Pierre J. COURTOY, Member
Christophe PIERREUX, Associate Member

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Sarah CARPENTIER, Graduate Student
Ludovic D'AURIA, Graduate Student
Anne-Christine HICK, Graduate Student
Thierry MEDTS, Graduate Student (till June 2010)
Thanh LAC, Technician (half-time)
Benoît MARIEN, Technician
Francisca N'KULI, Technician
Yves MARCHAND, Secretary

ENDOCYTOSIS AND EPITHELIAL DIFFERENTIATION

Endocytosis, a central activity of all eukaryotic cells, allows for cell nutrition, regulates the composition of the cell surface and controls transfer of macromolecules across epithelial barriers. This research group has made significant contributions in the dissection of endocytic pathways and in unravelling their contribution to numerous physiopathological processes. Our current investigations focus on endocytosis at the apical membrane of polarized kidney proximal tubular cells (PTC). We surmise that this most active endocytic surface will allow defining rate-limiting components of the different subcellular steps. These should also emerge from the dissection of various genetic defects causing low-molecular weight proteinuria in appropriate mouse models. Recent achievements include: (i), the elucidation of the signalling cascade whereby the paradigmatic oncogene, v-Src, and the master catalyst, Rab5a, impact on the apical actin cytocortex, so as to induce macropinocytosis, like enteropathogens (2); (ii) the elucidation of a deficit of apical endocytosis in ClCn5 KO mice, which mimick Dent's disease, a genetic predisposition to kidney stones (1); (iii) the serendipitous discovery that apical endocytosis of ultrafiltrated lysosomal proteases is a central mechanism for PTC lysosomes biogenesis (3); and (iv) the demonstration that the transcription factor, ZONAB, is a key component for the switch between proliferation and apical differentiation in various epithelia (7). While looking at fluorescent lipid markers to follow "bulk" trafficking and fate of the plasma membrane lipids by live cell imaging, we noticed their propensity to form micrometric domains, distinct from lipid rafts. Multiple approaches indicated a relation with native, endogenous lipid organization. We further provided the first evidence that sphingomyelin forms cytoskeleton-independent but temperature-dependent micrometric domains, which are regulated by cholesterol and membrane tension (8, 10). The group of C. Pierreux studies epithelial tubulogenesis and differentiation, using developing pancreas, salivary and thyroid glands, as complementary models of controlled interconversion between multilayered cell masses and polarized monolayers, with emphasis on paracrine and transcriptional control (6, 9). Two strong assets of our group are a several-decade expertise in structural biology and a versatile cellular and tissular imaging platform that offers conventional and multiphoton microscopy for vital imaging (5), as well as transmission and scanning electron microscopy.

PLASMA MEMBRANE LIPIDS FORM STRUCTURALLY AND KINETICALLY DISTINCT MICROMETRIC DOMAINS, REGULATED BY MEMBRANE TENSION AND AFFECTED IN HAEMATOLOGICAL DISEASES

L. D'Auria, P. Van Der Smissen, P.J. Courtoy and D. Tyteca

Lipids are the most abundant constituents of biological membranes. For several decades, plasma membrane lipids were considered to form a homogenous two-dimensional barrier, acting as solvent for membrane proteins. However, it is now well-accepted that some membrane lipids spontaneously cluster into discrete nanometric "lipid rafts" (liquid-ordered domains), thereby creating small-scale lateral asymmetry. In addition, micrometric domains have been reported using fluorescent lipids incorporated into artificial liposomes, erythrocyte ghosts, and in living cells for non-natural lipids, but the occurrence of micrometric endogenous lipid domains in the plasma membrane of living cells is still questioned.

Our group is addressing whether micrometric lipid domains naturally occur at the plasma membrane, using fluorescent analogs (BODIPY) of sphingomyelin (SM), an abundant outer plasma membrane sphingolipid. Both in erythrocytes and CHO cells, BODIPY-SM insertion labelled (sub)micrometric fluorescent plasma membrane domains. BODIPY-SM domains were randomly distributed in control erythrocytes at 37°C (Fig 1a) and coalesced upon hypotonic shock, excluding structural surface features and endocytosis, and indicating control by surface tension. In CHO cells, direct plasma membrane insertion of BODIPY-SM and intracellular enzymatic conversion of BODIPY-ceramide into BODIPY-SM produced similar surface BODIPY-SM domains. Depletion of endogenous SM by sphingomyelinase cleavage or biosynthetic inhibition impaired (sub)micrometric domains, strongly suggesting that fluorescent domains of exogenous BODIPY-SM reflected endogenous SM compartmentation. Thus, BODIPY-SM spontaneously clusters at the outer leaflet of the plasmalemma of living cells into ordered micrometric domains, defined in size by membrane tension and cholesterol (ref. 8).

Using fluorescent analogs of other outer leaflet plasma membrane lipids, we next show the co-existence of three segregated micrometric phases. Indeed, BODIPY-SM differed from BODIPY-glycosphingolipids and -phosphatidylcholine (PC) domains in temperature dependence, propensity to excimer formation, association with a glycosylphosphatidylinositol (GPI)-anchored fluorescent protein reporter, and lateral diffusion by FRAP, thus demonstrating different lipid phases and boundaries. This was further supported by double labelling experiments and was confirmed by additive occupancy, up to 70% cell surface coverage. Specific alterations of BODIPY-analogs domains by manipulation of corresponding endogenous sphingolipids suggested that distinct fluorescent lipid partition might reflect differential intrinsic propensity of endogenous membrane lipids to form large assemblies (ref. 10).

We have further dissected the mechanism accounting for the formation and maintenance of micrometric lipid plasma membrane domains in living erythrocytes as the most simple model. Experimental or genetic modulation of erythrocyte stretching, cholesterol content (by methyl-beta-cyclodextrin; Fig. 1b) and suppression of membrane-cytoskeleton anchorage via 4.1R complexes (hyperphosphorylation by PKC; Fig. 1c) and band3 complexes (splenectomyzed patient with spherocytosis, a genetic disease of red blood cells characterized by anemia due to erythrocyte fragility; Fig. 1d) differentially affected BODIPY-SM, -glycosphingolipids and -PC micrometric domains. Altogether, these results confirm that different phases coexist at the plasma membrane and point to a key role of membrane tension in micrometric lipid domain maintenance and formation.

Whether the lipid domains play a role in cell physiology and whether endogenous membrane lipids can also form large assemblies are some of our other challenges.

In collaboration with the Cliniques universitaires Saint-Luc, we are now addressing whether and how the organization of fluorescent lipids in micrometric domains is affected in patients with spherocytosis. To find a way to re-establish the erythrocyte membrane of the patients is one of our future goals.

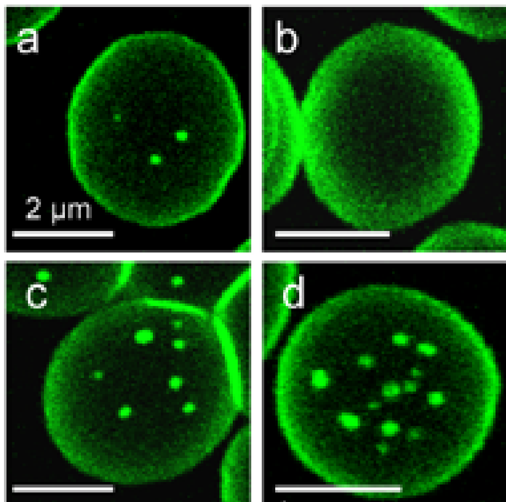

Fig. 1. Modulation of fluorescent sphingomyelin micrometric domains on erythrocytes by pharmacological (b,c) or genetic (d) perturbation of membrane architecture. (a) control erythrocyte at 37°C; (b) + methyl-beta-cyclodextrin (-25% cholesterol); (c) + calyculin A + PMA (hyperphosphorylation of 4.1R complex); (d) untreated erythrocyte from a spherocytotic patient.

DIFFERENTIAL MEMBRANE RECRUITMENT OF SRC MAY SPECIFY ITS DOWNSTREAM SIGNALLING TO MAPK AND PI3K PATHWAYS

Ph. de Diesbach, T. Medts, S. Carpentier, L. D'Auria, P. Van Der Smissen, A. Platek, M. Mettlen, D. Tyteca and P.J. Courtoy

Most Src family members are diacylated and constitutively associate with membrane "lipid rafts" that coordinate signalling. Whether the monoacylated Src also localizes at "rafts" remains controversial. Using polarized MDCK cells expressing the thermosensitive v-Src/tsLA31 variant, we here addressed how Src tyrosine-kinase activation may impact on its (i) membrane recruitment, in particular to "lipid rafts"; (ii) subcellular localization; and (iii) signalling. The kinetics of Src-kinase thermoactivation correlated with its recruitment from the cytosol to sedimentable membranes where Src largely resisted solubilisation by non-ionic detergents at 4 °C, and floated into sucrose density gradients like "lipid rafts". By immunofluorescence, activated Src showed a dual localization, at the apical plasma membrane and at adjacent endocytic vesicles. The plasma membrane Src pool did not colocalize with caveolin-1 and flotillin-2, but extensively overlapped with GM1 labelling by cholera toxin. Severe (~70%) cholesterol extraction with methyl-β-cyclodextrin (MβCD) did not abolish "rafts" floatation, but strongly decreased Src association with floating "rafts" and abolished its localization at the apical plasma membrane.

Src activation independently activated first the MAP-kinase-ERK1/2 pathway, then the PI3-kinase-Akt pathway. MAP-kinase-ERK1/2 activation was insensitive to Mβ CD, which suppressed Akt phosphorylation and apical endocytosis induced by Src, both depending on the PI3-kinase pathway. We therefore suggest that activated Src is recruited at two distinct membrane compartments allowing differential topological signalling, first via ERK1/2 at "non-raft" domains (possibly endosomes), then via PI3-kinase-Akt on a distinct set of "rafts" at the apical plasma membrane. Whether this model is applicable to c-Src remains to be examined (4). Current investigations are addressing which PI3K isoform regulates apical membrane recycling in differentiated PTC and how this works.

CONTRIBUTION OF APICAL ENDOCYTOSIS TO THE BIOGENESIS OF LYSOSOMES AND PATHOGENESIS OF CYSTINOSIS IN KIDNEY PROXIMAL TUBULAR CELLS

P.J. Courtoy, W.R. Lima and H.P. Gaide Chevronnay, in collaboration with C. Antignac (Paris, F), S. Cherqui (Scripps, CA, USA), E.I. Christensen (Aarhus, DK), O. Devuyst (NEFR, UCL)

Recruitment of acid hydrolases to lysosomes generally occurs by intracellular sorting based on recognition of a common mannose 6-phosphate signal in the trans-Golgi network and selective transport to late endosomes/lysosomes. We have obtained direct evidence for an alternative, efficient secretion-recapture pathway mediated by megalin, exemplified by cathepsin B in kidney proximal tubular cells (PTC). We found that in mouse kidneys with defective megalin expression [megalin knockout (KO)] or apical PTC trafficking (ClC-5 KO, see below), the (pro)cathepsin B mRNA level was essentially preserved, but the protein content was greatly decreased and the enzyme was excreted in the urine as mannose 6-phosphate-devoid species.

In polarized PTC monolayers, purified cathepsin B was avidly and selectively taken up at the apical membrane; uptake was abolished by the "receptor-associated protein", a megalin competitor. Direct interaction of cathepsin B with megalin was demonstrated by surface plasmon resonance. Circulating procathepsin B was detected in normal mouse serum. Purified cathepsin B injected into mice was preferentially taken up by kidneys and targeted to lysosomes where it remained active, as shown by autoradiography and subcellular fractionation. A single cathepsin B injection into cathepsin B KO mice could reconstitute full lysosomal enzyme activity in the kidneys. These findings demonstrate a pathway whereby circulating lysosomal enzymes are continuously filtered in glomeruli, reabsorbed by megalin-mediated endocytosis, and transferred into lysosomes to exert their function, providing a major source of enzymes to PTC. These results also extend the significance of megalin in PTC and have several physiopathological and clinical implications (3). Current investigations are addressing the pathophysiology of cystinosis, a multisytemic lysosomal disease due to a defective lysosomal membrane cystine/H+ antiporter. This disease first manifests itself in kidney as a generalized PTC dysfunction, referred to as kidney Fanconi syndrome. Endocytosis of ultrafiltrated plasma proteins rich in disulfide bridges rich must be the main source of lysosomal cystine in PTC. We expect that analysis of Ctns-/- mice (Nevo et al, 2009) will help understanding how cystine accumulation causes apical PTC atrophy and how cystinosis can be corrected by grafting of hematopoetic stem cells (Yeagy et al, 2011).

THE TRANSCRIPTION FACTOR, ZONAB, IS CONTROLLED DURING EPITHELIAL POLARIZATION AND IS A KEY REGULATOR IN THE PROLIFERATION/ DIFFERENTIATION SWITCH

W.R. Lima, S. Dupasquier, C.E. Pierreux and P.J. Courtoy, in collaboration with K. Parreira and O. Devuyst (NEFR)

Epithelial polarization depends, and impacts on, gene expression. The transcription factor, ZONAB, can shuttle between tight junctions and the nucleus to promote expression of cyclin D, and thus participate in the control of proliferation. We have examined whether ZONAB simultaneously represses differentiation, using renal proximal tubular cells (PTC) as a model. During mouse kidney ontogeny and polarization of PTC monolayers in vitro, decreasing ZONAB level inversely correlated with differentiation of the apical endocytic receptors, megalin/cubilin, brush border and primary cilium markers. Conversely, ZONAB was reexpressed in dedifferentiated renal carcinomas.

Sparsely plated PTC formed small islands: peripheral cells, necessarily lacking external tight junctions, strongly expressed nuclear ZONAB, proliferated and failed to differentiate; central cells, able to form continuous junctional belts, lost nuclear ZONAB, stopped proliferating and engaged in apical differentiation (Fig. 2). In confluent PTC monolayers, stable ZONAB transfection repressed expression and function of the endocytic receptors and impaired brush border and primary cilium maturation. Reporter and chromatin immunoprecipitation assays demonstrated that megalin and cubilin are ZONAB target genes. ZONAB expression was regulated by polarity at pre- and posttranscriptional levels (proteasomal degradation). In PTC islands, proteasome inhibition extended nuclear ZONAB to central cells, which reversed their choice from differentiation to proliferation.

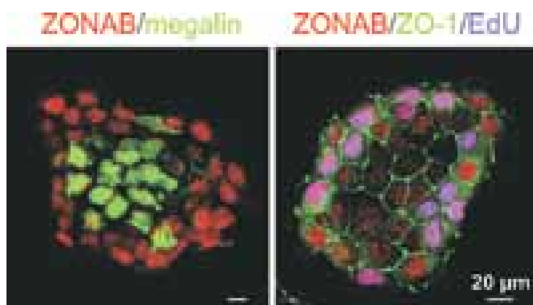

Figure 2. ZONAB is a key regulator in the switch between epithelial proliferation and differentiation. In colonies of kidney proximal tubular cells, ZONAB (immunolabelled in red) is absent in central differentiated cells (expressing megalin, in green, left) but is selectively detected in nuclei of peripheral proliferating cells (simultaneous DNA synthesis, labeled in blue, yields a pink color at right, from ref. 7).

Thus, ZONAB is down-regulated by epithelial polarity at both mRNA and protein levels and acts simultaneously to promote proliferation and repress differentiation. In vitro and in vivo data suggest that ZONAB is a sensor of epithelial density, involved in their switch from proliferation to differentiation (7).

EPITHELIAL DIFFERENTIATION

A.-C. Hick, S. Dupasquier, P.J. Courtoy and C.E. Pierreux, in collaboration with F.P. Lemaigre (LPAD)

Several endoderm-derived organs, such as the pancreas, salivary and thyroid glands, are composed of polarized epithelial monolayers. The pancreas and salivary glands are made of open branched tubules specialized in external secretion (exocrine), while the thyroid glands is organized in closed follicles for internal secretion (endocrine). During embryogenesis, the epithelial cells of these developing organs first form a proliferating mass before reorganizing in specialized monolayers (Fig. 3). Once fully differentiated, epithelial monolayers possess three distinct membrane domains, each able to transmit signals to the nucleus. First, the basal domain ensures adhesion to the extracellular matrix. Next, the lateral domain allows direct interactions with adjacent cells via junctional complexes. Finally, the free apical domain is now facing a lumen and bears a mecano-sensory cilium.

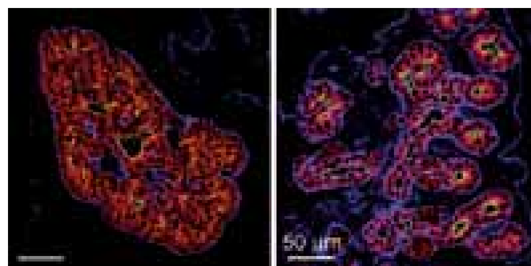

Fig. 3. Branching morphogenesis in exocrine glands. Reorganization in the early pancreatic bud of the multicellular mass of epithelial cells labelled for E-cadherin (left, red) into polarized monolayers with distinct apical domains (mucin, green) and their merging to create tubules (right). Laminin (blue) delineates basement membranes (From Ref 6).

Formation of epithelial monolayers requires a coordinate and dynamic interaction with their environment, composed of mesenchymal and endothelial cells. This process is gland-auto-

nomous, as it is faithfully reproduced in explant cultures. We have shown that embryonic salivary and pancreatic epithelial cells highly express receptors for SDF-1 (Stromal cell-Derived Factor-1), itself abundantly produced by immediately adjacent mesenchymal cells. Pharmacological inhibition of SDF-1:receptor interaction in explants of the pancreas or salivary glands fully abrogated the reorganization of the epithelial masses into monolayers (6). By three-dimensional analysis of the developing pancreas, we also uncovered a dense and close association of the epithelium with the endothelium (Fig. 4). Our in vivo and in vitro data show that endothelial cell recruitment is dependent on VEGF production by the pancreatic epithelium and that endothelial cells, in turn, limit acinar differentiation (9). These data demonstrate that paracrine epithelial:mesenchyme and epithelial:endothelial interactions are crucial for organ differentiation and bring a proof-of-principle that molecular components of these interactions can be readily dissected in explant cultures (6, 9).

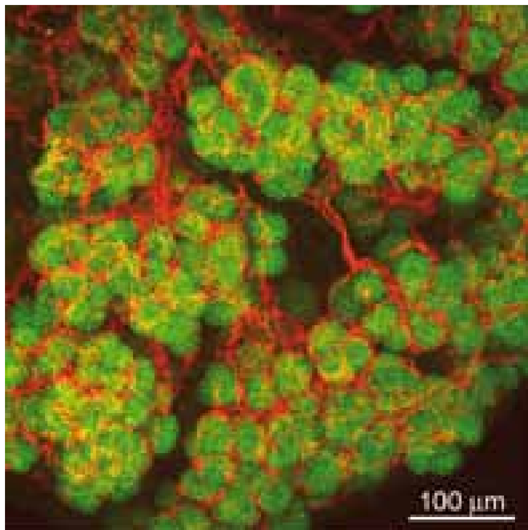

Fig. 4. Epithelial:endothelial interactions in the pancreas. Projections of 40 confocal images of an embryonic pancreas showing the dense and close association of pancreatic epithelial cells, labelled for E-cadherin (green), with endothelial cells, labelled for PECAM (red).

COLLABORATIONS ON MEMBRANE TRAFFICKING AND BRIEF REPORT ON THE CELL AND TISSUE IMAGING PLATFORM

P. Van Der Smissen, D. Tyteca, T. Lac, P.J. Courtoy

Besides sharing the same laboratory and continuing a two-decade fruitfull collaboration with the group of E. Marbaix and P. Henriet (see their report p 74), we have pursued our long-term commitment to promote collaborations by sharing expertise in endocytosis and cellular imaging. For the recent years, see our contribution to the study of the endocytic trafficking of syndecans and its role in signalling by its heparin-sulfate ligands (Zimmermann et al., Dev Cell 2005;9:377-88); of the subcellular trafficking of the thrombopoietin receptor (Royer et al., J Biol Chem. 2005;280:27251-61; see report by S. Constantinescu, p 127) and the amyloid precursor protein, APP (Feyt et al., J Biol Chem 2005;280:33220-7); elucidation of the disputed subcellular localization of aspartate N-acetyltransferase (NAT8L) and its congener, NAT8 (Wiame et al., Biochem J 2010;425:127-36; Veiga da Cunha et al., J Biol Chem J Biol Chem 2010;285:18888-98 see report by E. van Schaftingen, p 32); first evidence for dispersion of the actin cytoskeleton in epithelial cells by AMP-activated kinase (Miranda et al, Biochem Biophys Res Comm 2010;396:656-661; see report by M. Rider, p 51); ultrastructural analysis of differentiating hepatoblasts (Clotman et al., Genes Dev 2005;19:1849-54; see report by F. Lemaigre, p 21) and the biogenesis of glycosomes in Trypanosoma brucei (Galland et al., Biochim Biophys Acta Mol Cell Res 2007;1773:521-35; see report by P. Michels, p 57), or the morphological evidence by FRET of tight interaction between key players of CTL, that is interrupted during their anergy in cancer but can be reversed by galectins (Demotte et al., Immunity 2008;28:414-24; Cancer Res 2010;70:7476-88 see report by P. Van der Bruggen, p 107).

TEN SELECTED PUBLICATIONS OF THE PAST TEN YEARS

1. Christensen EI, Devuyst O, Dom G, Nielsen R, Van Der Smissen P, Verroust P, Leruth M, Guggino WB, Courtoy PJ. *Loss of chloride channel ClC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules.* **Proc Natl Acad Sci USA** 2003;100:8472-7.

2. Mettlen M, Platek A, Van Der Smissen P, Carpentier S, Amyere M, Lanzetti L, de Diesbach Ph, Tyteca D, Courtoy PJ. *Src triggers circular ruffling and macropinocytosis at the apical surface of polarized MDCK cells.* **Traffic** 2006;7:589-603.

3. Nielsen R*, Courtoy PJ*, Jacobsen C, Dom G, Rezende Lima W, Jadot M, Willnow TE, Devuyst O, Christensen EI. *Endocytosis provides a major alternative pathway for lysosomal biogenesis in kidney proximal tubular cells (*, equal first authors).* **Proc Natl Acad Sci USA** 2007;104:5407-12.

4. de Diesbach Ph, Medts Th, Carpentier S, D'Auria L, Van Der Smissen P, Platek A, Mettlen M, Caplanusi A, van den Hove MF, Tyteca D*, Courtoy PJ*. *Differential membrane domain recruitment of Src specifies its downstream signalling (*, equal senior authors).* **Exp Cell Res** 2008;314:1465-79.

5. Caplanusi A, Parreira KS, Lima WR, Marien B, Van Der Smissen P, de Diesbach Ph, Devuyst O, Courtoy PJ. *Intravital multiphoton microscopy reveals several levels of heterogeneity in endocytic uptake by mouse renal proximal tubules.* **J Cell Mol Med** 2008;12:351-4

6. Hick AC, van Eyll JM, Cordi S, Forez C, Passante L, Kohara H, Nagasawa T, Vanderhaeghen P, Courtoy PJ, Rousseau GG, Lemaigre FP, Pierreux CE. *Mechanism of primitive duct formation in the pancreas and submandibular glands : a role for SDF-1.* **BMC Dev Biol** 2009;9:66

7. Lima WR, Parreira KS, Devuyst O, Caplanusi A, N'Kuli F, Van Der Smissen P, Alves PM, Verroust P, Christensen EI, Terzi F, Matter K, Balda MS, Pierreux CE*, Courtoy PJ*. *ZONAB is controlled during epithelial cell polarization and is a key regulator in the switch between proliferation and differentiation (*, equal senior authors).* **J Am Soc Nephrol**, 2010;21:478-88.

8. Tyteca D, D'auria L, Van Der Smissen P, Medts T, Carpentier S, Monbaliu JC, de Diesbach P, Courtoy PJ. *Three unrelated sphingomyelin analogs spontaneously cluster into plasma membrane micrometric domains.* **Biochim Biophys Acta Biomembranes** 2010;1798:909-27.

9. Pierreux CE, Cordi S, Hick AC, Achouri Y, Ruiz de Almodovar C, Prévot PP, Courtoy PJ, Carmeliet P, Lemaigre FP. *Epithelial:endothelial cross-talk regulates exocrine differentiation in developing pancreas.* **Dev Biol** 2010; 347:216-27.

10. D'Auria L, Van Der Smissen P, Bruyneel F, Courtoy PJ*, Tyteca D*. *Segregation of fluorescent membrane lipids into distinct submicrometric domains: evidence for large-scale phase compartmentation of natural lipids.* **PlosOne**, 6 e17021.

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