p27Kip1 in cell-cell adhesion and cell polarity
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Apical plasma membrane organization and late endosome/lysosome dynamics in hepatocytes requires balanced phosphorylation of p27Kip1 on Ser-10

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Abstract

We have previously demonstrated that a sufficient expression level of the cyclin-dependent kinase (CDK) inhibitor p27Kip1 (p27) is obligatory for the biogenesis of apical bile canalicular plasma membranes (BC) in hepatic HepG2 cells (van IJzendoorn et al., 2004, chapter 2). Here, we have examined the involvement of p27 and its Ser-10 phosphorylation site in BC organization at the ultrastructural level. Overexpression of wild-type p27 or p27S10A results in an increase in the frequency of apical microvilli and an approximately 2-fold increase in microvilli length. By contrast, overexpression of p27S10D results in the appearance of typically electron-lucent BC with shortened and highly disorganized microvilli. Moreover, in p27S10D-expressing cells, a striking subapical accumulation of large, electron-dense late endosomes/multivesicular bodies (MVB) and secondary lysosomes is observed. Whereas in parental cells typically few internal vesicles occupy MVB, in p27S10D-expressing cells MVB are fully packed with LAMP-1-containing internal vesicles. Inhibition of Rho signaling, a previously reported target of p27, causes the accumulation of electron-dense lysosomes but morphologically distinguishable from those seen in p27S10D-expressing cells, and does not affect the structural integrity of BC. Together, our data implicate p27 and its Ser-10 phosphorylation site as a novel determinant in apical plasma membrane organization and late endosome/lysosome dynamics.
**Introduction**

p27\(^{Kip1}\), hereafter called p27, is a well-known cyclin-dependent kinase (cdk) inhibitor, the expression and subcellular localization of which is closely correlated to cancer survival prognosis (Belletti et al., 2005). The regulation of p27 function occurs mostly at the post-transcriptional level and includes phosphorylation at multiple sites by multiple kinases. For instance, phosphorylation of p27 on Thr187 by cdk2 contributes to the degradation of p27. Further, phosphorylation of p27 on Thr157 by Protein kinase B (PKB)/Akt prevents nuclear import of p27 (Viglietto et al., 2002; Liang et al., 2002; Shin et al., 2002; Sekimoto et al., 2004), thereby preventing its inhibitory effects on cdk2 in the nucleus. PKB/Akt can also prevent nuclear import of p27 by phosphorylating Thr198 (Motti et al., 2004). Phosphorylation of p27 on Ser-10 by Mirk/dyrk1b has been implicated in p27 stability (Deng et al., 2004; Kotake et al., 2005; Ishida et al., 2000). In addition to Mirk/dyrk1b, human kinase interacting with stathmin (hKis) phosphorylates p27 on Ser-10 upon mitogenic stimulation, resulting in shuttling of p27 from the nucleus to the cytoplasm (Boehm et al., 2002; Rodier et al., 2001) in a CRM1-dependent manner (Ishida et al., 2002). The nuclear exclusion of p27 may serve to spatially restrict it from binding and inhibiting cdk2 activity, thereby promoting cell cycle progression. In addition, cytoplasmic p27 may serve additional functions that are not directly linked to regulation of the cell cycle. Indeed, despite its extensive characterization as a cyclin-dependent kinase inhibitor, disruption of this function is inadequate to explain its role in tumorigenesis (Nho and Sheaff 2003). Additional regulatory functions of p27 that have been reported include cell migration and motility (McAllister et al., 2003; Besson et al., 2004) and cell-cell adhesion (Théard et al., manuscript submitted, chapter 3), which may involve the interaction of p27 with the microtubule-depolymerizing protein stathmin (Baldassarre et al., 2005), the cytoskeleton-regulating
small GTPase RhoA (Besson et al., 2004) and/or β-catenin (Théard et al., manuscript submitted chapter 3).

Interestingly, the p27 homologue in *Drosophila melanogaster*, Dacapo, was shown to interact with the cytoplasmic small GTPase rap1 (de Nooij et al., 1996) which, through modulating cell-matrix and cell-cell adhesion, is thought to function in the spatial and temporal control of cell polarity (Bos 2005). A yeast homologue of p27, Far1p, interacts with and translocates the guanine nucleotide exchange factor for cdc42, cdc24, from the nucleus to the cytoplasm, and functions as an adaptor that recruits polarity proteins to the site of extracellular signaling marked by Gβγ to polarize assembly of the cytoskeleton (Butty et al., 1998). In addition to a role for p27 in regulating cell polarity in *Drosophila melanogaster* and yeast, recent evidence suggest that p27 is also involved in cell polarity development in mammalian cells. Exposure of hepatocytes to signaling molecules that stimulate hepatocyte polarity development, e.g. cAMP analogs and the interleukin 6-family cytokine Oncostatin M (Zegers and Hoekstra, 1998; van der Wouden et al., 2003), stimulates p27 expression (Klausen et al., 2000; van IJzendoorn et al., 2004, chapter 2), through inhibition of p27 degradation (Klausen et al., 2000). Moreover, it was demonstrated that sufficient p27 expression is obligatory for Oncostatin M and cAMP signaling to stimulate cell polarity development, i.e. the biogenesis of apical surface domains in hepatocytes (van IJzendoorn et al., 2004, chapter 2). Sufficient p27 expression appears to be necessary to limit cdk2 activity, as the activity of (presumably non-nuclear (Gaulin et al., 2000)) cdk2 negatively interferes with the regulated supply of membranes from the endosomal system to the developing apical surface domain (van IJzendoorn et al., 2004, chapter 2). Although the requirement of p27 expression for subapical endomembrane dynamics and the biogenesis of apical surface domains is evident, nothing is known about the consequences of p27 expression levels and phosphorylation of p27 on Ser-10 on the (ultra)structural
organization of the apical plasma membrane domain. In this study, we have addressed this issue by light and electron microscopy approaches and present evidence for a role for p27 and its Ser-10 phosphorylation status in apical cell surface organization and late endosome/lysosome dynamics at the apical domain.

**Figure 1** Overexpression of wild-type p27 increases the number and length of apical microvilli. Transmission electron microscopy pictures of the BC of parental HepG2 cells (A) and p27WT-overexpressing cells (B), in more details in (A’) and (B’). Enlargement of the villi of the BC of the parental HepG2 (A’’) or p27WT (B’’) cells. Bars are 500nm.
Results

Overexpression of wild-type p27 increases the number and length of apical microvilli

We have previously demonstrated that a sufficient expression level of p27 is required for the biogenesis of apical plasma membrane domains in HepG2 cells (van IJzendoorn et al., 2004, chapter 2). In order to investigate the potential involvement of p27 expression on the organization of the apical plasma membrane domain, HepG2 cells were stably transfected with wild-type human p27 as described in Materials and Methods. p27-expressing clones (~2-fold overexpression) were used for electron microscopy analyses. As shown in figure 1A (enlarged in A’), parental HepG2 cells display typical slender microvilli at the apical surface which are on average 1 µm in length and ~80 nm in diameter (figure 1A’’). In contrast to parental HepG2 cells, HepG2 cells expressing wild-type p27 display an increase in microvilli number, resulting in a packed appearance of microvilli at the apical domain (figure 1B, enlarged in B’). In addition, microvilli appear more rigid and display a ~2-fold increase in length, while the average diameter is unchanged (figure 1B’’). These data show that an elevated level of p27 promotes microvilli formation and stimulates microvilli length, in this way increasing apical cell surface area with at least a factor 2, which is consistent with the previously reported positive correlation between p27 expression levels and cell differentiation (Quaroni et al., 2000; Deschenes et al., 2001).

The Ser-10 phosphorylation site of p27 is involved in apical microvilli organization

The functioning of p27 is controlled by several phosphorylation sites, among which Ser-10. Phosphorylation of Ser-10 has been implicated in p27 stability and the shuttling of p27 from the nucleus to the cytoplasm, where p27 may perform non-cell cycle-
related functions. In order to investigate the role of Ser-10 phosphorylation on apical plasma membrane organization, we stably transfected HepG2 cells with a construct encoding human p27S10A, in which Ser-10 is mutated to Ala-10 thereby preventing phosphorylation, or p27S10D, in which Ser-10 is mutated to Asp-10 thereby mimicking phosphorylation. Expression of either mutant p27 resulted in a ~2-fold increase in total cellular p27 levels (not shown; c.f. Théard et al., manuscript submitted, chapter 3). Overexpression of p27S10A results in an increased frequency of microvilli similar as observed in cells overexpressing wild-type p27, thereby enhancing the apical
p27kip1, Apical Organization and Lysosome Dynamics

A  ph c  A'  F-actin
B  ph c  B'  radixin
C  ph c  C'  DPP IV
D  ph c  D'  5'NT
E  ph c  E'  ZO-1
Apical-basolateral cell polarity is maintained in cells expressing p27S10D. A’, Phalloidin localization using immunofluorescence in cells overexpressing p27S10D around the BC visualized in phase contrast (ph c, A). In the same manner, radixin (Ph c B, IF B’), dipeptidyl peptidase IV DPPIV (Ph c C, IF C’), 5’-nucleotidase 5’NT (Ph c D, IF D’) and the tight junction protein ZO-1 (Ph c E, IF E’) present a proper localization in or around the BC.

surface area (figure 2A). In HepG2 cells expressing p27S10A, microvilli occupy the entire lumenal space. In addition, the apical lumen displays a marked increase in electron-dense amorphous deposits, which likely reflects an increase in apical secretion (figure 2A and A’). In striking contrast to cells expressing p27S10A, cells expressing p27S10D display a dramatic reduction and disordered appearance of apical microvilli (figure 2B and B’). In addition, the apical lumens of p27S10D expressing cells are typically electron-lucent (figure 2B’), which may reflect a decrease in apical secretion. These data show that the expression of p27S10A and p27S10D exerts opposite effects with regard to the structural organization of the apical surface and, consequently, the surface area of the apical domain, and suggest that the phosphorylation status of p27 on Ser-10 is an important determinant of apical surface organization and, possibly related, the secretion of electron-dense amorphous BC deposits.

**Apical-basolateral cell polarity is maintained in cells expressing p27S10D**

Because of the inhibitory effect of p27S10D expression on the organization and surface area of the apical domain, we next examined the subcellular distribution of apical resident proteins. As shown in figure 3A and A’, the BC lumens in p27S10D-expressing cells were surrounded by a cortical F-actin network, similar as observed in parental HepG2 cells (c.f. Zegers et al., 1998). In addition, the ERM protein radixin, which links F-actin to the plasma membrane and the loss of which has been reported
to result in a loss of apical microvilli (Kikuchi et al., 2002; Kitajiri et al., 2004), was localized correctly at the BC surface (figure 3B, B’). Similarly, the ecto-enzymes dipeptidyl peptidase IV (figure 3C and C’) and 5’ nucleotidase (figure 3D and D’), and the canalicular ABC transporters MRP2 and MDR1/3 (not shown) were properly localized at the apical surface domain. Finally, the tight junction marker ZO-1 displayed a proper localization, bordering the apical domain (figure 3E and E’). Together, these data suggest that in spite of the disorganized apical surface at the ultrastructural level, apical-basolateral cell polarity remains intact.

Expression of \( p27^{kip1} \) results in the appearance of large electron-dense multivesicular bodies

In addition to the appearance of electron-lucent apical lumens in cells expressing \( p27^{S10D} \), large and highly electron-dense structures were observed in close proximity of the disordered apical surface (figure 2B). Electron-dense structures in electron microscopy often represent late-endosomes/lysosomes. Indeed, incubation of \( p27^{S10D} \)-expressing cells with lysotracker DND-99, a red-fluorescent dye that stains acidic compartments in live cells, shows a significant increase in the number and size of acidic organelles, when compared to parental HepG2 cells (figure 4A and B). Lysotracker DND-99-positive organelles in \( p27^{S10D} \)-expressing cells were often found concentrated around the BC (figure 4E, F, compare to 4C, D). Further electron microscopy examination revealed that the electron-dense structures represent secondary lysosomes (figure 5A) but mostly multivesicular bodies (MVB; figure 5B, C). MVB are of variable sizes but typically 1-2 \( \mu \text{m} \) in diameter (MVB diameter in parental HepG2 cells is \( \sim 500 \) nm). Most MVB lumens display high electron-density whereas the lumen of the internal vesicles is electron-lucent (figure 5C, enlarged in 5C’). Also MVB in which the internal vesicles appeared more electron-dense than the MVB lumen could be detected (figure 5B). In addition to their large size, MVB are typically fully packed with internal vesicles (figure 5C’), which is
Figure 4 Expression of p27S10D results in the appearance of large and abundant vesicles positives for the late endosomal/lysosomal marker Lysotracker Red DND-99. Compared to the parental HepG2 cells (A), the p27S10D overexpressing cell line display a large amount of lysotracker positive vesicles (B) under 40X magnification. In more detail (60X), while in HepG2 cells the BC distinguishable in phase contrast (ph c) (C) is surrounded by only a few number of these structures (D), in the case of p27S10D cells, the BC observed in phase contrast (E) is encircled by a large number of these endosomal/lysosomal vesicles (F).
Figure 5 Expression of p27S10D results in the appearance of large electron-dense multivesicular bodies. In transmission electron microscopy, p27S10D cells present a minority of secondary lysosomes (A) or multivesicular bodies displaying highly electron-dense vesicles and electron-lucent lumens (B). In these cells, MVB exhibit mainly electron-dense lumen while the lumen of the internal vesicles is electron-lucent (C, enlarged in C'). In HepG2 cells, MVB only present few internal vesicles and display irregular shapes (D1, D2).
Figure 6 MVB are positive for the endosomal/lysosomal marker LAMP-1 staining but do not accumulate the cholesterol marker filipin. While in HepG2, only small vesicles are labeled by the endosomal/lysosomal protein LAMP-1 (A, phase contrast; B, IF), the p27S10D cells, LAMP-1 is also found in MVB (C, phase contrast; D, IF, enlarged in D'). Filipin is predominantly found in the basolateral and apical plasma membrane but little cholesterol is localized in MVB (F, arrows) distinguished with LAMP-1 (E, arrows). Around the BC, enlarged from F and E, the LAMP-1-positive structures (E', arrows) are not completely co-localized (G, arrows) with the filipin (F', arrows).
in contrast to parental HepG2 cells, in which only few internal vesicles are typically observed in MVB (figure 5D1 and D2). Indentation of the limiting MVB membrane is occasionally observed and may reflect the inward budding of vesicles (figure 5C’, arrows). The late endosomal/lysosomal nature of the MVB was verified by the presence of LAMP-1, a marker of late endosomes and lysosomes (Chen et al., 1985). Indeed, a clear accumulation of LAMP-1-containing bodies is readily observed in cells expressing p27S10D (figure 6D, phase contrast in 6C), in contrast to parental cells (figure 6A, B) or cells expressing p27S10A (not shown; c.f. figure 6B). Interestingly, LAMP-1 is predominantly associated with the internal vesicles of the large MVB whereas little staining is observed at the limiting MVB membrane (figure 6D, and enlargement of boxed area in 6D’), in contrast to previous reports (reviewed in Raiborg et al., 2003). The large MVB also contain little cholesterol, evidenced by filipin staining. Filipin-stained cholesterol, however, is predominantly found in the basolateral and apical plasma membrane (figure 6F) and does not appear to accumulate in MVB. Filipin-stained cholesterol in MVB is predominantly observed at the limiting MVB membrane and not in the internal vesicles (figure 6F), once again in contrast to previous reports (reviewed in Raiborg et al., 2003). Furthermore, the smaller LAMP-1-positive structures surrounding the apical lumen in p27S10D expressing cells did not completely overlap with other structures containing filipin-stained cholesterol (figure 6E’, F’ and G). Taken together, the expression of p27S10D results in the appearance of large, electron-dense multivesicular bodies that may display atypical distribution patterns (i.e. limiting vs. internal membranes) of proteins and lipids.
Figure 7 Inhibition of Rho kinase activity does not affect apical surface organization and causes the accumulation of lysosomal structures that are distinct from those seen in p27S10D expressing cells. 3 days-old p27WT cells grown in the presence of the ROCK inhibitor Y-27632 present electron-dense structures around the bile canaliculus (BC, A) but these are not MVB which are also observed (B, enlarged in B’). These electron-dense vesicles appear very irregular in shape (B) and sometimes multi-lamellar (C). Surprisingly, MVB are occasionally found within the bile canaliculus (D).
Inhibition of Rho kinase activity does not affect apical surface organization and causes the accumulation of lysosomal structures that are distinct from those seen in p27S10D expressing cells

Cytoplasmic p27 has recently been reported to interact with and inhibit RhoA (Besson et al., 2004). To investigate whether the affected apical surface organization and late endosomal body accumulation in p27S10D expressing cells may have occurred via inhibition of Rho signaling, cells expressing wild-type p27 were treated with Y-27632, a specific inhibitor of the Rho effector protein Rho kinase (ROCK). Cells were then fixed and processed for electron microscopical analysis.

The organization of the apical surface and microvilli appearance is not visibly affected in Y-27632-treated cells (figure 7A), in contrast to p27S10D expressing cells (c.f. figure 2). Y-27632 treatment resulted in the appearance of a large number of large pleiomorphic electron-dense lysosomes in the apical cytoplasm (figure 7A). Detailed examination of these electron-dense structures, however, revealed that these were different from those observed in p27S10D expressing cells. Indeed, whereas in p27S10D expressing cells the electron-dense structures were mostly MVB (c.f. figure 2), in Y-27632-treated cells MVB appeared normal with regard to size and internal vesicle content (figure 7B’), whereas most electron-dense structures represented secondary lysosomes and multi-lamellar bodies (figure 7C). The latter were never observed in p27S10D-expressing cells. Intriguingly, MVB were occasionally observed in the BC lumen (figure 7D). Because inhibition of Rho signaling does affect apical surface organization and causes the accumulation of late endosomal/lysosomal structures that are clearly distinct from those observed in p27S10D expressing cells, it is not likely that the phenotype presented by p27S10D expressing cells results from impaired Rho signaling.
Discussion

The data in this study implicate p27 as a novel determinant of structural apical plasma membrane organization. It is demonstrated that the overexpression of wild-type p27 promotes the frequency and length of microvilli at the apical surface of HepG2 cells and, in this way, enhances the apical cell surface area, which is consistent with a reported role for p27 in epithelial differentiation (Quaroni et al., 2000; Deschenes et al., 2001). Interestingly, the N-terminal Ser-10 phosphorylation site of p27 appears to play a crucial role in this. Thus, whereas the expression of p27S10A, a mutant that can not be phosphorylated, mimics and may even surpass the stimulatory effect of wild-type p27 overexpression on microvilli appearance and morphology, the expression of p27S10D, a phospho-mimetic mutant, causes a loss and disorganization of apical microvilli and, consequently, a reduction in apical cell surface area. In addition, the apical lumens of cells expressing p27S10D were electron-lucent, which is in striking contrast to the apical lumens formed between parental cells or cells expressing wild-type p27 or p27S10A, which typically contain a core of electron-dense amorphous deposits (figure 2).

In parallel to the absence of electron-dense deposits in their apical lumens, p27S10D-expressing cells show the presence of many large and highly electron-dense structures subjacent to the apical surface (figure 2) which, taken together, may suggest a defect in apical secretion. Electron and (immuno)fluorescent microscopy experiments show that these structures represent mostly acidic multivesicular bodies (MVB), fully packed with internal vesicles that contain LAMP-1. Some filipin-stained cholesterol can be observed at the limiting membrane of MVB but accumulation of cholesterol in these structures is not apparent. Indeed, most filipin-stained cholesterol is observed at the basolateral and apical cell surfaces. The distribution of LAMP-1 at the internal membranes and cholesterol at the limiting
membrane of MVB is not in agreement with earlier reports (Mobius et al., 2002; 2003; Raiborg et al., 2003), raising the possibility of altered protein and lipid sorting at MVB in p27S10D expressing cells. The >2-fold increase in MVB diameter, in parallel with the increase in internal vesicle content suggest an extended residence time of MVB in the cytoplasm (in agreement with van Deurs et al., 1993). In theory, this may be the result of impaired MVB fusion with primary lysosomes or, alternatively, due to an inhibition of MVB fusion with the cell surface. However, given the lack of primary lysosome accumulation, and given the electron density of the MVB lumen (excluding the lumens of internal vesicles) which may suggest prior fusion of the MVB with lysosomes, impaired MVB fusion with the cell surface is a more likely explanation. This would also be consistent with the pronounced sub-apical accumulation of the electron-dense MVB and the absence of electron-dense deposits in the apical lumens of p27S10D-expressing cells. However, we can not exclude the (not necessarily mutually exclusive) possibility that, in p27S10D expressing cells, endocytic membrane trafficking is altered in a way that promotes MVB formation. The parallel reduction of electron-dense deposits in BC lumens, accumulation of subapical electron-dense MVB, and shortening and disordered organization of apical microvilli in p27S10D expressing cells may suggest that apical secretion and microvilli organization are connected processes. This is supported by the reported requirement of external ligands for apical microvilli organization and length in Drosophila follicle cells (D’Alterio et al., 2005).

Phosphorylation of p27 on Ser-10 has been implicated in the shuttling of p27 from the nucleus to the cytoplasm (Ishida et al., 2002; Boehm et al., 2002; McAllister et al., 2003). A cytoplasmic localization of p27 may favor cell cycle progression or, alternatively, allow other non-cell cycle-related functions of p27 (Besson et al., 2004; Théard et al., manuscript submitted, chapter 3). The molecular mechanism by which p27-Ser10
regulates apical surface organization and MVB dynamics (this study) does not appear to be linked to cell cycle dynamics, as cells expressing the p27S10D mutant do not significantly differ from parental cells with respect to cell cycle distribution, cdk2 binding efficiency, and proliferation rate (Théard et al., manuscript submitted, chapter 3). Also p27-mediated inhibition of RhoA (Besson et al., 2004), the latter of which stimulates apical microvilli formation by way of ROCK-mediated phosphorylation of moesin (Oshiro et al., 1998), could be excluded as molecular mechanism by which p27-Ser10 regulates apical surface organization and MVB dynamics. Indeed, inhibition of ROCK with Y-27632 failed to mimic the effect of p27S10D expression on apical surface organization (figure 7). In addition, although accumulation of electron-dense structures was easily observed in Y-27632-treated cells, consistent with previous reports (Nishimura et al., 2002), these were distinct from those seen in p27S10D-expressing cells and did not include MVB.

Cytoplasmic p27 has recently been shown to interact with and inhibit the microtubule-depolymerizing protein stathmin, resulting in the accumulation of stable microtubule bundles (Baldassarre et al., 2005). Microtubules have been shown to be involved in MVB dynamics (Saito et al., 1997), as well as in secretion of lysosomal enzymes into the bile (LeSage et al., 1993). Furthermore, stathmin has a region of sequence similar to Lyst (Barbosa et al., 1997), mutations in which cause Chediak-Higashi syndrome, an autosomal recessive disease affecting the secretion of granules and lysosomes-like organelles (Burkhardt et al., 1993). Very interestingly, stathmin was reported to interact with tumor susceptibility gene (tsg) 101 (CC2; Maucuer et al., 1995; Li and Cohen, 1996). Tsg101 is a member of the ESCORT-I complex that sorts ubiquitinated substrates into MVB and, in this way, promotes MVB formation (Babst et al., 2000). Future studies are needed to investigate the interplay between p27 and its phosphorylation on Ser-10, stathmin, and the tsg101/ESCORT-I
complex in the regulation of endomembrane dynamics and apical cell surface organization.

While the responsible mechanism awaits further studies, the data in this study strongly suggest that a limited phosphorylation of p27 on Ser-10 is essential for subapical MVB dynamics on the one hand, and organizing and generating apical surface area through formation and elongation of microvilli on the other, which may be closely related events.

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Experimental procedures

Cells culture
HepG2 cells were grown in DMEM medium with 4500 mg/L glucose and supplemented with 10% FCS and antibiotics. Culture media were changed every other day. The p27WT, p27S10A and p27S10D cell lines were created as described in chapter 3 and maintained in selection medium containing G418 antibiotic (800µg/ml).

Electron Microscopy
3 days-old HepG2, p27WT (treated or not with Y-27632 (Sigma)), p27S10A and p27S10A were fixed and processed as described in chapter 3.

Fluorescence microscopy
Immunofluorescence was performed as described in the chapter 3 using the following antibodies: polyclonal anti-radixin (Sigma), monoclonal anti-DPPIV (gift from HP Hauri), monoclonal anti-5’-nucleotidase, monoclonal anti-ZO-1 (Zymed), monoclonal anti-LAMP-1 (H4A3, Developmental Studies Hybridoma Bank), anti-Alexa Fluor 488 or 596 goat anti-rabbit or -mouse (Molecular Probes) as secondary antibodies, together with Hoechst 33528 (5ng/ml) to label the nuclei. The staining procedure used to label the cells with TRITC-labeled phalloidin (100ng/ml, Sigma) was described in chapter 3. The Filipin staining procedure was the same than the one used for the previous antibodies at the exception of the permeabilisation step, carried out with saponin 0.1% in HBSS. The Filipin (50µg/ml, kind gift from Dr. Kobayashi) was added after 30min of incubation with the first and the secondary antibodies in the case of the LAMP-1 colabelling.

Lysotracker
3-days old cells were washed in HBSS and incubated in HBSS containing 1µM Lysotracker Red DND-99 (Molecular Probes), at 37°C during 30min. After extensive washes, living cells were observed with an Olympus Provis AX70 fluorescence microscope.