LPHN2 controls vascular morphogenesis by inhibiting endothelial cell adhesion and YAP/TAZ signaling

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Running title: *LPHN2 inhibits endothelial adhesion and YAP/TAZ*

SUMMARY

Modulation of endothelial cell (EC) adhesion to extracellular matrix (ECM) in response to mechanostimuli is essential for blood vessel patterning and functioning, yet the underpinning molecular mechanisms are deciphered only in part. We identify the adhesion G protein-coupled receptor Latrophilin 2 (LPHN2) as a novel determinant of vascular morphogenesis and endothelial barrier function. In cultured ECs, endogenous LPHN2 localizes at ECM adhesions, signals through cAMP/Rap1, and negatively regulates ECM-elicited YAP/TAZ mechanosignaling and haptotaxis. ECs also express endogenous fibronectin-leucine-rich transmembrane 2 (FLRT2) ligand that promotes cAMP/Rap1 signaling and hinders haptotaxis *via* LPHN2. To validate these findings *in vivo*, we generated *lphn2a* mutant zebrafish embryos in which vascular ECs are abnormally stretched, display YAP/TAZ hyperactivation, and lack proper intercellular junctions. Consistently, intravascularly injected cancer cells extravasate more easily in *lphn2a* null embryos compared to control animals. Overall, we unveil in vascular ECs a novel crosstalk between LPHN2 and the Hippo pathway, that may be therapeutically exploited to interfere with cancer metastatic dissemination.

KEYWORDS

Angiogenesis, Vascular permeability, Cancer, Guidance cues, Mechanosensing, YAP/TAZ, G protein coupled receptors

INTRODUCTION

The small GTPase Rap1 (Wang *et al*, 2012) is known for its ability to drive vascular morphogenesis (Chrzanowska-Wodnicka, 2013) and to exert opposite effects on cell adhesion and motility (Coló *et al*, 2012; Hong *et al*, 2007; Lagarrigue *et al*, 2016; Lagarrigue *et al*, 2015; Lyle *et al*, 2008), likely due to the involvement of different subcellular pools of Rap1 regulators and effectors (Bos & Pannekoek, 2012). For instance, the Rap1-GTP-interacting adapter molecule (RIAM) can either promote, through talin, the conformational activation of integrin adhesion receptors at the leading edge of migrating cells (Lagarrigue *et al.*, 2016) or support, via the mitogen-activated protein kinase kinase MEK, the disassembly of centrally located focal adhesions (FAs) (Coló *et al.*, 2012). Furthermore, major repulsive guidance cues such as secreted class 3 Semaphorins control blood vessel patterning (Valdembri *et al*, 2016; Wälchli *et al*, 2015) through the cytosolic GTPase activating protein domain of Plexin receptors (Worzfeld *et al*, 2014) that negatively regulates Rap1 signaling (Gioelli *et al*, 2018; Wang *et al.*, 2012). On the contrary, GPCRs promote Rap1 GTP loading *via* different pathways, the adenylyl cyclase (AC)/cyclic adenosine monophosphate (cAMP)/exchange protein directly activated by cAMP (EPAC) guanine nucleotide exchange factor cascade being a foremost one (Algire, 1943; Chang *et al*, 2007). However, cAMP/EPAC/Rap1 activation by different GPCRs also inhibits EC motility (Avanzato *et al*, 2016; Hong *et al.*, 2007).

The ability of ECs to adapt their shape and motile behavior to ECM patterning and rigidity is coupled to the Hippo pathway and its effectors Yes-associated protein (YAP) and WW domain-containing transcription regulator protein 1 (WWTR1) or transcriptional coactivator with PDZ- binding motif (TAZ), which translocate to the nucleus to control gene transcription (Dupont *et al*, 2011; Totaro *et al*, 2018), angiogenic blood vessel formation and function (Choi *et al*, 2015; Elaimy & Mercurio, 2018; Kim *et al*, 2017; Nakajima *et al*, 2017; Neto *et al*, 2018; Wang *et al*, 2016; Wang *et al*, 2017). As in most cell contexts, endothelial

FAs and F-actin cables allow mechanosensing by acting as physical and functional bridges between ECM and the activation of gene transcription programs (Elaimy & Mercurio, 2018; Kechagia *et al*, 2019). Indeed, actin stress fibers mechanically connect ECM-bound integrin-based adhesions to the nucleus *via* the linker of nucleoskeleton and cytoskeleton (LINC) complex (Kechagia *et al.*, 2019). These events change the conformation of nuclear pores, allowing YAP/TAZ nuclear translocation (Elosegui-Artola *et al*, 2017; Kechagia *et al.*, 2019; Totaro *et al.*, 2018). Indeed, YAP/TAZ activation correlates with the stability of actin cytoskeleton and cell tension, as controlled by myosin light chain II and Rho small GTPases (Kechagia *et al.*, 2019; Totaro *et al.*, 2018). Moreover, the Hippo pathway participates in a positive feed-back in which YAP and TAZ drive the transcription of genes involved in FA assembly (Nardone *et al*, 2017).In addition, the formation of cell mechanosensing complexes required to transmit guidance cue responses in ECs requires cAMP/EPAC/Rap1 activation (Chrzanowska-Wodnicka, 2017).

Here, we unveil in vascular ECs a novel crosstalk between ECM adhesions and the Hippo pathway that, being regulated by the inhibitory signaling of the adhesion G protein-coupled receptor (ADGR) Latrophilin 2 (LPHN2), controls Rap1 activation, FA disassembly, cell mechanics and *in vivo* blood vessel function.

RESULTS AND DISCUSSION

In order to identify novel guidance receptors that may play key roles in the regulation of vascular morphogenesis, we sought to focus on ADGRs that interact with several extracellular matrix (ECM) ligands and integrin receptors (Langenhan *et al*, 2013), whose adhesive functions are regulated by prototypic guidance cues such as Semaphorins that signal *via* Plexin receptors (Serini *et al*, 2003; Worzfeld & Offermanns, 2014). In this context, LPHN2 emerged as an ideal candidate because, similarly to Plexins, it was originally identified as a neuronal

receptor (Südhof, 2001) and its mRNA was reported to be highly expressed in *ex vivo* isolated blood vascular, but not lymphatic ECs (Valtcheva *et al*, 2013).

To characterize the mechanisms by which endogenous LPHN2 protein controls ECM-driven EC directional migration, we investigated its subcellular localization and function in cultured human EC. First, we confirmed that LPHN2 protein is expressed on the surface of human ECs (**Fig. S1A**) and fluorescence confocal microscopy experiments revealed a strong enrichment of endogenous LPHN2 in vinculin containing ECM adhesions of ECs (**Fig. 1A**). Due to autocatalytic processing, ADGRs exist as noncovalently associated heterodimers comprising an extracellular and a transmembrane subunit (Langenhan *et al.*, 2013). To verify the relationships of both LPHN2 subunits with ECM adhesions, we transfected ECs with an N-terminally HA-tagged and C-terminally EGFP-tagged mouse Lphn2 construct (HA-Lphn2-EGFP; **Fig. S1B**). We found that, similarly to endogenous LPHN2, both the extracellular and the intracellular moieties of HA-Lphn2-EGFP co-localized with vinculin at cell-to-ECM adhesion sites (**Fig. 1B**). Next, we evaluated the outcome of LPHN2 silencing (**Fig. S2A**) on the ECM-elicited migration of ECs. Impedance-based time-lapse haptotaxis assays revealed that LPHN2 silenced (siLPHN2) ECs display a much faster directional motility towards type I collagen (Coll I) (**Fig. 1C**) or fibronectin (FN) (**Fig. S2B**) than control silenced (siCTL) ECs. Thus, in cultured ECs LPHN2 mediates inhibitory signals that are likely initiated by autocrine loops of endogenous LPHN2 ligands.

Several GPCRs activate the cAMP/Rap1 pathway that regulates cell adhesion and migration (Gloerich & Bos, 2011). Thus, we assessed whether in ECs LPHN2 may impact on basal cAMP production and Rap1 GTP loading by pulling it down with a glutathione-S-transferase (GST) fusion protein carrying the Rap1-binding domain of human Ral guanine nucleotide dissociation stimulator (Franke *et al*, 1997). Compared to siCTL ECs, siLPHN2 ECs showed a decrease in the basal amount of both cAMP (**Fig. 1D**) and active Rap1 (**Fig. 1E**)

by 52.6 ± 8.7% (n= 9; p≤ 0.001) and 36.35 ± 4.8% (n= 5; p≤ 0.001), respectively. Moreover, the introduction of a silencing resistant mouse Lphn2 construct (**Fig. S2C**) effectively rescued active Rap1 levels in siLPHN2 ECs (**Fig. 1E**). These findings were consistent with the notion that cAMP/EPAC/Rap1 activation can inhibit migration by modulating cell adhesion dynamics (Lyle *et al.*, 2008). Therefore, we next evaluated the impact of LPHN2 silencing on FAs and the associated F-actin stress fibers. Confocal microscopy analysis revealed that in ECs the lack of LPHN2 results in a significant increase in the number of vinculin-containing FAs (by 125.0 ± 32.1% vs. siCTL; n= 40 cells per experimental group in 5 independent experiments; p≤ 0.01) and F-actin stress fiber corrected total cell fluorescence (CTCF; by 107.5 ± 21.9 % vs. siCTL; n= 40 cells per experimental group in 4 independent experiments; p≤ 0.01) (**Fig. 1F**). The small GTPase RhoA controls stress fiber formation and FA turnover (Lawson & Burridge, 2014), and ADGRs, such as GPR56/ADGRG1, were previously reported to activate Rho GTPase (Paavola & Hall, 2012). However, we did not detect any reduction in Rho activation after LPHN2 silencing in ECs (**Fig. S2D**). Hence, LPHN2 activates cAMP/Rap1 signaling and negatively regulates both FA number and haptotactic cell migration in a RhoA-independent manner in ECs.

In cultured ECs the silencing of LPHN2 results in a greatly increased number of FAs coupled to F-actin filaments (**Fig. 1**), which act as mechanical sensors promoting the translocation of the Hippo pathway effectors and transcriptional co-activators YAP and TAZ into the nucleus (Totaro *et al.*, 2018; Yu *et al*, 2015) by releasing them from their binding to the inhibitory SWI/SNF complex (Chang *et al*, 2018). Endothelial YAP/TAZ mechanosignaling controls angiogenesis, vascular barrier maturation, and blood vessel maintenance (Kim *et al.*, 2017; Nakajima *et al.*, 2017; Wang *et al.*, 2017). Therefore, we investigated whether LPHN2 may impact on YAP/TAZ signaling and found that, in ECs plated on hydrogels with a soft (5kPa) elastic module, LPHN2 silencing significantly increases YAP

and TAZ nuclear translocation along with the transcription of YAP/TAZ target genes *CTGF* and *CYR61* (**Fig. S3**). Hence, in ECs LPHN2 inhibits FA and stress fiber formation along with the ensuing nuclear translocation of YAP/TAZ that controls gene transcription in response to mechanosignaling.

Next, we reasoned that the higher FA number and ECM-driven migration rate caused by LPHN2 silencing in ECs (**Fig. 1C** and **Fig. S2B**) may be due to a loss of cell responsivity to LPHN2 ligands endogenously produced by ECs themselves, such as FLRT1-3 proteins (Seiradake *et al*, 2016). Indeed, the ectodomains of transmembrane FLRTs (Jackson *et al*, 2016) can be shed and act *in vivo* as repulsive factors capable of steering both axons (Yamagishi *et al*, 2011) and blood vessels (Seiradake *et al*, 2014). By means of real-time quantitative reverse transcription PCR (qRT-PCR) analysis, we observed that cultured human umbilical vein ECs actively transcribe *FLRT2*, but neither *FLRT1* nor *FLRT3* genes (**Fig. 2A**). In addition, we found that LPHN2 silencing results in a two-fold upregulation of *FLRT2* mRNA in ECs (**Fig. 2B**), hinting that *FLRT2* and *LPHN2* gene transcription are reciprocally regulated. To investigate the potential role of FLRT2 binding in the inhibitory activity of LPHN2 on EC migration, we generated a mouse Lphn2 construct devoid of the olfactomedin domain (ΔOLF Lphn2; **Fig. 2C**), which mediates the binding of FLRT ligands to LPHN receptors (Seiradake *et al.*, 2016). Accordingly, ligand-receptor *in situ* binding assay on COS-7 cells demonstrated that FLRT2 interacts with high affinity with wild type (WT), but not ΔOLF Lphn2 (**Fig. 2C**). Next, we compared the abilities of WT and ΔOLF Lphn2 constructs to rescue the increased haptotactic migration of siLPHN2 ECs (**Fig. 2D**). Since WT, but not ΔOLF Lphn2 rescued the abnormal haptotaxis of siLPHN2 ECs (**Fig. 2D**), we concluded that LPHN2 inhibition of ECM-elicited EC motility relies on the binding of its olfactomedin domain to endogenous ligands, such as FLRT2. Consistently, haptotaxis assays showed that ECM-elicited EC directional migration is respectively increased upon silencing of endogenous FLRT2 (siFLRT2; **Fig. S4A,**

B) (**Fig. 3A**) and decreased by stimulation with exogenous recombinant FLRT2 protein, which acts *via* LPHN2 (**Fig. 3B**). In agreement to what we observed upon LPHN2 silencing (**Fig. 1D-E**), siFLRT2 ECs displayed a decrease of the basal amount of both cAMP (**Fig. 3C**) and active Rap1 (**Fig. 3D**) by 12.9 ± 2.9 % (n= 6; $p \leq 0.05$) and 35.1 ± 6% (n= 4; $p \leq 0.01$) respectively, compared to siCTL ECs. In addition, stimulation with exogenous FLRT2 rescued active Rap1 GTP-loading in siFLRT2 ECs (**Fig. 3D** and **Fig.S4B**). Furthermore, confocal microscopy analyses revealed that, compared to siCTL ECs, siFLRT2 ECs display a substantially higher number of vinculin^{+} FAs (by 108.07 ± 28.8 % vs. siCTL; n= 60 cells per experimental group in 4 independent experiments; $p \leq 0.05$) and F-actin stress fiber corrected total cell fluorescence (CTCF; by 99.9 ± 32.3 % vs. siCTL; n= 69 cells per experimental group in 4 independent experiments; $p \leq 0.05$) (**Fig. 3E**). Therefore, FLRT2 is one of the endogenous ligands sustaining autocrine LPHN2-mediated chemorepulsive signals that negatively regulate the formation of FAs and associated and F-actin stress fibers in ECs.

In agreement with what we (**Fig. 1A** and **Fig.S1A**) and others (Valtcheva *et al.*, 2013) observed in cultured ECs, we found that Lphn2a protein is enriched in vascular ECs of dorsal aorta (DA) and posterior cardinal vein (PCV; **Fig. 4A**) of developing transgenic *Tg(kdrl:EGFP)^{s843}* zebrafish embryos. Hence, to directly investigate the functional role of LPHN2 in the *in vivo* vasculature, we generated a CRISPR/Cas9-mediated *lphn2a* zebrafish knock-out embryos with *Tg(kdrl:EGFP)^{s843}* genetic background (**Fig. S5**). The loss of Lphn2a did not grossly affect vascular patterning or blood circulation (**Fig. 4B**). However, we noticed that ECs forming the trunk vasculature were much thinner and stretched along the main vascular axis in *lphn2a$^{-/-}$* than in wild type embryos (**Fig. 4B,** *lower panels*). To better characterize this aberrant vascular phenotype, we examined EC morphology of *lphn2a* zebrafish mutants by means of transmission electron microscopy (TEM) on ultra-thin cross-sections (**Fig. 4C**). TEM analyses of DA of 48 hours post-fertilization (hpf) of *lphn2a$^{-/-}$*

zebrafish embryos unveiled how, despite a normal vascular lumen, the lining ECs are extremely thin and devoid of properly organized tight junctions (TJs), compared to wild type animals (**Fig. 4C**). Thus, LPHN2 signaling drives EC morphogenesis and adhesion in living blood vessels.

Next, we further characterized the molecular mechanisms involved in the control of EC shape and adhesion by LPHN2 in developing embryonic blood vessels. In agreement with what observed in cultured human ECs (**Fig. 2A-B**), real time qRT-PCR confirmed that, also *in vivo,* the *flrt2* gene is actively transcribed in ECs sorted from wild type zebrafish embryos and *flrt2* mRNA levels significantly increase upon *lphn2a* gene knock-down (**Fig. S6**). *In vitro*, siLPHN2 ECs display a greatly increased number of FAs coupled to F-actin filaments (**Fig. 1**), YAP/TAZ nuclear translocation and transcription of target genes, such as *CTGF* and *CYR61* (**Fig. S3**). Moreover, *in vivo* YAP/TAZ control EC shape and the formation of well-organized TJs (Kim *et al.*, 2017), both of which we found to be altered in *lphn2a* null zebrafish embryos (**Fig. 4C**). Therefore, we assessed the impact of *lphn2a* knockdown on the *in vivo* activation of the Hippo pathway in ECs by exploiting the *Tg(Hsa.CTGF:nlsmCherry)*^{ia49}/ *Tg(kdrl:EGFP)*^{s843} double transgenic zebrafish fluorescent reporter line of Yap1/Taz activity (Astone *et al*, 2018). Of note, *lphn2a* null embryos displayed a significantly increased Yap1/Taz activation in vascular ECs (**Fig. 4D**) and moreover real-time qRT-PCR analysis revealed a robust upregulation of *ctgfa* and *cyr61* target gene mRNAs in *lphn2a*$^{-/-}$ sorted ECs compared to controls (**Fig. 4E**). Altogether, our findings support a model in which FLRT2-activated LPHN2 GPCR controls physiological vascular morphogenesis and endothelial barrier maturation by inhibiting EC-to-ECM adhesion and YAP/TAZ-mediated mechanotransduction.

The entry and exit of solutes, leukocytes, and cancer cells from the bloodstream is regulated by the endothelial barrier that relies on the controlled remodeling of intercellular junctions (Wettschureck *et al*, 2019). Since we found that the lack of Lphn2a results in the

abnormal organization of TJs between ECs *in vivo* (**Fig. 4C**), we sought to investigate the impact of LPHN2 on the extravasation of cancer cells that can be effectively tracked and quantified by using the zebrafish embryo (Follain *et al*, 2018; Osmani & Goetz, 2019). To this aim, fluorescently-labeled mouse (B16F10) or human (SK-MEL-28) melanoma cells were injected in the duct of Cuvier of *Tg(kdrl:EGFP)*^{s843} wild type or *lphn2a*$^{-/-}$ zebrafish embryos and their metastatic extravasation potential was quantified by measuring the number of tumor cells outside the vessels in the caudal plexus where the circulating tumor cells (CTCs) preferentially arrested and extravasated (Follain *et al.*, 2018; Hyenne *et al*, 2019). We discovered that, likely due to their abnormal inter-EC junctions, *lphn2a* null zebrafish embryos are more permissive to extravasation of both mouse B16F10 (**Fig. 5A**) and human SK-MEL-28 (**Fig. 5B**) melanoma cells compared to wild type embryos.

To conclude, we identified the endothelial ADGR LPHN2 as a novel repulsive guidance receptor that controls *in vivo* vascular morphogenesis and function. Our data suggest that, upon activation by ligands, such as FLRT2, LPHN2 elicits the synthesis of cAMP and Rap1-GTP pools that, in turn, may promote the turnover and keep the homeostatic balance in the number of ECM adhesions in vascular ECs (**Fig. 5C**). Our findings support the concept that, similarly to Rho small GTPases (Pertz, 2010), Rap1 is functionally organized into qualitatively distinct spatiotemporal signaling modules, which, at different subcellular locations and through distinct effectors, can elicit opposite effects. Indeed, Rap1 was described to both promote (Lagarrigue *et al.*, 2016; Lagarrigue *et al.*, 2015) and inhibit (Coló *et al.*, 2012; Hong *et al.*, 2007; Lyle *et al.*, 2008) cell adhesion and motility. It is conceivable that, based on its positioning at the leading edge or in centrally located mature adhesion sites Rap1-GTP may respectively signal to support ECM adhesion formation or turnover.

We also uncovered that LPHN2 signals by inhibiting the Hippo effectors YAP and TAZ in vascular ECs, both *in vitro* and *in vivo* (**Fig. 6**). Although other GPCRs were described to

modulate YAP/TAZ activation *via* Rho GTPase-regulated F-actin dynamics (Totaro *et al.*, 2018; Yu *et al.*, 2015), we did not detect any effect of LPHN2 silencing on Rho activation. The inhibition of the formation of FAs and F-actin stress fibers that we report here appears as the most conceivable mechanism by which LPHN2 negatively regulates YAP/TAZ signaling in ECs (**Fig. 6**). In agreement with what previously reported [reviewed in (Park & Kwon, 2018)], the aberrant hyperactivation of YAP/TAZ caused by *lphn2a* knockdown in zebrafish embryos is associated with TJ disarrangement in blood vessel ECs. Our data show that, likely due to the loosening of endothelial intercellular adhesions, the lack of Lphn2a might favor tumor melanoma cells extravasation (**Fig. 6**). Such findings indicate that LPHN2 ligands, *e.g.* FLRT2, might be therapeutically exploited to strengthen the vascular barrier and to counteract cancer cell metastatic dissemination.

MATERIALS AND METHODS

DNA constructs

Mouse hemagglutinin (HA) tagged Lphn2 construct was synthesized by GeneArt Gene Synthesis (Thermo Fisher Scientific) and cloned into the Gateway system pENTR221 vector (Thermo Fisher Scientific). HA-Lphn2 cDNA was then subcloned into the pEGFP-N1 (Enhanced Green Fluorescent Protein (EGFP)) mammalian expression vector by standard PCR protocols. HA-Lphn2 ΔOLF pEGFP-N1 mutant construct lacking the Olfactomedin domain (amino acids 142-398) was obtained by PCR, according to the Taq polymerase manufacturer's instructions, using Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific). HA-Lphn2 WT and ΔOLF were also subcloned into the pCCL.sin.cPPT.polyA.CTE.eGFP.minhCMV.hPGK.Wpre (pCCL) lentiviral vector. Lentiviral particles were produced as described previously described (Gagliardi *et al*, 2012).

Cell culture

Primary human ECs were isolated from the umbilical cords as previously described (Jaffe *et al*, 1973). Briefly, umbilical vein was cannulated with a blunt 17-gauge needle that was secured by clamping. The umbilical vein was then perfused with 50 ml of phosphate-buffered saline (PBS) to wash out the blood. Next, 10 ml of 0.2% collagenase A (Cat. # 11088793001, Roche Diagnostics) diluted in cell culture medium were infused into the umbilical vein and incubated 30 min at room temperature. The collagenase solution containing the ECs was flushed from the cord by perfusion with 40 ml of PBS, collected in a sterile 50 ml centrifuge tube, and centrifuged 5 min at 800 *g*. Cells were first resuspended in M199 medium completed with cow brain extract, heparin sodium salt from porcine intestinal mucosa [0,025 mg/500 ml], penicillin/streptomycin solution, 20% fetal bovine serum (FBS) (Sigma Aldrich), and subsequently plated in cell culture dishes that had been previously adsorbed with 1% gelatin

from porcine skin (G9136, Sigma Aldrich). Cells were tested for mycoplasma contamination by means of Venor GeM Mycoplasma Detection Kit (MP0025-1KT, Sigma Aldrich) and grown in M199 complete medium. The isolation of primary venous ECs from human umbilical cords was approved by the Office of the General Director and Ethics Committee of the Azienda Sanitaria Ospedaliera Ordine Mauriziano di Torino hospital (protocol approval no. 586, Oct 22 2012 and no. 26884, Aug 28 2014) and informed consent was obtained from each patient. COS-7 (ATCC code CRL-1651) and HEK 293T cells were grown in DMEM medium completed with glutamine, penicillin/streptomycin solution, and 10% FBS (Sigma Aldrich). Both COS-7 and HEK 293T were transfected by means of Lipofectamine and PLUS reagent (Thermo Fisher Scientific). The calcium phosphate transfection method was instead used to transfect WT or ΔOLF Lphn2-HA-pCCL in HEK 293T cells and to produce the corresponding viral particles subsequently employed to transduce ECs.

Antibodies and reagents

2Y4824 rabbit polyclonal antibody (pAb) anti-LPHN2 was produced by Eurogentec by immunizing animals with peptide GGKTDIDLAVDENGL (amino acids 259-274)(Herberth *et al*, 2005). Rabbit 2Y4824 anti-LPHN2 was diluted 1:300 for immunofluorescence analysis. 2Y4824 rabbit anti-pre-immune serum (PIS; Eurogentec) was used for control purposes. Mouse monoclonal antibody (mAb) anti-HA tag (clone F-7), used 1:200 for immunofluorescence analysis, was from Santa-Cruz Biotechnology. Mouse mAbs anti-Vinculin (hVIN-1) and anti-α tubulin (clone B-5-1-2) were from Sigma-Aldrich and were diluted 1:400 and 1:8000 for immunofluorescence and Western blot analyses respectively. Rat mAb anti-HA High Affinity (clone 3F10), was from Roche and it was diluted 1:1000 for Western blot analysis. Mouse mAb anti-6xHis tag (clone HIS.H8) was from OriGene

Technologies. Goat pAb anti-FLRT2 (AF2877), from R&D Systems, was diluted 1:100 and 1:1000 for immunofluorescence and Western blot analysis, respectively.

Horseradish peroxidase (HRP) conjugated donkey anti-goat (sc-2056), goat anti-rabbit (sc-2054) and goat anti-rat secondary Abs were from Santa Cruz Biotechnology, while HRP-goat anti-mouse (115-035-003) was from Jackson ImmunoResearch Laboratories. Alexa Fluor 555 donkey anti-mouse (A31570), Alexa Fluor 555 donkey anti-rabbit (A31572), Alexa Fluor 555 Phalloidin (A34055), Alexa Fluor 488 donkey anti-rabbit (A21206) and donkey anti-goat (A11055), Alexa Fluor 647 donkey anti-mouse (A31571), and DAPI (D3571) were from Thermo Fisher Scientific. Alkaline phosphatase (AP)-labeled goat anti-mouse IgG (H+L) cross-adsorbed secondary Ab (G-21060) was from Thermo Fisher Scientific.

Uncropped scans of all blots are provided as Figures S7 and S8.

Recombinant proteins

Human fibronectin was from R&D Systems. Coll I from calf skin was produced by Sigma-Aldrich. Recombinant human carrier-free FLRT2 protein (2877-FL) was from R&D Systems. Streptavidin-conjugated HRP was from GE Healthcare.

Gene silencing in cultured ECs

The day before oligofection, ECs were seeded in six-well plates at a concentration of 12 x 10^4 cells per well. Oligofection of siRNA duplexes was performed according to the manufacturer's protocols. Briefly, human ECs were transfected twice (at 0 and 24 h) with 200 pmol of siGENOME Non-Targeting siRNA #1 (as control) or siGENOME SMART pools siRNA oligonucleotides (GE Healthcare Dharmacon), using Oligofectamine Transfection Reagent (Thermo Fisher Scientific). 24 h after the second oligofection, ECs were lysed or tested in functional assays. Knockdown of LPHN2 was achieved through the siGENOME SMART pool

siLPHN2 M-005651-02, while, in the case of FLRT2, was the siGENOME SMART pool siFLRT2 M-009104-00.

Western Blot analysis

Cells were lysed in buffer containing 25 mM Tris–HCl pH 7.4, 100 mM NaCl, 5% glycerol, 0.5 mM EGTA, 2 mM $MgCl_2$, 1 mM PMSF, 1 mM Na_3VO_4, protease inhibitor cocktail (Sigma Aldrich) and detergent 1% NP40. Cellular lysates were incubated for 20 min on ice, and then centrifuged at 15,000 *g*, 20 min, at 4 °C. The total protein amount was determined using the bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific). Specific amounts of protein were separated by SDS–PAGE with precast Bolt 4-12% Bis-Tris gel (Thermo Fisher Scientific) or Mini PROTEAN TGX precast 7,5% gel (Bio-Rad). Proteins were then transferred to a nitrocellulose membrane (Bio-Rad), probed with Abs of interest and detected by enhanced chemiluminescence technique (PerkinElmer).

Immunoprecipitation

To immunoprecipitate and analyze by Western blot endogenous LPHN2, 5 x 10^6 HUVEC were transferred to ice, washed 3 times in cold PBS 1X, and surface-labeled at 4 °C with 0,2 mg/ml sulfo-NHS-SS-biotin (Thermo Scientific) in PBS1X for 30 min. Cells were then washed 3 times and lysed with a buffer containing 25 mM Tris–HCl pH 7.4, 100 mM NaCl, 1% Triton X-100, 5% glycerol, 0.5 mM EGTA, 2 mM $MgCl_2$, 1 mM PMSF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Sigma Aldrich). Cellular lysates were incubated for 20 min on ice and then centrifuged at 15,000 *g*, 20 min, at 4 °C. The total protein amount was determined using the bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific). Equivalent amounts (1 mg) of protein were precipitated for 1 h at 4 °C with protein A-Sepharose beads and then centrifuge for 1 min 15,000 *g* at 4 °C. Protein A-Sepharose beads were collected to

prepare the pre-clearing sample and the lysate were then immunoprecipitated for 1 h at 4 °C with the rabbit pAb anti-LPHN2 or pre-immune serum. Immunoprecipitates were washed four times with lysis buffer and then separated by SDS–PAGE. Proteins were then transferred to a nitrocellulose membrane (Bio-Rad), probed with Abs of interest and detected by enhanced chemiluminescence technique (PerkinElmer).

Rap1 and Rho-GTP pull-down assay

Rap1-GTP and Rho-GTP were respectively analyzed by means of the active Rap1 (Cat. #16120, Thermo Fisher Scientific) and Rho (Cat. #16116, Thermo Fisher Scientific) pull-down and detection kits. The assays were performed according to manufacturer's protocols. Total Rap1 or Rho proteins, detected in the input fractions, were used to calculate the normalized amount of active Rap1-GTP or active Rho-GTP. Bands were quantified with ImageJ software and normalized optical density (N.O.D.) was calculated relative to control.

Confocal scanning microscopy on cultured ECs

Cells were plated on 0.17 mm glass coverslips (no. 1.5) pre-coated with FN [5 μg/ml] and allowed to adhere for 3 h. Cells were washed in phosphate-buffered saline (PBS), fixed in 4% or 2% para-formaldehyde (PFA), permeabilized in 0.1% Triton or Saponin 0,01% PBS 1X for 2 min or 5 min on ice respectively, incubated with different primary Abs and revealed by appropriate Alexa-Fluor-tagged secondary Abs (Thermo Fisher Scientific). Cells were analyzed by using a Leica TCS SPEII confocal laser-scanning microscope (Leica Microsystems) employing the Leica LAS AF software for image acquisition. The acquisition was performed by adopting a laser power, gain, and offset settings that allowed maintaining pixel intensities (gray scale) within the 0–255 range and hence avoid saturation. Analysis of confocal images (Fig. 1F and 3E) was performed using the ImageJ software package. The

fluorescence intensity of F-actin has been calculated measuring the corrected total cell fluorescence (CTCF) parameter: CTCF = Integrated Density – (Area of selected cell x Mean fluorescence of background readings) (Gavet & Pines, 2010). FA number was measured applying for each cell a default threshold; moreover, FA number was normalized on cell area. Nuclear/cytoplasmic YAP and TAZ ratios were calculated by measuring the mean intensity fluorescence of each protein coming from the nucleus (overlapping with the DAPI staining) divided by its cytoplasmic localization (cell area – DAPI signal).

Binding assays

Empty vector, WT Lphn2 and ΔOLF Lphn2 expression constructs were transfected in COS-7cells to be used in the binding assay. *In situ* binding assays were performed as described previously (Tamagnone *et al*, 1999). Briefly, Lphn2-expressing COS-7 cells were incubated for 1h at 37° with or without recombinant 6xHis-tagged human FLRT2, mouse mAb anti-6xHis, and AP-labeled goat anti-mouse. After five washes in DMEM diluted 1:2 in PBS, cells were fixed and incubated with the AP substrate nitro blue tetrazolium–5-bromo-4-chloro-3-indolyl-phosphate (NBT–BCIP; Promega).

Haptotactic endothelial cell migration assays

Real time directional EC migration of ECs was monitored with an xCELLigence RTCA DP instrument (ACEA Biosciences). The bottom side of the upper chamber (the side facing the lower chamber) of CIM-Plate 16 was coated with 30 µl of 1 µg/ml Coll I or 3 µg/ml FN for 30 min at room temperature. Each lower chamber well was first filled with 160 µl of M199 1% FBS (containing or not 800 ng/ml of rhFLRT2 protein) and then assembled to the upper chamber. Each upper chamber well was then filled with 30 µl of M199 1% FBS. The plate was put for 1h at 37°C. The experiment file was set up using the RTCA Software 1.2. ECs were

detached and resuspended to a final concentration of 30,000 cells/100 µl. The BLANK step was started to measure the background impedance of cell culture medium, which was then used as reference impedance for calculating CI values. 100 µl of cell suspension (30,000 cells) were then added to each well of the upper chamber. The CIM-Plate 16 was placed in the RTCA DP Instrument equilibrated in a CO_2 incubator. ECs migration was continuously monitored using the RTCA DP instrument. Average, standard deviation and p value were calculated on the CI data exported from RTCA instrument for the technical replicates of each experimental condition in the time. Migration data are represented as a percentage considering the control samples as 100%.

cAMP assay

The cellular amounts of cAMP were quantified by using the non-acetylated cyclic AMP competitive ELISA Kit (Thermo Scientific, Cat. #EMSCAMPL) following to the manufacturer's instructions. The concentration of cAMP in samples was expressed as pmol of cAMP per mg of total proteins.

mRNA isolation from cultured ECs and real-time RT-PCR analysis

Cells were washed three times with PBS and frozen at -80°C. RNA isolation and reverse transcription: cells were thawed on ice, total RNA was extracted following the manufacturer's recommended protocol (ReliaPrep RNA Miniprep Systems, Promega. Cat. # Z6011). The quality and integrity of the total RNA were quantified by the NanoDrop 1000 spectrophotometer (Thermo Scientific). cDNAs were generated from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan real-time RT-PCR assay: mRNA expression of *LPHN2*, *FLRT1-3*, and endogenous housekeeping control genes, *i.e.* glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and TATA binding protein

(*TBP*), was measured by real-time RT-PCR using TaqMan Gene Expression Assays run on a C1000 Touch thermal cycler (Bio-Rad). The following assays were used: Hs00202347_m1 (*LPHN2*), Hs00534771_s1 (*FLRT1*), Hs00544171_s1 (*FLRT2*), Hs01922255_s1 (*FLRT3*), Hs00170014_m1 (*CTGF*), Hs00155479_m1 (*CYR61*), Hs99999905_m1 (*GAPDH*), and Hs00427620_m1 (*TBP*). For each sample, three technical replicates of each gene were run in a 96-well plate (cDNA concentration 50 ng/well) according the manufacturer's protocol. Between the two measured housekeeping genes, we chose a normalization factor calculation based on the geometric mean of *GAPDH* and *TBP* gene transcript for most of real-time RT-PCR experiments, while *GAPDH* only was employed in Fig.2A (Vandesompele *et al*, 2002). The experimental threshold (Ct) was calculated using the algorithm provided by the Bio-Rad CFX Manager 3.1 software (Bio-Rad). Ct values were converted into relative quantities using the method described by Pfaffl (Pfaffl, 2001) and amplification efficiency of each gene was calculated using a dilution curve and the slope calculation method (Pfaffl, 2001).

Zebrafish embryo lines and handling

Zebrafish were handled according to established protocols and maintained under standard laboratory conditions. Experimental procedures related to fish manipulation followed previously reported recommendations (Workman *et al*, 2010) and conformed to the Italian regulations for protecting animals used in research, including DL 116/92. The Ethics committee of the University of Torino approved this study. Larvae were anesthetized and then sacrificed by ice chilling. Following zebrafish lines were used for these studies: wild-type AB, *Tg(kdrl:EGFP)*^{s843} and *Tg(Hsa.CTGF:nlsmCherry)*^{ia49}/*Tg(kdrl:EGFP)*^{s843}.

Generation of *lphn2a* null zebrafish embryos

Lphn2a$^{-/-}$ zebrafish mutants were generated by CRISPR/Cas9-mediated genome editing at ZeClinics (Barcelona, Spain). A single guide RNA (sgRNA) was designed using the online tool http://crispor.tefor.net/, based on exon site, high efficacy, and not off-target published algorithms to specifically target an optimal CRISPR sequence on exon 2 of *lphn2a* gene (ENSDARG00000069356). The *lphn2a*-targeting sgRNA, with the specific targeting sequence CAACCGTCAAGACGAATACA-AGG, was injected in one-cell stage embryos in a solution containing Nls-CAS9 protein (PNA BIO). The mutagenesis efficacy was evaluated on pools of 30 injected embryos, whose genomic DNA was PCR amplified and analyzed via T7 endonuclease system. F0 injected embryos were raised to adulthood and screened, by genotyping the F1, for germline transmission of the mutation. Heterozygous mutants harboring the mutation were then incrossed to obtain homozygous mutants (F5 generation). The genomic region surrounding the CRISPR target site was PCR-amplified using the following primers: *lphn2a*-Fw (5'- TCTCAGAGTGACTTCCCCGGATC -3'), *lphn2a*-Rv (5'- GCAGCCATTATTTATCCCAGCTACC -3'). *Lphn2a*$^{-/-}$ heterozygous and homozygous mutants were identified by analyzing in agarose gel the PCR product digested with TasI restriction enzyme (TSP509I, Thermo Fisher Scientific) and by sequencing.

Transmission electron microscopy (TEM)

Embryos were anaesthetized and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, then dehydrated, embedded in epoxy resin and sectioned, following a standard TEM sample preparation protocol (Santoro *et al*, 2009). All steps after fixation were performed by the TEM service of the Department of Biology (University of Padova, Italy).

Larvae dissociation and fluorescence activated cell sorting (FACS)

Wild type and *Tg(kdrl:EGFP)*^{s843} larvae at 2 days post-fertilization (dpf) were dissociated as previously described (Zancan *et al*, 2015) using 1x PBS, 0.25% trypsin phenol red free, 1 mM EDTA pH 8.0, 2.2 mg/ml Collagenase P (Sigma). Digestion was stopped by adding $CaCl_2$ to a final concentration of 1 mM and fetal calf serum to 10%. Dissociated cells were rinsed once in PBS and resuspended in Opti-MEM (Gibco), 1% fetal calf serum and 1X Penicillin-Streptomycin solution (Sigma). Cells were filtered through a 40 µm nylon membrane. For sorting, we used FACS Aria IIIu sorter (BD Biosciences, San José, USA) with the following settings for EGFP: argon-ion Innova Laser (Coherent, USA) (488 nm, 100 mW); 100 µM nozzle; sorting speed 500 events/s in 0-32-0 sort precision mode. We performed data acquisition and analysis with the BD FACSDiva software (BD Biosciences, San José, CA, USA). GFP^+ and GFP^- cells were separately collected in resuspension medium, and RNA was extracted using the RNA isolation kit Nucleospin® RNA XS (Macherey-Nagel). cDNA was made with RT High Capacity kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative real-time RT-PCR on ECs from zebrafish was performed with CFX384 Touch Real-time PCR Detection System (Biorad) using 5x HOT FIREPol®EvaGreen® qPCR Mix Plus (Solis BioDyne), the following primer sequences were used *lphn2*-F: 5'-AGTATCCCTCATCTGCCTGG-3' *lphn2*-R: 5'-AGCTGAACTCCTTCCAGACA-3'; *flrt2*-F: CATTGCATGGCTCAGGTCTC, *flrt2*-R: ATGAGTTGGCCAGGGATGAA, *cyr61*-F: 5'-GCGGAGACTCGGAGAAAGAAC-3', *cyr61*-R 5'-CGATGCACTTCTCCATCTGATG3'; *ctgfa*-F: 5'-CTCCCCAAGTAACCGTCGTA-3', *ctgfa*-R: 5'-CTACAGCACCGTCCAGACAC-3; *ctgfb*-F: 5'-CCCACAAGAAGACACCTTCC-3', *ctgfb*-R: 5'-ATTCGCTCCATTCAGTGGTC-3'. Results are expressed as relative mRNA abundance and normalized to actin beta1 (*actb1*) or eukaryotic translation elongation factor 1 alpha 1, like 1 (*eef1a1l1*) as endogenous reference genes, which were amplified by employing the following

primer sequences *actb1*-F: 5' GTATCCACGAGACCACCTTC-3', *actb1*-R: 5'-GAGGAGGGCAAAGTGGTAAAC-3'; *eef1a1l1*-F 5'-GACAAGAGAACCATCGAG-3', *eef1a1l1*-R 5'-CCTCAAACTCACCGACAC-3'.

Intravascular injection of cancer cells in zebrafish embryos

At 48 hpf, embryos were dechorionated at 2 dpf, anesthetized with tricaine 0.16 mg/ml and placed along plastic lanes immersed in 2% methylcellulose/PBS. Sub-confluent cells in 10cm culture dishes were rinsed twice with warm serum free medium and then incubated for 30 minutes at 37°C (B16F10 or SK-MEL28) with MemBright-560 (Lipilight-IDYLLE) and quickly diluted in serum free medium (200nM final). To eliminate all possible traces of unbound MemBright-560, cells were then rinsed three times with serum free medium, then with EDTA, and trypsinized. Stained cells (at $100 \cdot 10^6$ cells per ml) were loaded in a glass capillary needle and microinjected into the duct of Cuvier of the embryos under the stereomicroscope using a WPI PicoPump apparatus. Xenotransplanted embryos were grown at 32° C, monitored daily and analyzed starting from one day post injection (dpi) up to 3 days.

Images acquisition and analysis on zebrafish embryos

Double transgenic fluorescence of *Tg(kdrl:EGFP)*^{s843} and Tg *(Hsa.CTGF:nlsmCherry)*in *lphn2a*$^{-/-}$ mutant background was visualized under a AZ100 stereomicroscope equipped with AxioCam (Zeiss) dissecting microscope and then with a Leica SP8 confocal microscope. Larvae were anaesthetized and mounted in 1% low-melting- point agarose gel. Endothelial cells targeted EGFP and nuclear mCherry fluorescence (Yap/Taz) was visualized by using 488 nm and 561 nm lasers. All images were analyzed with Fiji software (https://fiji.sc/). Yap/Taz signal were automatically segmented on the mCherry fluorescence image stacks, inside a EGFP fluorescent mask identifying endothelial cells, and total signal intensity was calculated,

according to previous work (Facchinello *et al*, 2016). After confocal acquisition, heterozygous and homozygous siblings were genotyped by PCR on DNA previously extracted from a single larva, as described by (Gagnon *et al*, 2014).

Statistical analysis

For statistical evaluation of *in vitro* experiments, parametric two-tailed heteroscedastic Student's t test was used to assess the statistical significance when two groups of unpaired normally distributed values were compared; when more than two groups were compared, parametric two-tailed analysis of variance (ANOVA) with Bonferroni's post hoc analysis was applied; with the only exception for Fig. 2A, all bar plots represent relative values of the corresponding control.

For statistical evaluation of *in vivo* experiments, neither randomization nor blinding was applied for samples or zebrafish embryo analyses. No statistical method or criteria was used to predetermine sample size or to include/exclude samples or animals. The Shapiro-Wilk normality test was used to confirm the normality of the data. The statistical difference of Gaussian data sets was analyzed using the Student unpaired two-tailed t test, with Welch's correction in case of unequal variances. For data not following a Gaussian distribution, the Mann-Whitney test was used. Illustrations of statistical analyses of *in vivo* experiments are presented as the means ± SEM.

ACKNOWLEDGEMENTS

We are grateful to Alberto Puliafito for help with data analysis. The research leading to these results has received funding from: Fondazione AIRC IG grants #13016, #16702, and #21315 (to G.S.), #19923 (to L.T.), #20366 (to D.V.), and #20119 (to M.M.S.); Fondazione AIRC under 5 per Mille 2018 - ID. 21052 program – P.I. Comoglio Paolo, G.L. Tamagnone Luca, G.L. Serini Guido, ; FPRC-ONLUS Grant "MIUR 2010 Vaschetto - 5 per mille 2010 MIUR" (to G.S.); Telethon Italy (GGP09175) (to G.S.); Associazione 'Augusto per la Vita' (to G.S.); ERC-CoN project 647057 - rEnDOx (to M.M.S.).

AUTHOR CONTRIBUTIONS

G.S. conceived the project; G.S., C.C., M.M.S., and N.F. designed the experiments; G.S., C.S., D.V., and M.M.S. supervised the research; L.T. and M.A. provided key reagents, methods, and technologies; C.C., N.F., D.G., G.V. and N.G. performed the experiments; C.C., N.F., D.G., G.V., N.G., C.S., D.V., M.A., L.T., M.M.S., and G.S. analyzed the data; C.C., N.F., D.G., G.V., N.G., C.S., D.V., M.A., L.T., M.M.S., and G.S. interpreted the results; C.C., N.F., M.M.S., and G.S. wrote the paper; all authors read and approved the manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest

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Figure legends

Figure 1. LPHN2 concentrates at ECM adhesions and negatively regulates ECM-elicited EC haptotaxis. (**A**) Confocal microscopy analysis of ECs indicates how endogenous LPHN2, as detected by an anti-LPHN2 antibody (*green*), co-localizes with vinculin (*red*) in ECM

adhesions of ECs as shown in merging (right panel). Lower row panels are magnifications of the corresponding upper row panels. Scale bar 20 μm. (**B**) Fluorescence confocal microcopy reveals that in ECs transfected with HA-Lphn2-EGFP both the extracellular, as detected by an anti-HA antibody (*red*), and the EGFP-fused intracellular (*green*) moieties co-localize with vinculin (*blue*) at ECM adhesions, as shown in merging (right panel). Lower row panels are magnifications of the corresponding upper row panels. Scale bar 20 μm. (**C**) Real time analysis of haptotactic migration control (siCTL) or LPHN2 (siLPHN2) silenced ECs toward Coll I was assessed with an xCELLigence RTCA DP system. Results are the average ± SEM of six independent assays. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test; $p \leq 0.001$ ***. (**D**) LPHN2 silencing in ECs decreases basal cAMP amount. siCTL and siLPHN2 absolute cAMP concentration values are 5.6 and 2.6 [pmol/ml] respectively. Results are the average ± SEM of nine independent assays. (**E**) LPHN2 silencing in human ECs decreases basal GTP loading of Rap1 small GTPase and pCCL lentivirus-mediated overexpression of silencing-resistant mouse Lphn2 rescues the decrease in Rap1-GTP levels. Total Rap1 was employed to calculate the normalized optical density (N.O.D.) levels of active Rap1-GTP. Results are the average ± SEM of five independent experiments, one of which is shown. Statistical analysis: parametric two-tailed analysis of variance (ANOVA) with Bonferroni's post hoc analysis; ANOVA p value <0.0001; Bonferroni's p value <0.0001 for CTL/siCTL vs. CTL/siLPHN2 and 0.0019 for CTL/siLPHN2 vs. Lphn2/siLPHN2. (**F**) Confocal microscopy analysis of endogenous LPHN2 (*green*), vinculin (*blue*), and phalloidin-labeled F-actin (*red*) reveals how, compared to siCTL ECs, LPHN2 silencing increases the number of vinculin-containing FAs and the amount of F-actin stress fibers (evaluated as CTCF) in siLPHN2 ECs. Lower row panels are magnifications of the corresponding upper row panels. Scale bars for siCTL panels and siLPHN2 panel are 10 μm and 15 μm respectively. Results concerning the number of vinculin-containing FAs and the amount of F-actin stress fibers are the average ± SEM of five independent experiments for a total of 45 (siCTL) and 38 (siLPHN2) ECs and four independent experiments for a total of 54 (siCTL) and 36 (siLPHN2) ECs respectively. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test; $p \leq 0{,}01$ **. See also Figures S1 and S2.

Figure 2. FLRT2-binding OLF domain of LPHN2 is necessary to inhibit ECM-elicited EC motility. (**A**) Real time quantitative PCR analysis of *FLRT1-3* mRNAs in human ECs relative to the house keeping gene *GAPDH*. Results are the average ± SEM of four independent

experiments (**B**) Real time quantitative PCR analysis of *LPHN2* and *FLRT1-3* mRNAs in siCTL and siLPHN2 human ECs. Results are normalized on siCTL values and are the average ± SEM of four independent experiments. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test; p≤ 0,01 **; p≤ 0,001 ***. (**C**) COS-7 cells transfected with empty vector or WT HA-Lphn2-EGFP or ΔOLF HA-Lphn2-EGFP constructs and their expression verified by Western blot analysis employing a rat mAb anti-HA. Next, COS-7 cells transfected with empty vector or WT HA-Lphn2-EGFP or ΔOLF HA-Lphn2-EGFP constructs were incubated or not with 6xHis-tagged rhFLRT2. The binding between the Lphn2 constructs and the rhFLRT2 ligand was revealed through sequential incubation with a mouse mAb anti-6xHis, an AP-conjugated goat anti-mouse pAb, and the AP substrate NBT–BCIP. Results show how WT, but not ΔOLF Lphn2 binds rhFLRT2. (**D**) Real time analysis of haptotactic EC migration toward Coll I was assessed with an xCELLigence RTCA DP system. Cells were first transduced with WT HA-Lphn2-pCCL, ΔOLF HA-Lphn2-pCCL and empty pCCL (CTL) lentiviral constructs and then oligofected with either siCTL or siLPHN2 siRNAs. Haptotactic migration toward Coll I of ECs was analyzed over time. SiLPHN2 ECs migrate faster in comparison to siCTL ECs and only transduction with WT, but not ΔOLF rescues the excessive migration siLPHN2 ECs. Results are the average ± SEM of three independent experiments. Results were analyzed by a two-tailed heteroscedastic Student's t-test comparing CTL/siCTL (*blue*) to CTL/siLPHN2 (*red*) and CTL/siLPHN2 to WT (*green*) or ΔOLF Lphn2/siLPHN2 (*purple*); p≤ 0,01 **; p≤ 0,001 ***. See also Figure S2.

Figure 3. FLRT2 negatively regulates ECM-elicited EC haptotaxis via LPHN2 and triggers cAMP/Rap1 signaling. (**A**, **B**) Real time analysis of haptotactic EC migration toward Coll I was assessed with an xCELLigence RTCA DP system. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test; p≤ 0,05 *; p≤ 0,01 **; p≤ 0,001 ***. (**A**) Endogenous FLRT2 silencing (siFLRT2) increases haptotactic migration compared to siCTL ECs. Results are the average ± SEM of six independent assays. (**B**) Exogenous rhFLRT2 [800 ng/ml] inhibits siCTL, but not siLPHN2 EC haptotaxis. For sake of simplicity data from the same experiments are plotted in two separated graphs: siCTL (left graph) and siLPHN2 (right graph). Results are the average ± SEM of four independent assays. (**C**) Endogenous FLRT2 silencing in ECs decreases basal cAMP amount. siCTL and siFLRT2 absolute cAMP concentration values are 4.7 and 3.69 [pmol/ml] respectively. Results are the average ± SEM of seven independent assays. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test; p≤ 0,05 *. (**D**) Endogenous FLRT2 silencing (siFLRT2) in

ECs decreases basal Rap1-GTP levels and treatment of siFLRT2 ECs with exogenous rhFLRT2 [800 ng/ml] rescues the decrease in Rap1-GTP levels. Total Rap1 was employed to calculate the normalized optical density (N.O.D.) levels of active Rap1-GTP. Results are the average ± SEM of four independent experiments, one of which is shown. Statistical analysis: parametric two-tailed analysis of variance (ANOVA) with Bonferroni's post hoc analysis; ANOVA p value 0.0019; Bonferroni's p value 0.0012 for siCTL vs. siFLRT2 and 0.0269 for siFLRT2 vs. siFLRT2 + FLTR2. (**E**) Confocal microscopy analysis of endogenous FLRT2 (*green*), vinculin (*blue*), and phalloidin-labeled F-actin (*red*) reveals how, compared to siCTL ECs, FLRT2 silencing increases the number of vinculin-containing FAs and the amount of F-actin stress fibers, evaluated as CTCF, in siFLRT2 ECs. Lower row panels are magnifications of the corresponding upper row panels. Scale bars for siCTL panels and siFLRT2 panel are 10 µm and 15 µm respectively Results concerning the number of vinculin-containing FAs and the amount of F-actin stress fibers are the average ± SEM of four independent experiments for a total of 63 (siCTL) and 59 (siFLRT2) ECs and four independent experiments for a total of 71 (siCTL) and 60 (siFLRT2) ECs respectively. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test; $p \leq 0,05$ *. See also Figure S3.

Figure 4. LPHN2 controls vascular morphogenesis and YAP/TAZ signaling in zebrafish embryo. (**A**) Confocal fluorescence microscopy analysis of trunk cross-sections at 72 hpf of *Tg*(*Kdrl:EGFP*)^{s843} zebrafish embryos carrying EC-specific EGFP expression. Lphn2 protein is enriched in ECs of dorsal aorta (DA) and posterior cardinal vein (PCV). Scale bar: 60 µm. (**B**) *Upper panels*. Lateral view in bright field and fluorescence confocal microscopy of wild type (WT) and CRISPR/Cas9-mediated *lphn2a* knock-out (*lphn2a*$^{-/-}$) zebrafish embryos in the *Tg(kdrl:EGFP)*^{s843} background. Scale bars: 100 µm. See also Figure S3. *Lower panels*. Confocal images of the trunk region of wild type *lphn2a*$^{+/+}$ and CRISPR/Cas9-mediated *lphn2a* knock-out (*lphn2a*$^{-/-}$) zebrafish embryos in the *Tg(kdrl:EGFP)*^{s843} background with Color code images of endothelial shapes in the dorsal aorta and cardinal vein at 72 hpf from *lphn2a*$^{+/+}$ and *lphn2a*$^{-/-}$ zebrafish embryos in which profiles of ECs were automatically segmented on EGFP fluorescence confocal microscopy image stacks. Scale bars: 50 µm. (**C**) TEM analysis of WT and *lphn2a*$^{-/-}$ dorsal aorta (n=3) at 48 hpf. Scale bars: 1 µm (upper images) and 5 µm (lower images). EC: endothelial cells, L: lumen, red arrow: EC tight junctions. Color code identification of EC lumen. (**D**) Yap1/Taz reporter activity is prominent in the endothelium during development from 20 to 48 hpf. *Left panels*, Representative confocal images of

Tg(Hsa.CTGF:nlsmCherry)^{ia49} /*Tg(kdrl:EGFP)*^{s843} double transgenic WT and *lphn2a*$^{-/-}$ siblings at 72hpf (n= 4). Yap1/Taz activation signal was automatically segmented on fluorescent confocal mCherry image Z-stacks, inside a EGFP fluorescent mask identifying endothelial cells, and only this endothelial cell-restricted Yap1/Taz signal intensity was calculated and represented in 3D analysis. Scale bars: 50 µm. *Right panel*, Relative quantification of integrated density of Yap1/Taz *Hsa.CTGF:nlsmCherry* reporter activity signal that co-localizes with *kdrl:GFP*. Statistical analysis: results were analyzed by a Mann-Whitney test; p≤ 0,05 *. (**E**) Real time quantitative PCR analysis of *ctgfa*, *ctgfb*, and *cyr61* mRNAs of FACS-sorted ECs isolated from *lphn2a*$^{+/+}$ or *lphn2a*$^{-/-}$ zebrafish embryos at 48 hpf relative to the house keeping gene *actb1* and normalized on the mRNA levels measured in *lphn2a*$^{+/+}$ animals. Results are the average ± SEM of ≥3 independent assays (n>80 embryos for condition). Statistical analysis: results were analyzed by a Mann-Whitney test; p≤ 0,001 ***. See also Figures S4, S5, and S6.

Figure 5. LPHN2 impairs cancer cell extravasation in zebrafish embryos. (**A, B**) MemBright-560-labeled melanoma cells were microinjected into the duct of Cuvier of 48 hpf *lphn2a*$^{+/+}$ or *lphn2a*$^{-/-}$ *Tg*(*Kdrl:EGFP*)^{s843} zebrafish embryos. After 36 hours, extravasated metastatic melanoma cells were imaged by confocal analysis of the caudal plexus. Mouse B16F10 (**A**, red) and human SK-MEL-28 (**B**, purple) melanoma cell extravasation is enhanced in *lphn2a*$^{-/-}$ compared to *lphn2a*$^{+/+}$ zebrafish embryos. Results are the average ± SEMA of 2 independent assays, in which 17 (B16F10) and 14 (SK-MEL-28) animals were analyzed. Scale bars: 50 µm. Statistical analysis: results were analyzed by a Mann-Whitney test; p≤ 0,05 *.

Figure 6. Integrin-mediated LPHN2-YAP/TAZ crosstalk. Vascular ECs synthesize the FLRT2 ligand of LPHN2 that localizes at integrin-based ECM adhesion sites. FLRT2-activated LPHN2 triggers a canonical heterotrimeric G-protein α subunit (Gα)/adenylate cyclase (AC)/ cAMP pathway that in turn, likely *via* the guanine nucleotide exchange factor EPAC, activates the small GTPase Rap1. LPHN2 signals to inhibit the formation of integrin-based ECM adhesions that are mechanically connected by stress fibers to the nucleus through the LINC complex. The lack of LPHN2 inhibitory signaling (LPHN2 KO) increases the formation of focal adhesion sites and actin stress fibers whose myosin II-mediated contraction release YAP/TAZ from their binding to the inhibitory SWI/SNF complex (not depicted) and transmit

force from the ECM to the nucleus, changing nuclear pore conformation, finally resulting in the translocation of YAP/TAZ into the nucleus and the transcription of target genes, such as CTGFA and CYR61. In agreement with previous studies (Kim *et al.*, 2017), the increased YAP/TAZ activation caused by *in vivo* LPHN2 knockdown also results in the abnormal assembly of vascular endothelial intercellular junctions (dark green bars), which favors cancer cell extravasation.

SUPPLEMENTAL INFORMATION

Supplementary figure legends

Figure S1, related to Figure 1. (**A**) Upon surface biotinylation LPHN2 was immunoprecipitated with a rabbit anti-LPHN2 pAb from EC lysates and revealed in Western blot by mean of HRP-streptavidin. Pre-immune serum (PIS) was employed for control purposes. The cleaved extracellular portion of LPHN2 appears as a ~130 kDa protein band. (**B**) HEK 293T cells were transfected with empty vector or increasing amounts (2, 4 and 6 µg) of HA-Lphn2-EGFP construct. Cells lysates were then analyzed by Western blot with either anti-HA (*left panel*) or anti-GFP (*right panel*) antibody. The 230 kD, 130 kD, and 97 kD protein bands correspond respectively to uncleaved full-length, cleaved N-terminal extracellular-only portion, and cleaved C-terminal portion of the transfected HA-Lphn2-EGFP protein. In the anti-GFP Western blot, the 27 kD band corresponds to the GFP whose cDNA was present in the control empty vector.

Figure S2, related to Figure 1. (**A**) Real time quantitative PCR analysis of *LPHN2* mRNA in siCTL or siLPHN2 human ECs relative to the house keeping gene *GAPDH* and normalized on siCTL levels. Results are the average ± SEM of nine independent assays. (**B**) Real time analysis of haptotactic migration control (siCTL) or LPHN2 (siLPHN2) silenced ECs toward FN was assessed with an xCELLigence RTCA DP system. Results are the average ± SEM of four independent assays. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test; $p \leq 0.01$ **. (**C**) Real time quantitative PCR analysis of *LPHN2* mRNA in siCTL or siLPHN2 human ECs rescued or not (CTL) with mouse WT or ΔOLF Lphn2 relative to the house keeping gene *GAPDH* and normalized on siCTL levels. Results are the average ± SEM of four independent assays. Statistical analysis: Statistical analysis: parametric two-tailed analysis of variance (ANOVA) with Bonferroni's post hoc analysis; ANOVA p value <0.0001; Bonferroni's p value is <0.0001 for each comparison CTL/siCTL vs. CTL/siLPHN2, CTL/siCTL vs. Lphn2/siLPHN2, CTL/siCTL vs. Lphn2ΔOLF /siLPHN2. Transduced and silenced ECs were also lysed and WT HA-Lphn2-pCCL and ΔOLF HA-Lphn2-pCCL protein expression levels analyzed by Western blot with anti-HA antibody. (**D**) LPHN2 silencing in human ECs does not affect basal GTP loading of RhoA small GTPase. Total Rho was used to calculate the normalized optical density (N.O.D.) levels of active Rho-GTP. Results are the average ± SEM of three independent assays. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test.

Figure S3, related to Figure 4, 5, and S5. (**A**) Confocal microscopy analysis of endogenous YAP (*red*) and TAZ (*green*) in siCTL or siLPHN2 human ECs to show how they increase their expression and co-localization with the nucleus (*DAPI*). Scale bar: 20 µm. (**B**) Quantification of the mean intensity fluorescence of nuclear/cytoplasmic YAP ratio in siCTL or siLPHN2 human ECs. Results are the average ± SEM of two independent experiments for a total of 187 cells, for a total of 96 (siCTL) and 91 (siLPHN2) ECs respectively. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test; p≤ 0,001 ***. (**C**) Quantification of the mean intensity fluorescence of nuclear/cytoplasmic TAZ ratio in siCTL or siLPHN2 human ECs. Results are the average ± SEM of two independent experiments for a total of 196 cells, for a total of 98 (siCTL) and 98 (siLPHN2) ECs respectively. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test; p≤ 0,001 ***. **(D)** Real time quantitative PCR analysis of CTGF and CYR61 mRNA in siCTL or siLPHN2 human ECs relative to the house keeping genes, GAPDH and TBP, and normalized on siCTL levels. Data of one of two independent assays are shown. Results are the average ± SD of three technical replicates. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test; p≤ 0,05 *.

Figure S4, related to Figure 3. (**A**) *Left panel*, real time quantitative PCR analysis of *FLRT2* mRNA in siCTL or siFLRT2 human ECs relative to the housekeeping gene *GAPDH* and normalized on siCTL levels. Results are the average ± SEM of six independent assays. Statistical analysis: results were analyzed by a two-tailed heteroscedastic Student's t-test; p≤ 0.001 ***. *Right panel*, Western blot analysis with an anti-FLRT2 Ab of lysates of siCTL or siFLRT2 ECs. (**B**) Western blot analysis with an anti-FLRT2 Ab of lysates of siCTL or siFLRT2 ECs or siFLRT2 ECs treated with exogenous rhFLRT2 [800 ng/ml]. Endogenous FLRT2 appears as a ~85 kDa protein, while the soluble extracellular portion of exogenous rhFLTR2 appears as a ~75 kDa protein band.

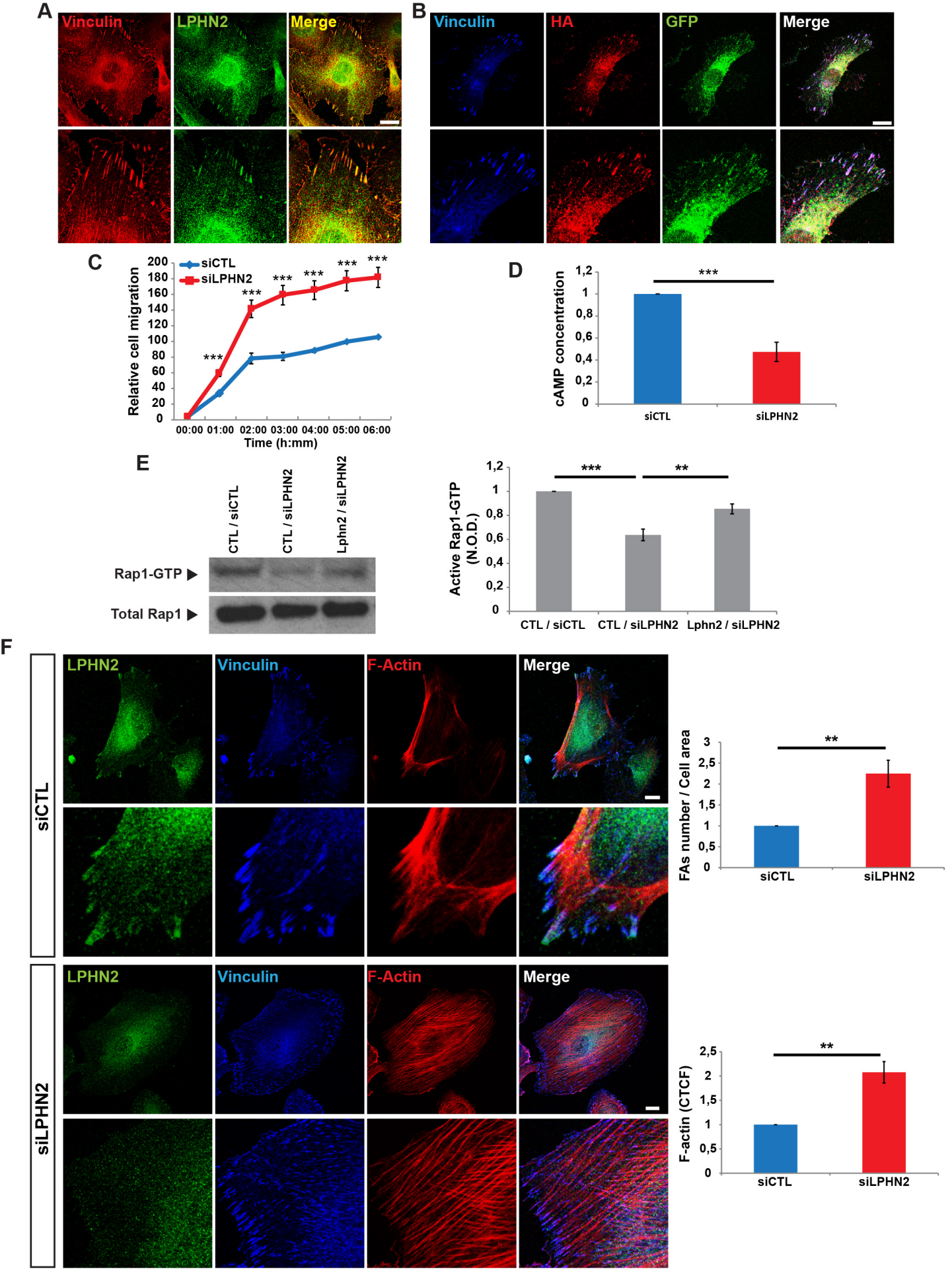
Figure S5, related to Figure 4, 5, and S6. Generation of a *lphn2* knock-out zebrafish strain by CRISPR/Cas9. (**A**) *lphn2a* (aka *adgrl2a*) zebrafish mRNA splicing variants displaying all exons. The black arrow outlines the *lphn2a* gene targeted region located in the second exon, which is common to all splicing variants. (**B**) DNA sequence details of the exon 2 *lphn2a* gene targeted region in *lphn2a*$^{+/+}$ and *lphn2a*$^{-/-}$ zebrafish embryos. Locus specific sgRNA is underlined and the protospacer-adjacent motif (PAM) sequence is labelled in bold italic. A 1-nucleotide insertion (A, red bold underscored), revealed by sequencing, and the consequent nine new amino acids (red) and STOP codon (red bold) are shown. (**C**) Alignment of *lphn2a*$^{+/+}$ and *lphn2a*$^{-/-}$ sequence obtained by Sanger

sequencing. (**D**) Schematic representation of genotyping. The one base insertion generates a second TasI restriction site in the diagnostic PCR, which has been used for genotyping.

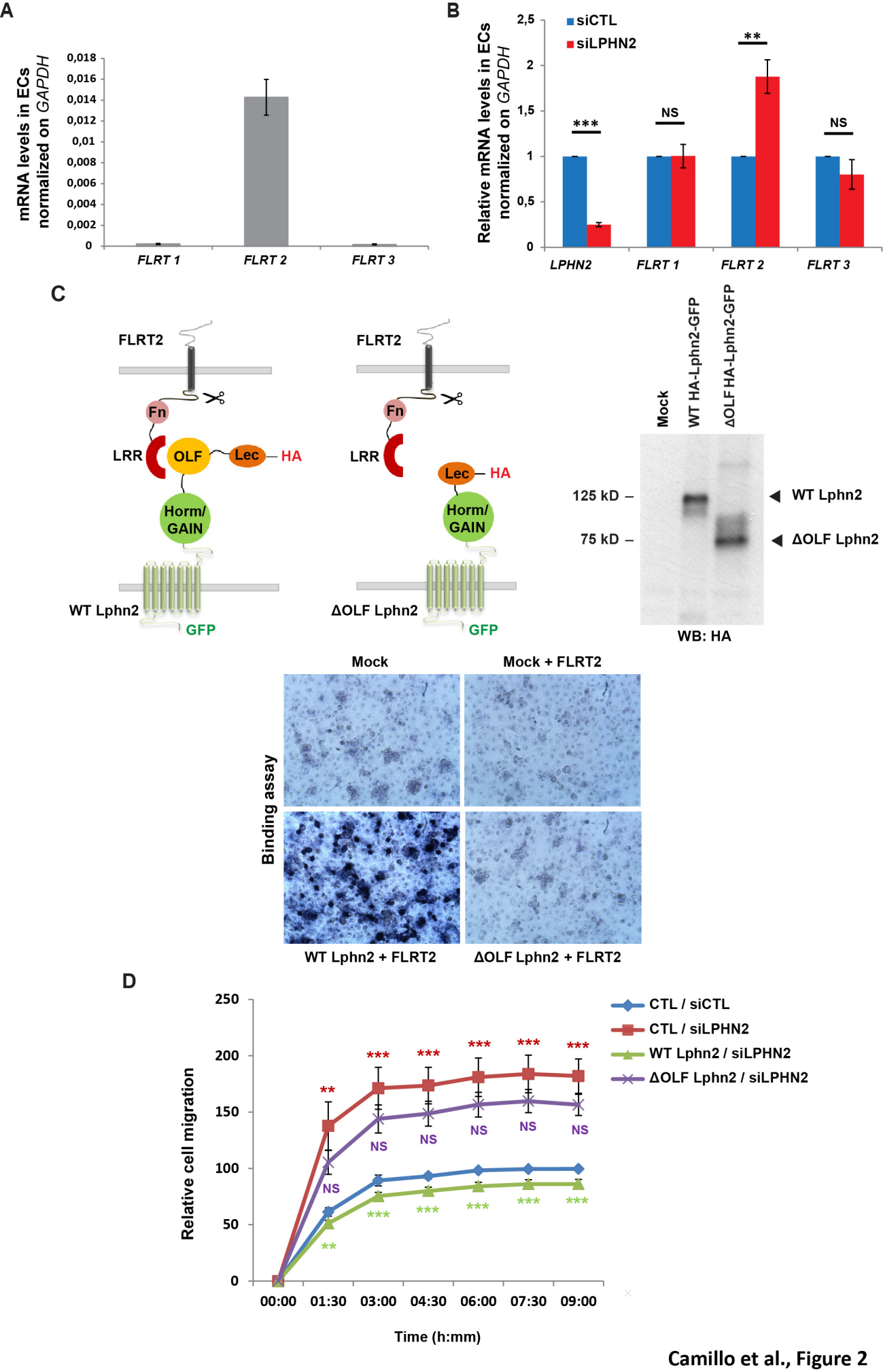
Figure S6, related to Figure 4, 5, and S5. Real time quantitative RT-PCR analysis of *flrt2* mRNA in *lphn2a*$^{+/+}$ or *lphn2a*$^{-/-}$ zebrafish embryos relative to the house keeping gene *eef1a1l1* and normalized on the mRNA levels measured in *lphn2a*$^{+/+}$ animals. Results are the average ± SEM of 5 independent assays (n>80 embryos for condition). Statistical analysis: results were analyzed by a Mann-Whitney test; $p \leq 0,05$ *.

Figure S7, related to Figure 1, 2, 3, and S1. Full images of blots that were cropped in main Figure 1, 2 and 3, and Figure S1. Yellow squares indicate the cropped images.

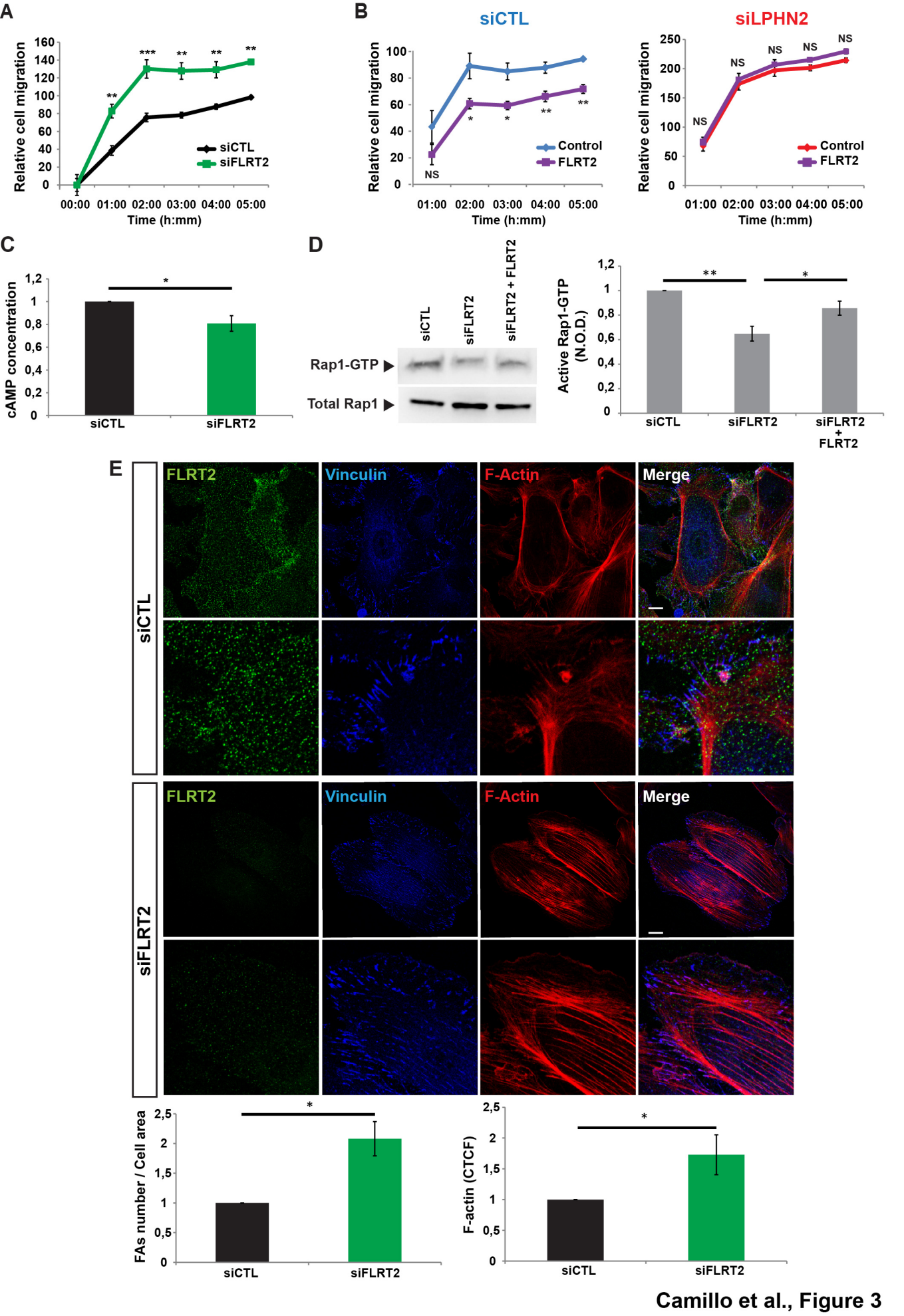
Figure S8, related to Figure S2 and S3. Full images of blots that were cropped in Figure S2 and S3. Yellow squares indicate the cropped images.



Camillo et al., Figure 1



Camillo et al., Figure 2



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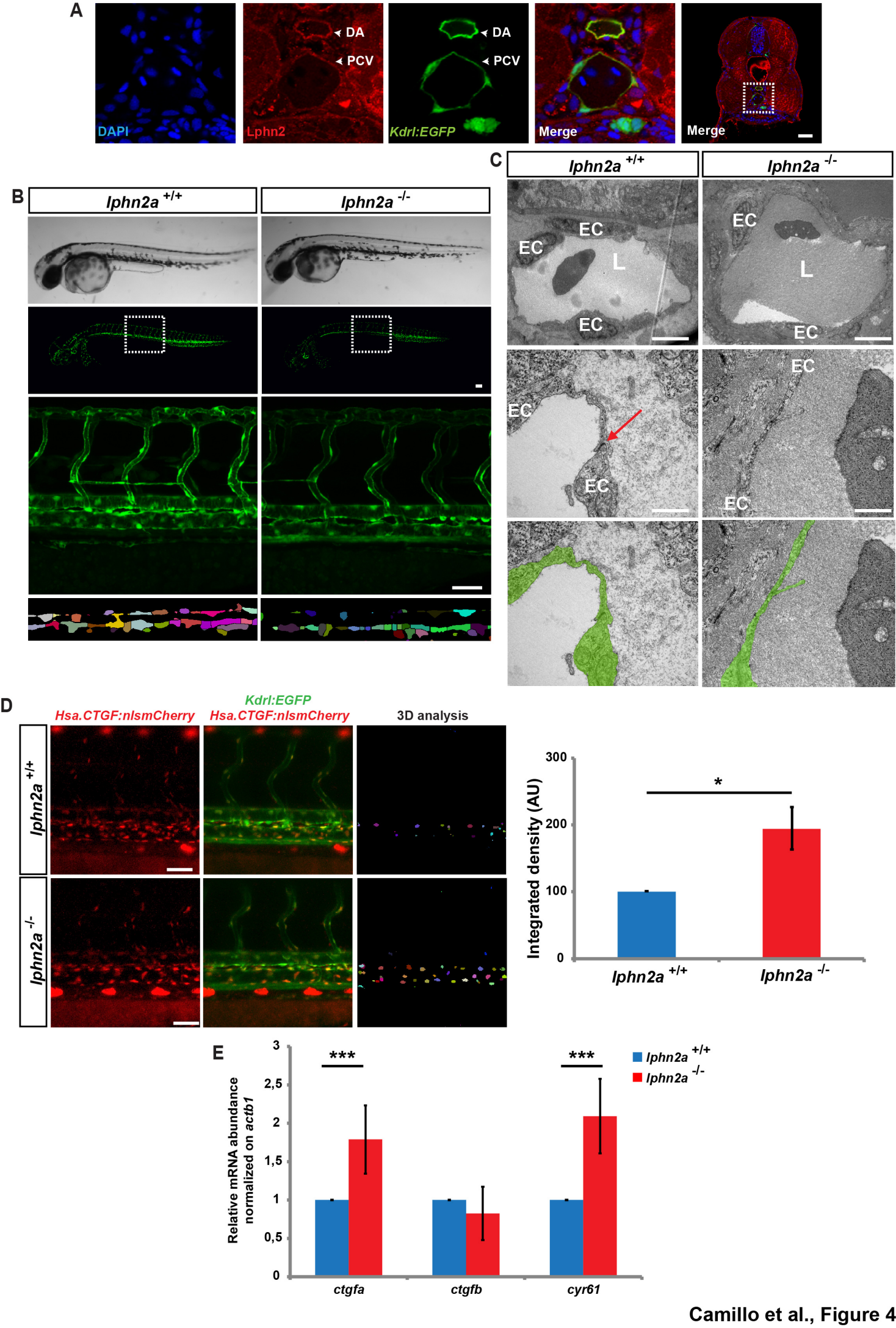
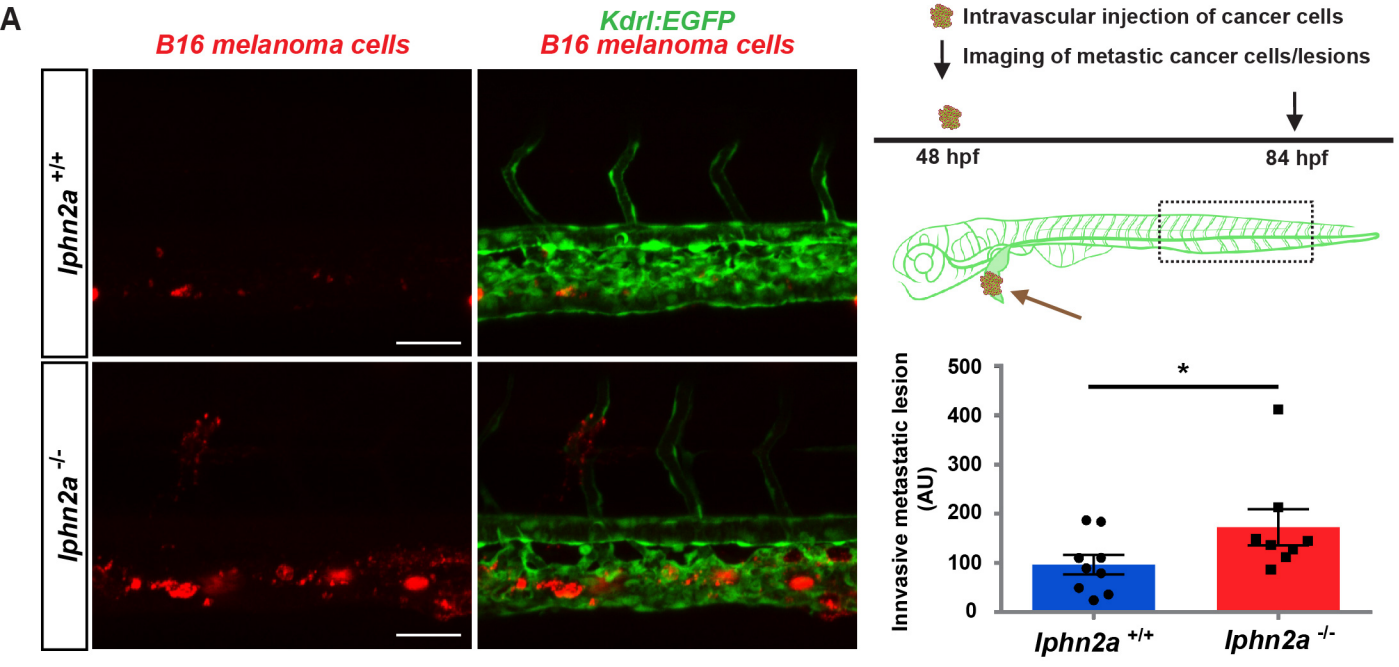
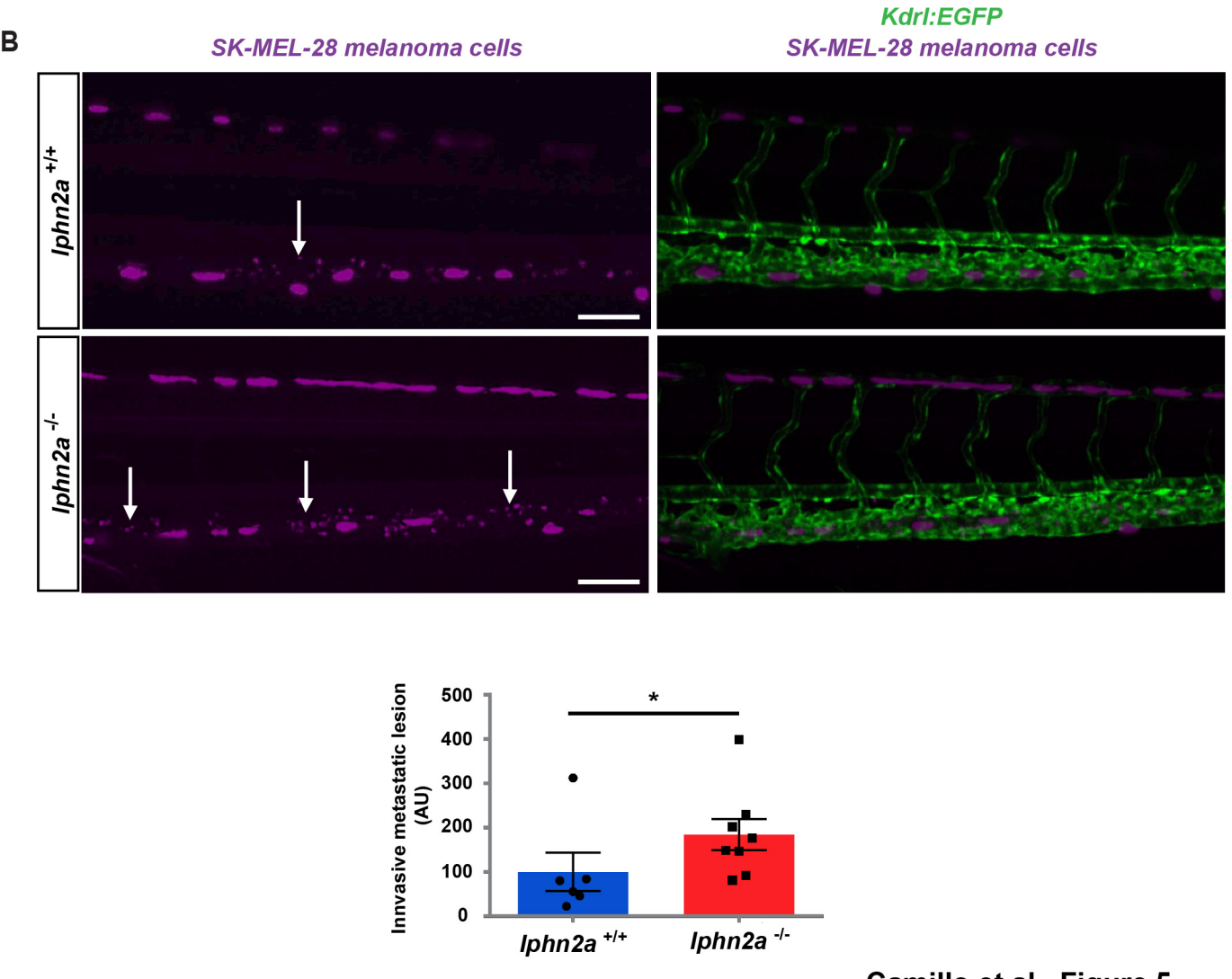


Camillo et al., Figure 4





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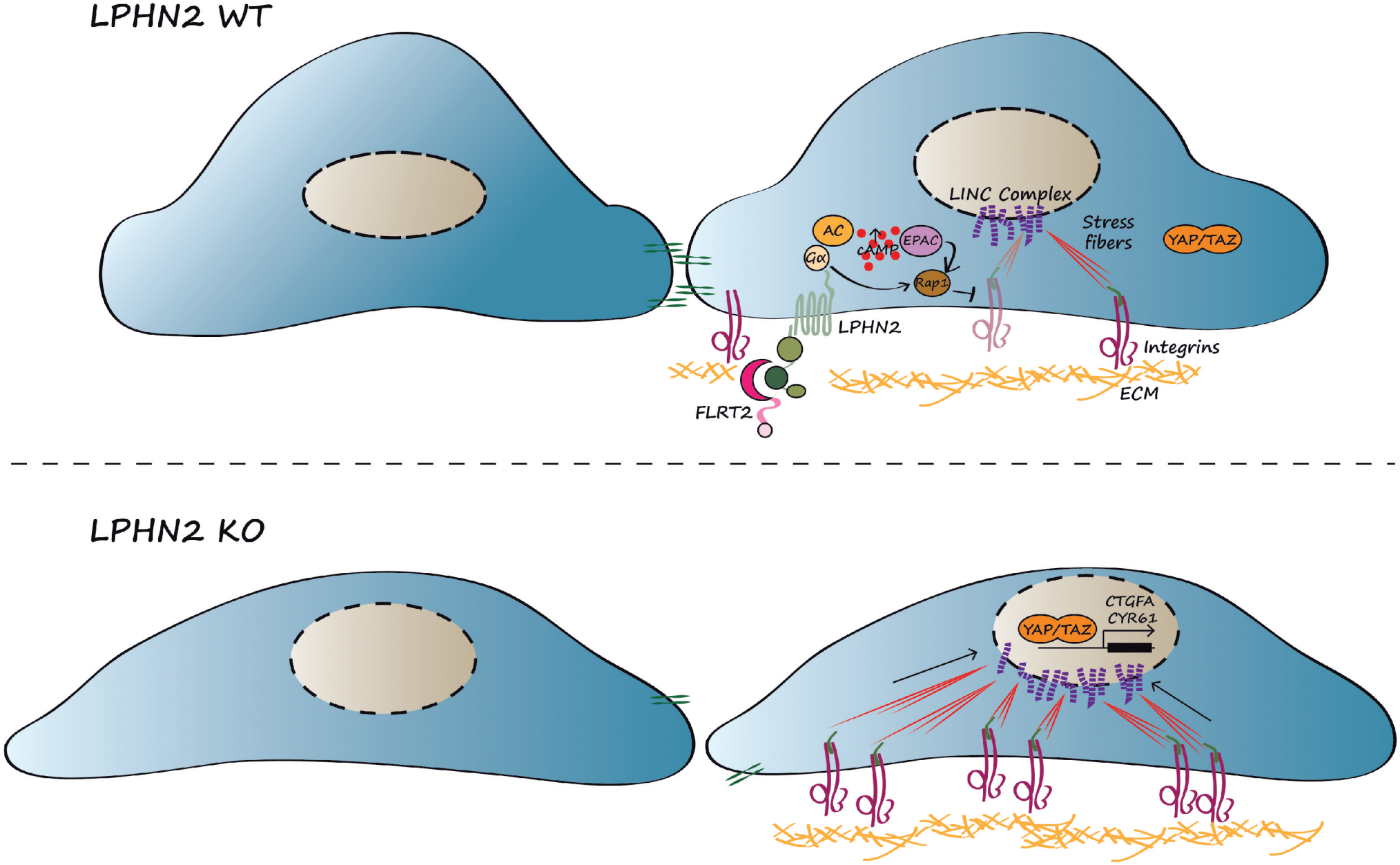

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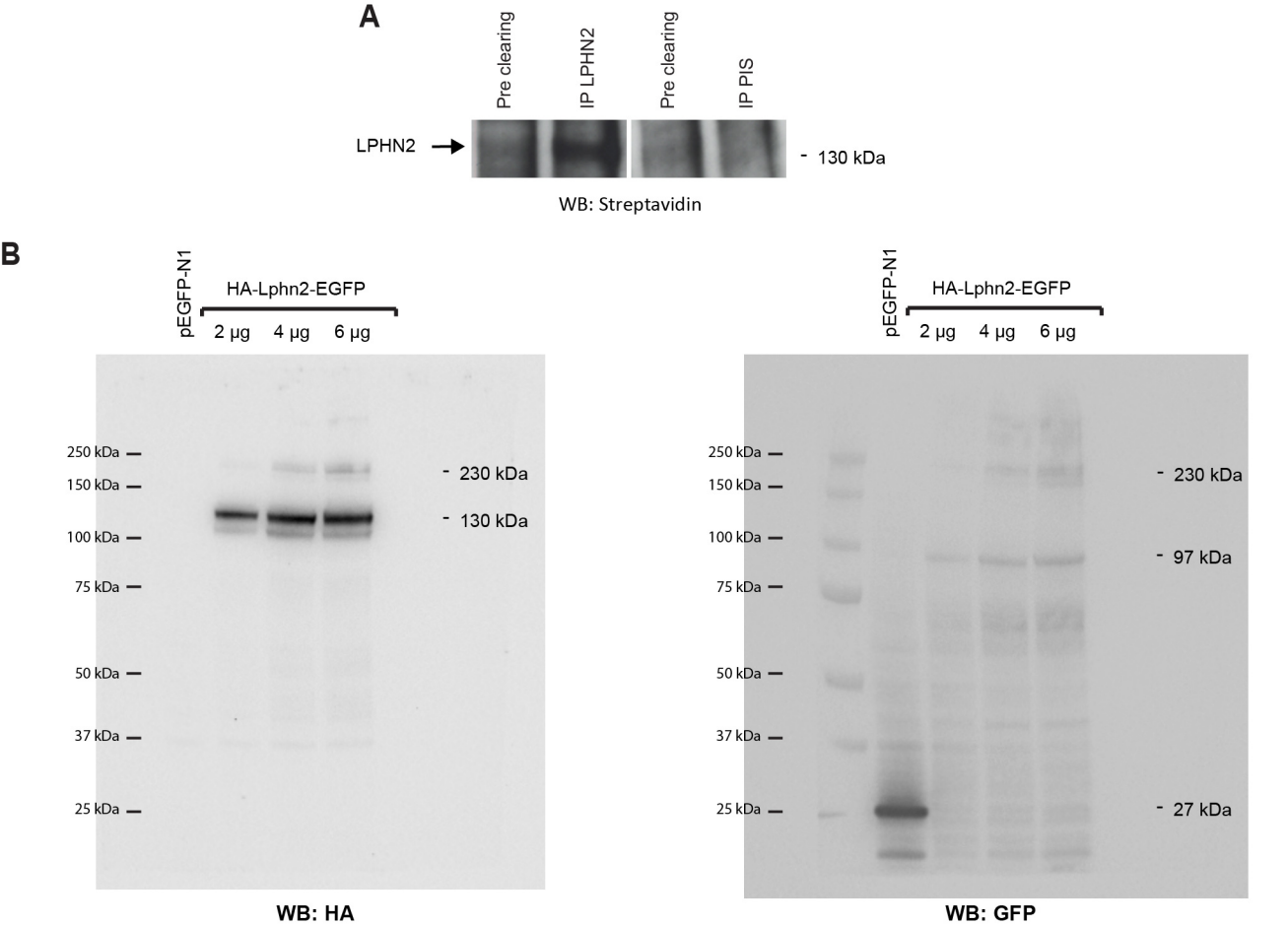
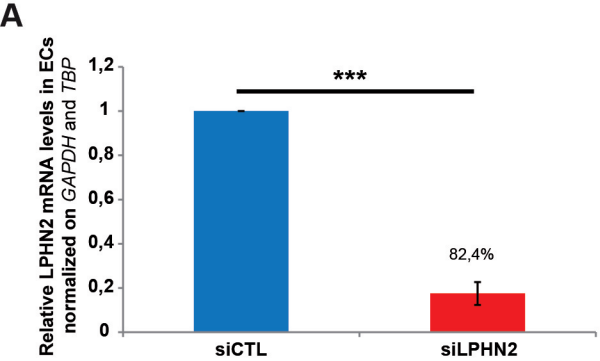
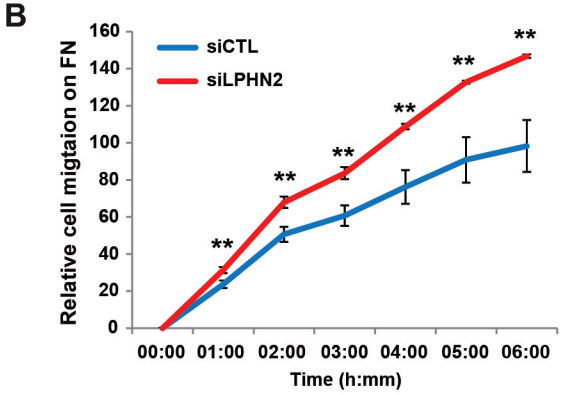
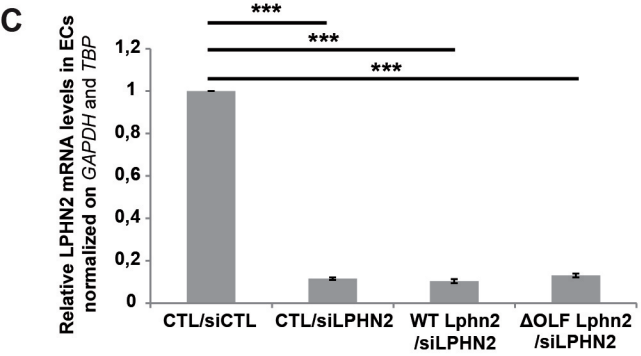
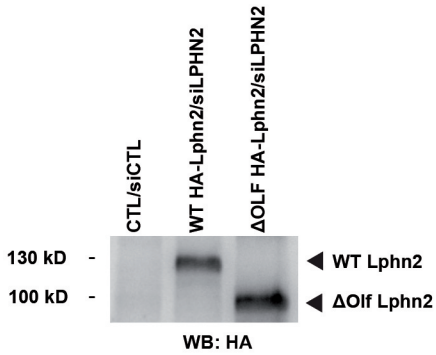


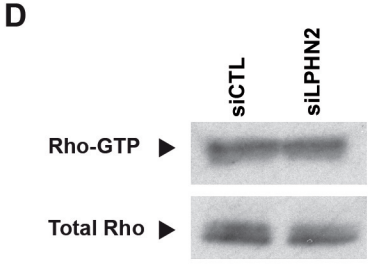
Camillo et al., Figure S1

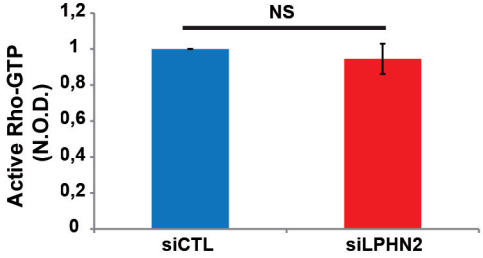












Camillo et al., Figure S2

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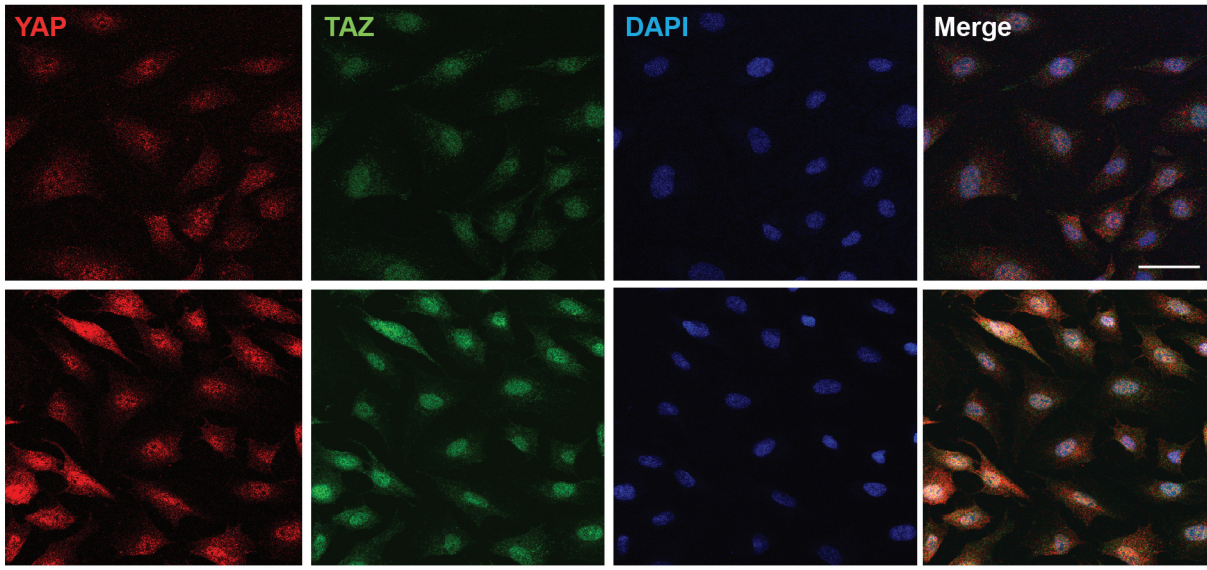

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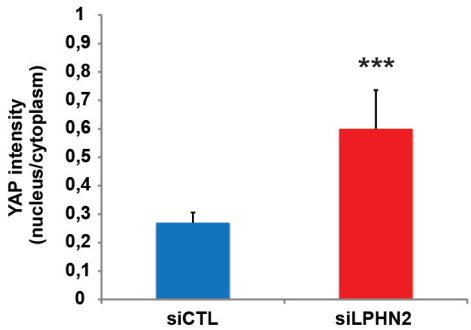

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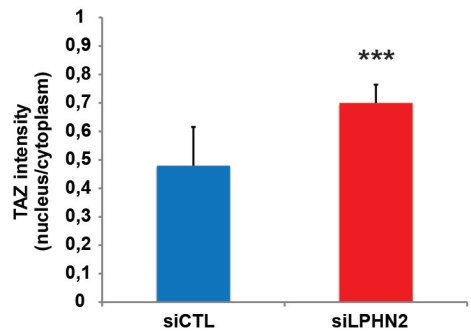

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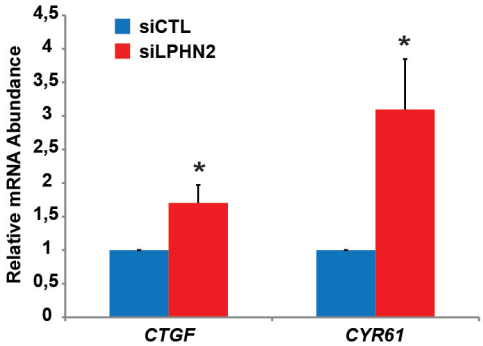

Camillo et al., Figure S3

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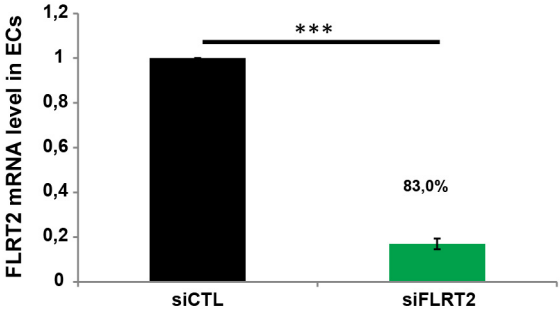

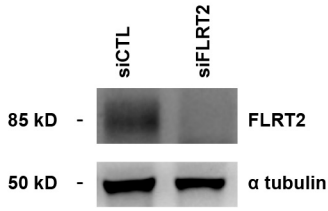

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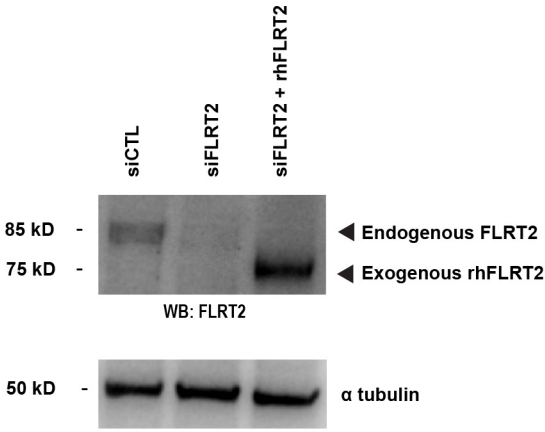
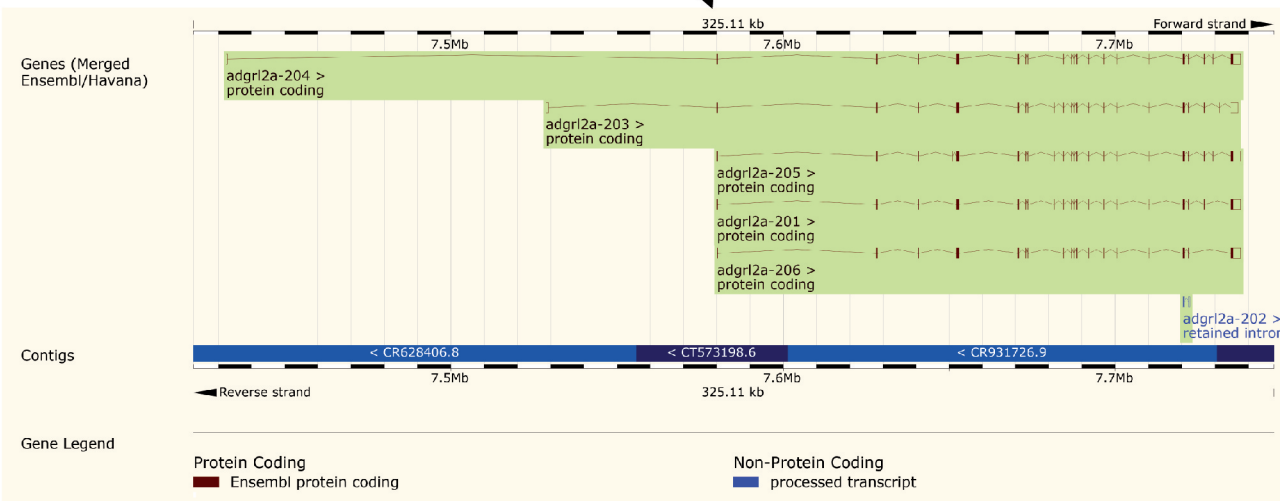


Camillo et al., Figure S4

A

Exon 2 CRISPR/Cas9 target



B

Iphn2a $^{+/+}$

```
61  GTCCTTTGTGGCCTTGTATTCGTCTTGACGGTTGTGCAGAGTTCTGACGGG  111
21   V  L  C  G  L  V  F  V  L  T  V  V  Q  S  S  D  G   37
```

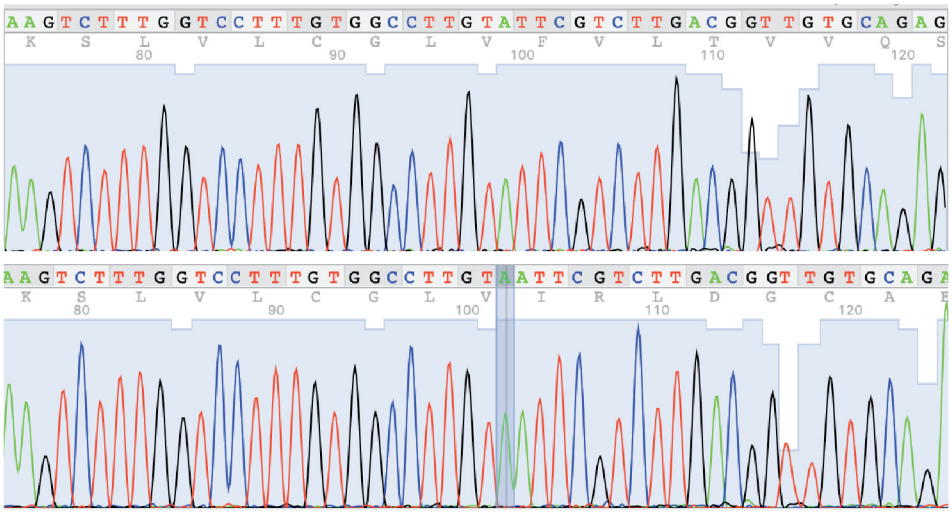
Iphn2a $^{-/-}$

```
61  GTCCTTTGTGGCCTTGTAATTCGTCTTGACGGTTGTGCAGAGTTCTGACGG  111
21   V  L  C  G  L  V  I  R  L  D  G  C  A  E  F  *      35
```

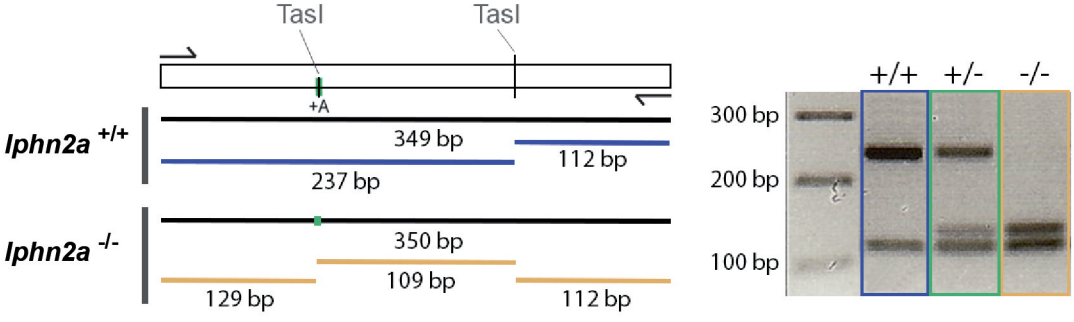
C

Iphn2a $^{+/+}$

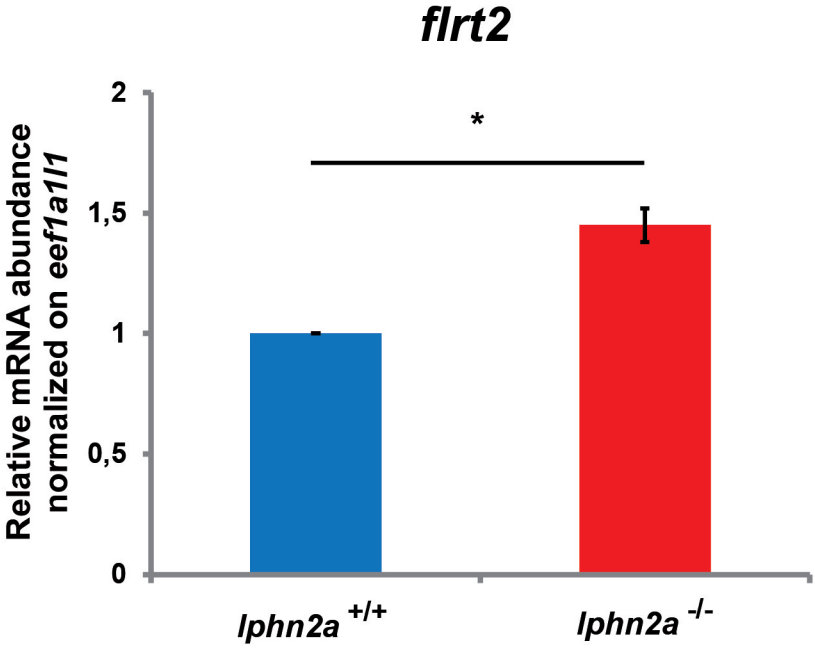
Iphn2a $^{-/-}$



D



Camillo et al., Figure S5



Camillo et al., Figure S6

Figure 1, panel E WB: Rap1

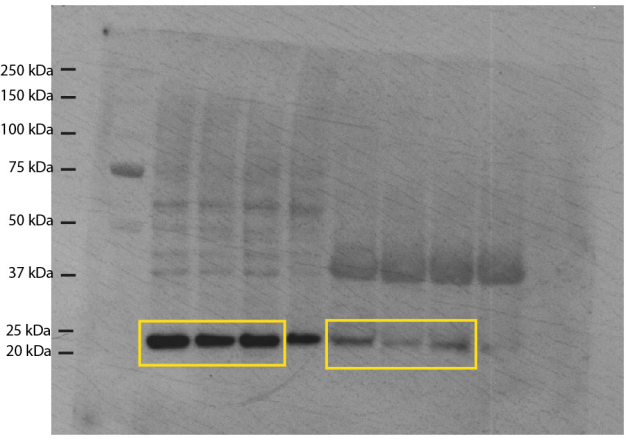


Figure 2, panel C WB: HA

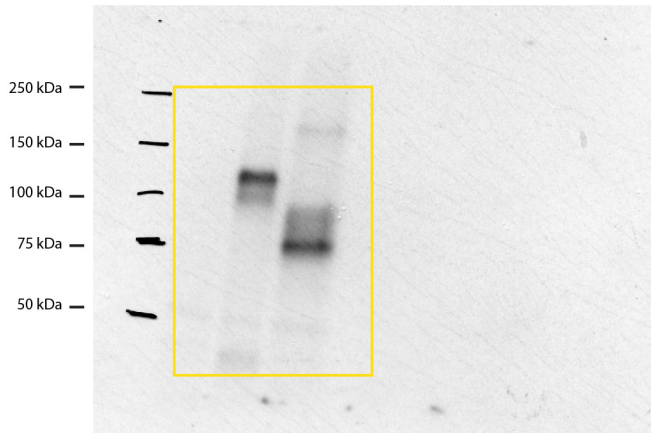


Figure 3, panel D WB: Rap1

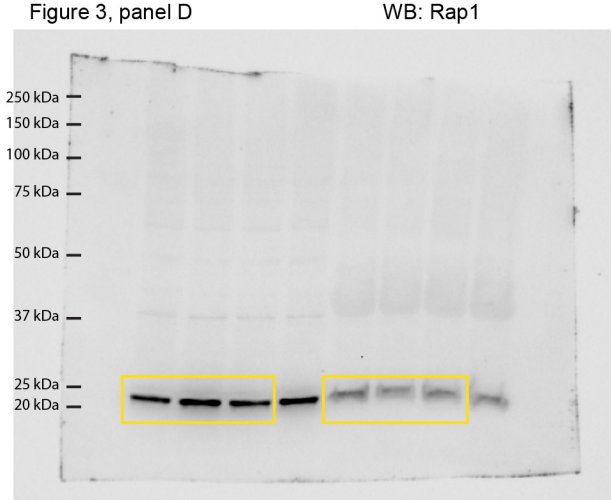
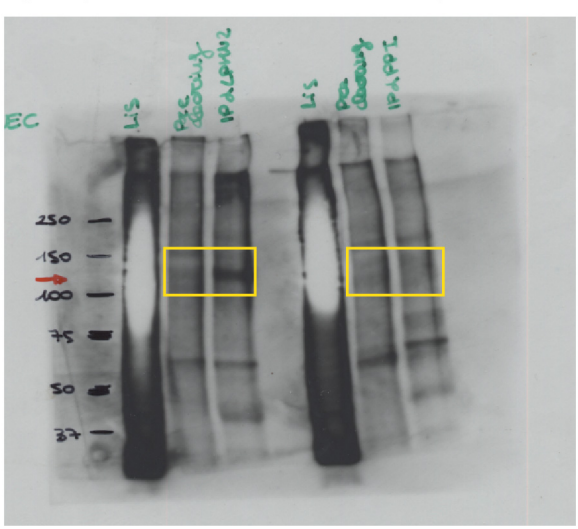


Fig. S1, panel A WB: Streptavidin



Camillo et al., Figure S7

Fig. S2, panel C

WB: HA

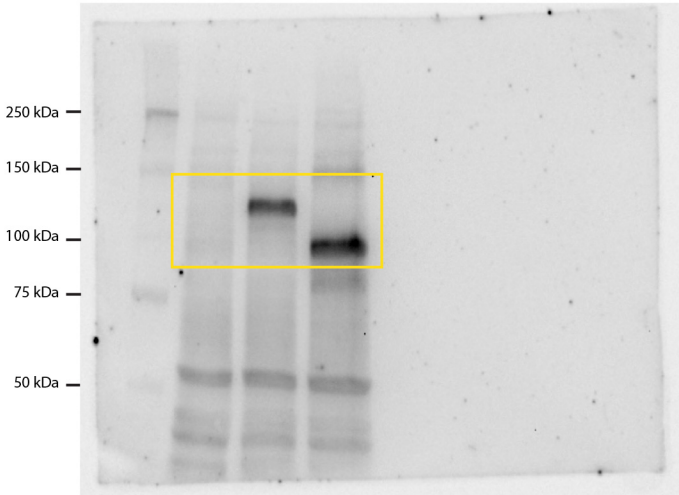
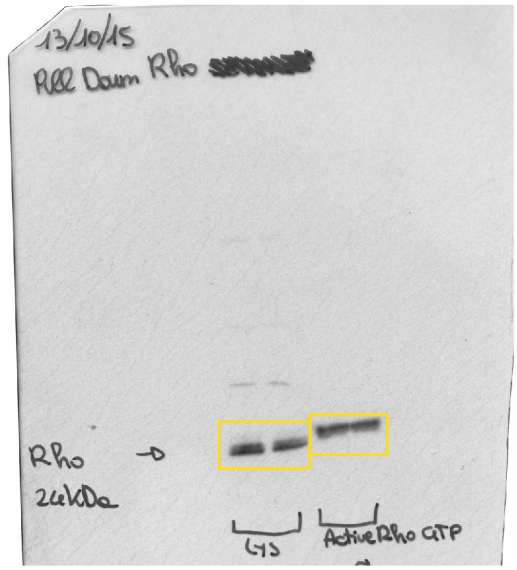


Fig. S2, panel D

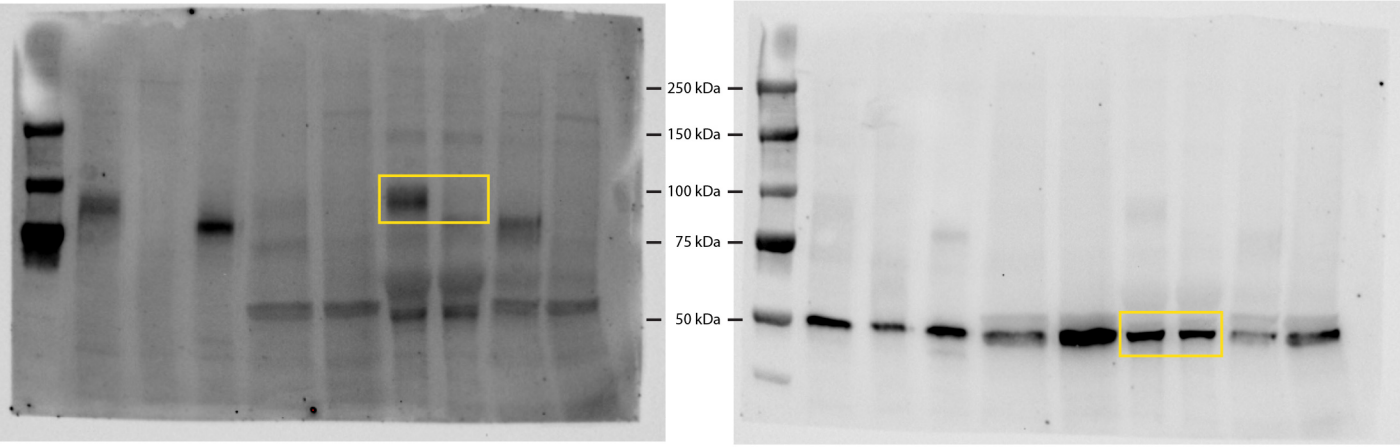
WB: Rho



WB: FLRT2

Fig. S3, panel A

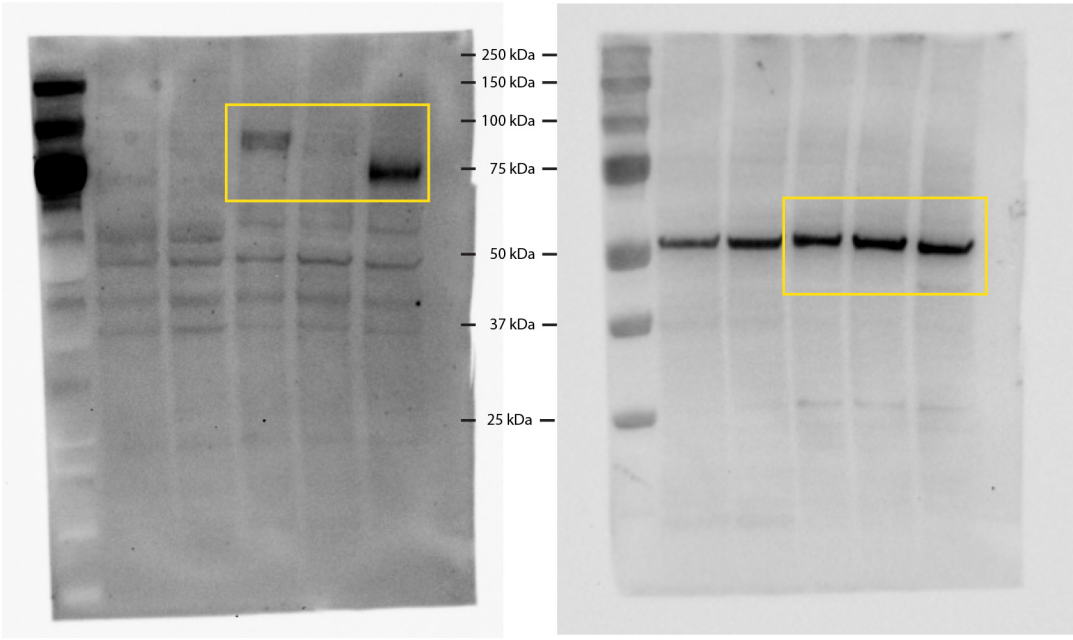
WB: α Tubulin



WB: FLRT2

Fig. S3, panel B

WB: α Tubulin



Camillo et al., Figure S8