Developmental Cell, Volume *48*

Supplemental Information

Studying the Fate of Tumor Extracellular Vesicles at High Spatiotemporal Resolution Using the Zebrafish Embryo

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Supplementary figure legends

Supplementary Figure 1 (Related to Figure 2): Analysis of MemBright labeled EVs (A) Histograms showing a spectroscopy analysis of MemBright and PKH describing the absorbance (left, y axis) and the fluorescence intensity (right, y axis) versus the wavelength (nm, x axis) of the two probes in water or methanol. The presence of aggregates of PKH in water is visible. Arrows indicate the presence of PKH aggregates in labeled EVs (left) as well as in control PKH alone (right). **(B)** Histograms showing the absorbance (left, y axis) and the normalized absorbance (right, y axis) of Zmel1 or 4T1 EVs labeled with PKH or MemBright versus the wavelength (nm, x axis). PKH aggregates are denoted with an arrow. **(C)** Histograms showing the intensity of the emitted fluorescence (left, y axis) and the normalized fluorescence intensity (right, y axis) of Zmel1 or 4T1 EVs labeled with PKH or MemBright versus the versus the wavelength (nm, x axis). PKH fluorescent aggregates are denoted with an arrow. **(D)** Representative fluorescent images of Zmel1 EVs labeled with PKH (at 2µM) or MemBright (at 200nM) and histogram showing the relative fluorescent intensity of individual puncta (p=0,001; Mann-Whitney test). **(E)** Representative fluorescent images of 4T1 EVs labeled with PKH (at 200nM) or MemBright (at 200nM) and histogram showing a higher fluorescent intensity of Zmel1-MemBright individual puncta compared to Zmel1-PKH puncta (p<0,0001; Mann-Whitney test). **(F)** Western blot on EVs labeled with MembrightCy3, or MemBright alone, separated on a density gradient (Left). It shows the presence of Alix and TSG-101 in the fractions 5-10 exclusively. No signal is observed in the control MemBright alone. Representative fluorescent images at low (upper) and high (lower) magnifications of the same samples than the western blots (right). Fluorescent MemBrightCy3 puncta accumulate in fractions 5-10.

Supplementary Figure 2 (Related to Figure 3): Characterization of MemBright EVs *in vivo*. (A) Representative confocal images of Zmel1 EVs labeled with MemBright-Cy5 and incubated with 100nm red fluorescent polystyrene beads *in vitro*. **(B)** Representative confocal Z projections of *Tg(pu1:GFP)* (lymphoid, monocytes/macrophages) embryos co-injected with Zmel1 EVs labeled with MemBright-Cy5 and with 100nm red fluorescent polystyrene beads imaged 3 hours post-injection. **(C)** Single plane zoom on embryos co-injected with Zmel1 EVs labeled with MemBright-Cy5 and with 100nm red fluorescent polystyrene beads. **(D)** Histogram showing the apparent diameters (left, nm) of MemBright labeled Zmel1 EVs and 100nm beads measured in confocal images *in vitro* and *in vivo* in zebrafish embryos (*in vitro*: p<0,0001; *in vivo*: p=0,6; Mann-Whitney test). **(E)** Confocal images from three different Z planes of Zmel1 EVs labeled with MemBright-Cy5 and incubated with human red blood cells *in vitro* for 10 minutes. **(F)** Confocal images from rapid time-lapses of *Tg(Gata1:RFP; Fli1:GFP)* embryos injected with MemBright-Cy5 labeled Zmel1 EVs, showing examples of EVs far (upper panel) or close (lower panel) from RBCs in the circulation. **(G)** Representative confocal Z projections of *Tg(Fli1:GFP)* embryos co-injected with Zmel1 EVs labeled with MemBright-Cy3 and with 4T1 EVs labeled with MemBright-Cy5. **(H)** Representative confocal single planes from a time-lapse imaged right after injection of *Tg(Fli1:GFP)* embryos co-injected with Zmel1 EVs labeled with MemBright-Cy3 and with 4T1 EVs labeled with MemBright-Cy5. **(I)** Time projection over 10 seconds of a time-lapse

imaged right after injection of *Tg(Fli1:GFP)* embryos co-injected with Zmel1 EVs labeled with MemBright-Cy3 and with 4T1 EVs labeled with MemBright-Cy5.

Supplementary Figure 3 (Related to Figure 4): Control Zebrafish embryo injected with MemBright-labeled EVs or with control MemBright alone. Representative confocal Z-projections of *Tg(mpeg1:GFP)* (macrophages) embryos injected with either 4T1 EVs labeled with MemBright-Cy3 or with MemBright-Cy3 without EVs and imaged 3 hours post injection.

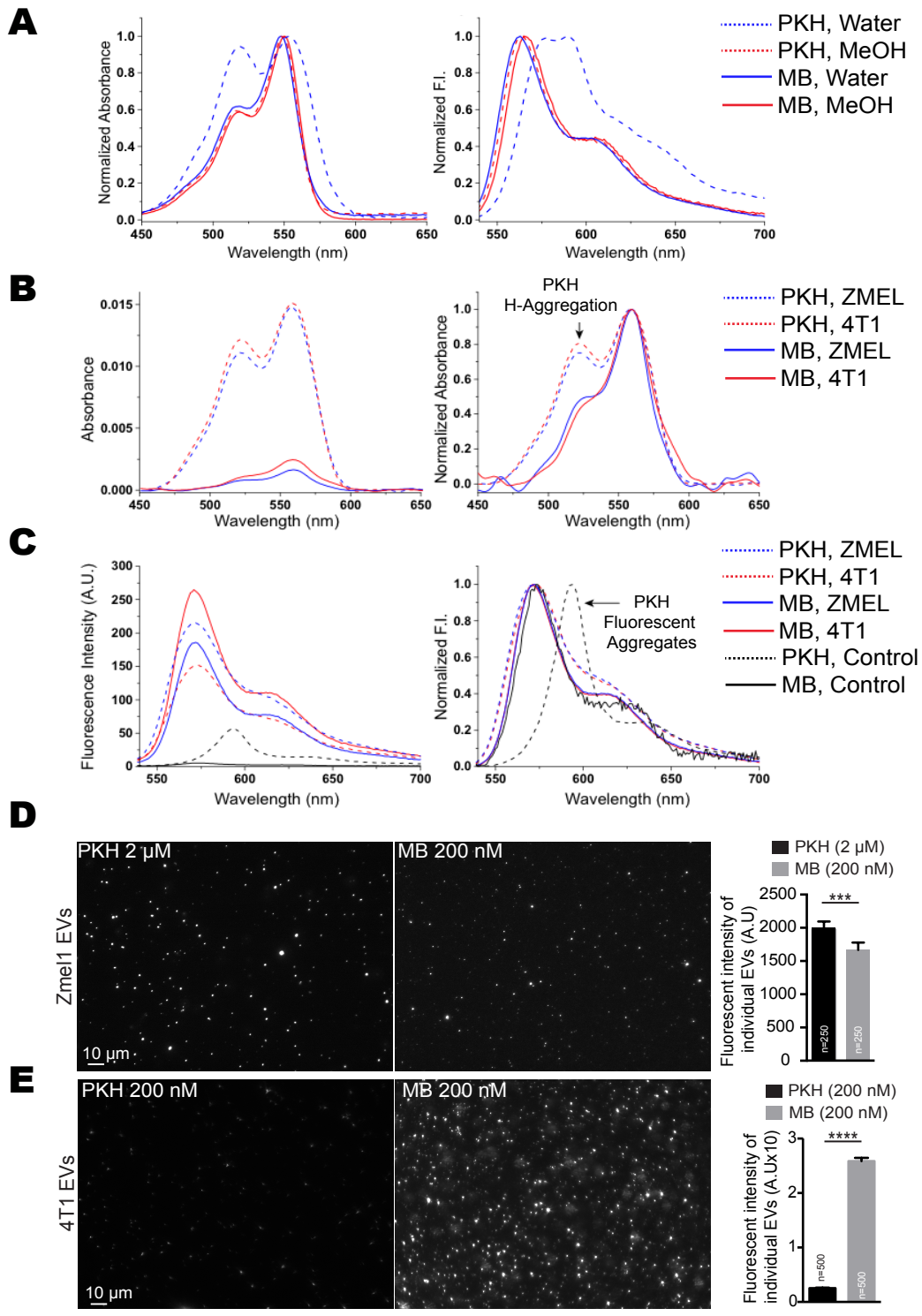
Supplementary Figure 4 (Related to Figure 5): Retrieval of the cells by CLEM and the putative journey of EVs in macrophages by electron microscopy (A) *Tg(mpeg1:GFP)* embryos were injected with 4T1 MemBright-Cy3 labeled EVs and imaged by confocal (upper panels). The upper right panel shows the position of the Region Of Interest (ROI) containing the two target cells, with respect to several embryonic landmarks imaged by confocal at low magnification. The lower left image shows the tail of the embryo after fixation and resin embedding imaged by microCT. The lower right image shows the position of the ROI in an electron microscopy section. **(B)** Higher magnification of the ROI imaged by confocal and electron microscopy. Common features between transmitted light in the living fish and electron microscopy on fixed fish are highlighted to allow a precise positioning of the ROI. The electron microscopy panel is stitched together from several individual images to allow a larger region to be visualized with better resolution. The asterisk points to a dirt speck on the EM section. **(C)** Electron microscopy images of EVs observed in the lumen of the vessel, in the close proximity of protrusions extending from the macrophage plasma membrane, which were identified by CLEM. **(D)** Electron microscopy images of putative EVs present in early endosomes close to the surface of macrophages. **(E)** Electron microscopy images of putative EVs present in MVBs.

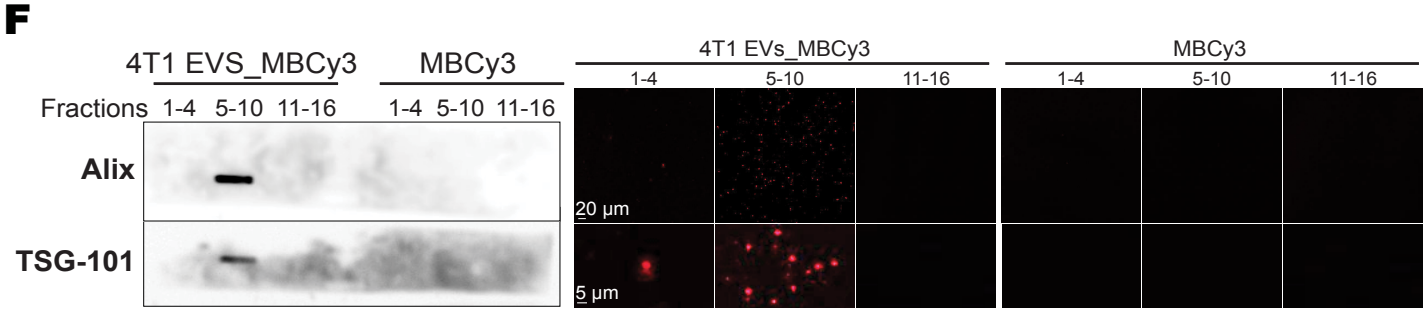
Supplementary Figure 5 (Related to Figure 7): 4T1 CD63-GFP cells pre-labeled with MemBright. (A) Representative confocal images of 4T1 CD63-GFP cells labeled with MemBright-Cy3 at different times before and after MemBright addition. **(B)** Zooms on confocal images of 4T1 CD63-GFP cells labeled with MemBright-Cy3 at 3h and 24h after MemBright addition. **(C)** Representative images of EVs isolated from the extracellular medium of 4T1 CD63-GFP cells pre-labeled with MemBright-Cy3.

Supplementary tables

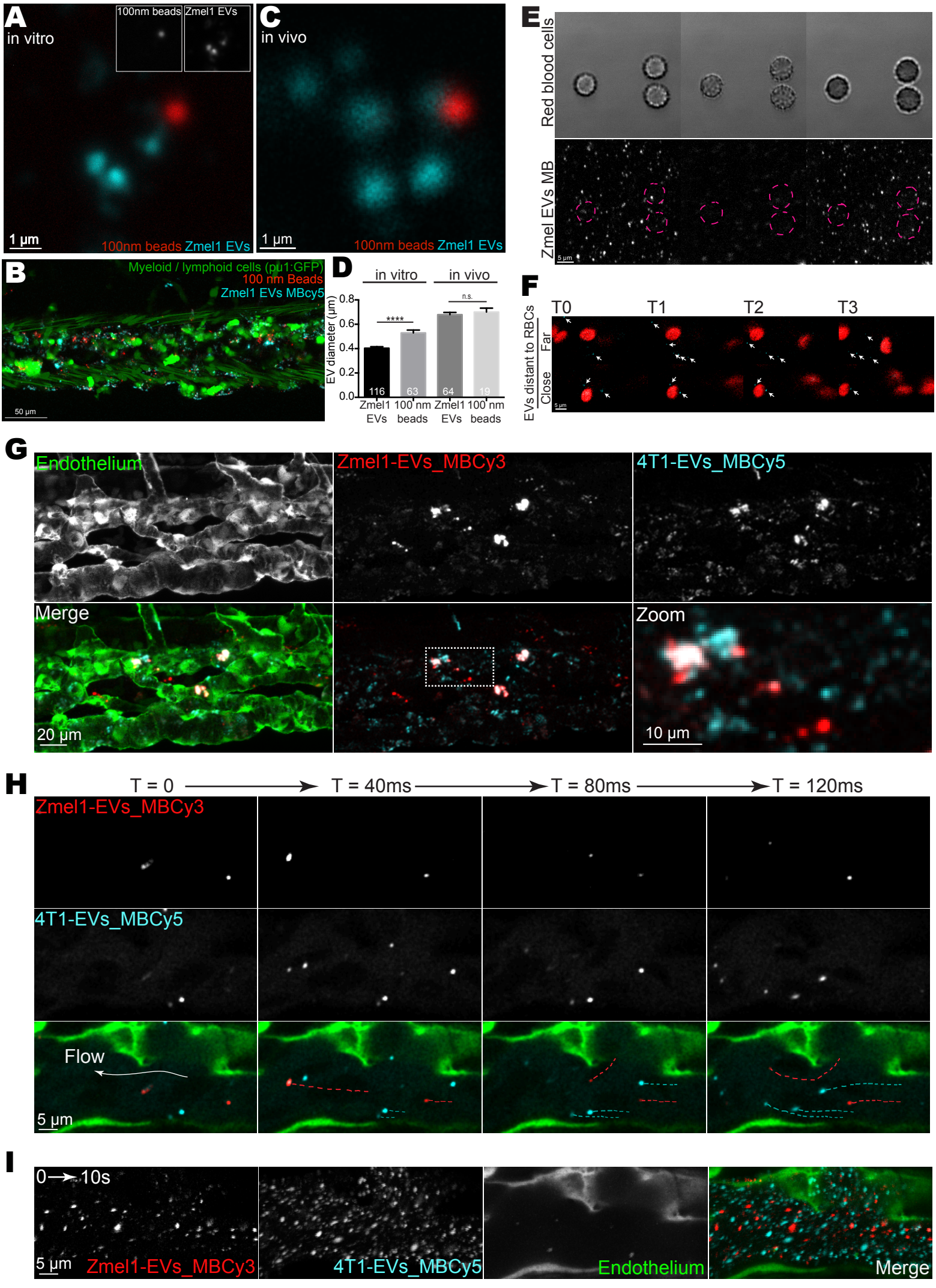
Table 1 (Related to Figure 1): proteins identified in EVs by mass spectrometry (A) proteins identified in EVs isolated from Zmel1 zebrafish melanoma cells (page 1-20); **(B-G)** proteins identified in EVS isolated from human melanoma 451-LU cells (page 21-68) **(B)**, SK-Mel28 cells (page 69-125) **(C)**, SK-Mel147 cells (page 126-167) **(D)**, SK-Mel103 cells (page 168-215) **(E)**, WM35 (page 216-258) **(F)** and WM164 cells (page 259-307) **(G)**; **(H-J)** proteins identified in EVs isolated from mouse melanoma B16-F0 cells (page 308-322) **(H)**, B16-F1 cells (page 323-349) **(I)** and B16-F10 cells (page 350-364) **(J)**; **(K)** proteins common to zebrafish, mouse and human melanoma EVs (page 365-367); **(L)** proteins common to Zmel1 EVs and AB9 EVs (page 368-371); **(M)** proteins common to Zmel1 EVs and YSL CD63-GFP positive EVs (page 372).

Table 2 (Related to Figure 2): Quantum yield of MemBright and PKH labeled EVs.

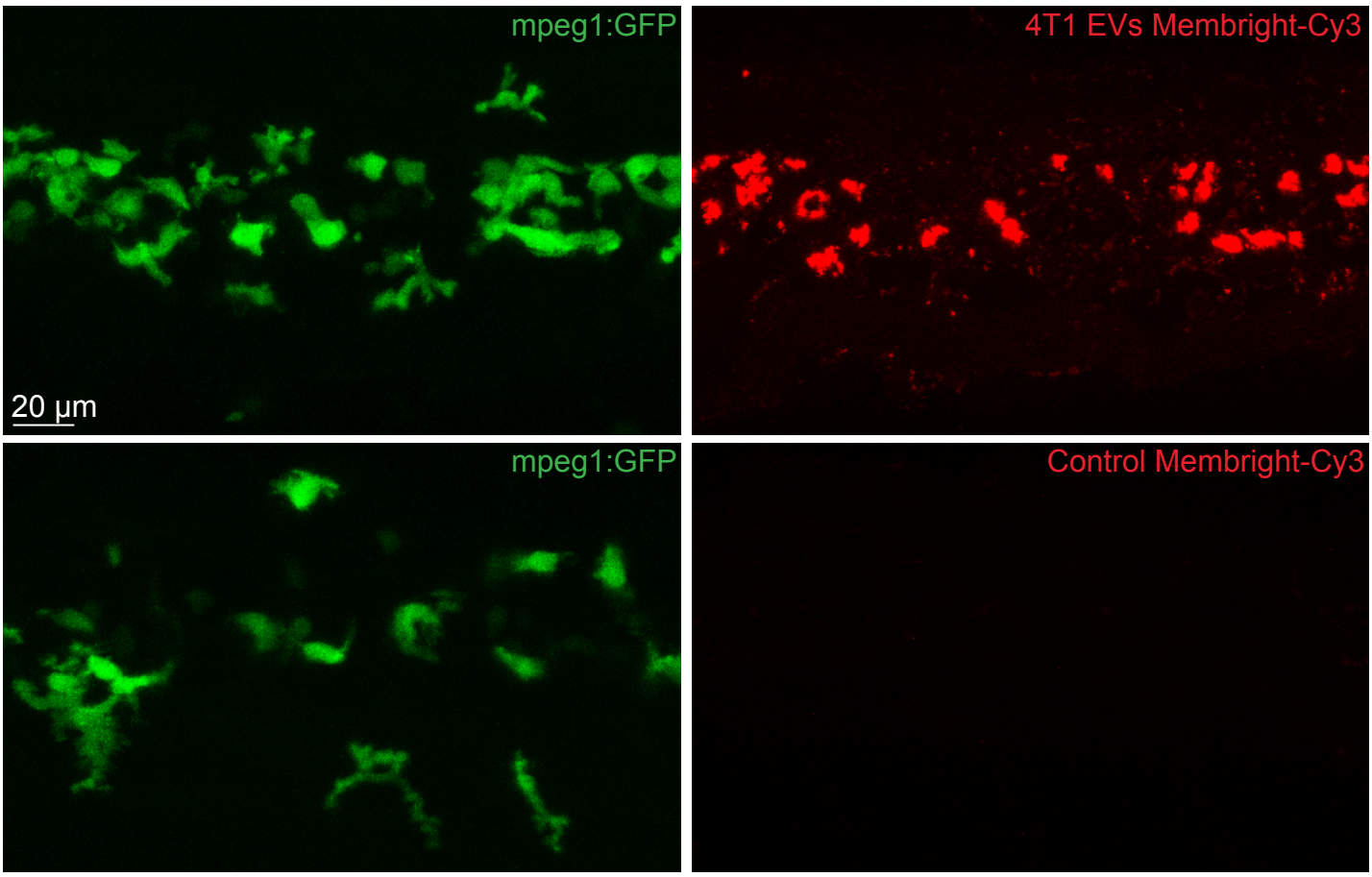




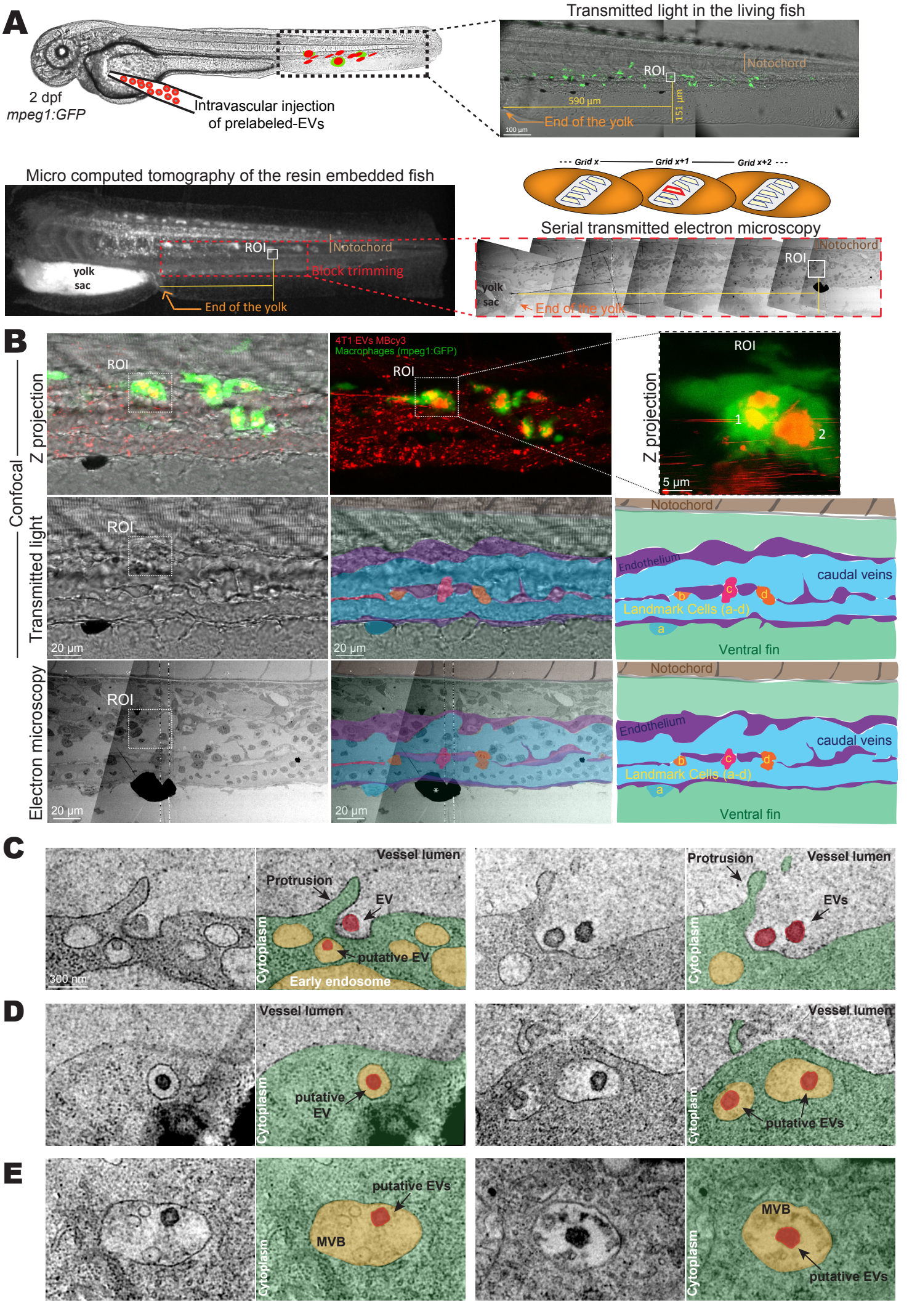
Supplementary Figure 1_Hyenne et al.



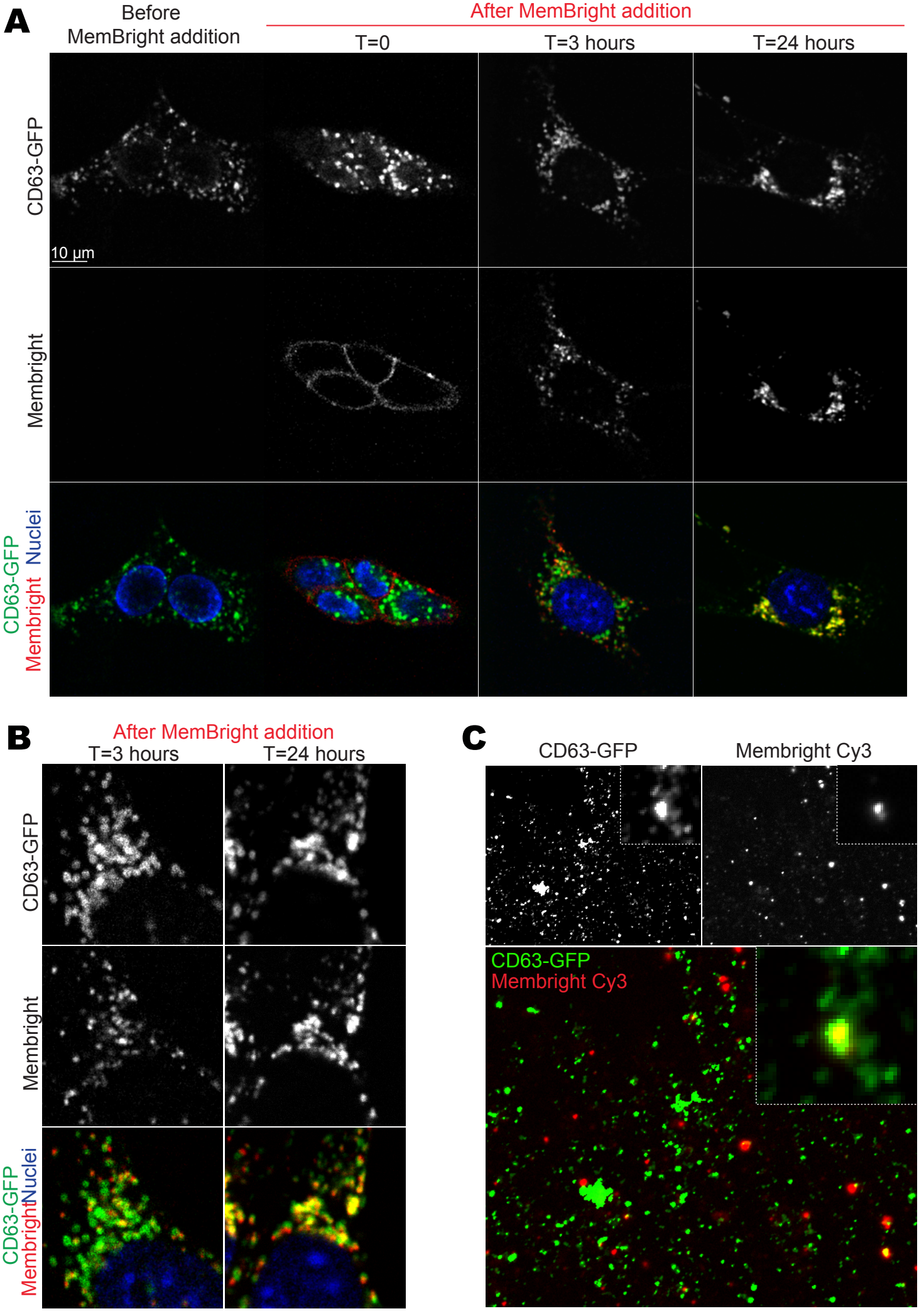
Supplementary Figure 2_Hyenne et al.



Supplementary Figure 3_Hyenne et al.



Supplementary Figure 4_Hyenne et al.



Supplementary Figure 5_Hyenne et al.

Table S2, related to Figure 2 : Photo-physical properties of labelled EVs.

	λ Abs (nm)	FWHM Abs (nm)	λ Em (nm)	FWHM Em (nm)	QY (ϕ)
PKH 4T1	559[a]	72	574	47	0.02
PKH Zmel1	558[a]	71	572	50	0.04
MB 4T1	559	42	572	33	0.42
MB Zmel1	560	42	571	34	0.41

[a] A second H-aggregation peak was observed at 522nm.