenylpyridinium (MPP) (Zhu et al., 2007) and the α-haemolysin of *S. aureus* (Mestre et al., 2010), or in cells infected with a *Vibrio parahaemolyticus* strain expressing the VopQ effector that is translocated into the cells via type-III secretion system 1 (T3SS1) (Burdette et al., 2009). In contrast, autophagy induced by either the *V. cholerae* cytolsin (Gutierrez et al., 2007) or by T2SS1-dependent POR3 effector of *V. parahaemolyticus* (Burdette et al., 2008) can be blocked by pharmacological inhibitors of PI3Ks. For the vacuolating VacA cytotoxin of *H. pylori*...
(Terebiznik et al., 2009), *V. cholerae* cytolysin (Gutierrez et al., 2007), and anthrax lethal toxin of *B. anthracis* (Tan et al., 2009), the induced autophagy causes cell survival as blockade of autophagy induces cell death or vacuolation or cell lysis. This cell survival response correlates with the presence of some of these toxins within the autophagic vacuoles that is indicative of their autophagy-dependent elimination (Gutierrez et al., 2007; Terebiznik et al., 2009).

In contrast, cell lysis develops both in cells showing POR3-induced, PI3K-dependent and VopQ-induced, PI3K-independent autophagy (Burdette et al., 2008; 2009). It is important to note that our findings demonstrate that in Sat-treated cells, the induced autophagy does not play any part in eliminating the intracellular toxin.

Following the early CFCS_{Sat}-induced acceleration of the autophagic flux, we observed that the conversion of the

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**Fig. 11.** Effect of MAPK inhibitors on CFCS_{Sat}-induced autophagy and cell detachment in sub-confluent GFP-LC3 HeLa cells. In A, observation by confocal microscopy of GFP-LC3 dots in cells exposed to CFCS_{Sat} (50 μl) (8 h) with or without Erk1/2 inhibitor, U0126 (10 μM) or p38 inhibitor, SB203580 (10 μM) or JNK inhibitor II (10 μM). In B, dose effect of MAPKs inhibitors on the CFCS_{Sat}-induced appearance of GFP-LC3 dots. In C, size distribution of GFP-LC3 dots in CFCS_{Sat}-treated GFP-LC3 HeLa cells in the presence or absence of the p38 inhibitor, SB203580 (10 μM). In D, analysis of GFP-LC3-I and GFP-LC3-II forms in cells exposed to CFCS_{Sat} (50 μl) for 6 h with or without p38 inhibitor, SB203580 (10 μM). In E, effect of Erk1/2, p38 and JNK inhibitors (10 μM) on CFCS_{Sat}-induced rearrangement of vinculin-positive FAs measured after immunolabelling with a pAb anti-vinculin antibody and observation by CLMS. In F, the effect of MAPKs inhibitors (10 μM) on CFCS_{Sat}-induced cell detachment measured at 24 h CFCS_{Sat} p.t. The immunoblots and micrographs are representative of two separate experiments. Each value shown is the mean ± SD of three separate experiments. In C and E, *P < 0.01 compared with CFCS_{Sat}. In F, *P < 0.01 compared with without inhibitor.
LC3-I form to LC3-II form did not increase any further at late times p-t in the presence of a specific inhibitor of vacuolar-type H^+ -ATPase, indicating a subsequent slowdown of the autophagic flux. This phenomenon was correlated with the appearance in CFCSSat-treated cells of large GFP-LC3-positive dots that are devoid of markers of autophagolysosomes and of degradative activity. Why the maturation of autophagosomes is blocked in CFCSSat-intoxicated cells remains to be determined. Delaying autophagosome maturation or impairing the fusion of autophagosome with the lysosome are strategies used by some intracellular bacteria that reside within autophagosomes, including *S. aureus* (Schnaith et al., 2007; Mestre et al., 2010), *Coxiella burnetii* (Romano et al., 2007), *Brucella abortus* (Pizarro-Cerdà et al., 1998), *Porphyromonas gingivalis* (Dorn et al., 2001) and *Anaplasma phagocytophilum* (Niu et al., 2008). This probably has the advantage of allowing them to utilize the nutrients present in the autophagosomes to grow and multiply. Moreover, accumulation of autophagosomes and slowdown the autophagy flux have been observed after treatment with lysosomotropic agents, hydroxchloroquine (Boya et al., 2005), the proton pump inhibitor, esomeprazole (Marino et al., 2010) and simaresine (Ostenfeld et al., 2008); the active metabolite of a drug used for treatment of psychiatric disorders, desmethylclomipramine (Rossi et al., 2009). It was noted that for such compounds, caspase-dependent cell death has been found associated with the induced autophagy. No feature of apoptotic cell death is observed here in attached cells treated with the toxin Sat. Moreover, we observe no cell lysis indicating the absence of CFCSSat-induced cell necrosis or pyroptosis. However, the attached CFCSSat-treated cells start to show cell death after that the large GFP-LC3 dots accumulated within the cell cytoplasm and all the CFCSSat-treated cells are died when they are detached (Fig. 12). Different types of cell death have been defined: type I (apoptosis), type II (autophagic cell death) and type III (necroptosis) (Kroemer et al., 2009). We found hallmarks of Type II autophagic cell death (Kroemer et al., 2009) in CFCSSat-treated cells. Indeed, agreeing with recently defined new functional criteria needed to ascertain autophagic cell death (Shen and Codogno, 2011), we report that CFCSSat-treated cells showed: (i) an acceleration of the autophagic flux at early time p-t; (ii) a blockade of the autophagic flux and maturation of autophagosomes that accumulated...
within the cell cytoplasm at late time p-t and (iii) an absence of caspase activation.

We found that the CFCSSat-induced PI3K-independent autophagy was regulated by both Erk1/2 and p38 MAPKs because specific Erk1/2 and p38 inhibitors blocked the CFCSSat-induced autophagy, including the appearance of LC3-I form and its conversion into LC3-II form and the accumulation of large GFP-LC3 dots within the cytoplasm of exposed cells (Fig. 12). Different signalling pathways regulate the PI3K-independent autophagy depending on whether it is associated with autophagic cell death or not. PI3K-independent autophagy in cells exposed to the neurotoxin MPP has been shown to be regulated by Erk1/2 (Zhu et al., 2007), whereas α-haemolysin S. aureus-induced PI3K-independent autophagy is regulated by calcium (Mestre et al., 2010). Depending on the cell phenotypes, the stimuli applied, and the degree and duration of activation, the MAP kinases Erk1/2 and p38, in combination or individually, all have dual roles in upregulating or downregulating autophagy pathways. Erk1/2 and p38 have both been found to regulate capsaicin-induced autophagy: at the sequestration step for p38, and at the maturation step for Erk1/2 (Choi et al., 2010). A release of calcium from the ER has been found to be associated with activation of Erk1/2, leading to predominantly autophagic cell death (Yang et al., 2009). Erk1/2 activation was also necessary for the induction of autophagy following ER stress in renal tubules (Kawakami et al., 2009). Activation of the Erk pathway by lindane, dichlorodiphenyltrichloroethane or biphenol A has a selective impact on autophagy at the maturation step, resulting in the accumulation of large defective autolysosomes (Corcelle et al., 2006).

One important observation is that autophagy induced by CFCSSat leads to detachment of the cells. This conclusion is supported by three observations (Fig. 12): (i) autophagy deficient cells, such as MEF Atg5−/−, did not detach when exposed to CFCSSat whereas CFCSSat-treated wild-type MEF Atg5+/+ cells were detached; (ii) CFCSSat induced a concomitant accumulation of large GFP-LC3-positive dots this is immediately followed by a severe disorganization of vinculin- and paxillin-positive FAs, both of which shortly preceded cell detachment and (iii) Erk1/2 and p38 blockers inhibited both the accumulation of the large GFP-LC3-positive dots and cell detachment. Epithelial cells matrix detachment promotes autophagy that protects epithelial cells from the stresses of matrix detachment, allowing them to survive provided they can reattach in a timely manner (Fung et al., 2008; Lock and Debnath, 2008). To the best of our knowledge, our data are the first observation that establishes a relationship between a bacterial toxin-induced autophagy and cell detachment. An immediate question that arises is that: whether Sat toxin causes directly or indirectly autophagy? Our results support that Sat does not directly cause autophagy. Indeed, we found that the disruption of the F-actin network in CFCSSat-intoxicated cells precedes the appearance of LC3-positive vesicles and that Erk1/2 and p38 blockers inhibiting the CFCSSat-induced, PI3K-independent autophagy failed to block the CFCSSat-induced disassembly of F-actin cytoskeleton (Fig. 12). We think that autophagy induced by CFCSSat soon after intoxication is a cell survival response that follows the dramatic disorganization of the cell cytoskeleton. But, the overstimulated autophagy followed by a slowdown of the autophagic flux and the cytoplasmic accumulation of autophagosomes, three hallmarks of Type II autophagic cell death, acted together to promote the cell detachment (Fig. 12). On the basis of our results and previous reports (Sandvig and van Deurs, 1992; Gutierrez et al., 2007; Zhu et al., 2007; Tan et al., 2009; Terebiznik et al., 2009), it is tempting to speculate that when the autophagic process is complete this leads to cell survival, whereas when it is incomplete or interrupted it leads to cytotoxic effects.

Class I SPATES exert a cytopathic effect on cells by disorganizing the cell cytoskeleton (Henderson and Nataro, 2001; Dutta et al., 2002). Consistent with Maroncle et al. (Maroncle et al., 2006), we found that after cell internalization Sat induces a dramatic disruption of the F-actin cytoskeleton. The mechanism by which the Class I SPATE toxins, Pet (Navarro-Garcia et al., 1999; 2001; Dutta et al., 2003) and SigA (Al-Hasani et al., 2009), induce disruption of the F-actin cytoskeleton after entering the cell is related to rearrangements of fodrin (Canizalez-Roman and Navarro-Garcia, 2003; Sui et al., 2003; Al-Hasani et al., 2009). Surprisingly, we observed that Sat, despite cleaving fodrin in vitro (Dutta et al., 2002), did not induce the redistribution of fodrin in HeLa cells. This resembles a previous observation for the class I SPATE, EspC, which altered the F-actin cytoskeleton without modifying the distribution of fodrin (Navarro-Garcia et al., 2004) despite cleaving fodrin in vitro (Dutta et al., 2002). Further experiments will be required in order to find out whether other Class I SPATES (Dutta et al., 2002) induce canonical or PI3K-independent autophagy that relies to cell detachment, or not.

The Sat gene is present in 85% of UPECs involved in UTIs (Guyer et al., 2000; Vila et al., 2002; Ruiz et al., 2002b; Guignot et al., 2007; Restieri et al., 2007). UPECs are able to invade, survive and multiply within the host urinary tract cells, leading to persistent and recurrent UTIs (Nielubowicz and Mobjley, 2010). As the scheme of pathogenicity proposed for UPECs implies the exfoliation of infected bladder epithelial cells (Mulvey et al., 2000), it is tempting to speculate that Sat, by inducing autophagic cell detachment, may be involved in cell exfoliation induced by Sat-expressing UPEC.
Experimental procedures
Reagents and antibodies
FITC- and RITC-phalloidin and DQ Red BSA were from Molecular Probes Inc. (Invitrogen, Cergy-Pontoise, France). Monoclonal antibody (mAb) directed against cytochrome C (Becton Dickinson Biosciences, Le Pont de Claix, France). Polyclonal rabbit antibody (pAb) directed against LC3, mAb anti-α-tubulin (clone DM1A), mAb anti-vinculin (clone hVIN-1) and mAb anti-paxillin (clone PXC-10) (Sigma-Aldrich Chimie SARL, L’Ile d’Abeau Chesnes, France), pAb anti-Pet toxin was from F. Navarro-Garcia (Department of Cell Biology, CINVESTAV-IPN, Mexico, Mexico). mAb anti-α-IL spectrin and pAb anti-AIF (sc-9416) were from Santa Cruz Biotechnology Inc (Tebu-bio, Le Perray en Yvelines, France). mAbs anti-Lamp-1 (clone H4A3) and Lamp-2 (clone H4B4) developed by J.T. August and J.E. Hildreth (The Johns Hopkins University, School of Medicine, Baltimore, MA, USA) (Mare et al., 1989), were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, and maintained by the University of Iowa (Department of Biological Sciences, Iowa, IA, USA). Rabbit pAb anti-cathepsin D antibody was from J.S. Mort (McGill University, Montreal, Canada). Rabbit anti-p44/42 Erk1/2 MAP (#9102), anti-phospho-p44/42 Erk1/2 (Thr202/Tyr204) (#4377), anti-p38 (#9202), anti-phospho-p38 (Thr180/Tyr182) (#9216), anti-SAPK/JNK (#9258) and anti-phospho-SAPK/JNK (Thr183/Tyr185) (#4668), and anti-phospho-Akt (Ser473) (#4060) pAbs were from Cell Signaling Technology (Beverly, MA, USA). LysoTracker Red, CellTracker Red, Mitotracker Red were from Invitrogen. Texas Red isothiocyanate (TRITC)- and Alexa-conjugated anti-rabbit, anti-goat and anti-mouse secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, USA) and Molecular Probes Inc. (Invitrogen). 3,4-diaminobenzidine (DAB) Peroxidase Substrate Kit was from Sigma-Aldrich (Saint Louis, MO, USA). Rabbit pAb anti-cathepsin D (7C10) was from J.S. Mort (McGill University, Montreal, Canada). Rabbit anti-p44/42 Erk1/2 MAP (#9102), anti-phospho-p44/42 Erk1/2 (Thr202/Tyr204) (#4377), anti-p38 (#9202), anti-phospho-p38 (Thr180/Tyr182) (#9216), anti-SAPK/JNK (#9258) and anti-phospho-SAPK/JNK (Thr183/Tyr185) (#4668), and anti-phospho-Akt (Ser473) (#4060) pAbs were from Cell Signaling Technology (Beverly, MA, USA). LysoTracker Red, CellTracker Red, Mitotracker Red were from Invitrogen. Texas Red isothiocyanate (TRITC)- and Alexa-conjugated anti-rabbit, anti-goat and anti-mouse secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, USA) and Molecular Probes Inc. (Invitrogen). 3,4-diaminobenzidine (DAB) Peroxidase Substrate Kit was from Sigma-Aldrich (Saint Louis, MO, USA). Rabbit pAb anti-cathepsin D (7C10) was from J.S. Mort (McGill University, Montreal, Canada).

Production of cell-free culture supernatants
Cultures (1000 ml) of bacterial strains grown in LB (OD600 of 1) were used (Guignot et al., 2007). Bacteria were removed by centrifuging at 12 000 g for 10 min at 4°C. Supernatants were filtered through a 0.22 μm pore-sized filter. Samples were concentrated using cross-filtration (10 000 molecular weight cut-off) to a volume of approximately 1 ml.

Cell lines, and culture and transfection conditions
Cells were seeded and grown in culture plates (TPP, ATGC Biotechnologie, Noisy Le Grand, France). HeLa cells and HeLa cells stably transfected with rat GFP-LC3 (Kabeya et al., 2000) kindly provided by Aviva M. Toikovsky, Department of Biochemistry, University of Cambridge, UK) were cultured at sub-confluence in RPMI-1640 with L-glutamine (Life Technologies, Cergy, France) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Life Technologies) at 37°C in an atmosphere containing 5% CO2. For autophagy induction by amino acid deprivation, HeLa and GFP-LC3 HeLa cells were incubated for 4 h in the presence of Earle’s balanced salt solution (EBSS) (Sigma) at 37°C in an atmosphere containing 5% CO2.

The mouse embryonic fibroblast (MEF Atg5+/−) and Atg5−/− MEF (Kuma et al., 2004) kindly provided by Noboru Mizushima, Tokyo Medical and Dental University, Tokyo, Japan), and the transiently transfected GFP-LC3-MEF Atg5−/− and GFP-LC3-MEF Atg5−/− were cultured by using 50% DMEM and 50% F12 (Life Technologies) supplemented with 10% vol/vol heat-inactivated (30 min, 56°C) FCS (Life Technologies), 2 mM L-glutamine, 1 mM pyruvate and 10 mM HEPES. Cells were grown in a humidified 5% CO2 atmosphere at 37°C. Transient transfection for the expression of GFP-LC3 generously provided by Tamotsu Yoshimori (Osaka University, Japan), in MEF cell lines was performed using the Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer’s protocol.

For the experiments, the cells were deprived of FCS for 18 h before use. Cells were washed twice with PBS and exposed to toxin in the presence of the appropriate culture medium in the absence of FCS. The plates were incubated at 37°C in 5% CO2/90% air for the indicated times. Before being examined the cells were washed three times with sterile PBS.

Quantification of cell detachment
For the quantification of cell detachment, the cells were observed under an Aristoplan phase-contrast microscope (Plan Achromat 100X/1.32-0.6 oil objective) (Leitz, Wetzlar, Germany). For each sample, more than 10 random fields each consisting of at least 100 cells were examined by two investigators. Cell detachment was also quantified by counting the detached cells present in the cell culture medium using a haemocytometer under a microscope.

Cytotoxicity assays
The viabilities of attached CFCSsat-treated cells were determined using 3’-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays according to the manufacturer’s instruc-
Fluorescence microscopy

For direct or indirect immunofluorescence labelling, cultured cells were prepared on glass coverslips, which were then placed in 24-well TPP tissue culture plates (ATGC, Marne la Vallée, France). To label the lysosomes, LysoTracker Red (50 nM) was added to the cultures for 60 min. For determination of cytoplasmic vacuoles, CellTracker Red (500 µM) was added to the cultures for 30 min. The labelled cells were washed three times with sterile PBS, and then fixed with 3% paraformaldehyde in PBS for 5 min at room temperature. For indirect immunolabelling, specimens were fixed with 3% paraformaldehyde in PBS for 5 min at room temperature, washed three times with PBS, treated with PBS containing 50 mM NH₄Cl for 10 min for aldehyde function neutralization and blocked by adding PBS containing 0.2% gelatin. Cells were permeabilized by incubating with 0.2% Triton X-100 in PBS for 4 min at room temperature and then washed three times with PBS. To observe apoptosis, the cells were stained with Hoechst 33258 (0.5 µg ml⁻¹). Cytochrome c release was observed using the mAb directed against cytochrome C antibody (1/100). Immunolabelling of the cytoskeleton and FA proteins was done as follows: fodrin was labelled using the pAb directed against α II-spectrin (1/50); F-actin with rhodamine-phalloidin (1/200); microtubules with the mAb directed against α-tubulin (1/500); vinculin with the mAb directed against vinculin (1/100); and paxillin with the mAb directed against paxillin (1/200). Cathepsin D immunolabelling was done using the pAb anti-cathepsin D (1/50). Lamp proteins were immunolabelled with the mAbs directed against Lamp-1 or Lamp-2 (1/200). Sat was immunolocalized using the pAb anti-Pet toxin that cross-reacts with Sat (1/50). After labelling with primary antibody, the immunolabellings were developed using appropriate FITC- or TRITC-conjugated secondary antibodies (Jackson Immunoresearch). Coverslips were mounted using Dako fluorescent mounting medium (DAKO).

Imaging and quantification

For confocal laser scanning microscopy (CLSM) examination, samples were visualized using a model LSM 510 META (Zeiss, Germany) equipped with an air-cooled 488 nm argon ion laser and a 543 nm helium neon laser, and configured with an Axiovert 100 M microscope using a Plan Apochromat 40X/1.2 NA Corr. water-immersion objective lens or a Plan, Apochromat 63X,1.4 oil-immersion objective lens. The pinhole was set at 1.0 Airy unit. For each microscopy image of a random region, 10 Z-section images were collected and stacked to form one 2D image by sum projection using the accompanying Zeiss software LSM510 2.5 on Windows NT4. The interval between each Z-section was 0.45 µm. Duplicate samples were used for each determination.

To quantify the positive GFP-LC3 cells and cells showing a disorganized F-actin cytoskeleton, and vinculin- and paxillin-positive FAs, cells were examined using an Aristoplan microscope (Leitz, Germany) with epifluorescence (Plan Aprochromat 100X/1.32-0.6 oil-immersion objective). For each sample, two investigators examined more than 500 cells in five random fields, each consisting of at least 100 cells. Only cells with at least five dots were scored as GFP-LC3 positive. To quantify the GFP-LC3 dots in a single cell, projections obtained by confocal microscopy of more than 50 random cells were examined and analysed, and the GFP-LC3 dots per cell were counted using Image J (version 1.42) software (NIH, USA). For the determination of the size of GFP-LC3 dots and vinculin- and paxillin-positive FAs in a single cell, the projections obtained by confocal microscopy were analysed using Imaris software (version 6.21) (Bitplane, Zurich). For each sample exposed to control and CFCSsat-treated conditions, more than 50 random cells were examined. The source of the images was hidden from the people counting the number of cells positive for GFP-LC3 dots, the GFP-LC3 spots/cell or evaluating cell detachment in order to eliminate any possible bias. Photographic images were resized, organized and labelled using Adobe Photoshop software (San Jose, CA, USA).

Video microscopy

To visualize the formation of GFP-LC3 dots, coverslips with GFP-LC3 HeLa cells were placed on a temperature-controlled stage and analysed using time-lapse confocal microscopy. A total of 24 slides were acquired every 1 h using a green filter and an AxioObserver Z1-Colibri (Zeiss) with a 20 plan Apochromat objective. Images were processed using the operating software for the Z1 microscope.

Western blot analysis

The cells were washed once with cold PBS, and then treated for 15 min at 4°C with extraction buffer (25 mM Heps, 0.5% triton, 150 mM NaCl, 2 mM EDTA) containing proteases and phosphatase inhibitors. Protein fractions were dissolved in the appropriate volume of Laemml buffer and held at 100°C for 5 min. The proteins were immediately separated on 12% SDS-polyacrylamide gels. For Western blot analysis, gels were transferred to polyvinylidene difluoride membrane (Perkin Elmer, Les Ulis, France). Primary rabbit anti-LC3 antibody was revealed with an anti-rabbit horseradish peroxidase-conjugated secondary antibody and detected using the ECL detection system under + detected using time-lapse confocal microscopy. The proteins were immediately separated on 12% SDS-polyacrylamide gels. For Western blot analysis, gels were transferred to polyvinylidene difluoride membrane (Perkin Elmer, Les Ulis, France). Primary rabbit anti-LC3 antibody was revealed with an anti-rabbit horseradish peroxidase-conjugated secondary antibody and detected using the ECL detection system under conditions recommended by the manufacturer (Amersham). Western blot signals were quantified using Scion image software (Scion Corporation, Frederick, MD, USA), and densitometry data from at least three independent experiments were averaged for statistical analysis.

Statistical analysis

All experiments were repeated at least three times. The statistical significance was determined using Student’s t-test. Significance was established when P < 0.01.
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Author contributions

VL-LM, YC, RA, and VR performed the experiments. VN conducted image analysis. VL-LM and ALS analysed the data. ALS designed the overall research and wrote the manuscript.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Inhibitors of PI3K did not inhibit CFCS<sub>sat</sub>-induced autophagy in sub-confluent GFP-LC3 HeLa cells. In A, observation (left panel) and quantification (right upper panel) of persistent GFP-LC3 dots in cells exposed to CFCS<sub>sat</sub> (50 µl) with 3-MA (10 mM) or wortmannin (200 nM). In B, a control experiment shows the disappearance of the GFP-LC3 dots in starved GFP-LC3 HeLa cells exposed to 3-MA (10 mM) or wortmannin (200 nM) treatment. In C, cell detachment develops in HeLa cells exposed to CFCS<sub>sat</sub> in the presence of wortmannin (200 nM). Micrographs are representative of three separate experiments. The immunoblots are representative of two separate experiments. Each value shown is the mean ± SD of three separate experiments. In A and C, *P < 0.01* compared with CFCS<sub>AEC165</sub>-L. In B, *P < 0.01* compared with starved cells.

**Fig. S2.** Absence of vacuolation in CFCS<sub>sat</sub> (50 µl)-treated GFP-LC3 HeLa cells. Cells were stained with CellTracker Red (CTR). The micrographs are representative of three separate experiments.

**Fig. S3.** CFCS<sub>sat</sub>-treated sub-confluent GFP-LC3 HeLa cells displayed an unchanged microtubule network. In A, microtubule network in CFCS<sub>AEC165</sub> (50 µl)- and CFCS<sub>sat</sub> (50 µl)-treated GFP-LC3 HeLa cells. In B, colocalization between LysoTracker Red (LTR) and GFP-LC3 dots observed in untreated starved GFP-LC3 cells (upper panel) did not occur in nocodazole treated, starved (left lower panel) GFP-LC3 HeLa cells. The boxed area shows an LTR-positive vacuole and a GFP-LC3-positive vacuole. The graphs (Profile) represent the fluorescence distribution determined for the section of the cell, as indicated by the orientation of the bar. A micrograph shows the disorganization of microtubules revealed by immunolabelling with an anti-tubulin antibody and CLMS observation, in nocodazole-treated starved cells (right lower panel). The micrographs and each of the values shown are from three separate experiments.

**Fig. S4.** Absence of Type I cell death and necrosis in CFCS<sub>sat</sub>-treated GFP-LC3 HeLa cells. In A; labelling of cells with MitoTracker Red (MTR) shows no change in mitochondria distribution in CFCS<sub>sat</sub> (50 µl)-treated compared with CFCS<sub>AEC165</sub> (50 µl)-treated. No release of cytochrome C or AIF in CFCS<sub>sat</sub>-treated cells was observed by indirect immunolabelling with an anti-cytochrome C or an anti-AIF antibody. In B, representative images after Hoechst 33342 staining demonstrating the absence of nuclear fragmentation in CFCS<sub>sat</sub>-treated cells. In C, z-VAD-fmk treatment (25 µM) had no effect on the CFCS<sub>sat</sub>-induced cell detachment and increase in GFP-LC3 dots. In D, glycine (10 µM) does not modify the CFCS<sub>sat</sub>-induced cell detachment and increase of GFP-LC3 dots. The micrographs are representative of three separate experiments.

**Fig. S5.** Erk1/2 and p38, but not JNK MAPKs, are activated in HeLa cells exposed to CFCS<sub>sat</sub>- CFCS<sub>AEC165</sub> (50 µl)- and CFCS<sub>sat</sub> (50 µl). MAPK inhibitors UO126 (Erk1/2 blocker, 10 µM) and SB203580 (p38 blocker, 10 µM). Representative immunoblots of two independent experiments are shown. Blotting with anti-actin pAb demonstrates equal gel loading. Phospho-p44/42 forms of Erk1/2 (p-p44Erk1/42Erk2) and p44/42 forms of Erk1/2 (p44<sup>Erk1/42Erk2</sup>).

**Fig. S6.** MAPK inhibitors UO126 (Erk1/2 blocker, 10 µM) and SB203580 (p38 blocker, 10 µM) fail to block the CFCS<sub>sat</sub>-induced disassembly of the F-actin network in sub-confluent HeLa cells. CFCS<sub>sat</sub> treatment (8 h, 50 µl) and MAPKs inhibitors (10 µM) F-actin stress fibres stained using RITC-phalloidin and observation by CLMS. The micrographs and each of the values shown are from three separate experiments.

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