Supplemental Figure Legends

Supplemental Figure 1. Mature DGs, immature DGs, and MVBs observed by different electron microscopy techniques in MEG-01 cells. (A) A low magnification image of a HPF MEG 01 cell showing numerous immature and mature DGs as well as MVBs (bar 1 µm). (B-D) Higher magnification images of conventionally fixed MEG 01 cells in varying stages of DG formation. MVBs are also visible in both figures B and C. The cytoplasm is not as well preserved in these cells as in the HPF cells, but the DGs themselves appear structurally similar. Bar in B 200nm; Bar in C-D 500nm. (E) HPF MEG-01 cell. Compare DG structures to figures B and C (bar 500nm).

Supplemental Figure 2. Dense granules observed by immuno-fluorescence microscopy in MEG-01 fixed cells. (A-C) MEG-01 cells were fixed, permeabilized, and immunostained with LAMP2 and MRP4 antibodies as dense granules markers (bar 5 µm). (B-C) The close ups indicated in panel A allow the visualization of the dense granules markers as doughnut shape structures.

Supplemental Figure 3. Controls for colocalization experiments. (A-B) For the no-colocalization control, the Manders’ Overlap Coefficient (MOC) was determined for fluorescent peroxisomal markers and fluorescent LAMP2. (A) MEG-01 cells were co-transfected with LAMP2-Cherry and GFP-Peroxisomal Membrane Protein 34 (GFP-PMP34), MOC= 0.08 ± 0.01 (n = 22 cells). (B) MEG-01 cells were co-transfected with LAMP2-GFP and the peroxisomal marker protein RFP-SKL, MOC= 0.09 ± 0.01 (n = 25 cells). (C-D) The MOC between dense granule and alpha granule markers was investigated by immunostaining of MEG-01 cells followed by confocal fluorescence microscopy. (C) MEG-01 cells were immunostained with antibodies against the alpha granule marker von Willebrand factor (vWF) and the dense granule marker MRP4, MOC= 0.12 ± 0.01 (n = 15 cells). (D) MEG-01 cells were also immunostained with
antibodies against the alpha granule marker vWF and dense granule marker LAMP2, MOC = 0.18 ± 0.01 (n = 21 cells). Bars indicate 5 µm.

**Supplemental Figure 4. Dense granules derive from late endocytic structures.** (A) MEG-01 cells late endocytic structures were labeled with the fluid phase marker fluorescent dextran and the dense granules with mepacrine. Structures containing both markers are presented in the inset A’ (same as Figure 2B). (B) Dense granules were labeled with the green fluorescent dye mepacrine in MEG-01 cells transfected with LAMP2-Cherry DNA. The inset B’ shows colocalization of the two dense granule markers. (C) MEG-01 cells transfected with the dense granule marker LAMP2-GFP were labeled with fluorescent dextran. Inset, LAMP2-GFP is present in organelles containing fluorescent dextran. Bars indicate 5 µm.

**Supplemental Figure 5. Surface expression of LAMP2 upon stimulation of MEG-01 cells with Thrombin.** MEG-01 cells were transfected with LAMP2-GFP to label DGs and treated with buffer (A) or 1 U/mL of Thrombin (B-C). The plasma membrane accumulation of LAMP2-GFP in the Thrombin treated cells indicates Thrombin triggers dense granule release in these cells.

**Supplemental Figure 6. Fluorescent Dextran follows the endocytic pathway in MEG-01 cells.** Transferrin Alexa 647 was used as a marker of early/recycling endosomes and Cherry-Rab7a as a marker of MVBs/late endosomes. For the transferrin experiment, attached MEG-01 cells were cool down on ice and incubated with 0.16 mg/mL of Transferrin Alexa Fluor 647 for 30 min on ice. Dextran Alexa Fluor 488 was added to reach a concentration of 0.80 mg/mL and the cells were incubated 5 min at 37°C. The cells were washed 3 times with cold media and image immediately. For the Cherry-Rab7a experiment, MEG-01 cells transfected with Cherry-Rab7a DNA were treated with Dextran Alexa Fluor 488 as described above. The graph indicates the
percentage of structures that contain both Dextran Alexa Fluor 488 and either Transferrin Alexa Fluor 647 or Cherry-Rab7a at each time point (10 cells per treatment). Notice the rapid decrease in colocalization with transferrin and progressive increase in colocalization with Rab7.

**Supplemental Figure 7. AP-3 also colocalizes with Rab32 in MKs and MEG-01 cells.** (A) Primary MKs were immunostained with antibodies against AP-3, Rab32, and Clathrin. Close up view of individual structures allows to observe colocalization of AP-3 and Rab32 (Merge panel, MOC = 0.41 ± 0.01, n = 14 cells). Note Clathrin is also present in several of those structures (Rab32 and Clathrin MOC = 0.30 ± 0.01, n = 14 cells). (B) MEG-01 cells were immunostained with antibodies against AP-3, Rab32, and Clathrin. The inset shows that similar to the results obtained with MKs, AP-3 and Rab32 colocalize in structures that in some cases contain Clathrin (Rab32 and AP-3 MOC = 0.36 ± 0.01, n = 3 cells; Rab32 and Clathrin MOC = 0.32 ± 0.01, n = 3 cells). Bars indicate 5 µm.

**Supplemental Figure 8. Rab32 and Rab38 are primarily present in immature dense granules.** (A) Dense granules were labeled with mepacrine in MEG-01 cells previously transfected with Cherry-Rab32 DNA. 99 ± 1% of structures containing Cherry-Rab32 (37 cells) also contain mepacrine. A Structure containing high amount of mepacrine is indicated with a green arrowhead, high concentration of Cherry-Rab32 with a red arrowhead, and intermediate amount of both markers with a yellow arrowhead (bar 5 µm). (B-C) Quantification of inversely proportional fluorescence intensity. MEG-01 cells transfected with Cherry-Rab38 (B) or Cherry-Rab32 (C) and simultaneously either transfected with LAMP2-GFP or stained with mepacrine. The mean fluorescence intensity for the entire cell was measured on both channels for each cell together with the fluorescence intensity of the 3 brightest structures in each channel for each cell (at least 34 cells per treatment) using Slidebook 5.0. The selection of the brightest structures was carried out one channel at a time in order to avoid bias and the
fluorescence intensity of the structures was normalized by the one of the whole cell to compensate for differences in transfection levels. The charts were constructed using the ratio between the normalized intensities of the green and red channels for each single structure: Green Intensity>Red Intensity indicates a ratio of green vs. red >1.2, Green Intensity=Red Intensity indicates a ratio of green vs. red >0.8 and <1.2, and Green Intensity<Red Intensity indicates a ratio of green vs. red <0.8. An average of the frequencies obtained for these three categories for each cell are expressed as percentage of brightest structures in each category. * p<0.05; ** p<0.001

Supplemental Figure 9. LAMP2 is also present inside immature dense granules and MVBs. (A) Immunogold electron microscopy image of MEG-01 cells show LAMP2 (18 nm) could be present both on the limiting membrane or the inside of organelles (Bar 500 nm). (B) LAMP2 (18 nm) is present in an internal vesicle inside a MVB (Bar 200 nm).

Supplemental Figure 10. Immunoprecipitation controls. (A) The specificity of the immunoprecipitation procedure was tested by comparing the ability of the LAMP2 antibody vs. an irrelevant antibody to pull down structures containing MRP4 from sucrose gradient fractions (SGF) 8 and 9. (B) The absence of PF-4 in SGF 8 and 9 immunoprecipitated with an anti-LAMP2 antibody indicates AGs are not pulled down together with DGs in these fractions. PF-4 is detected in the extract submitted to sucrose gradient fractionation. IP, immunoprecipitation; IB, immunoblotting.

Supplemental Figure 11. Rab32 and Rab38 knock-down in MEG-01 cells. Rab32 and Rab38 knock-downs were confirmed by immunoblotting analysis of total extracts of the treated cells using the corresponding antibody. The levels of tubulin in the same blot were used to confirm equal loading. Arrows indicate specific bands as evidenced by their dramatic decrease upon knock down.
Supplemental Movie 1. Dense granules in MEG-01 can be studied by live cell microscopy. MEG-01 cells were transfected with VMAT2-Cherry and LAMP2-GFP as dense granule markers. This is a movie of the cell shown in Figure 1D that clearly demonstrates the colocalization of the two dense granule proteins (1 frame/sec).

Supplemental Movie 2. Dense granules contain fluid material from outside the cell. MEG-01 cells late endocytic structures were labeled with fluorescent dextran and dense granules with mepacrine. Both markers localize to the same structures indicating dense granule have a late endocytic origin. In this movie (and inset in Figure 2B) we see that mepacrine concentrates in structures inside the dense granule while dextran labels the lumen of the organelle. Panels are from left to right: unfiltered dextran; unfiltered mepacrine; Gaussian filtered dextran; Gaussian filtered mepacrine; Gaussian filtered merge.

Supplemental Movie 3. Cherry-Rab32 can also be visualized in vesicles. MEG-01 cells were transfected with Cherry-Rab32 DNA. The movie shows Cherry-Rab32 localizes to both organelles and vesicles. Note how some vesicles move a greater distance and much faster than the larger structures.

Supplemental Movie 4. LAMP2 localizes to dense granules in Control MEG-01 cells. The movie illustrates LAMP2-Cherry localization to organelle size structures that present a limited range of movement in Control siRNA (Figure 7A-B).

Supplemental Movie 5. LAMP2 localizes to smaller, more dynamic structures in Rab32 knocked-down MEG-01 cells. The movie of the cell presented in Figure 7C-D shows LAMP2-Cherry presence in structures more consistent in size with vesicles and
small organelles that on average move more and faster than the larger structures from control cells.

**Supplemental Movie 6. LAMP2 also localizes to smaller, more dynamic structures in Rab38 knocked-down MEG-01 cells.** The movie of the cell presented in Figure 7E-F shows LAMP2-Cherry mostly present in structures more consistent in size with vesicles and small organelles that on average move more and faster than the larger structures from control cells.
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Supplemental Figure 1
Supplemental Figure 3
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Supplemental Figure 4
Supplemental Figure 6

% of Dextran-Alexa488 structures containing either Transferrin-Alexa647 or Cherry-Rab7a

- Transferrin-Alexa647
- Cherry-Rab7a

Minutes

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Supplemental Figure 8
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A

\[\text{siRNA}\]

\[\text{Control} \quad \text{Rab32} \quad \text{Rab38}\]

IB:

Rab32

Tubulin

B

\[\text{siRNA}\]

\[\text{Control} \quad \text{Rab32} \quad \text{Rab38}\]

IB:

Rab38

Tubulin

Supplemental Figure 11