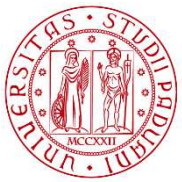


UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Head Office: Università degli Studi di Padova

Department of Biology

Ph.D. COURSE IN: BIOSCIENCES

CURRICULUM: BIOCHEMISTRY AND BIOTECHNOLOGY

SERIES XXX

The role of mitochondrial fission factor Drp1 in angiogenesis

Thesis written with the financial contribution of Fondazione Cariparo

Coordinator: Prof. Ildikò Szabò

Supervisor: Prof. Luca Scorrano

Co-Supervisor: Dr. Stéphanie Herkenne

Ph.D. student: Maya Chergova

Acknowledgements

First of all, I wish to thank Luca for giving me the opportunity to work with him and be a part of his lab. It was and still is a great experience from many points of view! I'm also very thankful to have worked on such an exciting and outstanding project! For that also a big "Thank you!" to Steph for having me on board and for everything during these 3 years. I feel extremely lucky to have had such great supervisors. Thank you for the advices, the discussions, the critics when needed, the freedom to make my own decisions and the trust you had in me and you always expressed, the knowledge and experience you've shared with me. It was great pleasure and fun working with you!

I want to thank all present and former lab members with whom I've had the chance to work. Thanks as well to everyone from Soriano, Giacomello and Ziviani's labs who were always around. You've all contributed to this great experience that it has been.

I wish to thank all the professors from the University of Padua who have supervised and discussed my work over the years, especially to Prof. Costa and Prof. Argenton. Many thanks for the help of everyone from microscope, mouse and informatics facilities from Valli and VIMM which was essential especially during times of "crisis".

I would like to express my gratitude to Prof. Francesco Cecconi and Prof. Heidi McBride who evaluated my thesis and whose suggestions certainly contributed to the improvement of the work.

Благодаря на всички близки и приятели, който са ме подкрепяли през тези години, който са вярвали мен, когато аз сама не съм. Благодаря на баба и дядо за винаги стиснатите палци! Нямаше да стане без вас! Благодаря на Томи, тате и Луис за подкрепата.

Благодаря ти, мамо! За всичко! За твоето „Всичко ще бъде наред!", което е било спасително толкова много пъти!

Обичам Ви!

Table of Contents

Summary

Angiogenesis is the process of new blood vessel formation from existing vasculature. Activation of endothelial cells (ECs) by vascular growth factors is an energy demanding process required for proliferation, migration and tridimensional rearrangements in order to form the new vessel tube. Vascular endothelial growth factor (VEGF) and its main receptor VEGFR2 are a crucial angiogenic signaling axis, involved in each step of the process and therefore precisely regulated on multiple levels. Over the last years basic research in the field of angiogenesis focused on alternative mechanism regulating VEGFR/VEGFR2 pathway. Thus, the role of mitochondrial dynamics and vesicular trafficking began to gain interest. This work focused on the role of mitochondrial fission factor Dynamin related protein 1 (Drp1) in ECs and blood vessel formation. Drp1 is a cytosolic GTPase whose activation leads to its translocation to the outer mitochondrial membrane where it forms ring-like multimeres that trigger GTP-dependent fission of the organelle. Initially, Drp1 has been described to localise at vesicle-like structure presumably along the cytoskeleton. Even though there have been evidences for its communication with the endosomal machinery this aspect of Drp1 functionality has been neglected.

Our work showed that angiogenic activation of HUVECs (Human Umbilical Cord Endothelial Cells) cells with pro-angiogenic factors (bFGF, VEGF, Tumor conditioned medium) modifies protein levels of mitochondrial shaping regulators. Levels of the pro-fusion protein Opa1 were upregulated, whereas Drp1 was downregulated. Short treatment with VEGF modulated Drp1 cytosolic distribution, which sparked our interest to test whether Drp1 could interact with VEGFR2. Indeed, immunoprecipitation and PLA analysis revealed an interaction between the two proteins following VEGF treatment. *DRP1* knock-down in HUVECs increased levels of activated VEGFR2 (P-VEGFR2 Y1175) and downstream ERK1/2. This pathway is crucial for endothelial cell proliferation and migration. Both angiogenic parameters were upregulated in *DRP1* knockdown cells compared to controls, suggesting that Drp1 negatively regulates basal angiogenesis *in vitro*.

To investigate the angiogenic role of Drp1 *in vivo*, we generated an endothelial specific mouse model for Drp1 ablation and analysed developmental vascularization in the mouse postnatal

retina. Consistently with our *in vitro* data, early stage (P2) vascular branching was significantly increased in *Drp1*$^{\Delta EC}$ pups. Showing that also *in vivo Drp1* ablation upregulates angiogenesis. However, at stage P6 no differences were observed between *Drp1*$^{\Delta EC}$ and *Drp1*$^{flox/flox}$ mice, suggesting that some compensatory mechanism take place to ensure normal vascular development. This conclusion was in line with the viability of *Drp1*$^{\Delta EC}$ adult mice.

The generation of an inducible endothelial *Drp1* knockout model, allowed us to approach the effects of acute *Drp1* deletion on P6 retinal vascularization. Surprisingly, *Drp1*$^{i\Delta EC}$ P6 vascularization was decreased due to a reduced number of tip cells at the angiogenic front and reduced branchings and width of microvessels. Showing that disregulated VEGFR2 activation could negatively affect the tip/stalk cell crosstalk and blood vessels maturation.

Mechanistically, we found that *DRP1* ablation does not affect VEGFR internalization, but interferes with the receptor's endocytic trafficking. Drp1 interacts with early endosomal marker Rab5 upon VEGF treatment. Localization of VEGFR2 in Rab5 positive early endosomes is crucial for its angiogenic signaling and downstream ERK1/2 activation. In line with the angiogenic upregulation, VEGFR2-Rab5 interaction is induced upon *DRP1* silencing.

In conclusion, we demonstarted that upon VEGF stimulation Drp1 interferes with VEGFR2-Rab5 communication and plays a negative role in Rab5-mediated VEGFR2 angiogenenic signaling. Drp1 ablation results in upregulated angiogenesis *in vitro* and *in vivo*, ultimately perturbing blood vessel stabilization.

Abbreviations and acronyms

ADOA	*Autosomal dominant optic atrophy*
ANG	*Angiopoietin*
ATP	*Adenosine tri-phosphate*
ADP	*Adenosine di-phosphate*
BAEC	*Bovine aortic endothelial cell*
BNGE	*BlueNative PAGE*
CaN	*Calcineurin*
CL	*Cristae lumen*
CL	*Cardiolipin*
CLW	*Cristae lumen width*
CJ	*Cristae junction*
CJW	*Electron transport chainElectron transport chain*
CMT2A	*Charcot-Marie-Tooth type 2*
CoA	*Coenzyme A*
CoQ	*Coenzyme Q*
CPT1a	*Carnitine palmitoyl-transferase 1A*
DAB2	*Disabled homologue 2*
DEP1	*Density-enhanced phosphatase 1*
Dll4	*Delta-like ligand 4*
DMEM	*Dulbecco's modified Eagle medium*
DNA	*Deoxyribonucleic acid*
Drp1	*Dynamin-related protein 1*
EC	*Endothelial cell*
ECM	*Extracellular matrix*
EEA1	*Early endosome antigen 1*
EGF	*Epidermal growth factor*
EGFR	*Epidermal growth factor receptor*
eNOS	*Endothelial nitric oxide synthase*
ERR	*Estrogen-related receptor*
ER	*Endoplasmic reticulum*
ETC	*Electron transport chainElectron transport chain*
$FADH_2$	*Flavin adenine dinucleotide*
FAO	*Fatty acid oxydation*
FAK	*Focal adhesion kinase*
FBS	*Fetal bovine serum*
FGF	*Fibroblast growth factor*
FM	*Flavin mononucleotide*
GDAP 1	*Ganglioside-induced differentiation-associated protein 1*
GED	*GTPase effector domain*
GLS	*Glutaminase*
G6P	*Glucose-6-phosphate*
G6PD	*Glucose-6-phosphate dehydrogenase*
GP	*Glycogen phosphorylase*
PhKG1	*Phosphorylase kinase subunit G1*
GTP	*Guanosine tri phosphate*

IBM	*Inner boundary membrane*
IMM	*Inner mitochondrial membrane*
IMS	*Intermembrane space*
INF2	*Inverted formin 2*
I/RP	*Inchaemia/Reperfusion*
HD	*Huntington's disease*
HIF	*Hipoxia-induced factor*
HSR	*Heparan sulfate proteoglycan*
Htt	*Huntingtin*
HUVEC	*Human umbilical vein endothelial cell*
kDa	*kilo Dalton*
KLF2	*Krüppel-like factor 2*
LDL	*Low-density lipoprotein*
LRP-1	*Low-density lipoprotein receptor-related protein 1*
LRRK2	*Leucine rich repeat kinase 2*
MAMs	*Mitochondrial associated membrane*
MAO	*Monoamine oxidase*
MAP	*Microtubules associated protein*
MAPL	*Mitochondria-anchored protein ligase*
Mff	*Mitochondrial fission factor*
Mfn1/2	*Mitofusin 1/2*
MICOS	*Mitochondrial contact site complex*
MMPs	*Matrix metalloproteinases*
mtDNA	*Mitochondrial DNA*
mRNA	*Messenger RNA*
mtROS	*Mitochondrial eactive oxygen species*
MTP18	*Mitochondrial protein 18kDa*
mtSSB	*Mitochondrial single strand binding protein*
NADH	*Nicotinamide adenine dinucleotide*
NCID	*Notch intracellular domain*
NEC	*Normal endothelial cell*
NO	*Nitric oxide*
NRF	*Nuclear respiratory factor*
NRPs	*Neuropilins*
O-GlcNAc	*O-linked N-acetylglucosamine*
OGT	*O-GlcNAc transferase*
OMM	*Outer mitochondrial membrane*
Opa1	*Optic atrophy 1*
OXPHOS	*Oxidative phosphorylation*
PA	*Phosphatidic Acid*
PAEC	*Pulmonary artery endothelial cell*
PASMC	*Pulmonary artery smooth muscle cell*
PAH	*Pulmonary artery hypertension*
PAR3	*Partitioning defective 3 homologue*
PARP	*Peroxisome proliferator-activated receptor protein*
PECAM	*Platelet endothelial cell adhesion molecule*
PD	*Parkinson's disease*
PDGF	*Platelet-derived growth factor*

PDGFR	*Platelet-derived growth factor receptor*
PDH	*Pyruvate dehydrogenase*
PFKFB3	*6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3*
PGC-1	*Proloferator-activated receptor gamma coactivator*
PHB	*Prohibitin*
PI3K	*Phosphatidyl-inositol 3-kinase*
PKA	*Protein kinase A*
PlGF	*Placenta growth factor*
3PG	*3-phosphoglycerate*
PM	*Plasma membrane*
PPP	*Penthose phosphate pathway*
PRPP	*Phosphorybosyl pyrophosphate*
PVR	*Pulmonary vascular resistance*
RCC	*Respiratory chain complexes*
RCS	*Respiratory chain supercomplexes*
RNA	*Ribonucleic acid*
ROCK1	*Rho-associated coil-containing protein kinase*
ROS	*Reactive oxygen species*
PRC	*PGC-1α-telated coactivator*
R5P	*Ribose 5-phosphate*
rRNA	*Ribosomic RNA*
SBP	*Serine biosynthesis pathway*
SC	*Stalk cell*
S1P	*Sphingosine-1-phosphate*
SIRT1	*Sirtuin 1*
SMC	*Smooth muscle cell*
SNARE	*Soluble N-ethylmaleimide-sensitive factor attachment protein receptor*
TC	*Tip cell*
TCA	*Tricarcoxylic acid*
TEC	*Tumoral endothelial cell*
TGF	*Transformation growth factor*
TIM	*Translocase of the inner membrane*
TH	*Thyroid hormone*
TOM	*Translocase of the outer membrane*
tPA	*Tissue plasminogen activator*
uPA	*Urokinase plasminogen activator*
uPAR	*Urokinase plasminogen activator receptor*
tRNA	*Transfer RNA*
VD	*Variable domain*
VEGF	*Vascular endothelial growth factor*
VEGFR	*Vascular endothelial growth factor receptor*
VEPTP	*Vascular endothelial protein tyrosine phosphatase*

Introduction

Mitochondrial-shaping proteins – known and unknown functions

Mitochondrial morphology has been established as key regulator of a variety of cellular processes such as differentiation and cell death. The shape of this organelle is constantly undergoing changes in order to respond to the physiological needs of the cell. Fusion and fission processes are ensuring this dynamic structure. Fusion is mediated by the outer mitochondrial membrane Mfn1 and 2 and the inner membrane Opa1, whereas fission is orchestrated by the cytosolic Drp1 and its outer mitochondrial membrane adaptor proteins (Fis1, Mff). Although the roles of these proteins in fusion and fission events are well established extra functions are being described as well. In the case of Opa1 it is an anti-apoptotic role by regulating the cristae junctions. Being an outer mitochondrial membrane also localized at the endoplasmic reticulum (ER), Mfn2 is participating in mitochondrial-ER contact sites important for Ca^{2+} and lipid exchange. This shows that, although essential in some cases, disruption of mitochondrial dynamics is not always the functional answer for phenotypes related to mitochondrial-shaping proteins. This idea should be considered when we think about Drp1, a cytosolic GTPase, known to regulate mitochondrial and peroxisomal fission, initially reported to localize at microvesicular compartments and highly regulated on multiple levels in a physiological and cell-dependent manner. Drp1 ablation is embryonically lethal in patients and mouse models and yet its activity is often considered to have a toxic effect. Reports on Drp1 functionality are controversial leaving the molecular mechanism behind certain Drp1-related phenotypes unclear. This could be due to the fact that Drp1 activity is mainly analyzed in the fission axis.

This work gives an overview on mitochondrial structure, functionality and dynamics and their role in the process of angiogenesis with a focus on Drp1. Using the physiological context of angiogenesis, we discovered a new function of Drp1 in endosomal trafficking which will contribute to the better understanding of its complex nature.

I. Mitochondria – much more than the power house of the cell

I.1. Origin and Structure

Mitochondria originated from the symbiosis between an eukaryote ancestor and the α-protobacterium approximately 1,5-2 billion years ago. While for many years mitochondrial structure relied on the use of conventional electron microscopy (EM) performed on ultrathin sections, the development of electron tomography allowed to generate a 3D reconstruction model of mitochondrial ultrastructure (Frey and Mannella, 2000; Frey et al., 2002). According to this model, mitochondria are organized in five compartments: the outer mitochondrial membrane (OMM), the inner mitochondrial membrane (IMM) further subdivided in the inner boundary membrane (IBM) and in cristae, the intermembrane space (IMS), and the matrix (Fig.1) (Cogliati et al., 2016).

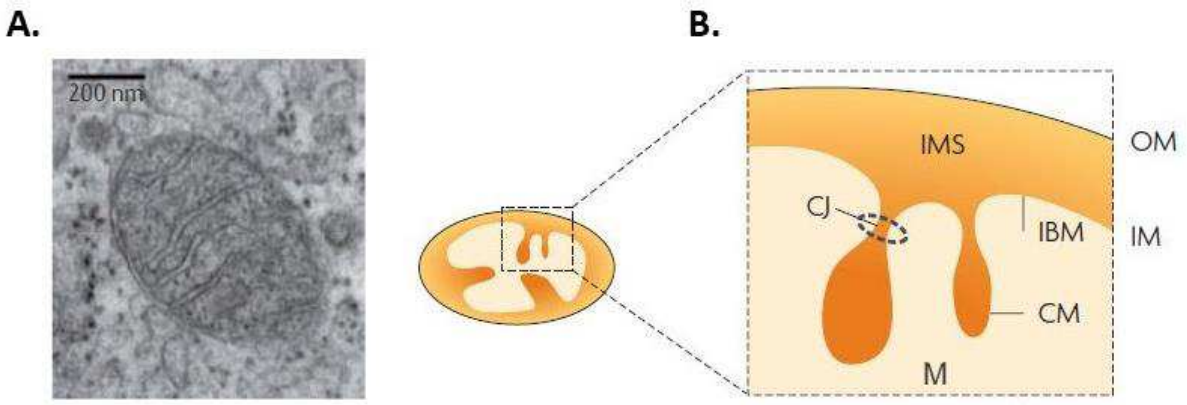


Figure 1. Mitochondrial ultrastructure. A) Electron microscopy image of the mitochondrion from a human fibroblast cell. B) Schematic representation of the structural organization of the mitochondria. OM-Outer membrane; IM-inner membrane; IMS-Intermembrane space; IBM-Inner boundary membrane; CJ-cristae junction; CM-cristae membrane, M-matrix. From (Detmer and Chan, 2007; Westermann, 2010).

I.1.1. The outer mitochondrial membrane (OMM)

The two mitochondrial membranes differ in their composition, reflecting their different functions. The OMM separates and connects the mitochondria to the cytosol and other organelles. Its composition is more homogenous compared to the IMM (see section I.1.2.) and it contains fewer proteins, which are all nuclear encoded. OMM proteins could be integral or peripherally bound and they are involved in a variety of processes such as: mitochondrial

import, which is mainly regulated by the translocase of the outer membrane (TOM) complex, pro (Bak and Bax) and anti-apoptotic (BCL-2) factors, pore forming proteins such as the voltage dependent anion channel (VDAC), organelle contact-mediating proteins such as Mfn1 and 2 (de Brito and Scorrano, 2008a, b; Ishihara et al., 2004).

I.1.2. The inner mitochondrial membrane (IMM)

The inner mitochondrial membrane (IMM) is characterized by high concentrations of cardiolipin and high protein-lipid ratio (de Kroon et al., 1997; Zinser et al., 1991) and it can be further subdivided in: inner boundary membrane (IBM) and the cristae (Vogel et al., 2006).

I.1.2.1. The inner boundary membrane

The IBM corresponds to the continuous and closely OMM-connected IMM (Fig.1), forming a continuous "contact site" between the two membranes (Frey et al., 2002). Functionally, this structure is reported to be involved in energy transfer from the matrix to the cytosol (Brdiczka et al., 2006) and in lipid exchange between OMM and IMM (Tatsuta et al., 2014). The IBM also contains the TIM (Translocase of the inner membrane) protein complex involved in mitochondrial protein import into the IMM or to the matrix (Reichert and Neupert, 2002).

I.1.2.2. The cristae

At certain regions the IMM membrane is forming deep invaginations into the matrix, defined as cristae (Perkins et al., 1997). Cristae are involved in iron-sulfur biogenesis (Zick et al., 2009), mitochondrial DNA maintenance (Vogel et al., 2006), assembly of respiratory chain complexes and supercomplexes (Cogliati et al., 2013a; Gilkerson et al., 2003) and apoptotic signaling (Scorrano et al., 2002). Crucial cristae regions are the cristae lumen (CL), which is the space inside the cristae invagination, and the cristae junction (CJ) (Fig.1), which corresponds to the opening of the cristae into the IBM (Frey and Mannella, 2000). The molecular regulation of cristae morphology as well as its physiological relevance are discussed in details in chapter II.1.2.

I.1.3. The matrix

The matrix corresponds to the space delimited by the IMM. It is relatively more viscous than the cytoplasm and it contains mitochondrial DNA and ribosomes as well as enzymes, co-factors, ions required for the metabolic reaction that take place there.

I.1.4. Mitochondrial DNA

In vertebrates, mitochondrial DNA consists of a double stranded circular DNA molecule of approx. 16,5 kb (Fig.2). Structure and sequence of mtDNA are highly conserved among mammals. The two strands of mtDNA molecule could be distinguished by their composition in Guanine (G) and Thymidine (T) bases, resulting in different buoyant densities of the “heavy” (H) and “light” (L) strands in denaturating cesium chloride gradients (Kasamatsu and Vinograd, 1974). Mitochondrial DNA encodes thirteen genes of proteins of the oxidative phosphorylation (OXPHOS) system, 2 ribosomal RNAs and 22 transport RNAs (Fig.2). The majority of the genes are located on the H strand (Yoon et al., 2010).

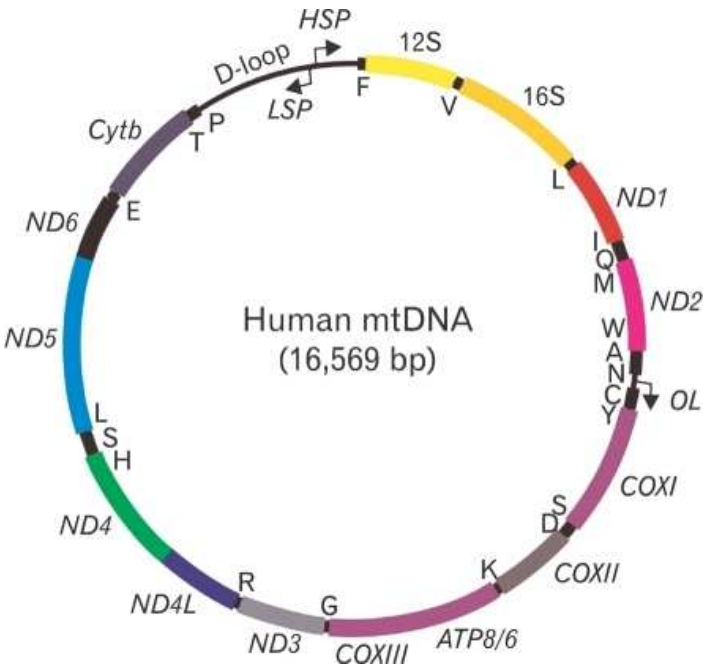


Figure 2. Schematic representation of the human mtDNA structure. The genome encodes two ribosomal RNAs (12S and 16S), 22 transfer RNAs (indicated by single letter abbreviation) between the coding genes, and 13 genes, encoding for subunits of the oxidative phosphorylation enzyme complexes. The major noncoding regions in the genome are the D-loop region which includes heavy and light-strand promoters (HSP and LSP), and the origin of L-strand replication (OL). From (Yoon et al., 2010).

Structurally, mtDNA is bound to proteins in structures called nucleoids (Legros et al., 2004). These proteins are playing both structural and functional roles in mitochondrial DNA replication and transcription (Bogenhagen, 2012; Bogenhagen et al., 2008). Nucleoid size and number may vary in response to physiological changes; moreover, maintenance of mtDNA integrity is essential and mtDNA mutations cause not only primary mitochondrial diseases, but are also associated to a variety of pathological conditions (Taylor and Turnbull, 2005).

I.2. Mitochondrial biogenesis

Mitochondrial content is a key factor to define the respiratory capacity of a cell and is therefore modulated in response to energy demands. For example, muscle exercise, browning of adipose tissue as well as neuronal activity stimulate mitochondrial biogenesis. As discussed in details in chapter IV, pro-angiogenic signals induce mitochondrial biogenesis in endothelial cells (Wright et al., 2008). Mitochondrial biogenesis relies on a precise cross-talk between mitochondrial and nuclear gene expression. The main pathways and players of mitochondrial biogenesis are summarized in figure 3.

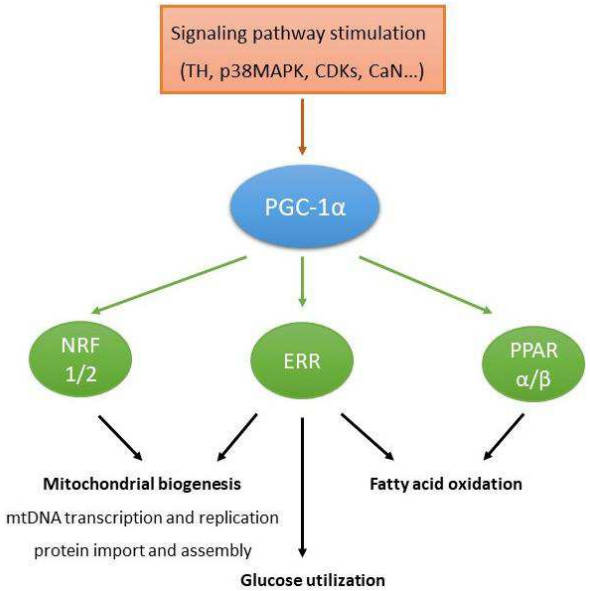


Figure 3. PGC1α signaling pathway. Depending on the physiological context a variety of signaling pathways, including Thyroid hormone (TH), p38 mitogen-activated protein kinase (p38MAPK), calcineurin (CaN) and many others have been shown to regulate expression and/or activity of PGC-1α. PGC-1α then co-activates transcription factors such as nuclear respiratory factors (NRFs), estrogen-related receptors (ERRs), and PPARs, known to regulate different aspects of energy metabolism including mitochondrial biogenesis and fatty acid oxidation. From (Ventura-Clapier et al., 2008).

I.3. The role of mitochondria in energy conversion

The major biochemical reactions taking place in the mitochondria are the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO). The link between them allows the fine metabolic tuning between ATP production, biosynthesis of fatty acids, amino acids, ketone-bodies and heme.

I.3.1. The tricarboxylic acid (TCA) cycle

Also known as Krebs cycle, the TCA cycle (Fig.4) leads to the complete oxidation of nutrient-derived carbons to CO_2 together with production of three Nicotinamide adenine dinucleotide (NADH), one Flavin adenine dinucleotide ($FADH_2$) and one Adenosine tri-phosphate (ATP) molecule per cycle. Acetyl-Coenzyme A (acetyl-CoA) is the starting molecule of the TCA cycle and it could originate from glycolysis-derived pyruvate or from β-oxidation of fatty acids. TCA cycle is linked to amino acids biosynthesis via its intermediate metabolites (Fig.4).

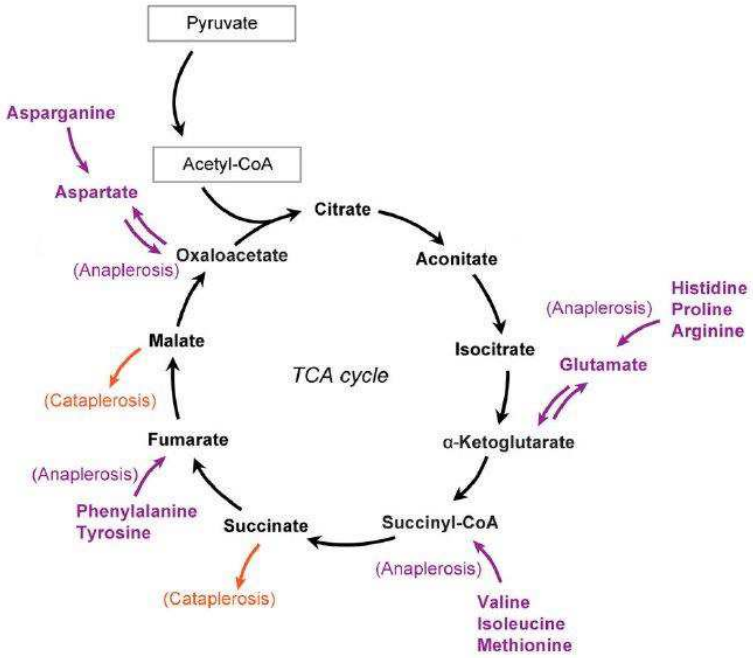


Figure 4. Schematic representation of the TCA cycle and its intermediate metabolites involved in cata and anaplerosis pathways. From (Diakos et al., 2016).

I.3.2. Electron transport chain (ETC) and Oxidative phosphorylation (OXPHOS)

Oxidative phosphorylation (OXPHOS) is the process whereby the electrons stripped from the reducing equivalents extracted from the catabolic intermediates by the TCA are transferred to molecular oxygen and the resulting energy is used to drive ATP biosynthesis. The electron transport chain (ETC), composed by multiprotein complexes localized within the IMM, is the enzymatic machinery behind OXPHOS (Fig.5).

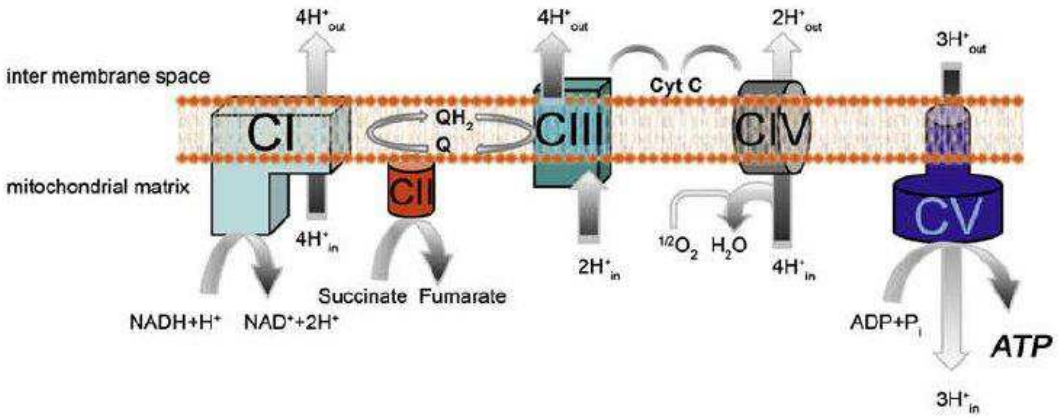


Figure 5. Model of the mitochondrial electron transport chain and OXPHOS process. Complex I, the NDH-CoQ oxidoreductase or NADH dehydrogenase, is the main entry point of electrons into the ETC. It oxidizes NADH, derived from TCA cycle, to NAD^+ and transfers electrons to coenzyme Q (CoQ), inducing the pumping of 4 protons across the IMM. Complex II, the succinate dehydrogenase complex, oxidizes carbons from succinate and transfers electrons through $FADH_2$, also derived from TCA cycle, to CoQ. Complex II does not pump protons into the IMS. Complex III, the cytochrome bc1 complex, is collecting electrons from $CoQH_2$ and transfers them to cytochrome c while pumping 4 protons across the IMM. Complex IV or the cytochrome c oxidase, transfers electrons from cytochrome c to the final electron acceptor O_2. This reaction is accompanied by the pumping of 2 protons. At the end of the ETC, ATP synthase generates ATP. The protons pumped into the IMS backflow through the ATP synthase into the matrix generating a rotation of the ATP synthase that initially produces mechanical force converted into chemical energy and allowing the coupling of adenosine di-phosphate (ADP) with inorganic Phosphor into adenosine tri-phosphate (ATP). From (Acin-Perez et al., 2008).

For a long time respiratory chain complexes were considered as individual complexes distributed randomly throughout the IMM – this was referred to as the fluid model (Hackenbrock et al., 1986). However, using BlueNative PAGE (BNGE) experiments in yeast, it has been demonstrated that respiratory complexes are organized into supercomplexes (SCs) (plasticity model) (Acin-Perez et al., 2008) (Fig.7). After construction of each single complex,

respiratory chain complexes assemble into dynamic functional structures depending on the energetic needs of the cell (Acin-Perez et al., 2008; Schagger, 1995).

I.3.3. Fatty acid β-oxidation

Fatty acid oxidation (FAO) is the catabolic process by which fatty acid molecules are broken down to generate acetyl-CoA, which enters the TCA cycle, and NADH and $FADH_2$, used in the ETC (Bartlett and Eaton, 2004). FAO is a key metabolic pathway for energy homoeostasis in organs such as the liver, heart and skeletal muscle. During fasting, when glucose supply becomes limited, FAO is essential. Mitochondria, as well as peroxisomes, harbor all enzymes necessary for FAO. Importance of FAO is supported by the fact that almost each enzyme of the pathway is related to a pathological condition (Rinaldo et al., 2002).

II. Mitochondrial dynamics

Mitochondria are highly dynamic organelles: their morphology varies from spherical isolated organelles to branched tubular networks. Mitochondrial morphology is dependent on the type and the physiological condition of the cell and is constantly modulated in response to the needs of the cell through fusion and fission processes. Over the last years, the balance between fusion and fission and the role of mitochondrial dynamics emerged as a key regulatory components in a plethora of cellular events. The mechanism of mitochondrial fusion and fission are regulated by large Dynamin-related GTPases.

II.1. The pro-fusion machinery

II.1.1. Mitofusins 1/2 and OMM fusion

The first pro-fusion protein was identified in *Drosophila melanogaster.* Fuzzy onion (Fzo1) is a large GTPase, located at the OMM and involved in the formation of giant mitochondria during fly spermatogenesis (Hales and Fuller, 1997). The Fzo1 orthologue in yeast has also been reported to mediate mitochondrial fusion (Hermann et al., 1998; Rapaport et al., 1998). In mammals two orthologues of Fzo1 exist– Mitofusin 1 and 2 (Mfn1/2) (Fig.6) (Santel and Fuller, 2001). Mfn1 and 2 are highly homologous, containing an N-terminal GTPase domain, two transmembrane domains and two coiled coil motifs, crucial for protein-protein interactions

(Chen et al., 2003; Rojo et al., 2002; Santel et al., 2003; Santel and Fuller, 2001). Ubiquitous knockout of the *Mfn1* or *Mfn2* gene results in embryonic lethality during midgestation due to placental dysfunction (Chen et al., 2003; Chen et al., 2007).

Structural differences between Mfn1 and 2 consist in the p21ras-binding domain at the N-terminal of Mfn2, which is absent in Mfn1 (Chen et al., 2014). This might be the explanation for the functional differences between the two proteins. Mfn1 has lower affinity for GTP but greater GTPase activity than Mfn2 (Ishihara et al., 2004). Thus, Mfn1 has stronger pro-fusion effect than Mfn2 (Chen et al., 2003). Moreover, is has been shown that the IMM pro-fusion protein Opa1 requires Mfn1 to induce fusion (Cipolat et al., 2004). Mfn1 is required on the surface of both tethered mitochondria in order to fulfil the fusion event (Koshiba et al., 2004). On the other hand, Mfn2 is also located on the endoplasmic reticulum (ER) and is involved in the tethering of the two organelles by establishing interactions with Mfn1 or 2 at the mitochondrial surface. This process is crucial for mitochondrial Ca^{2+} uptake from the ER (de Brito and Scorrano, 2008a; Naon et al., 2016). Although, the role of Mfn2 in the tethering of the two organelles has been challenged (Filadi et al., 2015), it is established that Mfn2 is involved in ER-mitochondria Ca^{2+} crosstalk. *Mfn2* ablation is reported to induce ER stress (Debattisti et al., 2014; Munoz et al., 2013; Ngoh et al., 2012). This mechanism was confirmed also in the fly model – flies lacking *Marf* (the orthologue of Mfn2), display functional and developmental abnormalities, which are rescued by preventing ER stress. Conversely, *MFN2* overexpression is shown to kill cancer cells by promoting Ca^{2+} uptake from the ER (Wang et al., 2015).

Mutated *MFN2* has been shown to be the cause for Charcot-Marie-Tooth type 2A (CMT2A) syndrome, neurodegenerative disorder, characterized with loss of sensory and motor axons resulting in neuronal degeneration (Lawson et al., 2005). Mechanistically, Mfn2 has been shown to interfere with mitochondrial transport along microtubules via interaction with Miro/Milton complex, perturbation of this process has been proposed to be underlying mechanism of CMT2A. No pathology has been linked to *MFN1* so far.

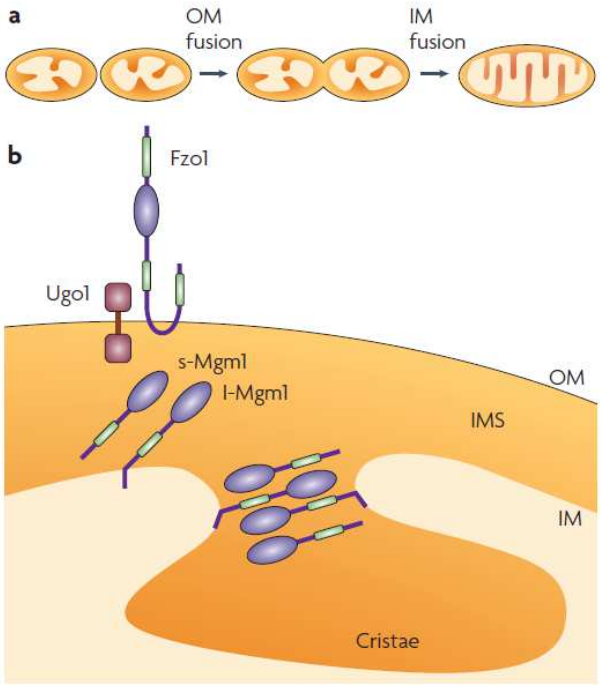


Figure 6. Schematic representation of the OMM and IMM fusion regulators. a) Mitochondrial fusion consists of OMM fusion, followed by fusion of the IMM. **b)** OMM fusion is orchestrated by Fzo1, which is the yeast orthologue of Mfn1 and 2; Mgm1 is the yeast orthologue of the mammalian IMM fusion regulator – Opa1. Proteolytic cleavage of the protein generates a long Mgm1 (l-Mgm1), which is IMM-bouded, and a short soluble (s-Mgm1) forms of the protein, both required for IMM fusion. Additionally to its fusion properties, Mgm1/Opa1 is required for cristae maintenance. Ugo1 is an OMM protein, which binds to both Fo1 and Mgm1 and is considered to coordinate OMM and IMM fusion processes. From (Detmer and Chan, 2007).

II.1.2. Opa1 in IMM fusion and cristae remodeling

Chemical treatment allows decoupling of OMM and IMM fusion processes (Malka et al., 2005). However, it is not completely clear whether *in vivo* the two are consequent or simultaneous processes. Optic atrophy 1 (Opa1, Mgm1p in yeast) is an IMM localized protein and is the key regulator of IMM fusion and cristae remodeling (Fig.6). Opa1 is composed of N-terminal mitochondrial targeting sequence, a central GTPase domain and two coiled coil domains, predicted on both sides of the GTPase domain. The C-terminal coiled coil is proposed to function as a GTPase effector domain (GED). Proteolytical cleavages of Opa1 produce the long form of Opa1 (l-Opa1), which possesses the N-terminal transmembrane domain and is IMM anchored, and the short soluble form (s-Opa1), which is further processed by the Rhomboid-like protease PARL, which generates the soluble IMS form of Opa1 (Cipolat et al., 2006).

Opa1 is ubiquitously expressed with highest levels in retina, brain, testis, heart and muscle (Alexander et al., 2000). The gene encoding for Opa1 has been identified as the major cause for development of autosomal dominant optic atrophy (ADOA), characterized with degeneration of the optic nerve, causing visual loss (Alexander et al., 2000; Delettre et al., 2000). In about 20% of the cases, defined as ADOA-plus, extra-ocular symptoms are present such as myopathy, ataxia, peripheral neuropathy, chronic progressive external ophthalmoplegia (Skidd et al., 2013). The crucial physiological role of Opa1 is supported by the observation that constitutive as well as conditional tissue-specific ablation of Opa1 in the mouse have lethal effects (Davies et al., 2007).

The effect of Opa1 in mitochondrial fusion has been debated because Opa1 overexpression was originally reported to cause mitochondrial fragmentation (Misaka et al., 2002). Later our group showed that this was likely a side effect of very high levels of Opa1 expression, because mild Opa1 overexpression enhances fusion, whereas *OPA1* downregulation represses it (Cipolat et al., 2004). Our group has recently confirmed these observations *in vivo* by generating mouse models for *OPA1* ablation and mild overexpression (Cogliati et al., 2013b; Varanita et al., 2015). Notably, Opa1 requires Mfn1 to drive fusion (Cipolat et al., 2004). It is still unclear which form of Opa1 (long, short of both) is required for the fusion events. Originally, both forms were thought to be required (Meeusen et al., 2006; Song et al., 2007); however subsequent work showed that the long form alone can drive fusion under stress conditions (Tondera et al., 2009). Moreover long non-cleavable forms of Opa1 could restore mitochondrial fusion (Song et al., 2009). On the other hand, *in vitro* experiments showed that the short form induces tubulation of cardiolipin containing liposomes, indicating a direct effect of this form on membrane shape (Ban et al., 2010). Very recently, a different model was put forward, whereby mitochondrial fusion requires long Opa1 interaction with cardiolipin in trans (Ban et al., 2017). Conversely, Opa1-Opa1 interaction in trans modulate cristae biogenesis.

The coordination of the OMM and IMM fusion processes remains unclear. In the yeast model it has been shown that Ugo1 physically links Fzo1 and Mgm1 (Fig.6). In the mammalian system SLC25A46, a member of the mitochondrial metabolite carrier family, found to be mutated in Leigh syndrome, an early-onset neurodegenerative disease, is the likely orthologue of Ugo1 (Janer et al., 2016).

Independently from its pro-fusion role, Opa1 plays a crucial role in cristae morphology (Fig.7) (Griparic et al., 2004; Olichon et al., 2003; Sesaki et al., 2003). Using biochemical and genetic approaches our group provided evidence that Opa1 mediated cristae remodeling through self-aggregation into high molecular weight complexes (Frezza et al., 2006) which are composed by l-Opa1 and s-Opa1 forms (Fig.6). Opa1 complexes keep CJs tight and prevent the release of cytochrome c normally stored inside the cristae, which are being remodeled during apoptosis (Scorrano et al., 2002). Mechanistically, this apoptotic cristae remodeling is a consequence of the disruption of Opa1 complexes leading to opening of CJs and release of cytochrome c (Cipolat et al., 2006; Frezza et al., 2006).

Another consequence of Opa1-mediated cristae stabilization is the formation and stabilization of respiratory chain supercomplexes *in vitro* and *in vivo* (Fig.7) (Cogliati et al., 2013a). Recently, our laboratory showed that a mouse model of mild *OPA1* overexpression resists skeletal muscle atrophy, heart and brain ischemic damage, and massive liver apoptosis by ameliorating mitochondrial performance and reducing cytochrome c release (Varanita et al., 2015). Moreover, controlled *OPA1* overexpression improves the phenotypes of two pre-clinical models of defective mitochondrial bioenergetics by correcting IMM architecture and promoting RCS assembly (Civiletto et al., 2015).

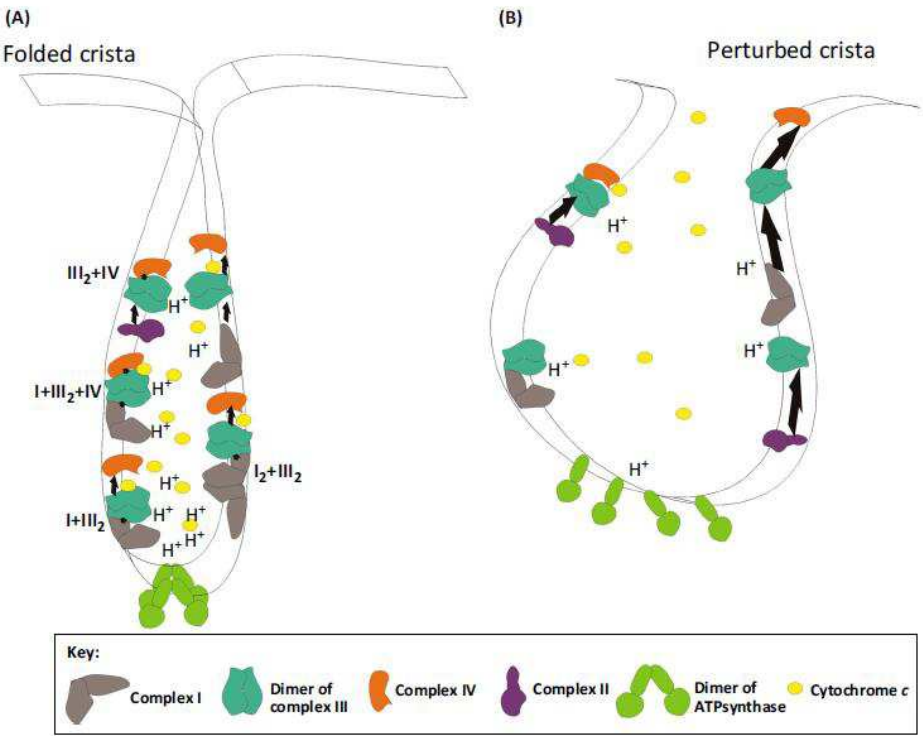


Figure 7. Schematic representation of the processes regulated by cristae morphology. A). Folded cristae maintain RCS organized and ATP dimers located at the edge of the cristae, ensuring efficiency of the OXPHOS system. Moreover, cytochrome c is kept inside in the cristae lumen; **B)** Unfolded cristae structure leads to RCS destabilization, reduction of ATP dimers and release of cytochrome c. From (Cogliati et al., 2013).

II.1.3. Other regulatory factors of cristae morphology

II.1.3.1. Mitofilin/Mic60 and the MICOS complex

Mitofilin, also called Mic60, is the mammalian homologue of the yeast Fcj1, responsible for formation of cristae junctions. Mitofilin is located within the IMM and enriched at cristae junctions (Gieffers et al., 1997; Odgren et al., 1996; Rabl et al., 2009). Loss of Mitofilin in human, worm and yeast cells leads to an extension of the IMM and massive loss of cristae junctions and to abnormal cristae, dissociated from the IBM (Hoppins et al., 2011a; John et al., 2005; Rabl et al., 2009; von der et al., 2011). Mitofilin is reported to participate in a larger highly conserved protein complex - mitochondrial contact site complex (MICOS), which is crucial for cristae maintenance and architecture of the IMM (Pfanner et al., 2014; Zerbes et al., 2012). Our group established that in mammalian cells Opa1 is epistatic to the MICOS complex in determining cristae junction stability and number, as well as diameter (Glytsou et al., 2016). Loss of any of the MICOS complex players is also characterized with strongly altered

IMM morphology, loss of CJs, disorganized cristae, leading to loss of respiratory activity and altered mtDNA stability. MICOS complex is also reported to be involved with communications with the OMM and in mitochondrial fusion events (Pfanner et al, 2014; Zerbes et al., 2012).

II.1.3.2. Prohibitins, SLP2 and cardiolipin

Prohibitin 1 and 2 (PHB1/2) are located within the IMM. Both PHB1 and 2 contain a highly conserved PHB domain (SPFH domain), shown to be a lipid raft marker and to participate in protein-protein interactions (Winter et al., 2007). Prohibitins are involved in many aspects of mitochondrial biology such as mtDNA maintenance (Kasashima et al., 2006), respiratory chain assembly (Schleicher et al., 2008) and mitochondrial morphology (Kasashima et al., 2006; Merkwirth et al., 2008). *Phb2*-depleted cells display mitochondrial fragmentation and cristae destabilization, probably as a result of altered OPA1 cleavage (Merkwirth et al., 2008). PHB1 and 2 have been shown to form a complex with Stomatin-like protein 2 (SLP2) from the highly conserved stomatin protein family. The complex is reported to bind and localize at cardiolipin enriched regions of the IMM (Bazan et al., 2013; Pineau et al., 2013).

Cardiolipin is important for formation of protein-protein and lipid-protein interactions (Holthuis and Ungermann, 2013) and also shown to increase fluidity of biological membranes and to facilitate curvature formation. Cardiolipin is enriched at the IMM and it has been shown to be essential for cristae formation – loss of mature cardiolipin causes disorganization of cristae structure and disassociation of SCs (Bottinger et al., 2012). Thus, showing the importance of lipid composition in the mechanism of IMM folding and functionality.

II.2. Mitochondrial fission

Mitochondrial fission is the process of mechanical constriction of the organelle. The cytosolic GTPase Dynamin related protein 1 (Drp1) is the master regulator of both mitochondrial and peroxysomal fission (Li and Gould, 2003; Smirnova et al., 2001). Drp1-dependent fission events are precisely regulated, multistep processes, essential for cell survival and specific to the cell type and the physiological context.

II.2.1. Drp1 - the master regulator of mitochondrial fission

II.2.1.1. Drp1's protein organization

DRP1 is a ~80kDa ubiquitously expressed protein (Yoon et al., 1998), with highest levels found in skeletal muscles, heart, kidney and brain. Similar to Dynamin, Drp1 is a multidomain GTPase (Fig.8) that consists of a N-terminal GTPase domain, a middle assembly domain (MID), a C-terminal GTPase-effector domain (GED). Unlike Dynamin, Drp1 does not contain the lipid-interacting pleckstrin homology domain. Instead, it has an uncharacterized variable domain (VD) of 95 amino acids in the human isoform, also called a B-insert (Zhang et al., 2011; Zhu et al., 2004).

In the cytosol Drp1 is predicted to exist as a T-shaped dimer or tetramer formed by a head (GTPase domain), the B-insert, located between these two domains allows the GED to back fold onto the Middle domain, forming the "leg" (Fig.8) (Zhu et al., 2004). When dimers or tetramers translocate to the OMM at fission sites they assemble into higher molecular complexes around the fission site. GTP binding and hydrolysis induces rearrangements of the head and the stalk, generating a mechanical force that constricts the membrane (Chang and Blackstone, 2010; Macdonald et al., 2014; Zhu et al., 2004). Mutation of a highly conserved Lysine into an Alanine (K38A) within the GTPase domain blocks GTP binding and is characterized as a dominant negative mutant of Drp1, whose overexpression causes the appearance of a hyperconnected mitochondrial network (Chang et al., 2010).

The middle domain is involved in intermolecular interaction and high molecular order complexes. A lethal mutation (described in section II.2.1.5.1.) within the middle domain has been characterized with decreased assembly of Drp1 oligomers at the OMM likely due to a decrease in GTPase activity, resulting in hyperfused mitochondrial network.

In the Drp1 molecule the GED domain back-folds onto the GTP-middle domain. Inhibition of this interaction by the K679A mutation within the GED domain leads to a decrease in the GTPase activity and the assembly of high order complexes resulting in downregulated mitochondrial fragmentation (Zhu et al., 2004).

Structurally, the B-insert is proposed to be unstructured with α-helices connections to the GED and the Middle domain. Functionally, the B-insert is still not well characterized but there are evidences that, similar to the pleckstrin domain, it is involved in lipid binding (Adachi et al., 2016; Bustillo-Zabalbeitia et al., 2014; Stepanyants et al., 2015; Ugarte-Uribe et al., 2014).

Deletions of the VD domain result in constitutive OMM translocation and activation of Drp1, therefore the VD domain has been described to have an autoinhibitory function (Cribbs and Strack, 2007).

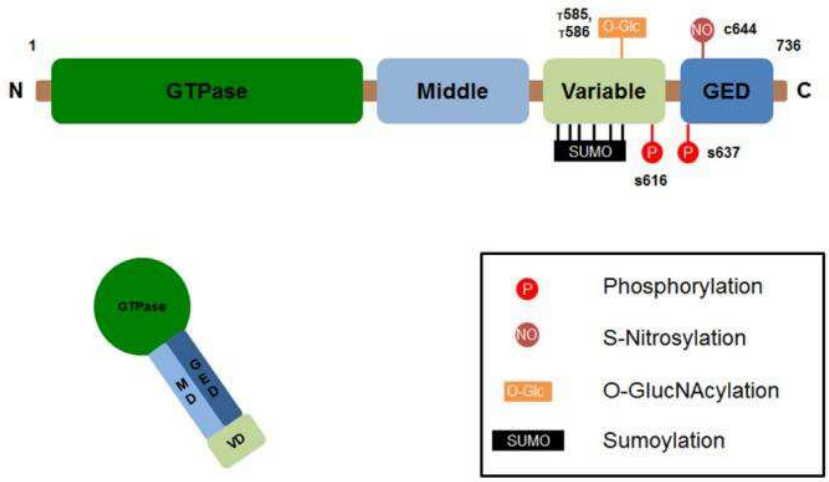


Figure 8. Protein structure and post-translational modifications of Drp1. Drp1 is a multidomain GTPase constituted by an N-terminal GTPase domain (green), followed by a Middle domain (light blue), a Variable domain also called B-insert (light green) and a GTPase-effector domain (GED, blue). In the cytosol Drp1 molecule is arranged in a T-shape manner, the "head" is formed by the GTPase domain and the "leg" by the Middle and GED domains, the variable domain (VD), located between these two domains allows the GED to back-fold onto the Middle domain (MD). Drp1 activation is controlled by a number of post-translation modifications, located within the Middle and the GED domains. From (Cho et al., 2013).

II.2.1.2. Genetic structure and splice variants

Drp1 is encoded by the *DNM1L* gene (12p11.21) composed by 31 exons. Alternative splicing of the pre-mRNA transcript gives rise to eight different splice forms. Yoon and colleagues described three splicing variants of 201, 180 and 162bp at the N-terminal GTPase domain, named A domain, localized within exon 3. The B splicing domain is located within exons 16 and 17 with splicing variants of 351, 318 and 240bp. The expression patterns of these splice variants is tissue specific – brain expresses longer forms (201 and 351 bp) of both A and B splicing domains, whereas other tissues (liver, lung, testicles) express predominantly shorter splice forms 162 and 240bp for A and B domains respectively (Yoon et al., 1998). Uo et al. showed that the neuro-specific splice form is important for cortical neurons survival (Uo et al., 2009). Strack and co-workers demonstrate that alternative splicing together with post-

translational phosphorylation regulates Drp1 subcellular localization between cytosol, microtubules and mitochondria (Strack et al., 2013). However, the functional importance of these splice variants has not been fully understood.

II.2.1.3. Drp1 subcellular localization

Generally, Drp1 is described as a cytosolic dimer or tetramer that translocates to the mitochondria or the peroxisomes upon fission stimulation (Li and Gould, 2003; Smirnova et al., 1998). However, its ability to bind biological membranes mostly via adaptor proteins suggests that other subcellular localizations should not be excluded. In 1998 Yoon et al. analyzed Drp1 subcellular localization. Their description of the immunofluorescence pattern of Drp1 perfectly illustrates the distribution of Drp1 we observe today: "antibodies to DLP1 stain punctate vesicular structures that are concentrated at the perinuclear region and extend out into the peripheral cytoplasm in linear arrays as if associated with cytoskeletal elements" (Yoon et al., 1998). Drp1 puncta colocalize indeed with tubules of the endoplasmic reticulum. Subfractionation analysis showed that indeed Drp1 associates with small non-endocytic vesicles and light membranes. Drp1 has also been described to colocalize with cytoskeletal microtubules (Strack et al., 2013; Varadi et al., 2004). Drp1 has been reported to interact with clathrin-coated vesicles via its adaptor Mff during synaptic vesicles endocytosis (Li et al., 2013). Actin filaments have also been described to directly bind Drp1 and target the protein to fission sites (Hatch et al., 2016; Ji et al., 2015). Recently, Jo et al. described Drp1 distribution to lamelipodia in MEFs in response to Platelet-derived growth factor (PDGF) stimulation. The authors suggest that Drp1 affects lamelipodia formation by interfering with actin polymerization rather than through modifying mitochondrial morphology (Jo et al., 2017).

II.2.1.4. Mechanisms of mitochondrial fission

Over the last years, the new discoveries in the field of mitochondrial constriction offered the model for Drp1-mediated mitochondrial fission. Fission occurs through the following major steps:

1) ER tubules and actin filaments facilitate constriction and serve as early markers of future fission sites;

2) Post-translation modifications of Drp1 induce its translocation to the OMM;

3) Drp1 binds to its adaptor proteins within the fission foci;

4) Drp1 dimers assemble into high molecular weight complexes encircling the fission site;

5) GTP-hydrolysis triggers mechanical membrane constriction;

6) Drp1 assembly and constriction facilitate recruitment of Dynamin2 to ensure efficient membrane constriction;

7) Drp1 and Dynamin2 oligomers disassemble.

II.2.1.4.1. Early Drp1-independent constriction step

II.2.1.4.1.a. Role of the endoplasmic reticulum in mitochondrial fission

Over the last years, the interest in the role of ER-mitochondria contact sites, the so called MAMs (mitochondria associated membranes), in a variety of physiological processes increased. The proximity between the two organelles is maintained by tether proteins. ER-localized Mfn2 interacts with Mfn1 and/or Mfn2 on the OMM (de Brito and Scorrano, 2008), PACS-2, Rab32, IP_3 receptor complexes have also been identified as tethers (Raturi and Simmen, 2012).

This interaction is mainly involved in the transfer of Ca^{2+} and lipids between the two organelles (Rizzuto et al., 1993; Rizzuto et al., 2012; Scorrano, 2003; Voss et al., 2012). MAMs are also involved in membrane constrictions, generating organelle division. In yeast and mammalian cells, ER-mitochondrial contact sites are early markers of mitochondrial fission foci (Friedman et al., 2011; Murley et al., 2013). The diameter of the mitochondria is constricted at ER contact sites and this is the position where Drp1 complexes preferentially form. Indeed, the diameter of the mitochondria is approximatively two-fold bigger that the diameter of Drp1 (Dnm1) oligomers. Thus, ER-mitochondrial contact sites have been defined as early markers of mitochondrial fission sites at which Drp1-dependent machinery preferentially forms (Fig.9). Similar role of the ER has been described also in fission of early endosomes during cargo sorting (Rowland et al., 2014).

The mechanism of ER-mediated mitochondrial constriction is an outstanding question. In yeast, ER-mitochondria contact sites colocalize with the position of the nucleoid and thus fission at this position ensures the equal distribution of nucleoids into daughter mitochondria (Murley et al., 2013). In mammalian systems, the precise tethering complex and the

mechanism driving Drp1 localization at ER-mitochondria contacts during mitochondrial fission is not completely understood.

In 2010 Bui and colleagues described that the small GTPase Rab32 regulates calcium signaling by anchoring calnexin to the MAMs. Rab32 also modulates intracellular protein kinase A (PKA) signaling and apoptosis (Bui et al., 2010). PKA is known to phosphorylate Drp1 at Ser637 which has an inhibitory effect on mitochondrial fission. Later on, the same group showed that Drp1 directly interacts with the Rab32 protein family (formed by Rab32A, Rab32B, Rab29 and Rab38) at ER-mitochondria contact sites (Ortiz-Sandoval et al., 2014). Rab32 and Drp1 are both found in light ER contact site membranes and dominant negative mutants of Rab32 lead to an increase of Drp1 on heavy membranes and to a collapsed perinuclear mitochondrial network. Therefore, Drp1 is proposed to be an effector of Rab32 at the ER-mitochondria interface.

The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein Syntaxin 17 has been also identified to interact with Drp1 at ER-mitochondria contact sites. Syn17 is localized on tubular smooth ER and has been previously reported to interact with ATG14L and to drive formation of functional autophagosomes upon starvation (Hamasaki et al., 2013). Arasaki and colleagues show that Syn17 colocalizes with ER-mitochondrial contacts and regulates mitochondrial fission by interfering with the activity of Mff-bound Drp1 (Arasaki et al., 2015). This interaction is dependent on microtubules and the lipid context of the MAMs environment – Syn17 and Drp1 indeed interact at lipid-rafts like structures. Moreover, the authors link Syn17-Drp1 interaction to the previously described Rab32-Drp1 communication at the MAMs. Syn17 prevents Drp1 deactivation by Rab32-mediated PKA phosphorylation at Ser637. Together these studies suggest that MAMs and raft-like structures could regulate the activity of Drp1 and mitochondrial fission.

II.2.1.4.1.b. Role of the cytoskeleton in early step mitochondrial constriction

Actin polymerization is also shown to drive Drp1 at the ER-mitochondrial contacts early in the fission process (Fig.9). This is regulated by both ER and mitochondria located proteins, such as inverted formin 2 (INF2) and Spire1C, respectively (Korobova et al., 2013; Manor et al., 2015). Recently, Hatch and coworkers reported that actin filaments are reservoirs for fission-competent Drp1 oligomers, ensuring a dynamic response to pro-fission signals (Hatch et al., 2016). Actin binding has also been reported for Dynamin 1 and 2 prior to their fission activity during endocytosis (Grassart et al., 2014; Gu et al., 2010). The work of Korbova and colleagues

showed that motor protein Myosin II is involved in the guiding of actin-bound Drp1 to the OMM (Korobova et al., 2014). Myosin II ablation results in a decrease of mitochondrial levels of Drp1 without affecting the interaction with actin.

Recently, the Myosin II - Septin 2 interaction has been described to communicate with Drp1 during fission (Pagliuso et al., 2016). Septins are highly conserved GTP-binding proteins involved in the formation of non-polar filaments of the cytoskeleton. Pagliuso and colleagues showed that Septin 2 localizes at fission sites and its ablation results in a hyperconnected mitochondrial network. Septin 2 directly interacts with Drp1 and is required for its localization at fission sites.

Another modulator of actin/Myosin II-mediated Drp1 translocation to mitochondria is the Tau protein in neurons (DuBoff et al., 2012). Tau is the major microtubule associated protein (MAP) in mature neurons and it stabilizes microtubules (Wang and Liu, 2008). Hyperphosphorylation of Tau and its abnormal localization have been associated to Alzheimer disease (Ittner and Gotz, 2011). Excessive actin stabilization by Tau alters mitochondrial localization of Drp1 leading to increased mitochondrial connectivity and neurotoxicity *in vivo* (Duboff et al., 2012). Interestingly, Tau has also been reported to regulate motor proteins like dynein and kinesin (Dixit et al., 2008) and organelle trafficking (Ebneth et al., 1998), however the role of Drp1 in these processes has not yet been investigated.

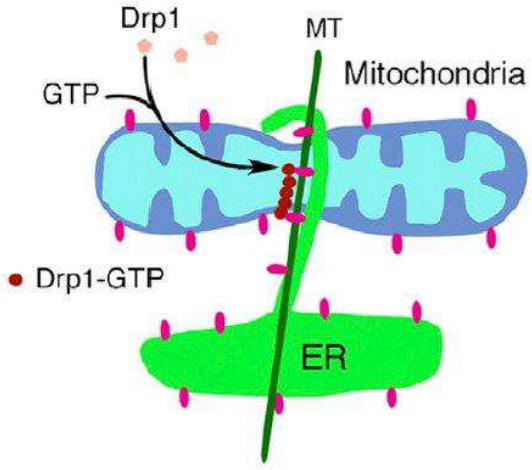


Figure 9. Cytoskeleton and ER contact sites in mitochondrial fission. Establishment of endoplasmic reticulum (ER)-mitochondrial contact sites is an early fission event. The constriction of the mitochondria by the endoplasmic reticulum encircling provides the platform for the Drp1-mediated fission machinery. Microtubules (MT) of the cytoskeleton are involved in the establishment of the organelle contact site and also bring GTP-bound Drp1 complexes to the fission site. From (Arasaki et al., 2015).

II.2.1.4.2. Post-translational modifications and regulation of Drp1 translocation to the mitochondria

A crucial step in mitochondrial fission is the translocation of Drp1 from the cytosol to the OMM. Post-translational modifications (phosphorylation, ubiquitination, SUMOylation) at different sites of Drp1 can impinge on the localization and activity of Drp1. The type and the effect of the modification are dependent on the signaling and tissue context (Table 1).

Modifications	Sites	Upstream regulators	Effects	References
Phosphorylation	S616	CDK1	Activation	Taguchi et al., 2007
		Erk1/2	Activation	Yu et al., 2011
		PKCδ	Activation	Qi et al., 2011
	S637	PKA	Inactivation	Chang and Blackstone, 2007; Cribbs and Strack, 2007b
		CaMK1α	Activation	Han et al., 2008a
		ROCK1	Activation	Wang et al., 2012c
Dephosphorylation	S637	Calcineurin (PP2B)	Activation	Cereghetti et al., 2008
S-nitrosylation	C644	Nitric oxide	Activation	Cho et al., 2009
SUMOylation	Multi-sites in variable domain	MAPL	Activation	Braschi et al., 2009
DeSUMOylation		SENP5	Inactivation	Zunino et al., 2007b
Ubiquitination		MARCH5	Inactivation	Nakamura et al., 2006a; Yonashiro et al., 2006b
		Parkin	Inactivation	Wang et al., 2011b
O-GlcNAcylation	T585, T586	O-GlcNAc-transferase	Activation	Gawlowski et al., 2012b

Table 1. Effects of post-translational modifications with their corresponding signaling pathways and effects on Drp1 activity. From (Cho et al., 2013).

II.2.1.4.2.A. Phosphorylation

The main post-translational regulation of Drp1 activity is phosphorylation. Phosphorylation by Calcium/calmodulin-dependent protein kinase 1α at Ser637 in human (Ser 600 in mouse and Ser656 in rat) promotes Drp1 OMM translocation and fission activity (Han et al., 2008). In contrast, cAMP-dependent protein kinase A (PKA) phosphorylation again at Ser637, which is located in the GED-domain, inhibits mitochondrial fission (Chang and Blackstone, 2010; Cribbs and Strack, 2007). Under nutrient starvation, the increase of cAMP levels stimulates phosphorylation at Ser637 resulting in elongated mitochondria, protected from mitophagy (Gomes et al., 2011; Rambold et al., 2011). Dephosphorylation by calcineurin at the same residue stimulates Drp1 translocation to mitochondria (Cereghetti et al., 2008). In podocytes hyperglycemia induces phosphorylation at the same residue (Ser637) by the Serine/threonine

kinase ROCK1 resulting in Drp1 translocation to the OMM and an increase in mitochondrial fission (Wang et al., 2012a).

During mitosis, Cdk1/cyclin B phosphorylates Drp1 at Ser616 (in human) within the B-insert domain, this promotes mitochondrial fission and ensures the proper distribution of mitochondria into the daughter cells (Kashatus et al., 2011; Taguchi et al., 2007). HIF1α induces Cdk1/cyclin B-dependent phosphorylation of Drp1 at Ser616 in pulmonary artery smooth muscles causing cellular hyperproliferation resulting in pulmonary arterial hypertension (Marsboom et al., 2012).

II.2.1.4.2.B. SUMOylation

SUMOylation is another way to post-translationally modulate Drp1 activity (Figueroa-Romero et al., 2009). SUMO1 protein (Small Uniquitine-like Modifier) stabilizes Drp1 on the OMM in a Bax/Bak-dependent manner during early stages of apoptosis (Wasiak et al., 2007). Mitochondrial SUMO E3 ligase MAPL (Mitochondrial-anchored protein ligase) also SUMOylates Drp1. MAPL overexpression stimulates mitochondrial fission (Braschi et al., 2009). Prudent et al. reported that MAPL-dependent SUMOylation stabilizes Drp1 at ER-mitochondrial contact sites, which actives ER-mediated calcium flux, cristae remodeling and cytochrome c release (Prudent et al., 2015). DeSUMOylation by the SUMO protease SENP5 rescues SUMO1 induced mitochondrial fission (Zunino et al., 2007). Conversely, during G2/M phase of cell cycle SENP5, reported to localize within the nucleoli, translocates to the mitochondria where it deSUMOylates Drp1 and induces mitochondrial fragmentation, knockdown of SENP5 causes cell cycle arrest (Zunino et al., 2009). The opposite effects of SENP5 on mitochondrial fission are still not known but they could be dependent on the cell type and status.

II.2.1.4.2.C. Ubiquitination

Ubiquitination also regulates Drp1 localization and activity. March5 (also known as MITOL) is a mitochondria-associated E3 ubiquitin ligase involved in mitochondrial quality control by ubiquitinating mutated or misfolded proteins on the OMM (Sugiura et al., 2011; Yonashiro et al., 2009). March5 ubiquitinates Drp1 on the OMM, however, the effect on mitochondrial morphology is controversial. March5 knockdown or overexpression of an inactive mutant lead to mitochondrial fragmentation (Nakamura et al., 2006; Yonashiro et al., 2006). However,

Karbowski et al. stated the opposite (Karbowski et al., 2007). Another E3 ubiquitin ligase, Parkin, associated with familial Parkinson's disease, interacts and ubiquitinates Drp1 for proteasomal degradation (Wang et al., 2011). Loss of PINK1 function promotes mitophagy in a Parkin- and Drp1-dependent manner. It is therefore possible that Parkin has dual roles for mitochondrial homeostasis and quality control.

II.2.1.4.2.D. S-Nitrosylation

S-nitrosylation of Cys644 within the GED-domain of Drp1 induces oligomers formation and GTPase activity in neurons leading to abnormal mitochondrial fragmentation which has been associated with development of Huntington's disease (HD) (Haun et al., 2013) et al., 2013). This mechanism of mitochondrial fragmentation appears to be specific to HD (Costa et al., 2010; Costa and Scorrano, 2012) and to other neurodegenerative disorders related to excessive mitochondrial fragmentation such as Alzheimer's disease (Wang et al., 2009).

II.2.1.4.2.E. O-GlcNAcylation

O-GlcNAcylation is a noncanonical glycosylation, involving the attachment of single O-linked N-acetylglucosamine (O-GlcNAc) groups to Ser and Thr residues of nuclear, cytosolic and mitochondrial proteins. O-GlcNAcylation signaling is sensitive to nutrient flux and stress conditions (hypoxia, heat shock). O-GlcNAcylation is regulated through the activity of two enzymes - the O-GlcNAc transferase (OGT), which catalyses the transfer of a GlcNAc moiety and the N-acetyl-glucosaminidase (OGA), which removes this modification. The mechanisms mediating this signaling pathway are still very unclear. In the case of Drp1, N-acetyl-glucosaminidase ablation in cardiomyocytes leads an increase in O-GlcNAcylations at Thr585 and Thr586 within the B-insert domain (Gawlowski et al., 2012). This post-translational modification decreases Ser637 phosphorylation, promotes the mitochondrial localization of Drp1 and its GTPase activity leading to increased fission and impaired mitochondrial fuction in cardiomyocytes (Gawlowski et al., 2012).

II.2.1.4.3. Anchoring Drp1 to the OMM – the adaptor proteins

Correct anchoring of Drp1 to the mitochondrial membrane and subsequent regulation of fission events are also determined by Drp1's adaptor proteins. Due to the lack of a pleckstrin

homology domain responsible for lipid-interactions, Drp1 is mainly described to bind to adaptor proteins located at fission foci.

II.2.1.4.3.A. Mitochondrial Fission Factor (Mff)

Mff is a small protein (25-35kDa) anchored to the OMM by its C-terminal. A central coiled-coil domain is important for targeting. At the N-terminal part, which is oriented towards the cytosol, two short repeats are suggested to act as a binding site for Drp1. Several studies concluded that Mff is the major receptor for Drp1 on the OMM (Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010). Silencing of Mff using siRNA results in mitochondrial elongation and reduction of mitochondrial bound Drp1. Consistently, overexpression of Mff induces Drp1 translocation to the organelle and subsequent fission (Otera et al., 2010). Mice lacking Mff die around 13 weeks post-birth from cardiomyopathy probably due mitochondrial fission defects (Chen et al., 2015). However this effect is milder than the embryonically lethal phenotype of the full Drp1 mouse knock-out (Ishihara et al., 2009; Wakabayashi et al., 2009a), suggesting that other adaptors are able to recruit Drp1. Indeed, Mff and the MiDs (see below) can coexist in the same fission foci (Elgass et al.; Palmer et al., 2011).

II.2.1.4.3.B. Mitochondrial dynamics proteins (MiD49/50)

Mitochondrial dynamics (MiD) are two orthologue proteins of 49 and 51kDa. They are anchored to the OMM by their N-terminal part and reported to act as adaptor proteins for Drp1 during fission (Otera et al., 2016; Palmer et al., 2011). High expression of the two proteins induces Drp1 translocation to the mitochondria but paradoxically the mitochondrial network becomes highly fused (Palmer at al., 2011). Zhao et al. also observed that upon overexpression of MiD51 Drp1 translocation is induced and mitochondria are hyper-fused. They described MiD51 as Mitochondrial Elongation Factor (MIEF1). This suggesting that MiD49/51 could anchor Drp1 to the OMM, but Drp1 is in a non-functional form. Parallel to this, MiD51 (MIEF1) was shown to interact with Fis1 (Zhao et al., 2011). This interaction is presumed to have a negative effect on mitochondrial fission complicating even more the regulation scenario of mitochondrial fission.

II.2.1.4.3.C. hFis1

Human Fis1 (hFis1) is a C-tail anchored OMM protein. In contrast to the puncta-like localization of Mff, mammalian hFis1 is localized evenly throughout the OMM. Its N-terminal containing multiple tetratricopeptide repeat motifs is oriented towards the cytosol and thought to be involved in recruiting Drp1 to the mitochondria similar to its yeast homologue (Fis1) (Stojanovski et al., 2004; Yoon et al., 2003). However, the precise function of Fis1 remains unclear. In yeast and plants, it is well established that Fis1 mediates mitochondrial fission (Zhang and Chan, 2007). However, in C. elegans Fis1 and Fis2 depletions does not affect mitochondrial morphology (Breckenridge et al., 2008). Several studies have shown that Fis1 downregulation increases mitochondrial connectivity and overexpression of the protein causes fragmentation of the organelle (James et al., 2003; Mai et al., 2010). In contrast, silencing or overexpression of hFis1 does not affect Drp1 activity or its recruitment to the OMM (James et al., 2003). Physiologically, Fis1 has been reported to participate in apoptosis by transmitting a signal from the mitochondria to the ER (Iwasawa et al., 2011) and by regulating mitochondrial cytochrome c release (James et al., 2003; Breckenridge et al., 2003). Gomes and colleagues linked Fis1 induced mitochondrial fragmentation to autophagy (Gomes and Scorrano, 2008).

Finally, the role of Fis1 in Drp1-dependent mitochondrial fission remains an enigmatic question. Recent reports state that Fis1-dependent fission is mostly linked to stressed-induced fragmentation and mitophagy (Shen et al., 2014), whereas Mff is the major adaptor for physiological fission (Otera et al., 2010). Drp1 immunoprecipitates Mff with higher affinity that Fis1, moreover Mff and Fis1 exist in separate high molecular weight complexes on the mitochondrial membrane, once again suggesting that they operate in different fission pathway depending on the cellular context (Otera et al., 2010).

II.2.1.4.3.D. Endophilin B1

Endophilins are fatty acyl transferases. They are reported to be involved in membrane curvature and membrane constriction during endocytosis and organelle biogenesis. They are formed by an N-terminal Bar domain, interacting with membranes, and a C-terminal SH3 domain for protein binding (Schmidt et al., 1999). During apoptosis Endophilin translocates to mitochondria. Knocking down of Endophilin leads to formation of OMM vesicles and tubules (Karbowski et al., 2004). This morphology is prevented upon Drp1 donwregulation or

transfection with dominant-negative Drp1K38A mutant (Karbowski et al., 2004), indicating Drp1 acts upstream of Endophilin.

II.2.1.4.3.E. Ganglioside-induced differentiation-associated protein (GDAP) 1

GDAP1 is anchored to the OMM via C-terminal hydrophobic transmembrane domain, the N-terminal domain is facing the cytoplasm (Niemann et al., 2005). It is expressed in myelinating Schwann cells, motor and sensory neurons. Mutations affecting GDAP1 lead to Charcot-Marie-Tooth peripheral neuropathy (Pedrola et al., 2005). Mutated GDAP1 is not targeted to the mitochondria and lacks fission activity. Knocking-down Drp1 or overexpressing a dominant negative mutant prevent GDAP1 induced mitochondrial fragmentation, indicating that GDAP1 fission activity requires Drp1 (Niemann et al., 2005).

II.2.1.4.3.F. Mutant Huntingtin

Abnormal polyglutamine expansions (polyQ) within Huntingtin (Htt) are responsible for Huntington's disease (HD), an autosomal dominant disease, characterized with progressive loss of striatal and cortical neurons. Bossy-Wetzel and colleagues have shown that mutated Htt (Q46-Q97) directly interacts with Drp1 and stimulates its pro-fission activity leading to massive mitochondrial fragmentation which alters axonal mitochondrial transport and neural cell death. This phenotype is rescued by overexpression of the dominant negative mutant of Drp1 K38A (Song et al., 2011). Moreover, it has been shown that mutated Htt hyperactivates the phosphatase calcineurin which dephosphorylates Drp1 at S637 and triggers mitochondrial fragmentation (Costa et al., 2010; Costa and Scorrano, 2012). Haun et al. reported that the translocation of Drp1 to mutant Htt in HD is induced specifically by S-nitrosylation of Cyc644 (Haun et al., 2013).

II.2.1.4.3.G. LRRK2

Leucine rich repeat kinase 2 (LRRK2) is a large multi-domain kinase. Mutations in LRRK2 are related to familial cases of Parkinson's disease (PD). In the cell LRKK2 is found to be cytosolic and bound to the mitochondrial membrane. Overexpression of LRRK2 directly interacts with Drp1 and stimulates its recruitment to the mitochondria leading to fragmentation, dysfunction and susceptibility to stress-induced cell death (Niu et al., 2012; Wang et al., 2012b). However, the exact mechanism underlying this process remains unclear.

II.2.1.4.3.H. Sacsin

Sacsin is a high molecular weight multi-domain protein with strong expression in the central nervous system, also expressed in the skin, skeletal muscles, and with low expression in the liver and pancreas. Mutations in its gene (*SACS*) cause child-onset autosomal recessive spastic ataxia of Charlevoix-Saguenay (Anderson et al., 2010). Sacsin localizes with the mitochondria and is reported to partially overlap with Drp1 foci. Moreover, immunoprecipitation analysis detects an interaction between the two proteins. In line with this observation, Sacsin knock-out mice and Charlevoix-Saguenay ataxia patients are characterized with a hyperfused balloon-like mitochondrial network (Girard et al., 2012), a phenotype observed in *Drp1*/*DRP1*-depleted cells, suggesting a function for Sacsin in mitochondrial fission process. Sacsin-deficient neuronal cells have enlarged mitochondria, accumulating in the soma.

II.2.1.4.3.J. Mitochondrial protein 18kDa (MTP18)

The IMS located protein MTP18 has been characterized as a downstream effector of phosphatidyl-inositol 3-kinase (PI3K) signaling and is mediating mitochondrial fission and apoptosis in response to PI3K activation (Tondera et al., 2005; Tondera et al., 2004). MTP18 mediated fission is Drp1 dependent (Tondera et al., 2005), however the precise mechanism and context of the pathway are not completely understood.

II.2.1.4.3.K. Drp1 and membrane lipids interactions

Although, Drp1 is anchored to the OMM via its adaptor proteins due to the lack of the pleckstrin homology domain responsible for lipid-interactions, *in vitro* experiments from several studies have shown that Drp1 can bind to liposomes in the absence of those proteins. The precise mechanism is still not well understood but membrane composition at fission sites is emerging as another control step in Drp1-dependent mechanisms of membrane remodeling.

II.2.1.4.3.K.a. Cardiolipin

In 2010 Montessuit and colleagues described a mechanism through which Drp1 is regulating Bax oligomerization during apoptosis (Karbowski et al., 2002; Montessuit et al., 2010). They show that Drp1 interacts directly with liposomes containing cardiolipin (CL). Cardiolipin is an unsaturated polyanionic phospholipid mainly localized in the IMM, however significant

amounts are also found in the OMM at the so called mitochondrial contact sites (Ardail et al., 1990). Montessuit and co-workers reported that the Drp1-CL interaction promotes Bax oligomerization by generating an intermediate state common for membrane remodeling called hemifusion or hemifission intermediate. Drp1 would pinch the membrane at CL-enriched fission sites and bring together the inner and the outer mitochondrial membranes. This membrane remodeling brings Bax oligomers together. Later on several studies investigated the Drp1-CL interaction (Bustillo-Zabalbeitia et al., 2014; Macdonald et al., 2014; Stepanyants et al., 2015; Ugarte-Uribe et al., 2014) and reported that Drp1 interacts with CL through its B insert and that this interaction stimulates Drp1 oligomerization and its GTPase activity.

II.2.1.4.3.K.b. Phosphatidic acid

A recent study (Adachi et al., 2016) reported a novel mechanism of lipid regulation of mitochondrial fission. Drp1 binds the head group of phosphatidic acid (PA) and the saturated acyl chains of other phospholipids. The interactions occur through the B-insert and the stalk domain (GED domain). Drp1 is inserted into the membrane bilayer to reach the acyl chains. In contrast to the fission-promoting effect of Drp1-CL interaction, the communication between Drp1 and PA has a negative effect on Drp1 fission activity. PA inhibits the GTPase activity of Drp1 oligomers, thus keeping them inactive, which blocks fission events. Moreover, Drp1 directly interacts with the PA-producing enzyme MitoPLD, suggesting that this interaction stimulates the production of PA in proximity to fission sites and thus negatively regulates the process. MitoPLD also immunoprecipitates pro-fusion proteins OPA1 and MFNs (Adachi et al., 2016), but in the case of Mitofusins-dependent fusion PA has been assigned a promoting function (Choi et al., 2006).

II.2.1.4.4. Mechanisms of fission

GTP interaction and hydrolysis are essential for mechanical membrane constriction by promoting conformational changes of Drp1 oligomers (Fig. 10) (Macdonald et al., 2014; Mears et al., 2011; Yoon et al., 2001). GTP-unbound oligomers on lipid layers have a diameter of 60 ± 12 nm (Francy et al., 2015). Addition of GTP causes immediate constriction of Drp1-lipid bound oligomers to 39 ± 9 nm. Dominant negative mutant of Drp1 K38A, defective in GTPase

activity proves that it is GTP hydrolysis that triggers membrane constriction. The K38A is able to bind GTP and form oligomers similar to WT Drp1. However, after addition of GTP diameter of Drp1K38A-lipid tubes remains ~ 60 nm (Francy et al., 2015). GTP hydrolysis not only triggers membrane constriction but is also a signal for the disassembly of Drp1 oligomers from the OMM back to the cytosol.

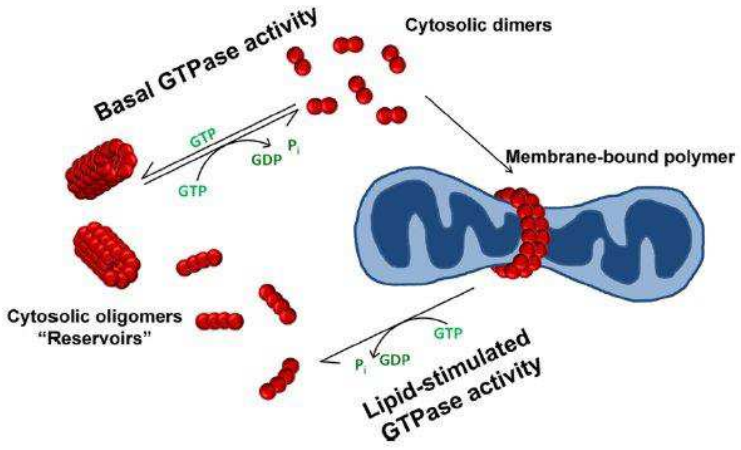


Figure 10. Schematic representation of Drp1 GTPase activity during membrane constriction. In the cytosol basal GTPase activity drives constant shifting between di-tetra or small oligomers of Drp1. Once activated GTP-bound Drp1 forms high molecular order oligomers. GTP hydrolysis drives conformational changes of the oligomers and membrane constriction. Finally, GDP-bound Drp1 disassembles from the membrane. From (Macdonald et al., 2014).

II.2.1.4.5. Drp1-Dynamin 2 collaboration in membrane constriction

Drp1 was long considered to be the only Dynamin-related GTPase constricting the mitochondria. Recently, Lee and colleagues have discovered that the ubiquitously expressed GTPase Dynamin 2 acts downstream of Drp1 constriction to ensure the complete fission of mitochondria (Lee et al., 2016). Dyn2 colocalizes at fission sites and its ablation in COS-7 cells leads to a hyperconnected mitochondrial network, independently from its function in clathrin-mediated endocytosis. The two proteins have different behavior of disassembly from the fission site: Drp1 together with Mff is distributed between the two daughter mitochondria whereas Dyn2 is bound to only of the newly formed organelles.

The mechanisms of Drp1 dissociation and turnover following constriction are not yet clarified. A recent study proposed a mechanism by with VPS35-contaning vesicle removes inactive Drp1

from the OMM. The process is regulated by EPS15-Homology Domain-containing (EHD) protein, involved in endocytic recycling of receptors from endosomes back to the plasma membrane, and rabankyrin-5, effector of early endosomes marker Rab5 (Farmer et al., 2017).

II.2.1.5. Physiological and pathophysiological relevance of Drp1

II.2.1.5.1. Pathological mutations

One case of *DRP1* heterozygous mutation has been reported so far. A single *de novo* mutation at a conserved residue 359 (A359D) at the Middle domain was identified in a newborn patient (Waterham et al., 2007), characterized with microcephaly, abnormal brain development, optic atrophy and lactic academia, the patient died 37 days after birth. Immunofluorescence analysis of fibroblasts showed a hyperconnected mitochondrial and peroxisomal network. Chang and colleagues went further to examine the molecular mechanism behind this phenotype and showed that mutations in the middle domain impaired the formation of higher order multimeres assembly, assembly dependent GTPase activity and mitochondrial retention of Drp1 multimeres resulting in abnormal mitochondrial connectivity and cellular distribution (Chang et al., 2010).

II.2.1.5.2. Insights gained from in *vivo* models

II.2.1.5.2.A.*Saccharomyces cerevisiae, Caenorhabditis elegans* and *Drosophila melanogaster*

Initially, mitochondrial division was described in *Caenorhabditis elegans* (Labrousse et al., 1999) and *Saccharomyces cerevisiae* where the process is controlled respectively by dynamin-related proteins called DRP-1 and Dnm1p (Bleazard et al., 1999; Sesaki and Jensen, 1999). Loss of Dnm1p/DRP-1 leads to an increase in mitochondrial connectivity ((Bleazard et al., 1999; Labrousse et al., 1999; Sesaki and Jensen, 1999).

In *Drosophila melanogaster* DRP1-mediated mitochondrial fission has been shown to regulate follicle cell differentiation during oogenesis by interfering with cell cycle regulation cyclin E signaling (Mitra et al., 2012; Parker et al., 2015). In the fly model DRP1 has also been connected to neuronal development - DRP1 mutant flies fail to distribute mitochondria at neuromuscular junctions leading to perturbed neurotransmission (Verstreken et al., 2005).

II.2.1.5.2.B. Mouse models of *Drp1* ablation

II.2.1.5.2.B.a. Full-body and neuronal-specific *Drp1* ablation

The physiological significance of DRP1 was shown independently by two groups who reported that full body ablation of *Drp1* is embryonically lethal around stage E11,5 - E12,5 (Ishihara et al., 2009; Wakabayashi et al., 2009b). Ishihara and colleagues showed that full *Drp1* knockouts have developmental abnormalities, especially in the forebrain. Neural cell-specific *Drp1* knock-out mice (NS-*Drp1*$^{-/-}$) die shortly after birth due to brain hypoplasia and apopotosis. *Drp1*$^{-/-}$ MEFs isolated from knockout mice are characterized with hyperconnected perinuclear mitochondrial network, but grow healthy with functional respiration and no signs of upregulated apoptosis. On the contrary, neuronal cells isolated from neuronal cell specific knock-out mice (NS-*Drp1*$^{-/-}$) have defects in synapse formation and are highly sensitive to Ca^{2+} -dependent apoptosis (Ishihara et al., 2009). This suggesting that *Drp1* ablation and deficient mitochondrial fission are dispensable in non-polarized cells such as fibroblasts, whereas they are compulsory for highly polarized cells such as neurons to ensure proper mitochondrial distribution and Ca^{2+} response.

Wakabayashi and coworkers report that *Drp1*$^{-/-}$ embryos have developmental problems most likely due to defects in placental formation. Similar to *Mfn2* null mice (Chen et al., 2003) the trophoblast giant cell layer is missing in *Drp1*$^{-/-}$ embryos, suggesting impaired nutrient and oxygen exchange between mother and fetus. Heart and blood vessel development were not affected, however isolated cardiomyocytes show lower beating rate compared to WT (Wakabayashi et al., 2009b). In line with the work of Ishihara et al., this study also shows that isolated MEFs have hyperconnected perinuclear mitochondrial network and reports the relevance of DRP1 in brain development. Cerebellum and mid-brain specific knock-out for *Drp1* (En1- *Drp1*$^{-/-}$) showed that knock-out mice have impaired cerebellum development due to hyperconnected mitochondrial morphology in Purkinje cells (PC). However, heterozygous *Drp1* mutants are normal at birth, growth and mating showing that partial *Drp1* ablation is tolerated (Manczak et al., 2012; Wakabayashi et al., 2009b).

DRP1 is important not only for brain development but also for survival of postmitotic neurons (Kageyama et al., 2012; Oettinghaus et al., 2016). Inducible *Drp1* knock-out in postmitotic Purkinje cells causes mitochondrial elongation, accumulation of oxidative stress and defective respiration leading to cell degeneration. Due to the loss of Purkinje cells mice develop problems in motor coordination (Kageyama et al., 2012). Inducible *Drp1* ablation in the adult

forebrain results in perinuclear hypeconnected mitochondrial residue without interfering with neuronal morphology, cell death and oxidative stress. However, ablation of *Drp1* was characterized with reduced ATP supply at the presynaptic terminal causing neurotransmission deficiency and early memory defects (Oettinghaus et al., 2016).

II.2.1.5.2.B.b. Heart-specific *Drp1* knock-out and muscle-specific overexpression

In the heart, inducible *Drp1* ablation leads to elongated mitochondria, impaired autophagy and accumulation of damaged mitochondria. Cardiac specific *Drp1* knock-out mice develop left ventricular dysfunction which is lethal (Ikeda et al., 2015). Another study shows that *Drp1* ablation in adult mouse cardiac myocytes perturbs mitochondrial fission and upregulates Parkin which impairs mitophagy and results in lethal cardiomyopathy (Song et al., 2015). Overexpression of *Drp1* specifically in the adult muscle impairs mitochondrial network remodeling specific for mature myocytes together with a decrease in mtDNA content. *Drp1* muscle overexpression downregulates protein synthesis and activates unfolded protein response (UPR) resulting in reduced muscle mass and exercise performance (Touvier et al., 2015).

II.2.1.5.2.B.c. *Drp1* ablation in mature oocyte

Udagawa and colleagues approached the role of DRP1 in oocytes maturation by generating a specific *Drp1* knock-out in growing oocytes ZP3-Cre-Drp1$^{-/-}$ (Udagawa et al., 2014). Oocytes of *Drp1* knock-out female mice show defects in ovulation and impaired follicular maturation. *Drp1*-deficient oocytes have hyperfused mitochondria, enlarged peroxysomes partially overlapping with mitochondria and clustered ER also overlapping with mitochondria. This observation is in line with a decrease in ER-stored Ca^{2+} implying that induced multiorganelle overlapping is a mechanism to compensate impaired Ca^{2+} flux. Moreover, this model highlights the role of DRP1 in organelle distribution and interorgenelle contact sites which appears to be cell type specific.

II.3. Physiological implications of mitochondrial dynamics

The regulation of mitochondrial dynamics through fusion and fission processes allows fast adaptation of the organelle to the needs of the cell. Both states – fused or fragmented, are essential and required during different physiological conditions (Fig.11).

II.3.1. Mitochondrial inheritance

Mitochondria have to be equally distributed from mother to daughter cells during cell division (Fig. 11). Thus, proliferation and content increase of the organelle during cell life is followed by segregation of the organelle during mitosis. The process is regulated by Drp1, activated by Cdk1/cyclin B (Kashatus et al., 2011; Taguchi et al., 2007).

II.3.2. Maintaining a healthy mitochondrial population

Loss of essential mitochondrial components may generate non-functional mitochondria, leading to a heterogeneous mitochondrial population in the cell. Fusion plays a crucial role in diminishing the effects of these non-functional mitochondria, by providing a pathway for them to regain essential components. Through fusion processes mitochondria can complement mtDNA errors or loss of membrane potential (Fig.11). The heterogeneous properties of mitochondria in fusion-deficient cells are consistent with this model (Chen et al., 2005a).

Clearing of damaged mitochondria is another mechanism for maintaining a healthy mitochondrial network. It occurs via autophagic degradation of damaged or nonfunctional mitochondria, also called mitophagy (Fig.11). Mitochondrial fission is an important step in that process, generating smaller mitochondrial, carrying the damages, which could be engulfed by the autophagosome. Calcineurin-dependent activation of Drp1 has been shown to be the underlying mechanism (Cereghetti et al., 2008). All this showing the importance of the precise fusion/fission balance in the preservation of healthy mitochondria, which is essential for preventing damage accumulation in the cell.

II.3.3. Mitochondrial motility

Proper mitochondrial distribution is important responding to bioenergetics needs of the cell. However, certain cell types such as neurons are highly dependent on proper mitochondrial distribution. Fission provides the split of the mitochondrial network into smaller units, which

could be transported to their destination along the cytoskeleton (Fig.11). In neurons mitochondria are located at pre and post-synaptic sites, where high energy supplies are needed (Li et al., 2004). Neurons that lack Milton, Miro or DRP1 show defective mitochondrial transport and have sparse mitochondria at axon terminals, leading to reduced capacity for synaptic transmission (Guo et al., 2005; Stowers et al., 2002; Verstreken et al., 2005).

II.3.4. Regulation of apoptosis

Both mitochondrial fusion and fission have been reported to regulate apoptosis (Fig.11). Drp1-mediated mitochondrial fragmentation is reported to occur at early stages of apoptosis in a wide range of organisms, including yeast (Fannjiang et al., 2004), worms (Jagasia et al., 2005), flies (Goyal et al., 2007) and mammals (Frank et al., 2001). Furthermore, the observation that the pro-apoptotic proteins BAX and Bak interact with DRP1 and mitofusins points out the importance of the crosstalk of mitochondrial shapping machineries and apoptosis (Brooks et al., 2007; Karbowski et al., 2002; Karbowski et al., 2006; Wasiak et al., 2007). As a final step apoptotic cristae remodeling and cytochrome c release are regulated by Opa1 and its complexes (Cogliati et al., 2013a; Frezza et al., 2006; Glytsou et al., 2016; Scorrano et al., 2002).

II.3.5. Chemotaxis

Chemotaxis refers to the directed migration of cells in response to chemical stimuli. It requires polarization of the cells and a localized energy production source at the migratory leading edge. This process is well studied in the immune system. Upon stimulation and in order to migrate, lymphocytes begin to polarize in into a leading edge that is the front of a migrating lymphocyte and a uropod. During this process mitochondria become fragmented in a Drp1-dependent manner and accumulate at the uropod, where ATP production is required (Campello et al., 2006). Overexpression of Opa1 or downregulation of Drp1 perturbs mitochondrial distribution to the uropod and downregulates migration. Similar to lymphocytes, increasing mitochondrial elongation by Opa1 overexpression or silencing Drp1 inhibits migration of epithelial cells and breast cancer cells (Desai et al., 2013; Zhao et al., 2013). Increased DRP1 levels have been observed in human invasive breast carcinomas and in malignant oncocytic thyroid tumors (Ferreira-da-Silva et al., 2015; Zhao et al., 2013). It has been shown that in cancer cells Drp1 is activated by MAPK signaling pathway. ERK1/2

phosphorylates Drp1 at Ser616, inducing mitochondrial fragmentation and tumor growth (Kashatus et al., 2015).

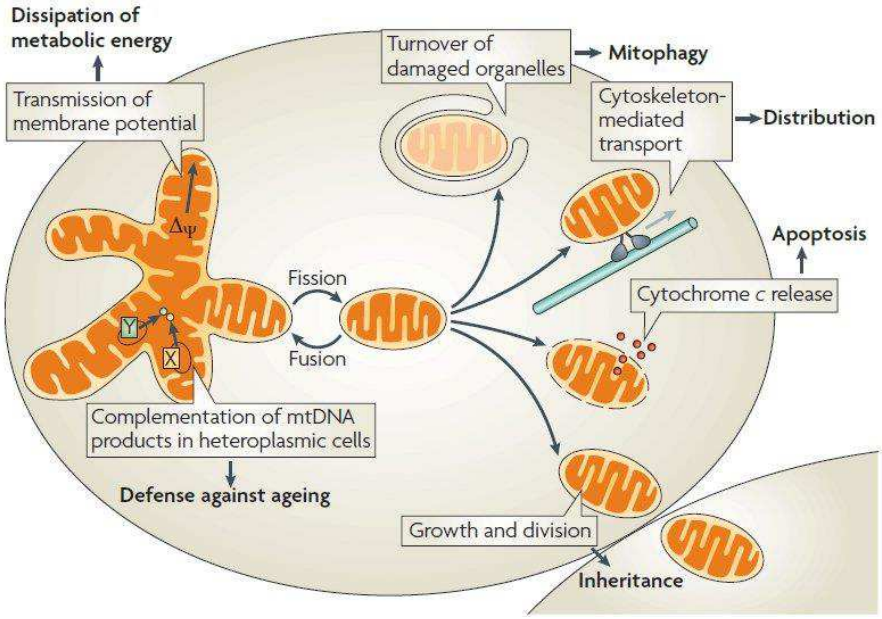


Figure 11. Mitochondrial fusion and fission and their biological implications. Division is required for inheritance of organelles during cell division, for the release of pro-apoptotic factors from the intermembrane space, for intracellular distribution by cytoskeleton-mediated transport and for turnover of damaged organelles by mitophagy. Fused mitochondrial networks are important for the complementation of mitochondrial DNA (mtDNA) gene products in heteroplasmic cells. From (Westermann, 2010).

Over the last years the mitochondrial dynamics and the protein machinery regulating them have been shown to participate in various physiological processes such as development, neuronal activity and homeostasis, cancer development, immune response, muscle biogenesis and homeostasis, thus opening the doors to explore mitochondrial dynamics in new biological fields.

III. Angiogenesis

During embryonic development, the primitive vascular system (vasculogenesis) is formed from migrating and differentiating mesoderm-derived endothelial cell (EC) progenitors called angioblasts. Once vasculogenesis is completed, the process of angiogenesis takes place (Fig.12). Angiogenesis corresponds to the sprouting and remodeling of new blood vessels from

preexisting ones (Fig.12), leading to the development of an elaborate and hierarchically organized system of arteries, arterioles, capillaries, venules and veins that transport oxygen, nutrients, hormones and signaling molecules as well waste products through the body. Angiogenesis is a complex multistep process in which precise regulation of EC behavior is critical.

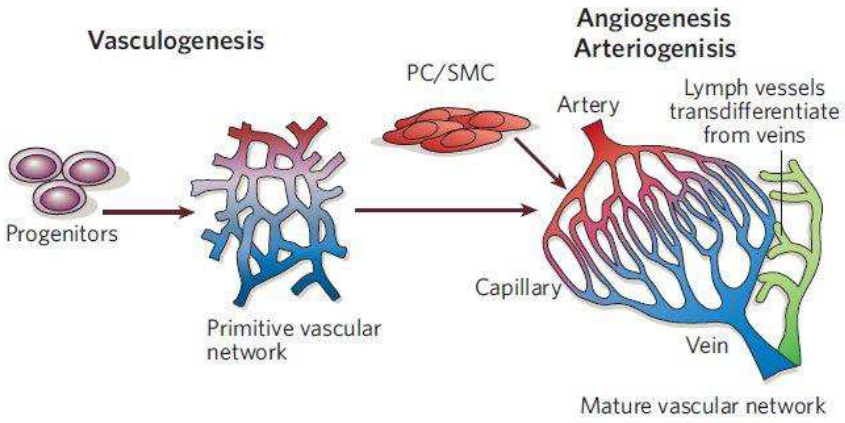


Figure 12. Schematic representation of vasculogenic and angiogenic stages of blood vessel formation. During vasculogenesis, endothelial progenitors give rise to a primitive vascular network, during subsequent angiogenesis the network expands, pericytes (PCs) and smooth muscle cells (SMCs) cover nascent endothelial channels, and a stereotypically organized vascular network emerges. Lymph vessels develop via transdifferentiation from veins. From (Carmeliet, 2005).

III.1. Types of angiogenesis

There are three main types of angiogenesis, involved in the formation of microvessels (Fig.13). Bridging angiogenesis consists in the interception of a vascular tube into smaller separated vessels by migration of ECs towards the center of the vessel tube. Intussusception consists of separation of enlarged vessels. Sprouting angiogenesis initiates with a sprout of EC in response to an angiogenic stimulation such as Vascular Endothelial Growth Factor (VEGF) of Fibroblast Growth Factor (FGF). It is a relative slow process since it relies on proliferation of ECs. However, it is most representative and studied in details (Carmeliet, 2000).

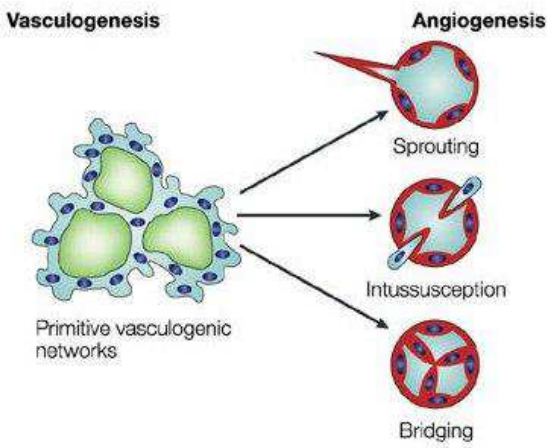


Figure 13. Schematic representation of the three types of angiogenesis. From (Carmeliet, 2000; Hendrix et al., 2003).

III.2. Stages of sprouting angiogenesis

Angiogenesis is very active during embryonic development whereas in the adult body it is kept quiescent. It could be reactivated during some physiological conditions such as tissue healing, endometrial and placental vascularization and muscle mass growth. But also during a number of pathologies, related to ischemic conditions, rheumatoid arthritis, diabetes-related microvessel complications as well as solid tumor formation and metastasis dissemination (Carmeliet, 2000, 2005). In these conditions, the reactivation of quiescent blood vessels occurs in response to pro-angiogenic signals coming from the surrounding environment (Fig.14). The cellular processes and signaling events will be discussed in detail in the following sections III.2.1.-5 (Fig.15).

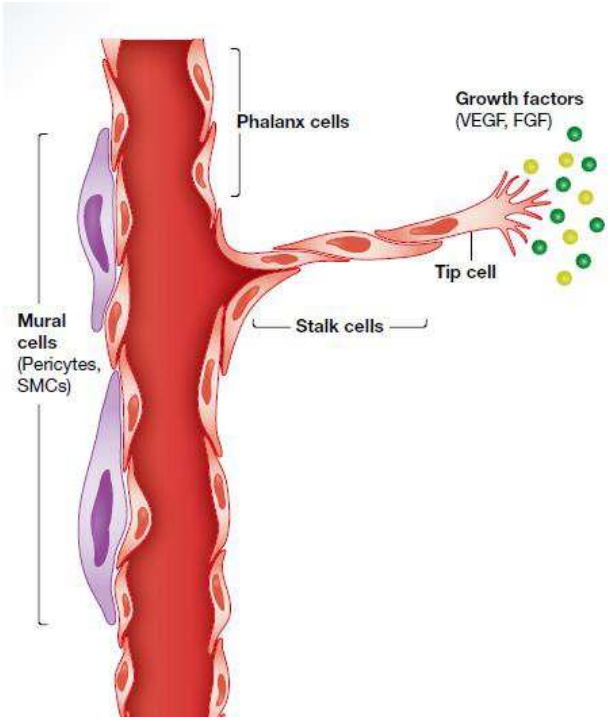


Figure 14. Schematic representation of sprouting angiogenesis. Endothelial cells (ECs) are quiescent in mature functional blood vessels. In these conditions Ecs are termed phalanx cells. Upon stimulus to extracellular cues (VEGF, FGF), ECs switch to an angiogenic phenotype towards the growth factor gradient. From (Wong et al., 2017).

III.2.1. Vasodilatation and vascular permeability

Sprouting angiogenesis initiates with an increase of blood vessel permeability mediated mainly by nitric oxide (NO) in response to VEGF stimulation (Bates et al., 2002). The increase in permeability is accompanied by a loss of cell-cell junction between ECs, by disrupting adhesion molecules such as Platelet endothelial cell adhesion molecule (PECAM) and Vascular endothelial cadherin (VE-cadherin) (Dejana et al., 2008; Herren et al., 1998). Permeability is also increased due to active ANG2, an antagonist of the ANG1-TIE2 signaling pathway that stabilizes blood vessels (Augustin et al., 2009).

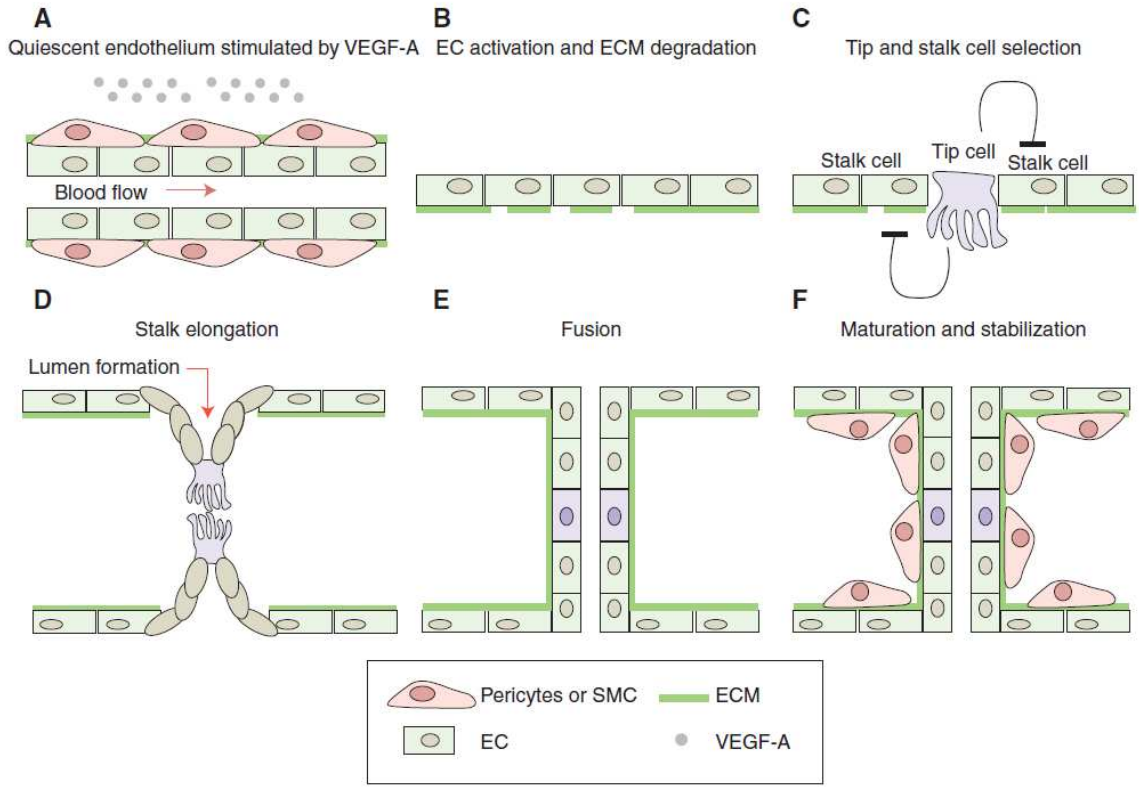


Figure 15. Schematic representation of the main cellular processes during angiogenic signaling. (A) and (B) Angiogenesis initiates with the activation of quiescent ECs and the degradation of the extra cellular matrix (ECM). Followed by specification of the (re)activated ECs into tip and stalk cells (C). ECs proliferate and collectively invade the hypoxic tissue while they remain connected to the original vascular network (D). The tip cells connect the new sprouts into a functional vessel loop (E). Formation of the vascular lumen initiates blood flow, increases tissue oxygenation, and, in turn, reduces the release of endothelial growth factors, supporting the establishment of quiescence. Vessel maturation and stabilization proceed with the recruitment of mural cells (pericytes) and the deposition of ECM (F). From (Blanco and Gerhardt, 2013).

III.2.2. Basement membrane degradation

The basement membrane or the extracellular matrix (ECM) is a layer of laminin and collagen-rich fiber supporting ECs and separating them from adjacent tissues. The ECM is degraded by matrix metalloproteinases (MMPs), allowing ECs to migrate and proliferate (Fig.15). Additionally, to ECM degradation MMPs act also on pericyte detachment and cell-cell junctions disruption by degrading VE-cadherin (Herren et al., 1998). MMPs stimulate angiogenesis by releasing ECM/basement membrane-sequestered angiogenic factors (VEGF, FGF) (Coussens et al., 1999).

Another player in blood vessel permeabilization processes is Plasmin/Plasminogen system. Plasmin, the active enzyme, could affect basement membrane degradation by directly degrading matrix proteins or by activating various MMPs (Tkachuk et al., 2009).

III.2.3. Elongation of the sprout and Tip/Stalk cell selection – the role of Notch

Elongation of the endothelial sprout is strictly dependent on migration and proliferation of ECs. This requires the differentiation of the activated ECs into subpopulations of "tip" (TC) and "stalk" cells (SC) (Fig.15, 16). Tip cells are located at the very edge of the newly formed sprout and possess numerous filopodial extensions, which allow them to sense the angiogenic source and to orient migration of the sprout in the right direction (Gerhardt et al., 2003). Tip cells are followed by the stalk cells, which are highly proliferative and have fewer filopodia (Gerhardt et al., 2003). They allow the progression of the sprout and its connection to the parental vessel. Stalk cells are also reported to establish the vascular lumen in growing vessels (Iruela-Arispe and Davis, 2009; Kamei et al., 2006). Tip and stalk cells differ for many other morphological and functional characteristic, represented in figure 16. The crosstalk between the two subpopulations is crucial to establish the correct phenotype balance, required for sprouting angiogenesis (Fig.14,16). Tip cells are highly responsive to VEGF since they express high levels of the VEGF main receptor VEGFR2 (described in details in section III.3.4.) whereas VEGFR2 signaling is suppressed in SCs.

Notch signaling pathway is a key regulator of the tip/stalk phenotype. Notch is a highly conserved pathway with established role is cell differentiation (Roca and Adams, 2007). ECs express multiple Notch receptors (NOTCH1, NOTCH3, NOTCH4) and transmembrane Notch ligands such as Delta-like ligand 4 (Dll4), Jagged 1 and Jagged 2. Ligand binding induces Notch receptor cleavage and the release of Notch intracellular domain (NICD) which functions as a transcription factor in the regulation of cell-fate decision-making. Upon VEGF stimulation tip cells express Dll4, which activates Notch in the adjacent future SC, where it suppresses VEGFR2 expression, thus allowing guided progression of the endothelial sprout (Hellstrom et al., 2007). Additionally to Dll4-inhibiting effect of TC fate, another ligand of Notch Jagged1, expressed specifically in SC, has been shown to promote TC formation and angiogenesis (Benedito et al., 2009). The authors propose a model in which Jagged 1 competes with Dll4 for binding Notch

in TCs and thus suppresses its signaling in TC and allows increased VEGFR2 signaling. EC-specific Jagged 1-knockout mouse model shows strongly reduced vascular sprouting and TC formation in retinal vascular plexus.

Recent studies propose additional regulators of Notch signaling during sprouting angiogenesis. Wnt and β-catenin pathway positively regulate Dll4 expression (Corada et al., 2010; Phng et al., 2009). Sirtuin 1 (SIRT1), which deacylates NCID in ECs and negatively modulates Dll4-Notch signaling (Guarani et al., 2011). All these finding show the importance of the complex and fine-tuned Notch signaling during vessel sprouting but also in vessel stabilization (Ehling et al., 2013).

Finally, recent data showed that ECs can rapidly shift between TC and SC *in vitro* and *in vivo* during spouting angiogenesis in the head of zebrafish (Jakobsson et al., 2010), suggesting that TC/SC fate is rather a plastic than rigid process.

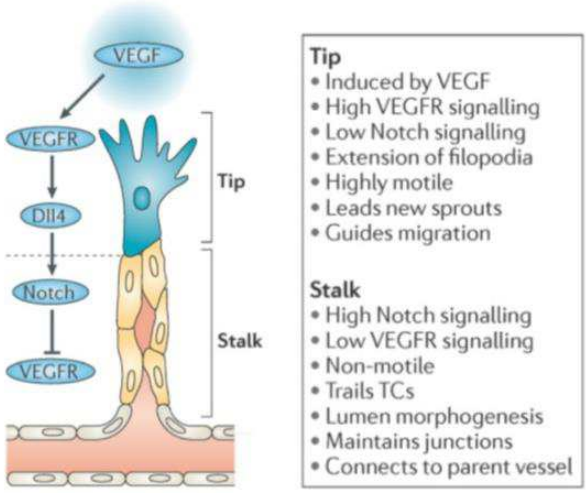


Figure 16. Schematic representation of endothelial tip and stalk cell morphological and functional differences. Tip cell formation is induced by VEGF signaling. On the contrary, in stalk cells high Delta-like 4 (DLL4)–Notch signaling represses VEGF receptor 2 (VEGFR2) signaling and thus suppresses development the tip cell phenotype. From (Herbert and Stainier, 2011).

III.2.4. Lumen formation

In addition to sprout elongation, new vessels require formation of the lumen to allow blood flow entry. There are two possibilities for lumen formation: cell hollowing, consisting in the coalescence of intracellular vacuoles (Kamei et al., 2006), or cord hollowing caused by fusion of the membranes of two adjacent ECs (Potente et al., 2011). Integrins can regulate lumen

formation by establishing interactions between ECs and the ECM and by the downstream signaling pathways such as Src, Focal adhesion kinase (FAK), MAPK, Rho GTPases (Avraamides et al., 2008; Iruela-Arispe and Davis, 2009; Liu and Senger, 2004). Establishment of apico-basal polarity in ECs polarity has been associated to lumen formation (Lampugnani et al., 2010; Strilic et al., 2009; Zovein et al., 2010).

III.2.5. Maturation of blood vessels

Progression of the endothelial sprout continues until it reaches adjacent vessels and connects with them through a process called anastomosis. Once this step is completed, ECs lose their pro-angiogenic features and begin to acquire quiescent phalanx ECs phenotype. Phalanx ECs are the ones forming the endothelial layer of quiescent blood vessels.

Over the years the crucial role of cell-cell junctions during this process has been demonstrated. ECs cell junctions are composed mainly by vascular endothelial cadherin (VE-cadherin). In addition to this function, VE-cadherin has been described as a co-receptor for VEGFR2, who acts as a negative regulator of VEGFR2 activity and downstream MAPK signaling (Lampugniani et al., 2003). Ablation of VE-cadherin is associated with an increase in VEGFR2 internalization (Lampugnani et al., 2006), supporting the role of VE-cadherin in promoting EC's quiescence. Moreover, EC's junctions are emerging as signaling units involved in ECs survival, activation and stabilization of blood vessels (Dejana et al., 1999; Lampugnani et al., 2017).

Stabilization is further induced by recruitment of mural cell – pericytes and smooth muscles cells (SMC) (Fig.15). Pericytes interact directly with EC of capillaries and newly formed vessels whereas SMCs stabilize veins and arteries and are separated by ECs by ECM. Recruitment of mural cells is regulated by Platelet-derived growth factor (PDGF) and its receptor PDGFR-1. PDGF is secreted by EC and binds to its receptor, expressed at the surface of mural cells, which induces their migration and proliferation (Gaengel et al., 2009). The crosstalk is completed by Angiopeitin 1 (ANG1), expressed by the mural cells, which activates its endothelial receptor TIE2 and stabilizes blood vessels (Augustin et al., 2009). Sphingosine-1-phosphate (S1P) and its receptor (S1PR) are also reported to be implicated in the communication between ECs and mural cells (Lucke and Levkau, 2010). Notch3 is described to regulate maturation and differentiation of arterial SMCs (Gridley, 2010). Transforming growth factor β (TGFβ) inhibits

proliferation and migration of ECs and induces differentiation of SMCs and production of ECM (Dickson et al., 1995; Pardali et al., 2010).

Once the blood vessel is formed and stabilized, the entry of the blood flow brings O_2 which downregulates VEGF signaling, terminating the angiogenic process (Chen et al., 2017; Chung et al., 2010).

III.3. Key angiogenic signaling pathways

Over the last 20 years vascular research has identified the key molecular pathways, regulating angiogenesis. Recent studies are now describing the fine-tuning of these pathways and the crosstalk between them.

III.3.1. Angiopoietin-Tie system

Tie2 receptors 1 and 2 (TIE1/2) and their ligands – angiopoietins 1 and 2 (ANG1/2), were the second identified EC-specific receptor tyrosine kinase signaling system, following VEGF/VEGFRs (Dumont et al., 1992; Iwama et al., 1993; Maisonpierre et al., 1993; Partanen et al., 1992; Sato et al., 1993). Tie/ANG axis is essential for vessel remodeling and maturation (Augustin et al., 2009).

Tie2 knockout mice die around E10,5-E12,5, due to a massive failure in the remodeling of the embryonic vasculature (Dumont et al., 1994; Patan, 1998; Sato et al., 1995). Even though initially it has been reported that hematopoiesis was not affected in the *Tie2* knockout (Rodewald and Sato, 1996), later on it has been shown that Tie2 has a crucial role in the process (Arai et al., 2004; Takakura et al., 1998). ANG1 has been identified as the ligand activating Tie2 receptor (Davis et al., 1996). *Ang1*- deficient mice phenocopy the lethality of *Tie2* knockouts (Suri et al., 1996). Tie2 binding to matrix-associated ANG1 induces EC migration. By contrast, Tie2-ANG1 trans-complexes formed between adjacent ECs promote quiescence. The signaling pathways activated by two types of interaction are different (Saharinen et al., 2008).

ANG2 is described to antagonize ANG1 on Tie2 and to destabilize vessels and promote angiogenic remodeling (Gale et al., 2002; Hackett et al., 2002; Maisonpierre et al., 1997). *In vivo* studies in mouse models for *Ang2* ablation showed that postnatal retinal vascularization is strongly affected with persistent hyaloid vessels (Gale et al., 2002; Hackett et al., 2002).

Although complex, the mechanism of ANG2/ANG1/Tie2 signaling has been studied in details, but the role of Tie1 receptor remains largely unexplored and it is still described as orphan receptor. In contrast to Tie2, in *Tie1* knockout mice the embryonic vasculature is not affected and they die later around E13,5-birth, due to massive edema caused by loss of vessel integrity (Puri et al., 1995; Sato et al., 1995). Moreover, Tie1 expression is induced upon hypoxia or VEGF-dependent angiogenic activation (McCarthy et al., 1998). Shear stress is also reported to modulate Tie1 expression (Porat et al., 2004; Woo and Baldwin, 2011). Tie1-Tie2 interactions have been implicated in the regulation of Ang1-induced Tie2 signal transduction (Saharinen et al., 2005; Seegar et al., 2010), indicating ligand-independent functions of Tie1. Recent studies propose a model in which Tie1 acts a context-dependent modulator of Tie2. Tie1 negatively regulates Tie2 in sprouting tip cells and positively during remodeling of stalk cells (Savant et al., 2015).

III.3.2. Notch signaling

As discussed in detail in section III.2.3. Notch and its ligands are crucial for sprouting angiogenesis by regulating tip/stalk cell selection. At a later stage of angiogenesis Notch signaling is reported to regulate artery formation (Pitulescu et al., 2017). Notch is emerging as e key regulator of blood vessels maturation and stability, mainly due to its inhibitory effect on VEGF/VEGFR2 signaling, but new molecular pathways are constantly being described (Kerr et al., 2016; Shah et al., 2017).

III.3.3. Wnt signaling

The Wnt signaling system is shown to regulate developmental processes such as cell-fate specification, proliferation, survival and organogenesis. Over the last years the role of the Wnt signaling in angiogenesis and ECs is emerging (Franco et al., 2009; Zerlin et al., 2008).

Wnt can signal in a canonic β-catenin-dependent way or in a non-canonical β-catenin-independent (Clevers, 2006; MacDonald et al., 2009). The canonical pathway is most well characterized one. In the absence of Wnt cytosolic β-catenin is degraded and Wnt target genes are not expressed. Binding of Wnt to Frizzled/Lrp receptor complex inhibits β-catenin degradation and promotes its translocation to the nucleus where it interacts with various transcription factors, inducing transcription of Wnt target genes. Canonical Wnt signaling is

reported to be important during vasculo and angiogenesis but repressed in healthy, quiescent adult vasculature (Corada et al., 2010; Ishikawa et al., 2001; Ye et al., 2009). Moreover, β-catenin links Wnt signaling to endothelial cell junctions complex signaling through its interaction with VE-cadherin (Stepniak et al., 2009).

Wnt can signal in a β-catenin-independent way by interacting with Frizzled and inducing release of intracellular Ca^{2+} and stimulating EC proliferation (Goodwin et al., 2007; Masckauchan et al., 2006). However, our knowledge on non-canonical Wnt during angiogenesis is incomplete.

III.3.4. VEGF/VEGFR2 - master regulators of angiogenesis

In vertebrates, vascular endothelial growth factors (VEGFs) include VEGFs A-D and placenta growth factor (PlGF). They are broadly expressed angiogenic cytokines that signal via their respective tyrosine kinase receptors (VEGFR2) – VEGFR1 (FLT1), VEGFR2 (Flk1/KDR) and VEGFR3 (FLT4). VEGF-A-VEGFR2 signaling axis is a master regulator of angiogenic signaling during development and pathology. This pathway is indispensable for vasculo and angiogenesis and is involved in EC survival as well as in the initiation and performance of each step of the angiogenic process – permeabilization and ECM degradation, proliferation, migration. However proper vascular development requires a degree of negative regulation of the pathway, specifically during maturation of blood vessels when ECs need to require quiescence properties (Ehling et al., 2013). As discussed earlier VE-cadherin and Notch signaling have negative effects of VEGFR2 angiogenenic effects.

III.3.4.1. VEGF-A

VEGF was initially described by Sanger and colleagues as vascular permeability factor, secreted by tumors (Senger et al., 1983; Senger et al., 1986). Later on, its crucial role in developmental angiogenesis has been elucidated using mouse models. Complete ablation of the gene encoding for VEGF-A leads to embryonic death at E9,5-10, due to defects in blood vessel formation. Single allele ablation has similar effect – embryos die around E11-12 (Carmeliet et al., 1996; Ferrara et al., 1996).

The gene encoding for VEGF-A is composed by 8 exons and alternative splicing generates various forms of the mature protein with a length from 121 to 206 amino acids. Splice variants

have different affinities for VEGFRs binding as well as for binding to co-receptors such as neuropilins (NRPs), heparan sulfate proteoglycans (HSRs) and the extracellular matrix (ECM), allowing precise tuning of VEGF-mediated signaling (Ferrara, 2010).

VEGF-A_{165} is the most prominent VEGF-A form in humans (Holmes and Zachary, 2008), existing in as homodimer of 46kDa. Expressed by a number of cell types such as smooth muscle cells, macrophages, tumor cells, it acts in a paracrine way on endothelial cells. VEGF-A could also signal in autocrine way, promoting EC survival (Lee et al., 2007; Maharaj et al., 2006)

III.3.4.2. VEGFRs

VEGFR 1-3 show a similar structural organization with seven extracellular immunoglobulin homology domain repeats, a transmembrane domain and a split tyrosine kinase domain (Fig.17). However, the receptors differ in their activation and signaling mode and in the biological processes they regulate. For example, VEGFR3 binds to unprocessed VEGF-C and is involved in lymphangiogenesis (Karkkainen et al., 2004). Recently, VEGFR3 has been shown to limit VEGF/VEGFR2 signaling axis in quiescent and angiogenic endothelial cells and thus preventing excessive vascular permeability (Heinolainen et al., 2017). VEGFR1 binds to VEGF-A, VEGF-B and PlGF. VEGFR1 has greater affinity for VEGF-A than VEGFR2 but weaker tyrosine kinase activity, thus by forming heretodimers with VEGFR2 it acts as a negative regulator of VEGFR2 signaling (Cudmore et al., 2012; Fong et al., 1995). VEGFR2 is the main signaling VEGFR in blood vessel endothelial cells. Ablation of VEGFR2 (*Flk1* $^{-/-}$*)* in the mouse leads to embryonic lethality at stage E9 (Shalaby et al., 1995), pointing out the importance of VEGFR2 in vasculo and angiogenesis during embryonic development. In the adult body VEGFR2 expression is strongly increased upon reactivation of angiogenesis during uterine vascularization or tumor-induced angiogenesis (Holmes and Zachary, 2008; Koch and Claesson-Welsh, 2012).

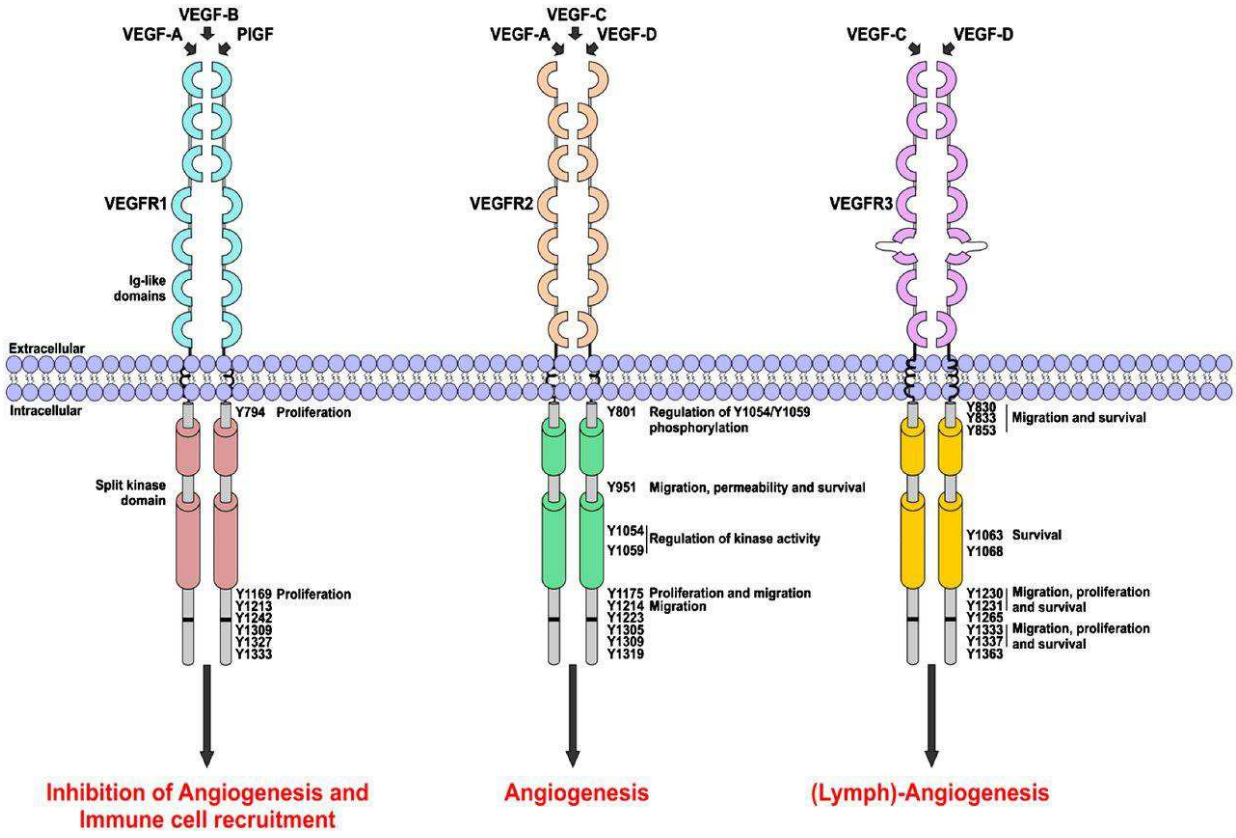


Figure 17. Receptor tyrosine kinase subfamily of VEGFR proteins. Schematic representation of VEGFRs homodimers, interacting VEGF ligands, phosphorylated tyrosine residues and their functionality. From (Smith et al., 2015).

III.3.4.3. VEGFR2 activation

Canonical VEGFR2 activation consists of VEGF-A binding to the extracellular immunoglobulin domains 2 and 3 of the receptor, which induces homo- or heterodimerization. The possibilities of heterodimerization and their functional properties are detailed in figure 7. Dimerization is followed by trans-phosphorylation of tyrosine residues located within the intracellular kinase domains (Fig.17), meaning that one molecule phosphorylates the other one in the dimer. Phosphorylation at tyrosine 1175 is crucial for VEGFR2 angiogenic signaling, by directly interacting with downstream signaling cascade effectors. This was shown *in vivo* by generating a mouse model in which the tyrosine at 1173 (corresponding to 1175 in human) is substituted by phenylalanine. *Flk1* ^{Y1173F} mice die at E8,5-E9,5 due to a disrupted vasculature similar to *Flk1* $^{-/-}$ mice (Sakurai et al., 2005). Phosphorylation of Y951 (Y949 in mouse) activates Src kinases (Li et al., 2016). The rest of the tyrosine residues are phosphorylated in response to VEGF-A but not as strongly as the Y1175 and their roles in signal transduction have not yet been clarified (Matsumoto et al., 2005).

VEGFR2 could also be phosphorylated on serine and threonine residues. The role of these post-translational modifications is not well studied. *In vitro* studies suggest that they are involved in signal attenuation (Meyer et al., 2002; Singh et al., 2005). Another parameter crucial for full activation of the receptor is represented by rotation of the dimer upon ligand binding (Anisimov et al., 2013; Lemmon and Schlessinger, 2010). Non-canonical VEGFR2 activation involves non-VEGF ligands and shear stress (Jin et al., 2013; Tzima et al., 2005).

Activated VEGFR2 mediates its angiogenic properties through a number of downstream signaling cascades including PLCγ- ERK1/2, which also involves Ca^{2+} signaling and is important for EC proliferation, migration as well as arterial differentiation and homeostasis (Hong et al., 2006; Simons and Eichmann, 2015). Activated VEGFR2, together with VE-cadherin and Src activates AKT pathway to regulate pathological and adult angiogenesis as well as vascular maturation and metabolism (Carmeliet et al., 1999; Chen et al., 2005b; Kerr et al., 2016; Lee et al., 2014a).

VEGFR2-dependent activation of Src regulates cytoskeletal, cell–cell and cell-ECM adhesion components, thus regulating vascular permeability (Ferrando et al., 2012). Mechanistically, in response to VEGF-A Src phosphorylates the focal adhesion kinase (FAK) and VE-cadherin (Chen et al., 2012; Weis et al., 2004; Westhoff et al., 2004).

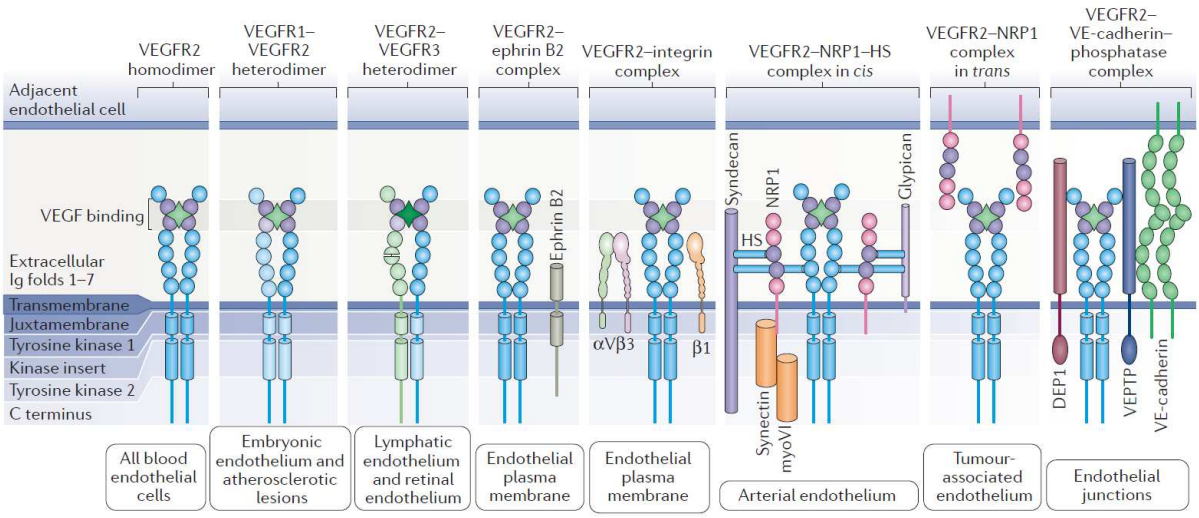


Figure 18. VEGFR2 homo and hetero dimers, signaling complexes and their functionality. Vascular endothelial growth factor (VEGF) ligands (VEGFA: light green; VEGFC or VEGFD: dark green) bind to the second and third extracellular immunoglobulin domain (purple) of VEGFR2, inducing receptor dimerization. VEGFR2 forms homodimer or heterodimers with VEGFR1 or VEGFR3. VEGFR2 homodimer signaling is modulated by different VEGF-binding co-receptors such as heparan sulfate (HS) proteoglycans (syndecan or glypican) and neuropilins (NRPs), as well as non-VEGF-binding auxiliary proteins, such as vascular endothelial cadherin (VE-cadherin), integrins, ephrin B2 and protein tyrosine phosphate (PTP). VEGFR2 may also form *trans*-complexes with NRP1 expressed on adjacent cells. The biological context in which each receptor complex is known to function is indicated. DEP1, density-enhanced phosphatase 1; myoVI, myosin VI; VEPTP, vascular endothelial protein tyrosine phosphatase. From (Simons et al., 2016).

III.3.4.4. The role of co-receptors in VEGFR2 activation and signal transduction

It has been long considered that VEGFR2 is the exclusive binding site for VEGF-A. However, various studies have shown the ability of the ligand to bind to co-receptors and molecular partners of VEGFR2. Moreover, it has been reported that formation of a molecular complexes is crucial for regulating VEGFR2 signaling.

III.3.4.4.A. VEGF-binding co-receptors

III.3.4.4.A.a. Neuropilins

Neuropilins are glycoprotein receptors initially described for binding axonal guidance molecules – semaphorins, in neurons (Chen et al., 1997; He and Tessier-Lavigne, 1997).

VEGF-A binds to NRP1 via specific motifs on exon 7 and 8 (Parker et al., 2012). NRP1-VEGF-A interaction is mediated by heparan sulfate, which also allows the interaction between NRP1 and VEGFR2 (Fig.18) (Plein et al., 2014). NRP1 plays an important role in VEGFR2 intracellular trafficking. NRP1 is able to bind VEGF-VEGFR2 complex in trans, between adjacent EC and this

interaction inhibits VEGFR2 internalization and suppresses angiogenesis (Koch et al., 2014). VEGFR2-unbound NRP1 is able to bind and sequester VEGF and thus negatively regulate its signaling (Okabe et al., 2014). As discussed in details in section III.3.4.5., NRP1 plays an important role in VEGFR2 endocytic trafficking.

III.3.4.4.A.b. Integrins

Integrins are heterodimeric transmembrane glycoproteins involved in cell-cell and cell-matrix contacts. Integrins are formed by a non-covalent interaction between the α (18) and β (8) subunits, each subunit contains a transmembrane and a small cytosolic subunit.

Integrins β1 and β3 are shown to interact with VEGFR2 signaling (Fig.18). VEGF-A induces VEGR2-Integrin β3 interaction, required for complete activation of the receptor (Byzova et al., 2000). VEGFR2-Integrin β1 complex is induced by ECM-bound VEGF-A and induces cell-surface translocation of VEGFR2 to EC-ECM contact sites called focal adhesions, which prolongs receptors activation (Chen et al., 2010).

Integrins can interact with adaptor proteins (talin, paxilin, vinculin) via the cytosolic domain of the β subunit and thus interfere with signaling pathways like MAPK, AKT and Rho GTPases or/and endosomal trafficking (Caswell et al., 2009; Hynes, 2002).

III.3.4.4.B. VEGFR2 molecular partners

III.3.4.4.B.a. VE-cadherin

In 2003 Lampugnani and colleagues reported that VE-cadherin interacts with VEGFR2 (Fig.18). This interaction negatively regulates VEGFR2 activation and suppresses downstream MAPK signaling. Mechanistically, VE-cadherin binding induces dephosphorylation of VEGFR2 by the phosphatese DEP1 (Lampugniani et al., 2003). Later the authors showed that VE-cadherin also regulates VEGFR2 internalization by stabilizing the receptor at cell-cell junctions. As discussed earlier, this observation is supported by the increase in VEGFR2 internalization upon VE-cadherin ablation (Lampugnani et al., 2006). A more recent study shows that VE-cadherin and VEGFR2 interact directly through their transmembrane domains (Coon et al., 2015).

A recent study reported a crosstalk between Integrins and VE-cadherin in the regulation of angiogenesis. The authors showed that Integrin β1 is indispensable for proper localization of VE-cadherin and thereby cell–cell junction integrity. This complex is required for normal vascular sprouting, but also negatively regulates EC proliferation during maturation of the

vessels, thus supporting the formation of a stable, non-leaky blood vasculature (Yamamoto et al., 2015).

III.3.4.4.B.b. Ephrin B2

Ephrin B2 is another molecular partner of VEGFR2 at the plasma membrane (Fig.18). Ablation of ephrin B2 leads to a complete block of VEGF-induced VEGFR2 endocytosis, resulting in strongly disrupted postnatal angiogenesis (Sawamiphak et al., 2010). Ephrin B2 regulates the movement of VEGFR2 from the plasma membrane into the cytoplasm of endothelial cells by interacting with cell polarity proteins disabled homologue 2 (DAB2) and the partitioning defective 3 homologue (PAR3) (Nakayama et al., 2013).

III.3.4.4.B.c. LRP and uPAR

As discussed in section III.2.2., plasmin is involved in ECM degradation during vascular permeabilization. Active plasmin results from the proteolytical cleavage of plasminogen by either tissue plasminogen activator (tPA) or by urokinase plasminogen activator (uPA). uPA needs to bind to its receptor urokinase Plasminogen Activator Receptor (uPAR) in order to become activated. VEGF-A induses uPA/uPAR interaction and the internalization and redistribution of uPAR and integrins (Alexander et al., 2012; Prager et al., 2004a; Prager et al., 2004b). A recent study described uPAR as a new co-receptor for VEGFR2 by showing the formation of VEGF-induced VEGFR2-integrin β1-uPAR-LRP1 at the plasma membrane with LRP1 being indispensable for complex internalization and mediation of VEGFR2 angiogenic effects (Herkenne et al., 2015).

III.3.4.5. Internalization and endosomal trafficking of VEGFR2

Following VEGF-A binding, activated VEGFR2 enters the cell predominantly via clathrin-dependent internalization (Damke et al., 1994; Lampugnani et al., 2006). As described in some previous chapters many co-factors and adaptor proteins regulate VEGFR2 internalization (integrins, VE-cadherin, ephrin B2, uPAR and LRP1). A recent study reported a role for Dynamin 2 in modulating Integrin β1 during VEGFR2 internalization (Lee et al., 2014b).

The effects of VEGFR2 internalization on its angiogenic signaling have been controversial. Ewan et al. showed that VEGFR2 is internalized into endosome and then degraded by the

lysosomes, thus describing internalization as a signal-attenuating process (Ewan et al., 2006). More recently, Low Density Lipoprotein (LDL) has been shown to induce VEGFR2 internalization and degradation, which has a negative effect on VEGF signaling (Jin et al., 2013). In contrast, many studies report that VEGFR2 internalization is a mechanism of signal perpetuation. Lampugnani and coworkers showed that endosome-located VEGFR2 is still phosphorylated and able to activate MAPK pathway (Lampugnani et al., 2006). This was supported by another study, showing that VEGF-A-induced receptor internalization is crucial for ERK 1/2 activation (Gourlaouen et al., 2013). These findings showed the importance of the intracellular spatio-temporal regulation of VEGFR2 signaling, which is orchestrated by the endosomal compartment and a subset of Rab GTPases (Bhuin and Roy, 2014; Jopling et al., 2009; Zerial and McBride, 2001a).

Once internalized VEGFR2-containing clathrin-coated vesicles fuse with early endosomes, which are Rab5 and early endosome antigen 1 (EEA1) positive vesicles (Fig.19) (Lampugnani et al., 2006; Lanahan et al., 2010). NRP1 plays a crucial role in the intracellular trafficking of VEGFR2 by binding its C-terminal domain to Synectin-Myosin IV complex. This interaction is crucial for endosomal translocation of VEGFR2 complex and VEGF-induced ERK1/2 activation (Lanahan et al., 2013; Lanahan et al., 2010). The interaction with motor proteins synectin and myosin IV allows the movement of VEGFR2-containing early endosomes away from the plasma membrane, protecting the Y1175 residue from dephosphorylation by the PM-located phosphatases. Thus, Y1175 can activate downstream ERK1/2 cascade and promote angiogenesis. The signaling could be terminated when endosomes get in proximity to the ER. ER-anchored PTP1B phosphatase preferentially dephosphorylates Y1175 thus selectively suppressing ERK 1/2 pathway. NRP1-bound VEGFR2 could be recycled back to the plasma membrane whereas the absence of NRP1 leads to its lysosomal degradation (Ballmer-Hofer et al., 2011).

Once the internalized VEGFR2 transduces its signal, it could be degraded via Rab7-positive late endosomes (Fig.19), which fuse with lysosomes (Rab9-positive). VEGFR2 could also be recycled back to the PM via Rab11-mediated slow or Rab4 fast pathway (Ballmer-Hofer et al., 2011). Additionally, VEGFR2 can undergo constitutive or ligand-independent endocytosis. The mechanism is poorly understood but it appears to consist of a rapid recycling of the internalized receptor back to the PM via Rab4 pathway (Fig.19) (Zhang and Simons, 2014).

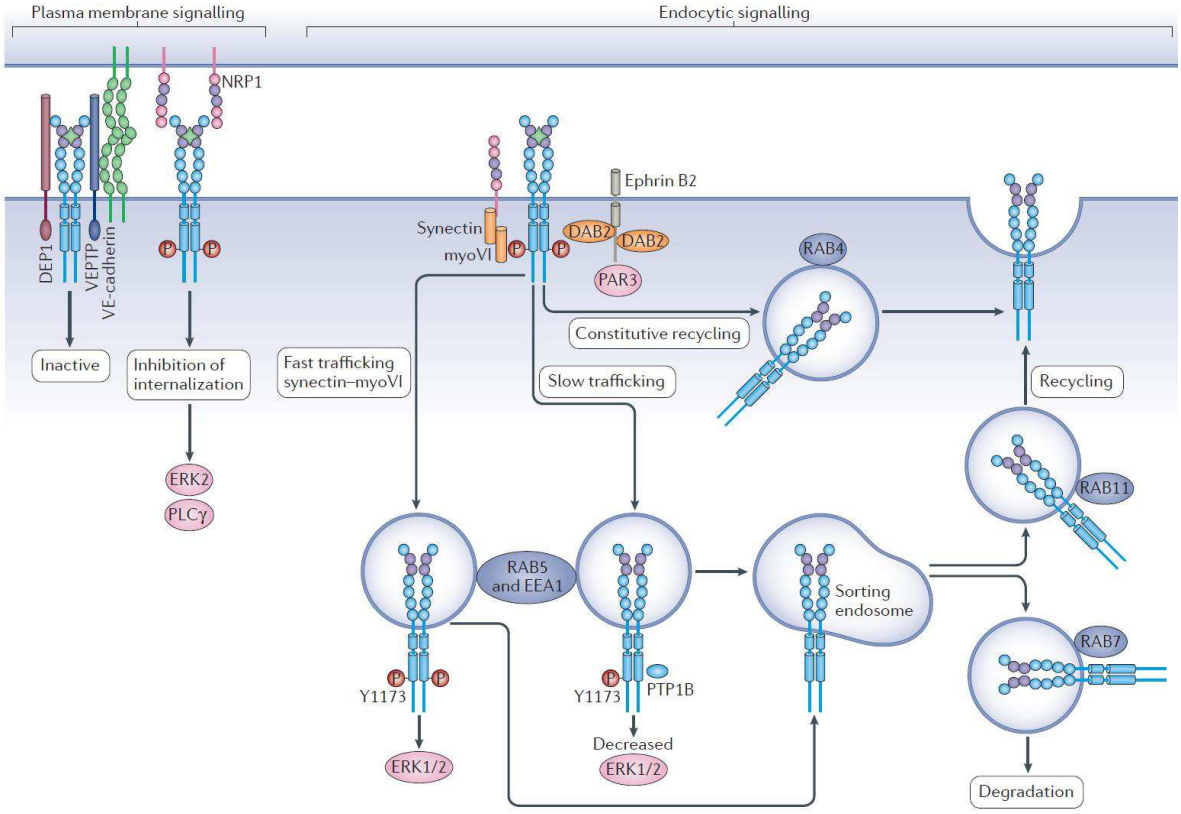


Figure 19. VEGFR2 signaling at the plasma membrane and endosomal trafficking. Internalized VEGFR2 can signal from the early endosomes. Trafficking continues towards the sorting endosome from which the receptor could be recycled back to the plasma membrane via Rab11-positive vesicles or degraded via Rab7 positive vesicles. Ligand-independent, constitutive recycling of VEGFR2 could be mediated via RAB4 endosomes. DAB2, disabled homologue 2; DEP1, density-enhanced phosphatase 1; EEA1, early endosome antigen 1; myoVI, myosin VI; NRP1, neuropilin 1; PAR3, partitioning defective 3 homologue; PLCγ, phospholipase Cγ; PTP1B, protein tyrosine phosphatase 1B; VE-cadherin, vascular endothelial cadherin; VEGFR2, vascular endothelial growth factor receptor 2; VEPTP, vascular endothelial protein tyrosine phosphatase; Y1173, Tyr1173. From (Simons et al., 2016).

III.3.4.6. Compartmentalization of VEGFR2 signaling

VEGFR2 signaling is compartmentalized within the cell and various pools of VEGFR2 exist (Gampel et al., 2006). This compartmentalization is far more complicated than just a simple division between plasma and intracellular VEGFR2 pools. Indeed, within the plasma membrane VEGFR2 could be enriched in regions like lipid rafts, focal adhesions and cell-cell junctions (Baumer et al., 2006; Saharinen et al., 2008). These plasma membrane regions are considered as hotspots of VEGFR2 signaling. Moreover, they support the idea of distinct specialized VEGFR2 signaling pathways, regulated by the corresponding co-receptors like for example VE-cadherin at cell-cell junctions. In addition to accessory proteins, regulation of

VEGFR2 internalization and signaling could be affected by the lipid composition of the PM. This mechanism is very likely to be occurring in lipid rafts-located VEGFR2. Moreover Labrecque and colleagues showed that depletion of cholesterol, a key component of lipid rafts, increases levels of activated VEGFR2 but suppressed downstream ERK 1/2 activation (Labrecque et al., 2003).The mechanisms regulating these processes are not well defined, but are becoming a topic of growing interest in angiogenic research.

III.3.4.7. Perspectives in VEGFR2 signaling research

Being a key regulator in tumor angiogenesis as well, VEGF-VEGFR2 axis became an interesting target in anti-angiogenic cancer therapy and led to the generation of a number of drugs inhibiting the pathway (Ferrara et al., 2004; Labrecque et al., 2003; Loges et al., 2009). However, tumor resistance is a persistent issue (Casanovas et al., 2005). Confronting it requires a better understanding of the complex biological mechanism, regulating VEGFR2 signaling. Therefore, over the last years, the basic research in the field of angiogenesis has been extremely focused on the fine-tuning and the crosstalk between canonical signaling pathways and other biological factors that regulate them, such as miRNAs expression in ECs, co-receptor complexes and lipid composition, post-translational modifications and trafficking of the receptor, microvesicular signaling between ECs and other cell types. Thus the mitochondria and its dynamic morphology, which have emerged separately as a key regulator in many cellular processes, began to gain interest in the angiogenic field.

IV. Mitochondria in angiogenesis

The role of mitochondria has been studied in detail in cell types known to rely on OXPHOS as source of energy such as neurons, skeletal muscles and cardiomyocytes. On the contrary, in ECs glycolysis is believed to be the main energy source. Therefore, the role of mitochondria in ECs has been neglected for a long time. However, the master regulator of mitochondrial biogenesis PGC1α is expressed in ECs and PGC1α-dependent mitochondrial biogenesis is a protective mechanism against oxidative stress (Afolayan et al., 2016; Valle et al., 2005). PGC1α is induced by a number of pro-angiogenic factors such as hypoxia and caloric restriction (Leone et al., 2005; Patten et al., 2012). VEGF has been reported to induce mitochondrial biogenesis

via Akt3 signaling. Akt3 regulates PGC1α nuclear translocation in response to VEGF stimulation (Wright et al., 2008).

Recently, with the emerging of the role of mitochondria as a key signaling component of the cell, its function in the endothelium has been revised.

IV.1. Redox signaling and angiogenesis

Reactive oxygen species (ROS) are receiving substantial interest in angiogenic signaling. The source of ROS in ECs could be cytosolic and mitochondrial. The main producer of cytosolic ROS is NADPH oxidase, composed of Nox1, Nox2, Nox4, p22phox, p47phox, p67phox and the small GTPase Rac1. Endothelial NADPH is activated by VEGF, Angiopoietin-1, cytokines, shear stress and Angiotensin II (Harfouche et al., 2005; Ushio-Fukai and Alexander, 2004; Ushio-Fukai et al., 2002) . NADPH oxidase-derived ROS signaling modulates angiogenesis both *in vitro* and *in vivo* (Al-Shabrawey et al., 2005; Harfouche et al., 2005; Ikeda et al., 2005; Ushio-Fukai et al., 2002; Yamaoka-Tojo et al., 2004).

Mitochondrial ROS (mtROS) are mainly produced through complexes I and III of the OXPHOS system. Cytosolic and mitochondrial ROS share Nox4. Nox4-mediated mtROS is shown to influence blood pressure (Santillo et al., 2015). Another source of mtROS is the growth factor adaptor protein p66Shc (Paneni and Cosentino, 2012; Paneni et al., 2012). Upon high glucose concentration or pro-apoptotic signals, p66Shc can translocate to the mitochondrial intermembrane space where it oxidases cytochrome c and generates H_2O_2 which could lead to ECs senescence and apoptosis (Camici et al., 2007).

Cytosolic ROS can trigger mtROS via “ROS-induced ROS release”. This mechanism is proposed to amplify ROS signaling in ECs increasing ECs dysfunction (Zorov et al., 2014). Angiotensin II-induced ROS could trigger mtROS release through mitoK_{ATP} channel opening as protective mechanism from excessive nitric oxide (NO) (Doughan et al., 2008).

In ECs NO is produced by the endothelial Nitric oxide synthase (eNOS). NADPH, arginine and O_2 are the substrates of eNOS, linking this pathway to both redox signaling and endothelial metabolism (Alderton et al., 2001; Forstermann and Munzel, 2006; Moncada and Higgs, 1993; Palmer et al., 1988; Sakuma et al., 1988; Schmidt et al., 1988).

eNOS can be activated through Ca^{2+} dependent and independent pathways. In the first case extracellular acetylcholine or histamines increase Ca^{2+} levels, activating Calmodulin, which

activates eNOS to produce NO. The Ca^{2+}-independent pathway involves VEGF-induced PI3K/AKT which activates eNOS by phosphorylating it at Ser1179 (Fulton et al., 1999). A major activation pathway for eNOS is shear stress, which activates AKT and/or protein kinase A (Forstermann and Sessa, 2012). The main effect of NO on EC and blood vessels is the induction of vasodilation (Dimmeler et al., 1999; Dimmeler and Zeiher, 1999; Fulton et al., 1999). eNOS mutant mice have been described to have reduced physiological angiogenesis, due to a decrease in ECs proliferation and impairments in pericytes recruitement (Lee et al., 1999). Impairments of NO synthesis, due to an increased activation of eNOS could cause ECs dysfunction (Graham and Rush, 2004; Li et al., 2007). Moreover, eNOS uncoupling has been related to hypertension and ECs dysfuncion (Heitzer et al., 2000).

IV.2. Endothelial metabolism and angiogenic signaling

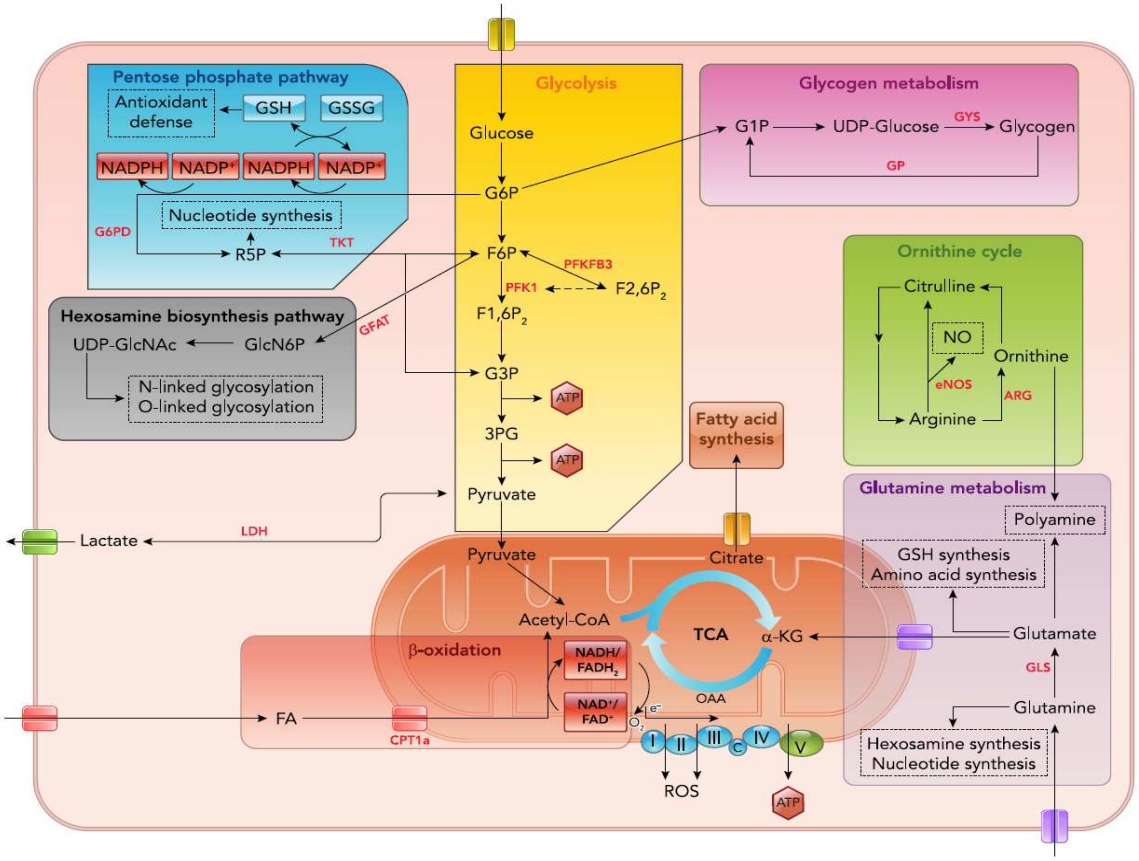


Figure 20. Schematic representation of endothelial metabolism. 3PG, 3-phosphoglycerate; αKG, alpha-ketoglutarate; acetyl-CoA, acetylcoenzyme A; ARG, arginase; ATP, adenosine triphosphate; CPT1a, carnitine palmitoyltransferase 1a; eNOS, endothelial nitric oxide synthase; FAD^+/FADH2, flavin adenine dinucleotide; F1,6P2, fructose-1,6-bisphosphate; F2,6P2, fructose-2,6-bisphosphate; FA, fatty acid; F6P, fructose-6-phosphate; G1P, glucose-1-phosphate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; G6PD, glucose-6- phosphate dehydrogenase; GFAT, glutamine fructose-6-phosphate aminotransferase; GlcN6P, glucosamine-6-phosphate; GLS, glutaminase; GP, glycogen phosphorylase; GSH, glutathione; GSSG, glutathione disulphide; GYS, glycogen synthase; LDH, lactate dehydrogenase; $NAD(P)^+$/NAD(P)H, nicotinamide adenine dinucleotide (phosphate); NO, nitric oxide; OAA, oxaloacetate;PFK1, phosphofructokinase-1; PFKFB3, phosphofructokinase-2/fructose-2,6-bisphosphatase isoform 3; R5P, ribose-5-phosphate; ROS, reactive oxygen species; TCA, tricarboxylic acid (cycle); TKT, transketolase; UDP-Glucose, uridine diphosphate glucose; UDP-GlcNAc, uridine diphosphate-*N*-acetylglucosamine. From (Bierhansl et al., 2017).

IV.2.1. Glycolysis

New blood vessel formation is an energy demanding process, therefore requires quick metabolic adaptations and ATP productions. ECs have high glycolytic activity which is the main source of ATP (De Bock et al., 2013). This is surprising given the fact that ECs are in an high oxygen environment and that OXPHOS generates 10 times more ATP per mole of glucose than glycolysis. The possible explanations would be the fact that glycolysis is a faster process which

allows better adaptation to energy demands. Moreover, it could be an adaptive strategy of ECs to generate ATP independently of oxygen when sprouting into avascular hypoxic tissues.

Apart from energy generation, glycolysis intermediate metabolites can participate in side anabolic pathways of macromolecules biosynthesis required for intense proliferation of ECs during angiogenesis. A key regulator of glycolysis in ECs is 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), that regulates production of fructose-2,6-bisphosphate via activation of phosphofructokinase-1 (De Bock et al., 2013) (Fig.20). VEGF stimulation of ECs upregulates levels of PFKFB3 and therefore promotes glycolysis (De Bock et al., 2013; Xu et al., 2014). Pharmocological and genetic inhibition of PFKFB3 results in decreased angiogenesis in the mouse postnatal retina and *in vitro* using EC spheroids model (De Bock et al., 2013; Schoors et al., 2014; Xu et al., 2014). Overexpression of PFKFB3 stimulates angiogenesis and the tip cell phenotype of ECs both *in vitro* and *in vivo* (De Bock et al., 2013). Upregulated glycolysis and glycolysis-derived metabolic pathways such as pentose phosphate pathway and serine biosynthesis are a signature of tumor endothelial cells (TEC) (Fig.21) (Cantelmo et al., 2015). Thus, targeting glycolysis appears to be a promising anti-cancer strategy (Cantelmo et al., 2016) .

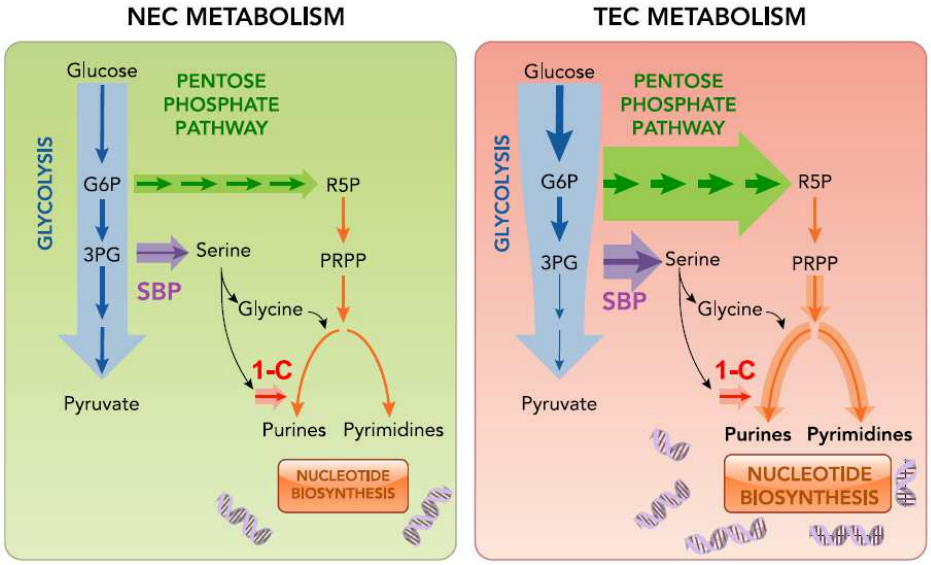


Figure 21. Metabolism of normal endothelial cell (NEC) versus tumor endothelial cell (TEC). Schematic representation of the metabolic regulation in normal endothelial cells (NEC) and tumor endothelial cells (TEC). PPP- pentose phosphate pathway; SBP-serine biosynthesis pathway (SBP), G6P-glucose-6-phosphate; 3PG-3-phosphoglycerate (3PG); PRPP-phosphorybosyl pyrophosphate; R5P-ribose 5-phosphate. From (Bierhansl et al., 2017).

IV.2.2. Glycogen metabolism

Another metabolic pathway linked to glycolysis is the Glycogen cycle. Glycogen is synthetized from Glucose-6-phosphate (Fig.20). The precise function of Glycogen in ECs is still unclear. It has been shown that when exposed to high glucose ECs increase the synthesis and accumulate glycogen (Artwohl et al., 2007) and the contrary (Vizan et al., 2009b). Inhibition of glycogen phosphorylase (GP), the enzyme responsible for glycogen degradation (Fig.20) leads to reduced migration and viability of ECs (Vizan et al., 2009b). Inhibition of the activator of GP – phosphorylase kinase subunit G1 (PhKG1), inhibits ECs migration, proliferation and tube formation *in vitro* as well as intersomitic vessel sprouting in zebrafish (Camus et al., 2012).

IV.2.3. Pentose Phosphate Pathway

The Pentose Phosphate Pathway (PPP) is another glycolysis side-pathway starting again from glucose-6-phosphate which generates pentose, a 5-carbon-sugar essential for nucleotides and nucleic acid synthesis (Fig.20). This pathway is composed by two phases: the irreversible oxidative PPP regulated by the rate-limiting glucose-6-phosphate dehydrogenase (G6PD) and the reversible non-oxidative pathway under the control of the transketolase (TKT) which depending on the needs of the cell produces either ribose-6-phosphate or the glycolysis intermediates fructose-6-phosphate and glyceraldehyde-3-phosphate (Fig.20). Inhibition of each of these pathways results in decreased ECs viability and migration (He et al., 2011; Stanton, 2012; Vizan et al., 2009a; Vizan et al., 2009b). The oxidative pathway of PPP produces NADPH, which reduces oxidase glutathione, therefore is involved in ROS scavenging. Another function of NADPH is in the synthesis of lipids, nucleotides and nitric oxide (NO) (Fraisl, 2013). Moreover, overexpression of G6PD increases NADPH and NO levels and has a promoting effect on ECs proliferation, migration and tube formation (Leopold et al., 2007; Leopold et al., 2003). Activation of protein kinase A due to hyperglycemic conditions downregulates G6PD and reduces EC survival (Giacco and Brownlee, 2010; Zhang et al., 2000).

IV.2.4. Hexosamine Biosynthesis Pathway

O and N-linked glycosylation are essential for protein functionality in ECs. Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) is the end-product of the Hexosamine Byosynthesis Pathway (HBP) and is required for glycosylation reactions. HBP is also a derivate pathway of

glycolysis. Glycolytic Fructose-6-phosphate is transformed in glucosamine-6-phosphate by the rate-limiting enzyme glutamine fructose-6-phosphate aminotransferase (GFAT) (Fig.20). Subsequently, UDP-GlcNAc is generated from glucosamine-6-phosphate, Acetyl-CoA and ATP (Mapanga and Essop, 2016; Willems et al., 2016). Upregulated glucosamine concentrations induce protein glycosylation *in vitro* and decreases ECs migration and tube formation. In parallel, overexpression of O-GlcNAcase, which removes glycosylations from proteins, induces migration and tube formation (Lu et al., 2007; Ngoh et al., 2010). Several studies show that glycosylation modifications have specific effects on key angiogenic molecules. N-glycosylation of VEGFR2 promotes its stability, surface expression and signaling activity (Croci et al., 2014; Rahimi and Costello, 2015). O-glycosylation affects Notch signaling by activating Notch/ligand interaction (Croci et al., 2014). However, the effects of these modifications on angiogenic properties of ECs require further examination.

IV.2.5. Fatty acid metabolism

FAO has also been reported to have a regulatory function in angiogenesis. Pharmacological and genetic inhibition of Carnitine palmitoyltransferase I (CPT1a), a key component of the pathway, leads to a decrease in EC proliferation *in vitro* and *in vivo*. Proliferating ECs use FAO as carbon source for DNA synthesis during vessel sprouting (Schoors et al., 2015). Moreover, blocking of CPT1a causes vessel leakage due to disrupted Ca^{2+} homeostasis (Patella et al., 2015).

Blocking FAO affects only proliferating stalk cells by nucleotide synthesis (Schoors et al., 2015), whereas blocking glycolysis via PFKFB3 affects both tip and stalk cells (De Bock et al., 2013), suggesting that different EC subtypes use different metabolic pathways and switch between them as a function of their requirements.

IV.2.6. Amino Acid Metabolism

Amino Acid metabolism in ECs is not well understood. However, new discoveries in the field are highlighting its role as a regulatory mechanism of ECs function. As described earlier, arginine is required for eNOS-dependent synthesis of NO (Fig.20) (Palmer et al., 1988; Wu and Meininger, 2000).

Glutamine is another amino acid of interest. ECs can obtain it from diet or synthetize it from glutamate using glutamine synthetase (Kobayashi et al., 2006). Glutamine can promote ECs viability and decrease cellular permeability induced by hyperglycemia by downregulating apoptosis-related proteins such as cytochrome c and thus attenuating mitochondrial stress (Safi et al., 2015). ECs express glutaminase (GLS) and can drive glutamine transformation in α-ketoglutarate which will enter the TCA cycle. Pharmacological inhibition of GLS decreases ECs viability and increases senescence (Unterluggauer et al., 2008).

A recent study reports a crosstalk between glutamine and asparagine in new blood vessel formation (Huang et al., 2017). Using the first mouse model for specific ablation of GLS1 in ECs (*GLS1*^{ECKO}*)* the authors inhibit the conversion of glutamine into glutamate, which later enters TCA cycle and generates α-ketoglutarate. Angiogenesis is decreased in *GLS1*^{ECKO} due to disruption of the tip cell phenotype and decreased proliferation. Because in ECs glutamine metabolism is needed for asparagine synthesis, asparagine supplementation in GLS1 deprived ECs can restore protein synthesis and cellular prolifetation.

IV.3. Mitochondrial distribution

Mitochondrial distribution is important in some cell types such as neurons, but this aspect has not been studied in details in ECs. In coronary arterioles mitochondria are anchored to the cytoskeleton and upon shear stress they release ROS which contributes to flow-mediated dilatation of vessels (Liu et al., 2008). In pulmonary artery ECs, hypoxia triggers perinuclear distribution of mitochondria leading to an increase in ROS accumulation in the nucleus and induction of hypoxia related genes (Al-Mehdi et al., 2012).

IV.4. Mitochondrial dynamics in angiogenesis

IV.4.1. Mitochondrial fusion

Schleicher and colleagues (Schleicher et al., 2008) were the first to investigate the role of mitochondrial shape in angiogenesis. They showed that mitochondrial located PHB1 was expressed in SMCs as well as in ECs. *PHB1* knock down in bovine aortic endothelial cells (BAECs) induced mitochondrial ROS production and loss of mitochondrial membrane potential. Moreover, silencing of *PHB1* in BAECs increased VEGF-stimulated Ser473 phosphorylation in a mtROS-dependent manner. Downstream AKT activation led to increased

NO release. Another downstream target of AKT signaling - the small GTPase Rac1, was also activated upon *PHB1* ablation in a PI3K-dependent way. Rac1 is involved in the maintenance of the cytoskeletal balance (Wang et al., 2010). Indeed, in *PHB1* knock-down cells, VEGF-induced formation of actin stress fibers, migration and tube formation were decreased. Persistent activation of AKT and its downstream Rac1 signaling lead to senescence of ECs. However, given the pleiotropic role of PHB1 on mitochondrial morphology and function, it is difficult to assign these changes specifically to an effect on mitochondrial shape.

Lugus and coworkers investigated the role of OMM fusion proteins Mfn1 and Mfn2 in ECs (Lugus et al., 2011). VEGF stimulation of human umbilical vein endothelial cells (HUVECs) upregulated mRNA and protein levels of *MFN1* and *2*. Consistently, VEGF-stimulated angiogenic performance of HUVECs *in vitro* was decreased upon both *MFN1* and *MFN2* ablation. Knocking-down *MFN1/2* led to a fragmented mitochondrial network and loss of membrane potential. Serum deprivation-induced apoptosis was increased in *MFN1/2* knock down cells. However, mtROS production was diminished specifically in MFN2 knock down cells. Downstream JNK activation, which could be ROS-dependent, was ablated in *MFN1* knock down but with greater effect in *MFN2*. In parallel, AKT (Ser473) and downstream eNOS (Ser1177) activation were shown to be specific to *MFN1* ablation. This suggests that perturbing mitochondrial dynamics by ablating Mitofusins results in a common phenotype, driven however by two different mechanisms that can be ascribed to the specific differences in their function.

IV.4.2. Mitochondrial fission: Drp1 function in the endothelium and angiogenesis

Most of our knowledge on the role of Drp1 in angiogenesis comes for correlative analyses of mitochondrial shape in models of human disease.

Hyperglycemia, the major feature of diabetes, causes mitochondrial fragmentation, induces ROS production and disrupts Ca^{2+} signaling in a variety of cell types (Rube and van der Bliek, 2004; Yu et al., 2006; Yu et al., 2008) including ECs (Li et al., 2009; Paltauf-Doburzynska et al., 2004; Trudeau et al., 2010).

Studies using mouse models of diabetes and diabetic patients demonstrated that hyperglycemia induced mitochondrial fragmentation is Drp1-dependent (Makino et al., 2010;

Shenouda et al., 2011). In these contexts, Rho-associated coil-containing protein kinase (ROCK), which acts downstream of Rho during apoptosis and ROS production, activates DRP1 by directly phosphorylating it at Serine 600. This triggers Drp1 translocation to the OMM and subsequent mitochondrial fission. Consequently, mitochondrial ROS production and apoptosis of podocytes and kidney microvascular endothelial cells are increased leading to diabetic nephropathy (Wang et al., 2012a).

Pulmonary arterial hypertension (PAH) is a syndrome characterized with obstruction and inflammation of small pulmonary arteries leading to an increase in pulmonary vascular resistance (PVR), right ventricular hypertrophy and heart failure. These vascular impairments are due to hyperproliferation of pulmonary arterial endothelial cells (PAECs) and pulmonary artery smooth muscle cells (PASMCs) (Archer et al., 2010). The hyperproliferative phenotype of PASMCs in the context of PAH has been associated with normoxic activation of HIF-1α and mitochondrial fragmentation (Bonnet et al., 2006). HIF-1α mediated PASMCs hyperproliferation in PAH is Drp1-dependent in human PASMCs, lung sections from PAH patients and three rodent models of PAH. The authors propose a mechanism for HIF-1α induced mitochondrial fragmentation and PASMCs hyperproliferation in which Cdk1/CyclinB, which is also upregulated in PAH, activates Drp1 through a phosphorylation at Ser616 leading to cell cycle progression from G2 to mitosis. Pharmacological (Mdivi1) or siRNA inhibition of Drp1 restores mitochondrial morphology, downregulates hyperproliferation and regresses PAH. Thus, Drp1 has been proposed as a promising therapeutical target for PAH (Marsboom et al., 2012).

Ischemia/Reperfusion (I/RP) process is known to induce ECs dysfunction (Carden and Granger, 2000). Ischemia leads to oxygen deprivation and a hypoxia-like condition, whereas restoration of blood flow causes shear and oxidative stress to the blood vessel and the affected tissues. Mitochondrial-dependent apoptosis is activated in rat coronary vessels ECs following I/RP (Scarabelli et al., 2002). In post ischemic ECs the I/RP induced oxidative, nitrosative and shear stresses modify mitochondrial morphology by activating Drp1 through a phosphorylation at Ser616 resulting in fragmented mitochondrial network (Giedt et al., 2012).

Finally, in HUVECs *DRP1* silencing causes premature senescence and loss of angiogenic capacities: mitochondrial hyperfusion results in reduced autophagic flux and increased mtROS production (Lin et al., 2015).

Although a small number of papers have addressed the role of Drp1 in ECs and in angiogenesis, these studies are always performed in the context of a given pathology, and do not clarify the function of Drp1 in physiological angiogenesis.

V. Scope of the Thesis

The scope of this thesis was to investigate the function of Drp1 in the endothelium during physiological angiogenesis. To address this question *in vivo* we generated constitutive and inducible mouse models for endothelial-specific ablation of *Drp1*. Our work describes a new mechanism of action for Drp1 in ECs, providing the first insights into its function in new blood vessel formation.

Results

The mitochondrial fission factor Dynamin Related Protein 1 limits VEGF/VEGFR2 endocytic trafficking and angiogenesis.

Maya Chergova[1,2], Stéphanie Herkenne[1,2], Cécile Paques[3], Eliška Novotná[1], Ingrid Struman[3] and Luca Scorrano[1,2]

[1]Department of Biology, University of Padova, Padova, Italy

[2]Venetian Institute of Molecular Medicine, Via Orus 2, Padova, Italy

[3]Laboratory of Molecular Angiogenesis, GIGA-Cancer, University of Liège, Liège, Belgium

Character count 36 614

Address correspondence to

Luca Scorrano luca.scorrano@unipd.it

Summary

Because of the mostly glycolytic nature of endothelial cell metabolism, the role of mitochondria and mitochondrial shape in angiogenesis, the new blood vessel formation from existing vasculature, has not been studied. Here we show that the mitochondrial fission factor Dynamin related protein 1 (Drp1) unexpectedly limits endosomal VEGFR2 signaling and hence angiogenesis. Drp1 levels were reduced when Human Umbilical Vein Endothelial Cells (HUVECs) were activated, and angiogenesis was accordingly stimulated in HUVECs where *DRP1* was silenced. *In vivo*, constitutive and inducible *Drp1* ablation in endothelial cells increased early stage postnatal retina vascular sprouting. Mechanistically, upon VEGF stimulation Drp1 interacted with the internalized VEGFR2 and its early endosome partner Rab5 at the endosomal VEGFR2 signaling platform. Drp1 deletion unleashed VEGFR2 activation and its downstream signaling, indicating that the VEGFR2-Rab5-Drp1 interaction limits VEGFR2 mediated angiogenesis. Our data reveal an unexpected extramitochondrial function of Drp1 in endothelial cells, where it localizes also at the endosomes to constrain the endosomal VEGFR2 signaling platform.

Introduction

Angiogenesis, the new blood vessel formation from preexisting vasculature, is very active during embryonic development and during tissue healing, ovulation and placental vascularization (Carmeliet, 2005). In response to pro-angiogenic stimuli, quiescent endothelial cells (ECs) become activated, degrade their basal membrane, proliferate, migrate and eventually form the new vessel tubes (Blanco and Gerhardt, 2013). Intense research elucidated the key signaling nodes in the EC activation and angiogenesis. Vascular endothelial growth factors (VEGF A-D) and their respective tyrosine kinase receptors (VEGFR 1-3) are key components of ECs angiogenic signaling (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1995). The VEGF-A-VEGFR2 pathway regulates the initiation and performance of each step of the angiogenic process: VEGF-A and VEGFR2 gene deletion in the mouse leads to embryonic death at E9 due to defects in blood vessel formation (Ferrara et al., 1996; Shalaby et al., 1995). Notably, vascular development also requires negative angiogenic regulation, especially during blood vessel maturation when ECs must become quiescent (Ehling et al., 2013). This might occur by antagonizing signaling cascades, such as Notch (Kerr et al., 2016; Shah et al., 2017), Vascular endothelial cadherin (VE-cadherin) that stabilizes VEGFR2 at EC junctions (Lampugnani et al., 2006), and VEGFR1 that competes with VEGFR2 for VEGF (Cudmore et al., 2012; Fong et al., 1995), or by regulation of VEGFR2 activity itself by controlling its gene expression, stability, communication with co-receptor and accessory molecules, internalization and especially intracellular trafficking (Eichmann and Simons, 2012; Simons, 2012; Simons et al., 2016). Upon VEGF binding, VEGFR2 undergoes clathrin-dependent internalization and localizes in early Rab5-positive endosomes which then recruit the Rab5 effector Early endosome antigen 1 (EEA1) (Ballmer-Hofer et al.,

2011). This stage is crucial for downstream ERK1/2 activation (Lampugnani et al., 2006; Lanahan et al., 2013; Lanahan et al., 2010), and for modulation of signaling intensity: VEGFR2 could be degraded via the fusion of the late Rab7 positive endosomes with lysosomes, or be recycled to the plasma membrane via Rab4 or Rab11 positive vesicles (Ballmer-Hofer et al., 2011).

Angiogenic signaling pathways are regulated by a variety of factors, including miRNAs, co-receptors, receptor post-translational modifications and trafficking, microvesicular signaling between ECs and other cell types and last but not least ECs metabolism (Bierhansl et al., 2017). Despite the intensively energy-consuming processes that lead to angiogenesis, ECs display a largely glycolytic metabolism. Thus, perhaps not surprisingly, the role of mitochondria in angiogenesis has long been neglected. However, the function of these organelles extends much beyond their role in energy conversion, and they participate in complex processes, from cell death to signaling, and even to differentiation. All these mitochondrial roles are controlled by mitochondrial shape and morphology changes. Morphology and ultrastructure depend on the balance between fusion and fission processes, controlled by a set of mitochondria-shaping proteins (Pernas and Scorrano, 2016). Fusion is controlled by the outer mitochondrial membrane (OMM) Mitofusins 1 and 2 (Chen et al., 2003; Ishihara et al., 2004), and the inner mitochondrial membrane (IMM) Optic Atrophy 1 (Opa1) (Cipolat et al., 2004). Fission requires the cytosolic dynamin related protein 1 (Drp1) (Smirnova et al., 2001) that translocates from the cytosol to the OMM in response to post-translational modifications (phosphorylation, ubiquitination, SUMOylation). The type and the effect of the modification depend on the signaling and tissue context (Cho et al., 2013). On the OMM, Drp1 dimers or tetramers bind to the adaptor proteins Fis1, Mff, MiD49/50, located at the fission loci defined by the endoplasmic reticulum and

actin polimerization (Friedman et al., 2011; Hatch et al., 2016). At the fission site, Drp1 forms ring-like multimers that drive organelle constriction via GTP hydrolysis (Francy et al., 2015; Macdonald et al., 2014).

Functionally, Drp1-mediated fission is crucial for elimination of damaged mitochondria via mitophagy, equal distribution of mitochondria during mitosis and mitochondrial motility (Westermann, 2010). Moreover, Drp1 is also essential for fission and function of peroxisomes (Li and Gould, 2003). Loss of Drp1 is lethal in patients and animal models (Chang et al., 2010; Ishihara et al., 2009; Wakabayashi et al., 2009a; Waterham et al., 2007), substantiating the importance of Drp1 in mitochondrial and peroxisomal biology. However, Drp1 was initially discovered to be localized in vesicle-like structures along the cytoskeleton and to be retrieved in a Rab5-positive fraction (Yoon et al., 1998), raising the question of whether this multifunctional fission dynamin might be involved in functions not restricted to mitochondria and peroxisomes.

In our efforts to understand if angiogenesis utilizes mitochondrial shape changes, we discovered a role for Drp1. A combination of *in vivo* and *in vitro* data indicate that Drp1 acts as a negative regulator of VEGFR2 angiogenic signaling, unexpectedly acting at the level of Rab5-positive early endosomes.

Results

Angiogenic ECs stimulation affects Drp1 levels.

To investigate the role of mitochondrial morphological changes during angiogenesis, we inspected by confocal imaging TOM20-immunolabeled mitochondria of Human umbilical vein endothelial cells (HUVEC) exposed to a mixture of pro-angiogenic stimuli. Because we observed a remarkable elongation of the already interconnected network (Fig. 1A), we delved into the specific stimulus triggering mitochondria shape changes. A similar elongation was observed upon stimulation of quiescent ECs with human Fibroblast Growth Factor (bFGF), Vascular Endothelial Growth Factor (VEGF) and tumor conditioned medium (TCM) (Fig. 1B). Molecularly, this elongation was associated with an increase in Opa1 protein levels and with a concomitant decrease in levels of Drp1 and of its modulator Calcineurin (Fig. 1C). Visual and densitometric inspection of the immunoblots indicated early Drp1 downregulation, followed by a return to normal levels 24hrs post angiogenic stimulation. This early plummet in Drp1 protein levels was the result of reduced Drp1 mRNA, as indicated by RT-PCR (Fig. 1D). These data indicate that angiogenic EC distribution results in mitochondrial elongation, influencing Drp1 levels.

Drp1 represses angiogenesis *in vitro* and *in vivo.*

If the reduction in Drp1 levels upon angiogenic EC stimulation was part of angiogenic signaling, genetic Drp1 downregulation would be per se proangiogenic. *DRP1* specific siRNAs efficiently reduced Drp1 levels in HUVEC, without causing changes in the expression of other mitochondrial fusion and fission proteins (Fig. 2A) and resulting in mitochondrial elongation (Fig. 2B). Interestingly, the measured angiogenic parameters

(migration, Fig. 2C and proliferation, Fig. 2D) were increased in *DRP1*-silenced quiescent HUVEC.

To determine if DRP1 also affected angiogenesis *in vivo*, we generated a mouse model of endothelial-specific *Drp1* ablation. *Drp1*$^{flox/flox}$ mice were crossed with Tg(Tek-cre)2352Rwng mice expressing the Cre recombinase under the Tie2 promoter, expressed in ECs (Kisanuki et al., 2001), generating *Drp1*$^{\Delta EC/\Delta EC}$ mice (Fig. 3A and S1A and B). Constitutive endothelial *Drp1* knockout mice were born at normal Mendelian distribution and were viable, despite a complete deletion of *Drp1* in the EC compartment (Fig. 3B). We therefore turned to the established model of mouse postnatal retinal vascularization (Stahl et al., 2010). At birth the mouse retina is avascular and pups are blind. From day P1, a layer of blood vessels emerges from the optic nerve and progresses towards the edges of the retina. The first vascular layer is completed within day P7, allowing the study of different stages and facets of developmental angiogenesis, such as early sprouting or maturation and stabilization of blood vessels. In the early P2 stage, when angiogenesis is very active, the vascular plexus progression towards the retina's edges was not increased in *Drp1*$^{\Delta EC/\Delta EC}$ pups (Fig. 3C and quantification in D). Conversely, branching, a parameter of blood vessel network complexity, was significantly increased (Fig. 3E and F), indicating that also *in vivo Drp1* ablation affects angiogenesis. The modulatory nature of the role of Drp1 on angiogenesis emerged when we turned to later stage retinas. Indeed, at P6 we did not detect any difference between *WT* and *Drp1*$^{\Delta EC/\Delta EC}$ retinal vascular progression, capillary branching and arteriovenous patterning (Fig. 3G,H,I,J). These results are consistent with the viability of *Drp1*$^{\Delta EC/\Delta EC}$ mice and suggest that Drp1 acts as a modulator, rather than as a core component, of angiogenic signaling, or that compensatory mechanisms are in place.

Acute *Drp1* endothelial ablation affects blood vessels stability.

To overcome the potential compensatory effects occurring in *Drp1*$^{\Delta EC/\Delta EC}$ mice, we generated an inducible endothelial knockout model for *Drp1* (*Drp1*$^{i\Delta EC/i\Delta EC}$, Fig. 4A and S2A and B) by crossing the *Drp1*$^{flox/flox}$ mice with Cdh5(PAC)-CreERT2 mice, expressing the Cre recombinase under the tamoxifen-inducible Vascular endothelial (VE) cadherin promoter (Monvoisin et al., 2006). Acute *Drp1* ablation was induced by Tamoxifen injections from P2-P4, resulting in an almost complete reduction in *Drp1* mRNA at P6 in EC extracted from lungs (Fig.4B). However, because of the timing required to delete Drp1 in the EC compartment, we could not analyze the early retinal colonization, but we could inspect the effects of Drp1 ablation on later developmental stages, when angiogenic signaling must be efficiently tuned down to ensure proper angiogenesis (Ehling et al., 2013; Greenberg et al., 2008).

When we inspected retinas extracted at P5,5-P6 we observed that at a difference from the constitutive *Drp1*$^{\Delta EC/\Delta EC}$ mice, vascular progression was blunted in tamoxifen treated *Drp1*$^{i\Delta EC/i\Delta EC}$ mice (Fig. 4C and D), due to a reduction in tip cells (TC) at the angiogenic front (Figure 4E and F). TCs are the EC subtypes located at the very edge the vascular sprout, characterized by numerous filopodia, high angiogenic activity and leading vascular network progression towards the growth factors source. Furthermore, capillary branching was reduced in *Drp1*$^{i\Delta EC/i\Delta EC}$ retinas (Figure 4G and H). Microvessels were slender with an increase in the occurrence of pruning vessels, corresponding to newly formed vessels that were not stabilized and therefore regressed (Korn and Augustin, 2015) (Figure 4G). These phenotypes could be a consequence of dysregulated VEGFR2 activation, which should be downregulated during angiogenesis maturation (Chung et al., 2010; Greenberg et al., 2008; Kerr et al., 2016; Lampugnani et al., 2006; Shah et al., 2017).

Drp1 interacts with Rab5 to modulate VEGFR2 endosomal trafficking.

To understand how changes in Drp1 levels influence angiogenesis we turned back to the cellular HUVEC model. Given VEGF modulatory effects on Drp1 expression, we focused our interest on this master angiogenic signaling axis. Pro-angiogenic VEGF effects are mediated by its major receptor on the EC surface, VEGFR2. VEGFR2 engagement by VEGF results in Tyr1175 autophosphorylation, essential for activation of downstream ERK1/2 signaling (Hong et al., 2006; Matsumoto et al., 2005). In line with the upregulated EC's proliferation and migration, *DRP1* downregulation in HUVECs resulted in increased VEGFR2 Tyr1175 autophosphorylation, without changes in total levels of the receptor (Fig 5A) and in the expected ERK1/2 activation (Fig. 5A). Notably, this effect was specific for ECs, because when DRP1 was silenced using the same siRNA in the MDA-MB-231 breast cancer cell line, ERK1/2 phosphorylation was decreased (Fig. S3A), in line with the known upstream effect of ERK1/2 on Drp1 in cancer cells (Kashatus et al., 2015), suggesting that the mechanism is specific to ECs.

To understand the link between Drp1 reduction and VEGFR2 hyperactivation, we inspected endogenous Drp1 localization upon short (10 minutes) VEGF treatment, sufficient to activate VEGFR2 internalization and signaling (Fig. 5B). The largely cytosolic Drp1 staining of unstimulated HUVEC disappeared and Drp1 was retrieved in perinuclear puncta that resembled the vesicular structures originally identified as Drp1 positive (Yoon et al., 1998). 10 minutes of VEGF stimulation is sufficient to induce VEGFR2 internalization and cytosolic signaling. The changes in Drp1 cytosolic redistribution suggested that Drp1 might unexpectedly localize at VEGFR2 positive endocytic vesicles during angiogenic signaling. First, we noticed that following 10 minutes of VEGF stimulation Drp1 co-immunoprecipitated with VEGFR2 (Fig. 5C). The

interaction was not detected in VEGF-unstimulated HUVEC. To verify if this interaction occurred in an intact cellular milieu, we turned to a proximity ligation assay (PLA) that can reveal protein-protein vicinity *in vivo* (Soderberg et al., 2008). Consistently with our co-immunoprecipitation analysis, PLA analysis, shown to be specific (Fig. S3C), did not show any interaction between Drp1 and VEGFR2 in unstimulated EC and revealed extensive Drp1-VEGFR2 interaction in HUVECs briefly pulsed with VEGF (Fig. 5D). These experiments indicate that Drp1 interacts with VEGFR2 following its activation by VEGF

Because VEGF binding to VEGFR2 induces receptor's dimerization, autophosphorylation and internalization (Simons et al., 2016) and Drp1 ablation enhances VEGFR2 signaling, we reasoned that Drp1 could be a negative regulator of VEGFR2 endocytosis. To test this hypothesis, we measured if *DRP1* downregulation altered levels of VEGFR2 on the plasma membrane, but we did not detect any decrease in VEGFR2 surface levels (Fig. 5E). Thus, we concluded that the increase in VEGFR2 activation in *DRP1* depleted cells did not result from excessive receptor internalization.

Alternatively, Drp1 silencing might affect VEGFR2 fine tuning by impinging on the endocytic trafficking of the latter, a crucial step in angiogenesis regulation (Lampugnani et al., 2006). For example, Drp1 ablation might decrease Rab7-dependent VEGFR2 degradation and hence result in the observed VEGFR2 sustained signaling (Simons et al., 2016); however, Drp1 puncta did not colocalize with Rab7 positive endosomes in resting cells or upon short (10 minutes) VEGF stimulation (Fig. S3B). Because we excluded that Drp1 silencing impaired VEGFR2 degradation, we turned our attention to the possibility that Drp1 affected localization of the receptor in the early Rab5 positive endosomes that serve as a platform for VEGFR2 mediated ERK activation.

Interestingly, the low level of Rab5-Drp1 colocalization observed in unstimulated HUVEC was increased upon VEGF treatment (Fig. 5F). The colocalization resulted from true protein proximity as confirmed by a PLA between Drp1 and Rab5. Notably, the PLA signal was increased by VEGF supplementation, further supporting a role for Drp1 at Rab5 positive endosomes in stimulated ECs (Fig. 5G).

Following ligand-binding-induced internalization of tyrosine kinase receptors such as EGFR, early endosomes colocalize with the mitochondria (Demory et al., 2009). However, we did not detect any difference in colocalization of Rab5 positive vesicles with mitochondria upon VEGF stimulation (Fig. S3D), suggesting that the observed effects are specific to Drp1 interaction with Rab5-positive early endosomes. We therefore tested the possibility that Drp1 functions by negatively modulating the localization of VEGFR2 to Rab5 positive early endosomes. PLA revealed a low level of basal VEGFR2-Rab5 interaction that was strongly induced by VEGF treatment of HUVECs (Fig. 5H). In line with the hypothesis that Drp1 limits VEGFR2-Rab5 interaction, *DRP1* downregulation was per se sufficient to increase it in unstimulated HUVECs, without affecting protein levels of Rab5 and its effector EEA1 (Fig. S3E). Our data suggest that Drp1 regulates endosomal vesicle trafficking, not the expression of early endosomal markers.

Discussion

The role of mitochondria and of mitochondrial morphology, a node in several signaling cascades, in angiogenesis is unknown. Here we show that downregulation of the key mitochondrial fission protein Drp1 amplifies angiogenic signaling *in vitro* and *in vivo*. Unexpectedly, Drp1 is required to tune down VEGFR2 activation at early endosomes, highlighting a hitherto unknown function for this dynamin superfamily proteins outside of the control of mitochondrial shape.

That Drp1 might participate in ECs function and in angiogenesis has been suggested by a handful of studies on its role in disease (Giedt et al., 2012; Lin et al., 2015; Marsboom et al., 2012; Wang et al., 2012a). However, the function of Drp1 in the endothelium during physiological and developmental angiogenesis is unclear. We addressed this question by a combination of *in vitro* and *in vivo* experiments in ad hoc generated constitutive and inducible mouse models for endothelial-specific ablation of *Drp1*. Consistently with our *in vitro* data, we observed an upregulation of angiogenesis at stage P2 in *Drp1*$^{\Delta EC/\Delta EC}$ pups compared to *WT* littermates. The normalization of the phenotype at P6 was in line with the viability and fertility of adult *Drp1*$^{\Delta EC/\Delta EC}$ mice. Being a crucial process in embryonic development, lack of master angiogenic regulators is often lethal. In the case of proteins involved in the regulation of major signaling pathways, compensatory mechanisms often take place to ensure normal vascularization and viability. The inducible mouse model for endothelial *Drp1* ablation we generated allowed us to overcome these compensatory effects. In contrast to *Drp1*$^{\Delta EC/\Delta EC}$, P6 angiogenesis was decreased in *Drp1*$^{i\Delta EC/i\Delta EC}$ due to a decrease in endothelial tip cells (TCs) and capillary branching. The progressing vascular sprout is formed by two subtypes of endothelial cells the Tip cells (TCs) leading the angiogenic front, followed by the stalk cells (SCs). TCs have high VEGFR2 activity and high

number of filopodia, helping the migration of the sprout towards the source of VEGF (Gerhardt et al., 2003). SCs are highly proliferative, with no filopodia and low VEGFR2 activity. The crosstalk between the two cell types is crucial for the establishment of the correct phenotype and the normal angiogenic sprouting. Notch is the main signaling pathway regulating these events. TCs are characterized with low Notch activity, but they express the Notch ligand Dll4, which activates Notch in the adjacent SC. High Notch activity in the SCs inhibits VEGFR2 signaling (De Smet et al., 2009; Gerhardt et al., 2003; Hellstrom et al., 2007). Therefore, it is possible that an increase of VEGFR2 signaling in the SCs is perturbing the TCs phenotype in the *Drp1*$^{i\Delta EC/\ i\Delta EC}$ P6 retina, leading to the observed phenotype. Alternatively, Drp1 ablation might per se affect Notch signaling, as observed for other mitochondria shaping proteins in the course of cardiomyocyte differentiation (Kasahara et al., 2013). In this investigation line it would be interesting to measure the activation of calcineurin, a calcium/calmodulin dependent serine/threonine protein phosphatase shown to regulate both Drp1 and Notch. This hypothesis is further supported by the observation that calcineurin levels decrease upon angiogenic activation of ECs (Fig. 1C).

Mechanistically, during angiogenesis Drp1 negatively regulates VEGFR-Rab5 interaction. Like VEGF stimulation, lack of *DRP1* induces VEGFR2-Rab5 interaction, suggesting that VEGFR2 localization in Rab5 positive endosomes is prolonged, ultimately leading to increased ERK1/2 dependent angiogenic features of ECs. In arteriogenesis, complete ERK activation results from VEGFR2 located at the level of EEA1 positive structures. Our data suggest that during angiogenesis the same signaling might depend on Rab5.

Rab5 activity in endosomal docking, fusion and sorting is regulated through its effector proteins EEA1 and Rabenosyn-5 (Christoforidis et al., 1999; Nielsen et al., 2000;

Rubino et al., 2000). Rab5-EEA1, together with SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), lead to the transition of clathrin-coated vesicles into early endosomes. In the case of VEGFR2 internalization and early trafficking events, the endosomal compartments interact with the cytoskeleton via motor protein complex of synectin/myosin IV to allow fast movement of the activated VEGFR2 away from the plasma membrane and protect the Y1175 from plasma membrane-located phosphatases (Ballmer-Hofer et al., 2011; Lanahan et al., 2013; Lanahan et al., 2010) The precise mechanisms regulating this process are not fully understood, but it has been shown that VEGFR2 co-receptor NRP1 plays an important role by establishing the interaction between VEGFR2 complex and synectin via its PDZ domain. This interaction is critical for ERK1/2 activation in arteriogenesis (Chittenden et al., 2006).

Given the molecular and functional characteristics of Drp1 and its interaction with myosin II (Korobova et al., 2014), it is possible that Drp1 acts as an adaptor protein of endosomes, linking them to motor complexes and the cytoskeleton. Moreover, Drp1 interacts with proteins from the endocytic pathway Rab32 and Syntaxin 17, member of the SNARE family, at lipid raft-like structures at the ER-mitochondria contact sites surface (Arasaki et al., 2015; Ortiz-Sandoval et al., 2014). Lipid rafts structures and organelle contact sites are emerging as key signaling platforms, opening the possibility that Drp1 regulates VEGFR2 signaling via rafts and/or interorganellar contact sites. At this point it is essential to investigate whether Drp1 is a specific inhibitor of VEGFR2 signaling or could affect activation of other receptors involved in angiogenesis such as Fibroblast growth factor receptor (FGFR) or Epidermal growth factor receptor (EGFR). Another open question is whether Drp1 is involved in receptor trafficking in general or this function is restricted to tyrosine kinase receptors.

Our hypothesis that Drp1 regulates VEGFR2 signaling via an extra-mitochondrial function requires a deeper study on mitochondrial functionality in ECs lacking Drp1. Moreover, it is essential to establish the mechanism targeting Drp1 to Rab5 positive endosomes from the posttranslational modification to the adaptor proteins. Our preliminary data (not shown) demonstrates that in HUVECs classical Drp1 adaptors Mff and Fis1 are localized on both early endosomes and that short (10 minutes) VEGF treatment modulates Drp1 interaction with these molecules. Investigating Drp1 subcellular localization, posttranslational modification status and molecular partners following a time-course of VEGF stimulation is a crucial point to be addressed in our future work.

From a functional point of view, increased VEGFR2 signaling is likely to increase blood vessels permeability. Tumor blood vessels are characterized by reduced pericyte coverage and therefore higher permeability, which contributes to anti-angiogenic therapy resistance and metastasis dissemination. Therefore pathways regulating blood vessel's maturation and stability present a question of great interest in the field of anti-angiogenic cancer therapy (Potente et al., 2011). Promoting Drp1 anti-angiogenic function in the endothelial compartment during tumor development would be useful in increasing tumor blood vessel stabilization and responsiveness to anti-cancer therapies.

In conclusion, our results describe an unexpected function of Drp1 in angiogenesis as a negative regulator of VEGFR2 signaling axis, by interfering with the receptor endocytic trafficking. Our study not only gives insights into VEGFR2 intracellular signal regulation, but extends Drp1 function to the regulation of vesicular transport and of Rab GTPase-dependent membrane dynamics.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) (Lonza) were used from passages 5-11. Cells were seeded on 0,2% gelatin-coated dishes and maintained in culture in 75% angiogenic EGM-2 BulletKit medium (Lonza) supplemented with 5% fetal bovine serum (FBS) and 25% non-angiogenic Human endothelium serum-free medium (SFM) (Gibco). Experiments were performed in non-angiogenic SFM medium supplemented with 5% EGM2 medium or with the indicated angiogenic factor VEGF (50ng/ml) (RELIATech, Vinci-BiochemSrl), bFGF (10ng/ml) (Promega), Tumor-conditioned medium (20%), obtained by centrufugation of the medium of cultured cancer cell lines (Hela and MDAMB231).

siRNA transfection

SiRNA transfections were performed using Calcium Phosphate method. HUVECs (2,5 x 10^5) were seeded into each well of a six-well plate in serum-free medium (SFM) supplemented with basic fibroblast growth factor (bFGF; 5 ng/ml) or 5% of EGM2 angiogenic medium and allowed to adhere overnight. siRNA-CaCl2 complexes were prepared by first combining the 30nm siRNA (Sigma Aldrich) with 10 ml of 2.5M $CaCl_2$. One hundred microliters of HBSP(280 mM NaCl, 1.9 mM Na2HPO4, 12 mM glucose, 10 mM KCl, 50 mM Hepes, pH 7.05) (Sigma Aldrich) were added. The mixture was incubated for 1 min at room temperature. The mixture was added dropwise to the cells, and the cells were incubated for 16 hours. Cells were then collected and seeded for further tests. Experiments on transfected HUVECs and evaluation of transfection efficiency were performed after 48 hours.

Western blotting

Cells were lysed in lysis buffer and heated at 95°C for 10 min. Equal amounts of protein were resolved by 8% SDS PAGE and transferred to polyvinylidene fluoride membranes (PVDF) according to the manufacturer'sprotocol. The blots were blocked for 1 hour at room temperature with 5% BSA (Sigma Aldrich) in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and probed overnight at 4°C with 1:1000 mouse anti-Drp1 (BD-Biosciences); mouse anti-Opa1 (BD-Biosciences); rabbit anti-GRP75 (Santa Cruz Biotechnology); rabbit anti-VEGFR2 (Cell Signalling Technology); rabbit anti-Phospho-VEGFR2 Tyr1175 (Cell Signalling Technology); rabbit anti-ERK 1/2 (Cell Signalling Technology); rabbit anti-Phospho-ERK 1/2 (Cell Signalling Technology); mouse anti-Actin (Merck Millipore); rabbit anti-Calcineurin (Cell Signalling Technology); mouse anti-Fis1 (Enzo Life Sciences); rabbit anti-Mff (Proteintech); rabbit anti-Mfn2 (ABM); rabbit anti-Rab5 (Cell Signalling Technology); mouse anti-EEA1 (Thermo Fisher Scientific). After three washes with TBS-T, the appropriate secondary antibody at a 1:5000 dilution was added for 1 hour at room temperature. The bands were visualized by enhanced chemiluminescence ECL kit (Pierce).

Scratch wound migration assay

SiRNA transfected HUVECs (1 x 10^5cells/well) were seeded into each well of a 48-well plate in 350 ml of SFM with bFGF (5 ng/ml) (Promega) and incubated to reach confluence. Using a pipette tip, a "wound" was made in the monolayer (at time 0). The cells were then washed with PBS and incubated with SFM supplemented with bFGF (5 ng/ml). Migration of cells was evaluated after 4,5 hours.

Cell proliferation assay

SiRNA transfected HUVECs (5×10^3) were seeded into each well of a 96-well plate in 100 ml of SFM containing bFGF (5 ng/ml) (Promega) and incubated for 48 hours. Thymidine analogue -5-bromo-2-deoxyuridine (BrdU) was added and the culture was incubated for 16 hours. BrdU incorporation was measured with the Cell Proliferation ELISA BrdU (chemiluminescence) kit (Roche Applied Science) according to the manufacturer's protocol.

Immunofluorescence

HUVECs (3×10^5) were cultured in 35-mm dishes coated with gelatin (0,2%). After VEGF (50 ng/ml) incubation, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, blocked and permeabilized with PBS-5%BSA-0,5% saponin solution, and incubated overnight in PBS-1% BSA-0,1% saponin (Sigma Aldrich) containing 1:100 mouse anti-Drp1 (BD Biosciences), rabbit anti-Rab5 (Cell Signalling Technology), rabbit anti-Rab7 (Cell Signalling Technology). After 3 washes with PBS cells were incubated with the same solution containing the corresponding secondary fluorescent antibodies Alexa Fluor 488 and 568 (Thermo Fisher Scientific) for 1 hour at room temperature. Samples were washed 3 times with PBS and nuclei were stained with DAPI. The samples were analyzed by fluorescence microscopy (Zeiss LSM700).

Proximity ligation protocol

HUVECs (3×10^5) were cultured in 35-mm dishes coated with gelatin (0,2%). After VEGF (50 ng/ml) incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, blocked and permeabilized with

PBS-5%BSA-0,5% saponin solution, and incubated overnight with 1:100 mouse anti-Drp1 (BD Biosciences), rabbit anti-VEGFR2 (Cell Signalling Technology), rabbit anti-Rab5 (Cell Signalling Technology). 0,1% of saponin was added to the antibody solution from the *Duolink® In situ Red Starter Kit* (Sigma-Aldrich). The PLA was performed according to the manufacturer's instructions with an anti-mouse-MINUS and anti-rabbit-PLUS PLA probes for Drp1-VEGFR2 interaction and an anti-goat-PLUS and anti-rabbit-MINUS in case of VEGFR2-Rab5. DAPI was used to stain the nuclei. The slides were analyzed by fluorescence microscopy (Zeiss LSM700).

Flow cytometry

SiRNA transfected HUVECs (7,5 x 10^5) were scraped off of the plates in PBS containing 5% BSA. After 30 minutes blocking in this solution at 4°C, primary rabbit anti VEGFR2 antibody (1:100) was was added for 1 hour at 4°C. Then, the appropriate FITC-conjugated secondary antibody was added again for 1 hour at 4°C. The cells were analyzed with a BD FACSCalibur flow cytometer.

Mouse models

Endothelial *Drp1* knockouts were obtained by crossing *Drp1*$^{flox/flox}$ mice (generated by K. Mihara, Kyushu University, Japan) with Tg(Tek-cre)2352Rwng (Tie2Cre) mice (Jackson's Laboratory). For inducible ablation *Drp1*$^{flox/flox}$ mice were crossed with Cdh5(PAC)-CreERT2 mice (generated by Ralf Adams, Max Plank Institute, Münster, Germany). All breedings were performed in a BL6 genetic background. All animal experiments were performed according to the approved ethical protocols.

Genotyping

DNA was extracted from tail tissue samples using Alkaline lysis buffer (NaOH 25 mM, Na2-EDTA 2H2O 0.2 mM, pH=12). Amplification of *Drp1* locus was performed using Forward 5' CAGCTGCACTGGCTTCATGACTC 3' and Reverse 5' GTCAACTTGCCATAAACCAGAG 3' primers.

For the amplification of the *Cre* locus were used the following primers for both Tie2Cre and VeCadCre strains: Forward 5' GCGGTCTGGCAGTAAAAACTATC 3'; and Reverse 5' GTGAAACAGCATTGCTGTCACTT 3'.

Tamoxifen injections

To induce Tamoxifen-dependent ablation of *Drp1* in the endothelium pups were injected intraperitoneally for three consecutive days with 50µl Tamoxifen (Sigma-Aldrich) at a concentration of 1 mg/mL in corn oil (Sigma-Aldrich). Adults were injected intraperitoneally for five consecutive days with 100µl at a concentration of 10 mg/mL.

Retinal murine neovascularization model

To analyze postnatal neovascularization in the mouse retina, $Drp1^{\Delta EC/\Delta EC}$, $Drp1^{i\Delta EC/i\Delta EC}$ and $Drp1^{flox/flox}$ littermates were sacrificed at the indicated postnatal day. The eyes were fixed in 4% paraformaldehyde solution for 45 min, the retinas were dissected and post-fixed in PFA 4% for 1 hour. After 5 washes with PBS retinas were permeabilized in PBS with 1% BSA and 0,5% Triton over night at 4°C. The day after retinas were incubated with biotinylated isolectin B4 (Vector Laboratories) (1:100) for 4 hours 4°C and washed over night in the same conditions. Secondary streptavidin–Alexa 488 (Invitrogen) (1:300) was added for 1-2 hours at 4°C. The secondary antibody was also

washed overnight and retinas were flat-mounted for analysis. Flat-mounted retinas were analyzed using Leica DMI4000 microscope. Images were analyzed using ImageJ. The retinal radius (from the optic nerve to the edge of the retina) and the vascular radius (from the optic nerve to the vascular front) of each petal of the retina were measured. The vascular coverage was calculated as the ratio between the vascular radius and the retinal radius of each petal of a single retina. Branchings were quantified using ImageJ, quantification represents branchings per mm of retina. Images of tip cell's filopodia were taken by a confocal Zeiss LSM700 microscope and images were analyzed using ImageJ. Quantifications of tip cells represent tip cell per mm of retina.

Lung endothelial cells isolation

Whole lungs were extracted from *Drp1*$^{\Delta EC/\Delta EC}$, *Drp1*$^{i\Delta EC/\ i\Delta EC}$ and *Drp1*$^{flox/flox}$ littermates and collected in serum free DMEM supplemented with penicillin and streptavidin. Under a tissue culture hood, the lungs were removed from medium and minced using small scissors. Minced tissue was mixed with 1 ml of serum free DMEM into a 2ml Eppendorf tube. Collagenase/Dispase (Roche Applied Science) solution was added to the tube with a final concentration of 2mg/ml in 1ml. Enzymatic digestion was carried at 37°C for 45 minutes. Digested tissue was resuspended by mixing up and down through a 20G syringe. Tissue suspension was filtered through a 100µm cell strainer, washed with 15ml of Isolation medium (DMEM P/S + 10% of FBS) and centrifuged at 400xg for 5 minutes. Supernatant was eliminated and the pellet resuspended in 1ml of PBS-0,1% BSA containing anti-PECAM-1 (CD31) (BD Pharmingen) antibody conjugated Dynabeads. Cell suspension together with Dynabeads was mixed for 15

minutes at room temperature. Tubes were mounted on a Magnetic Particle Concentrators. After sedimentation of the beads, the supernatant was eliminated. Beads were washed 3 times with 1ml of PBS-0,1% BSA. Finally, the beads were resuspeded in EGM2 medium and plated on gelatin coated dishes. At 50% confluency a second purification step was performed using secondary anti-ICAM2 (BD Pharmingen)-conjugated Dynabeads (Invitrogen).

Analysis of gene expression by qRT-PCR

Total RNAs from lung endothelial cells and HUVECs were extracted using the kit miRNaesy (Qiagen). cDNA was synthetized from 1µg of RNA using *iScript DNA Synthesis Kit* (Biorad). Quantitave PCR reaction was performed using SYBR green method (Bioline). For *Drp1* amplification from mouse lung endothelial cells were used the primers proposed by Uo et al., 2009.

Amplification of human DRP1 from HUVECs was performed using the following primers forward: 5' CACCCGGAGACCTCTCATTC 3'; and reverse: 5' CCCCATTCTTCTGCTTCCAC 3'. Quantification of mRNA levels was done using the $2^{-\Delta Ct}$ and was normalized to Actin expression.

Statistical analysis

In vitro experiments (migration and proliferation) were displayed graphically (bar diagrams) as mean and SEM (Standard Error of the Mean). Values were tested for significance by the one-samplet T-test. *In vivo* data was displayed as box plots and values were tested for significance by a one way ANOVA with a Tukey's posttest. Results were considered significant when *p< 0.05.

Acknowledgements

This work was supported by Fondazione Cariparo, Italy.

Figure legends

Figure 1. Angiogenesis modulates mitochondrial dynamics.

(A) Confocal microscopy image of mitochondria immunostained for TOM20 protein (green) in HUVECs, grown in serum-free medium (left panel) and in pro-angiogenic, growth factor-supplemented medium (right panel).

(B) Confocal microscopy image of mitochondria immunostained for TOM20 protein (green) in HUVECs, grown in serum-free medium (first panel) and stimulated for one hour with bFGF (10ng/ml), VEGF (50ng/ml), 20% Tumor conditioned medium (TCM) from HeLa cells.

(C) Levels of the indicated proteins were assessed by Western blot analysis of total protein extracts from HUVECs, treated with bFGF (10ng/ml), VEGF (50ng/ml), 20% Tumor conditioned medium from HeLa cells for the indicated periods.

(D) *DRP1* mRNA levels assessed by RT-PCR from HUVECs, treated with VEGF (50ng/ml) for the indicated periods. Data represent mean ± SEM of three independent experiments, * p ≤ 0, 05 in a t-Test analysis.

Figure 2. *DRP1* silencing upregulates angiogenesis *in vitro.*

(A-D) HUVECs were transfected with scramble control non silencing (Ctl siRNA) or silencing *DRP1* siRNA (30nM) for 48 hours.

(A) Levels of the indicated proteins were assessed by Western blot analysis of total protein extracts from HUVECs, transfected with a scramble (Ctl) and *DRP1* silencing siRNA.

(B) Confocal microscopy image of mitochondria immunostained for TOM20 protein (green) in HUVECs, transfected with a scramble (Ctl) and *DRP1* silencing siRNA.

(C) Migration assay (scratch test) was performed on HUVECs 48h post siRNA transfection. Size of the wound was measured after 4,5h. A time point reflecting only migration of primary HUVEC, which require longer time to divide. Data represent mean ± SEM of four independent experiments, * p ≤ 0, 05 in a t-Test analysis.

(D) Proliferation assay by measuring the BrdU incorporation on HUVECs 48h post siRNA transfection. Data represent mean ± SEM of four independent experiments, * p ≤ 0, 05 in a t-Test analysis.

Figure 3. Endothelial specific *Drp1* ablation induces early stage angiogenesis *in vivo*.

(A) Schematic representation of the generation of $Drp1^{\Delta EC/\Delta EC}$. $Drp1^{flox/flox}$ mice were crossed with $Tie2Cre^{-/+}$ mice.

(B) Specificity of *Drp1* ablation in the endothelium. Protein levels of DRP1 in lung endothelial cells, extracted from $Drp1^{flox/flox}$ and $Drp1^{\Delta EC/\Delta EC}$ pups, were measured using Western blot analysis (n = 3 $Drp1^{flox/flox}$ and 3 $Drp1^{\Delta EC/\Delta EC}$ pups.

(C) Isolectin B4 staining of P2 retinas extracted from $Drp1^{flox/flox}$ and $Drp1^{\Delta EC/\Delta EC}$ pups.

(D) Box plot represents quantification of the ratio of the radial length from the optic nerve to the vascular front and the retinal surface colonized by vessels at P2 (n = 13 $Drp1^{flox/flox}$ and 7 $Drp1^{\Delta EC/\Delta EC}$ pups).

(E) Isolectin B4 staining of P2 retinas extracted from $Drp1^{flox/flox}$ and $Drp1^{\Delta EC/\Delta EC}$ pups.

(F) Box plot represents the quantification of vascular branchings per mm of retinal surface at P2 (n = 13 $Drp1^{flox/flox}$ and 7 $Drp1^{\Delta EC/\Delta EC}$ pups).

(G) Isolectin B4 staining of P6 retinas extracted from *Drp1*$^{flox/flox}$ and *Drp1*$^{\Delta EC/\Delta EC}$ pups.

(H) Box plot represents quantification of the ratio of the radial length from the optic nerve to the vascular front and the retinal surface colonized by vessels at P6 (n = 5 *Drp1*$^{flox/flox}$ and 3 *Drp1*$^{\Delta EC/\Delta EC}$ pups).

(I) Isolectin B4 staining of P6 retinas extracted from *Drp1*$^{flox/flox}$ and *Drp1*$^{\Delta EC/\Delta EC}$ pups.

(J) Box plot represents quantification of the ratio of the radial length from the optic nerve to the vascular front and the retinal surface colonized by vessels at P6 (n = 5 *Drp1*$^{flox/flox}$ and 3 *Drp1*$^{\Delta EC/\Delta EC}$ pups).

Figure 4. Acute *Drp1* endothelial ablation affects blood vessels stability.

(A) Schematic representation of the generation of *Drp1*$^{i\Delta EC/i\Delta EC}$. *Drp1*$^{flox/flox}$ mice were crossed with *VeCadCre*$^{-/+}$ mice. Cre expression is induced by intraperitoneal Tamoxifen injection.

(B) Specificity of *Drp1* ablation in the endothelium. *Drp1* mRNA levels in lung endothelial cells, extracted from *Drp1*$^{flox/flox}$ and *Drp1*$^{i\Delta EC/i\Delta EC}$ pups, were measured by RT-PCR. Data represent mean ± SEM of three independent experiments, * p ≤ 0, 05 in a t-Test analysis (n = 3 *Drp1*$^{flox/flox}$ and *3 Drp1*$^{i\Delta EC/i\Delta EC}$ pups).

(C) Isolectin B4 staining of P5,5-6 retinas extracted from *Drp1*$^{flox/flox}$ and *Drp1*$^{i\Delta EC/i\Delta EC}$ pups. Drp1 ablation was induced with Tamoxifen injections from P2-P4.

(D) Box plot represents quantification of the ratio of the radial length from the optic nerve to the vascular front and the retinal surface colonized by vessels at P6 (n = 5 *Drp1*$^{flox/flox}$ and 4 *Drp1*$^{i\Delta EC/i\Delta EC}$ pups).

(E) Isolectin B4 staining of P5,5-6 retinas extracted from *Drp1*$^{flox/flox}$ and *Drp1*$^{i\Delta EC/i\Delta EC}$ pups. Drp1 ablation was induced with Tamoxifen injections from P2-P4.

(F) Box plot represents the quantification of endothelial tip cells at the angiogenic front per mm of retinal surface vascular (n = 5 *Drp1*$^{flox/flox}$ and 5 *Drp1*$^{i\Delta EC/i\Delta EC}$ pups).

(G) Isolectin B4 staining of P5,5-6 retinas extracted from *Drp1*$^{flox/flox}$ and *Drp1*$^{i\Delta EC/i\Delta EC}$ pups. Drp1 ablation was induced with Tamoxifen injections from P2-P4.

(H) Box plot represents the quantification of vascular branchings per mm of retinal surface (n = 5 *Drp1*$^{flox/flox}$ and 5 *Drp1*$^{i\Delta EC/i\Delta EC}$ pups). White arrows indicate pruning vessels.

Figure 5. Drp1 regulates Rab5-dependent VEGFR2 signaling.

(A) HUVECs were transfected with scramble control non silencing (Ctl siRNA) or silencing *DRP1* siRNA (30nM) for 48 hours. Levels of the indicated proteins were assessed by Western blot analysis of total protein extracts.

(B) Confocal microscopy image of non-treated and VEGF-stimulated (50ng/ml, 10 minutes) HUVECs immunostained for Drp1 (green).

(C) Immunoprecipitation (IP) assay showing an interaction between endogenous VEGFR2 and Drp1 in HUVECs after VEGF stimulation (50ng/ml, 10 minutes). HUVEC lysates were immunoprecipitated with a mouse anti-Drp1 antibody and analyzed by Western blotting using rabbit anti-VEGFR2 antibody.

(D) Proximity ligation assay (PLA) showing an interaction between endogenous VEGFR2 and Drp1 in HUVECs after VEGF stimulation (50ng/ml, 10 minutes). Red spots indicate the proximity of the two proteins. (control samples are presented in Fig S3B).

(E) Cell surface abundance of VEGFR2 in non permeabilized HUVECs transfected with scramble (Ctl) and *DRP1* silencing siRNA (green). Left panel represents secondary Alexa 488 antibody alone as an internal control. Right panels represent VEGFR2 surface abundance (green) compared to Alexa 488 signal intensity (purple) (n=3).

(F) Confocal microscopy image of non-treated and VEGF-stimulated (50ng/ml, 10 minutes) HUVECs immunostained for Drp1 and Rab5. Colocalization analysis was performed using ImageJ.

(G) Proximity ligation assay (PLA) showing an interaction between endogenous Drp1 and Rab5 in HUVECs after VEGF stimulation (50ng/ml, 10 minutes). Red spots indicatethe proximity of the two proteins.

(H) HUVECs were transfected with scramble control non silencing (Ctl siRNA) or silencing *DRP1* siRNA (30nM) for 48 hours. HUVECs transfected with Ctl siRNA were additionally treated with VEGF (50ng/ml, 10 minutes). Proximity ligation assay (PLA) was performed between VEGFR2 and Rab5. Red spots indicate the proximity of the two proteins.

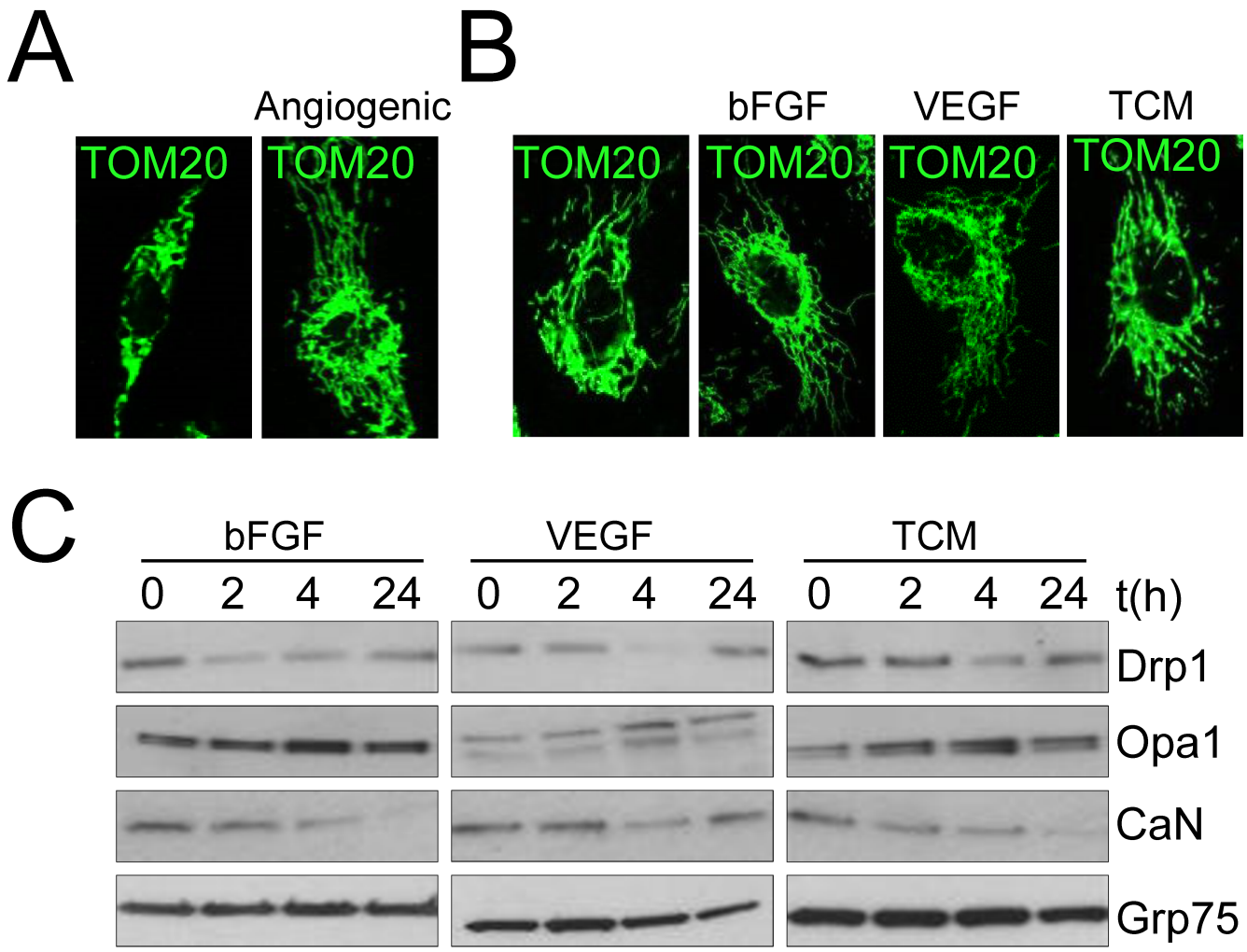

A
Angiogenic
TOM20
TOM20
B
bFGF
VEGF
TCM
TOM20
TOM20
TOM20
TOM20
C
bFGF
VEGF
TCM
0 2 4 24 0 2 4 24 0 2 4 24 t(h)
Drp1
Opa1
CaN
Grp75
D
Relative DRP1 mRNA levels following VEGF treatement (a.u.)
1.5
1.0
0.5
0.0
*
*
*
0h 1h 2h 4h

Figure 1.

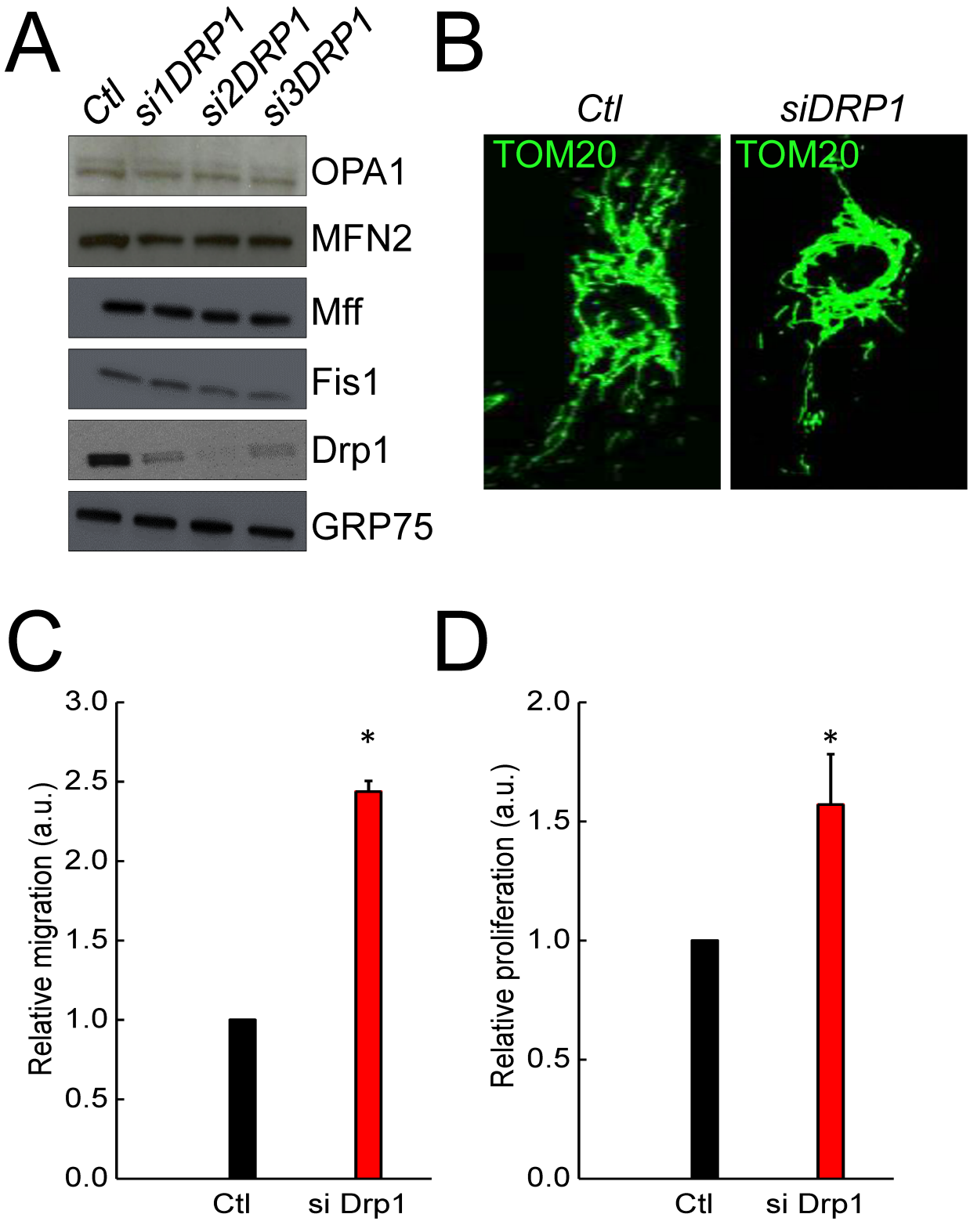

A
Ctl
si1DRP1
si2DRP1
si3DRP1
OPA1
MFN2
Mff
Fis1
Drp1
GRP75
B
Ctl
siDRP1
TOM20
TOM20
C
Relative migration (a.u.)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Ctl
si Drp1
D
Relative proliferation (a.u.)
2.0
1.5
1.0
0.5
0.0
*
Ctl
si Drp1

Figure 2.

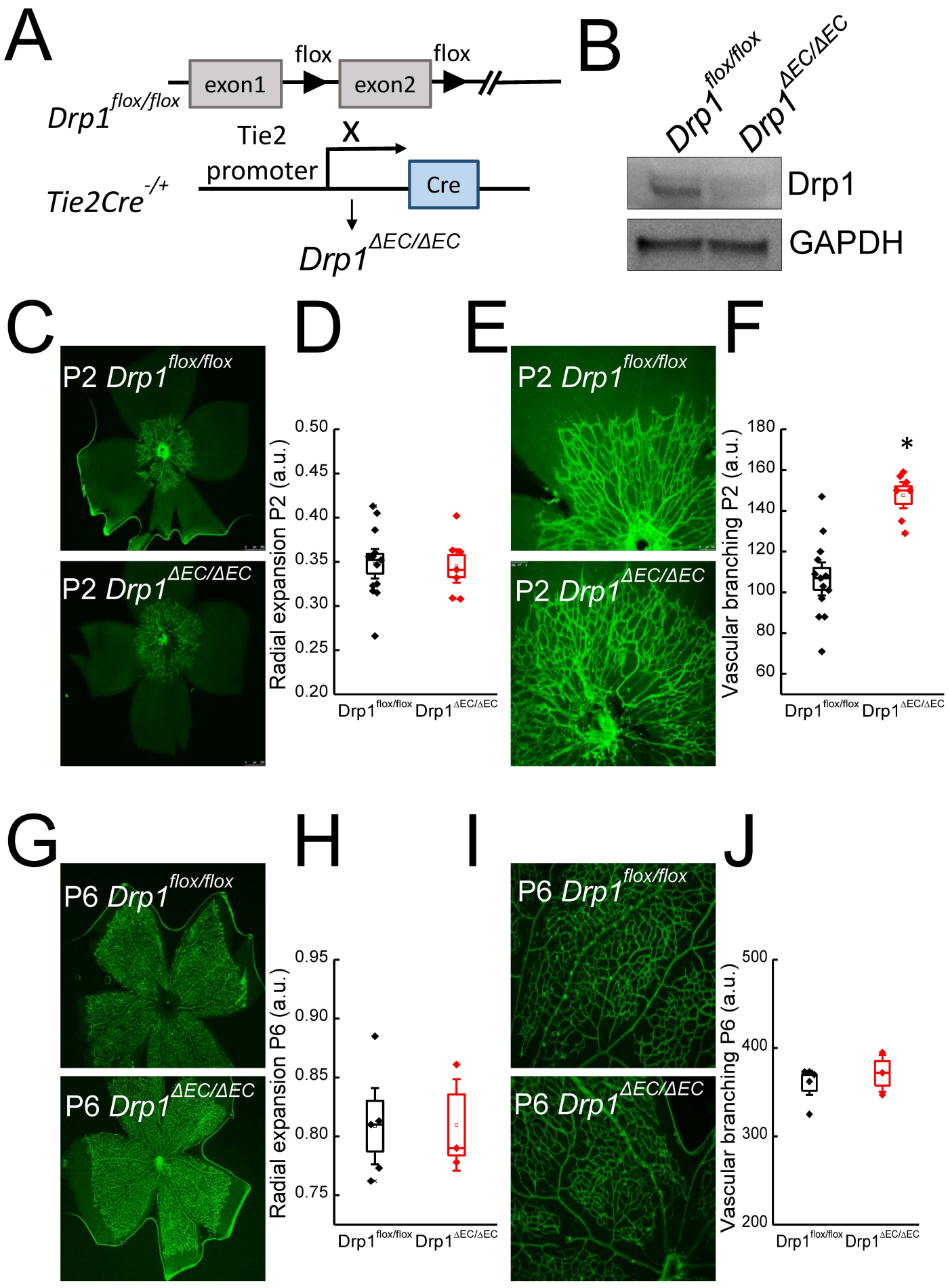

A
Drp1flox/flox
exon1
flox
exon2
flox
Tie2
promoter
X
Cre
Tie2Cre-/+
Drp1ΔEC/ΔEC
B
Drp1flox/flox
Drp1ΔEC/ΔEC
Drp1
GAPDH
C
P2 Drp1flox/flox
P2 Drp1ΔEC/ΔEC
D
Radial expansion P2 (a.u.)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
Drp1flox/flox
Drp1ΔEC/ΔEC
E
P2 Drp1flox/flox
P2 Drp1ΔEC/ΔEC
F
Vascular branching P2 (a.u.)
180
160
140
120
100
80
60
*
Drp1flox/flox
Drp1ΔEC/ΔEC
G
P6 Drp1flox/flox
P6 Drp1ΔEC/ΔEC
H
Radial expansion P6 (a.u.)
0.95
0.90
0.85
0.80
0.75
Drp1flox/flox
Drp1ΔEC/ΔEC
I
P6 Drp1flox/flox
P6 Drp1ΔEC/ΔEC
J
Vascular branching P6 (a.u.)
500
400
300
200
Drp1flox/flox
Drp1ΔEC/ΔEC

Figure 3.

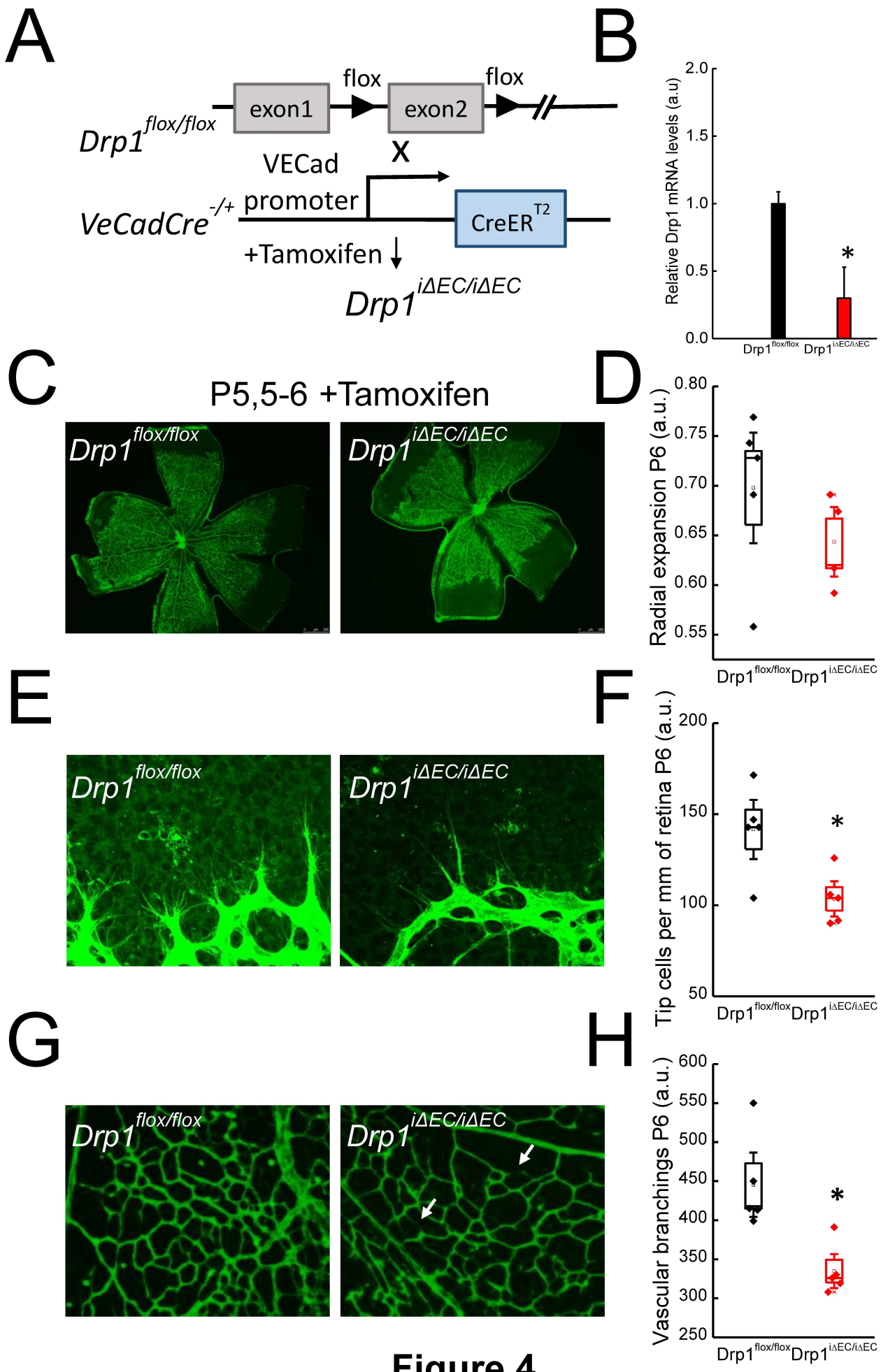

A
B
flox
flox
exon1
exon2
Drp1flox/flox
X
VECad
promoter
VeCadCre-/+
CreER T2
+Tamoxifen
Drp1iΔEC/iΔEC
Relative Drp1 mRNA levels (a.u)
2.0
1.5
1.0
0.5
0.0
*
Drp1flox/flox
Drp1iΔEC/iΔEC
C
P5,5-6 +Tamoxifen
Drp1flox/flox
Drp1iΔEC/iΔEC
D
Radial expansion P6 (a.u.)
0.80
0.75
0.70
0.65
0.60
0.55
Drp1flox/flox
Drp1iΔEC/iΔEC
E
Drp1flox/flox
Drp1iΔEC/iΔEC
F
Tip cells per mm of retina P6 (a.u.)
200
150
100
50
*
Drp1flox/flox
Drp1iΔEC/iΔEC
G
Drp1flox/flox
Drp1iΔEC/iΔEC
H
Vascular branchings P6 (a.u.)
600
550
500
450
400
350
300
250
*
Drp1flox/flox
Drp1iΔEC/iΔEC

Figure 4.

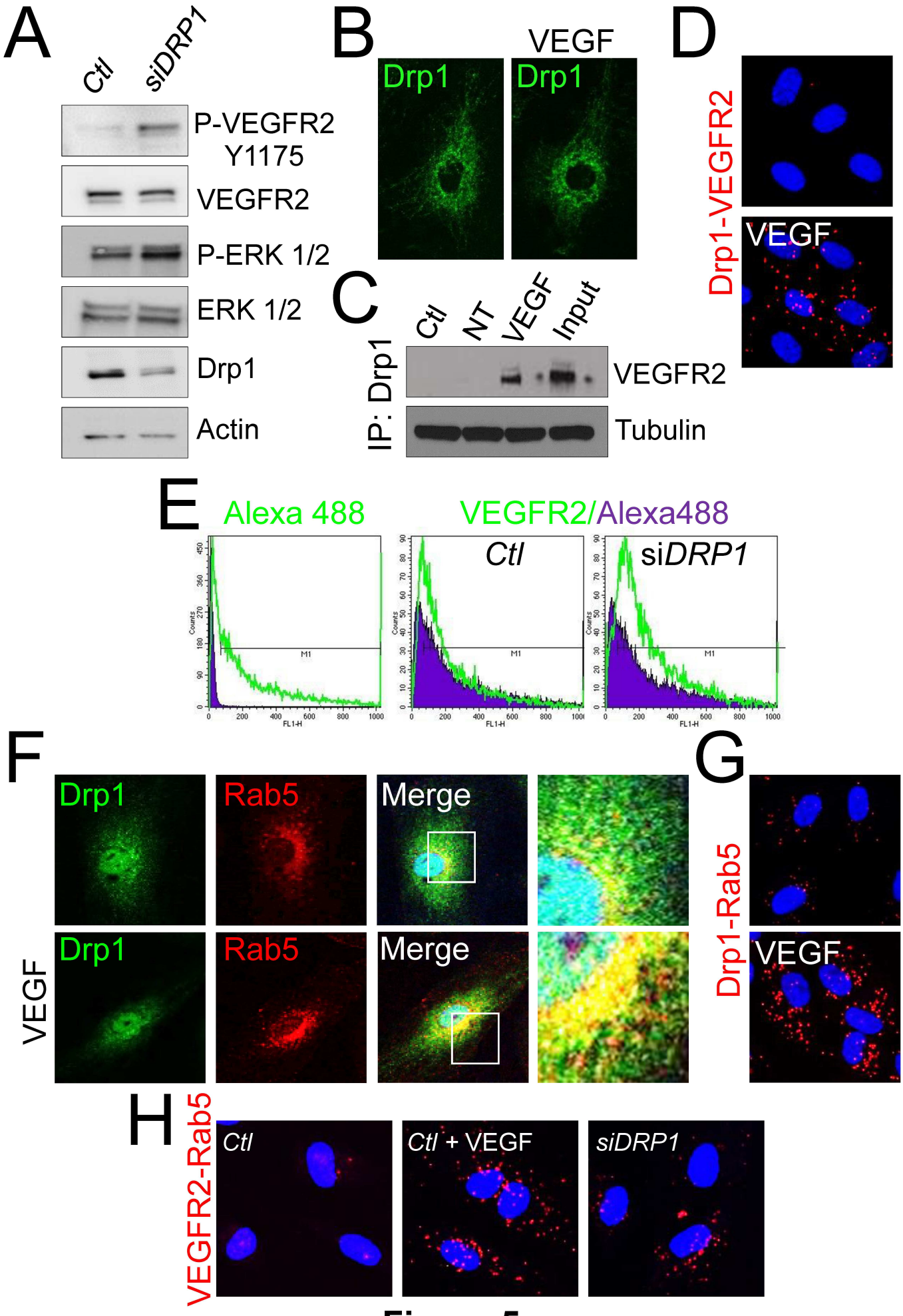

A
Ctl
siDRP1
P-VEGFR2 Y1175
VEGFR2
P-ERK 1/2
ERK 1/2
Drp1
Actin
B
VEGF
Drp1
Drp1
C
Ctl
NT
VEGF
Input
IP: Drp1
VEGFR2
Tubulin
D
Drp1-VEGFR2
VEGF
E
Alexa 488
VEGFR2/Alexa488
Ctl
siDRP1
M1
Counts
FL1-H
F
Drp1
Rab5
Merge
VEGF
Drp1
Rab5
Merge
G
Drp1-Rab5
VEGF
H
VEGFR2-Rab5
Ctl
Ctl + VEGF
siDRP1

Figure 5.

Supplementary figure legends

Figure S1. *Drp1$^{\Delta EC/\Delta EC}$* genotyping.

(A) The genotype of *Drp1$^{\Delta EC/\Delta EC}$ and Drp1$^{flox/flox}$* littermates was verified using PCR gene amplification. Homozygous *WT Drp1$^{+/+}$* mice are characterized with a single band at 200bp, homozygous *Drp1$^{flox/flox}$* mice with a single band at 290pb and heterozygous *Drp1$^{flox/+}$* mice present two band: one at 200bp and one at 290bp.

(B) Tie2Cre expression was detected by amplification of a single band at 100bp. Combination of the two PCR analysis gives the correct genotype of the animal.

Figure S3. *Drp1$^{i\Delta EC/i\Delta EC}$* genotyping.

(A) The genotype of *Drp1$^{i\Delta EC/i\Delta EC}$ and Drp1$^{flox/flox}$* littermates was verified using PCR gene amplification. Homozygous *WT Drp1$^{+/+}$* mice are characterized with a single band at 200bp, homozygous *Drp1$^{flox/flox}$* mice with a single band at 290pb and heterozygous Drp1$^{flox/+}$ mice present two band: one at 200bp and one at 290bp.

(B) VeCadCre expression was detected by amplification of a single band at 100bp. Combination of the two PCR analysis gives the correct genotype of the animal.

Figure S3. Drp1 regulates Rab5-dependent VEGFR2 signaling.

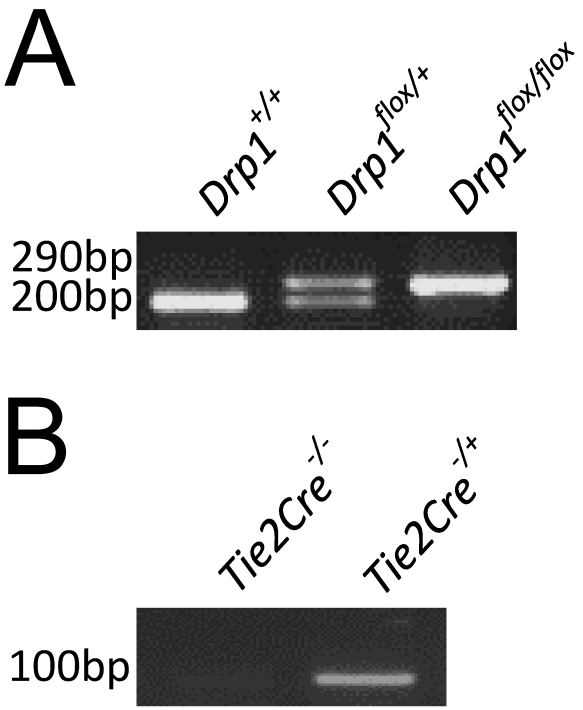
(A) Levels of the indicated proteins were assessed by Western blot analysis of total protein extracts from MDA-MB-231 breast cancer cells**,** transfected with a scramble (Ctl) and three different types of *DRP1* silencing siRNAs (30nm).

(B) Proximity ligation assay (PLA) in non-treated HUVECs using only the secondary antibodies anti-mouse and anti-rabbit (upper panel). The absence of red fluorescent signal confirms the specificity of the detected interactions in Fig. 5. Lower panel represents a PLA experiment in non-treated HUVECs between Drp1 and Mff, a well described interaction. Red spots indicate the proximity of the two proteins.

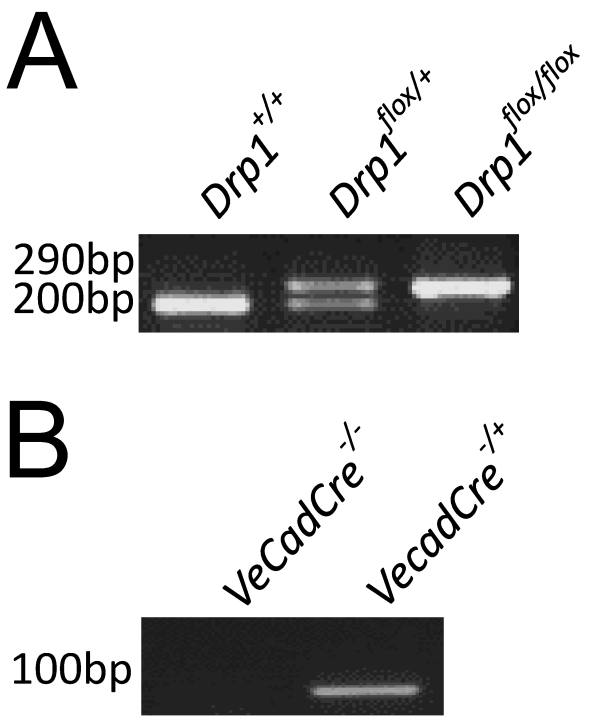
(C) Confocal microscopy image of non-treated and VEGF-stimulated (50ng/ml, 10 minutes) HUVECs immunostained for Drp1 (green) and Rab7 (red). Colocalization analysis was performed using ImageJ.

(D) Confocal microscopy image of non-treated and VEGF-stimulated (50ng/ml, 10 minutes) HUVECs immunostained for Rab5 (green) and TOM20 (red). Colocalization analysis was performed using ImageJ.

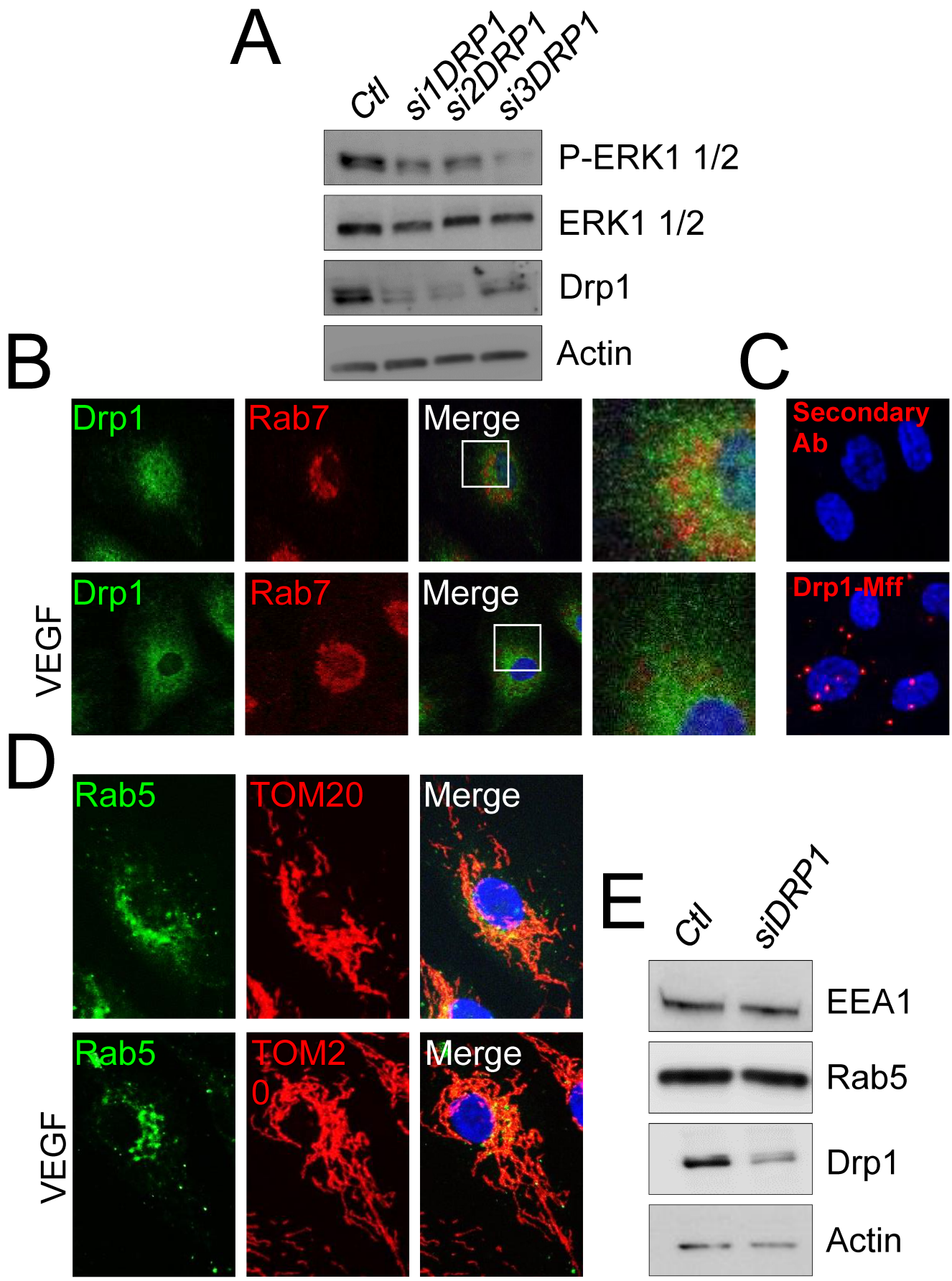
(E) Levels of the indicated proteins were assessed by Western blot analysis of total protein extracts from HUVECs, transfected with scramble (Ctl) and *DRP1* silencing siRNA.



Supplementary figure 1.



Supplementary figure 2.



Supplementary figure 3.

References

Arasaki, K., Shimizu, H., Mogari, H., Nishida, N., Hirota, N., Furuno, A., Kudo, Y., Baba, M., Baba, N., Cheng, J.*, et al.* (2015). A role for the ancient SNARE syntaxin 17 in regulating mitochondrial division. Developmental cell *32*, 304-317.

Ballmer-Hofer, K., Andersson, A.E., Ratcliffe, L.E., and Berger, P. (2011). Neuropilin-1 promotes VEGFR-2 trafficking through Rab11 vesicles thereby specifying signal output. Blood *118*, 816-826.

Bierhansl, L., Conradi, L.C., Treps, L., Dewerchin, M., and Carmeliet, P. (2017). Central Role of Metabolism in Endothelial Cell Function and Vascular Disease. Physiology *32*, 126-140.

Blanco, R., and Gerhardt, H. (2013). VEGF and Notch in tip and stalk cell selection. Cold Spring Harbor perspectives in medicine *3*, a006569.

Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. Nature *438*, 932-936.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C.*, et al.* (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*, 435-439.

Chang, C.R., Manlandro, C.M., Arnoult, D., Stadler, J., Posey, A.E., Hill, R.B., and Blackstone, C. (2010). A lethal de novo mutation in the middle domain of the dynamin-related GTPase Drp1 impairs higher order assembly and mitochondrial division. J BiolChem *285*, 32494-32503.

Chen, H., Detmer, S.A., Ewald, A.J., Griffin, E.E., Fraser, S.E., and Chan, D.C. (2003). Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. The Journal of cell biology *160*, 189-200.

Chittenden, T.W., Claes, F., Lanahan, A.A., Autiero, M., Palac, R.T., Tkachenko, E.V., Elfenbein, A., Ruiz de Almodovar, C., Dedkov, E., Tomanek, R.*, et al.* (2006). Selective regulation of arterial branching morphogenesis by synectin. Developmental cell *10*, 783-795.

Cho, B., Choi, S.Y., Cho, H.M., Kim, H.J., and Sun, W. (2013). Physiological and pathological significance of dynamin-related protein 1 (drp1)-dependent mitochondrial fission in the nervous system. Experimental neurobiology *22*, 149-157.

Christoforidis, S., McBride, H.M., Burgoyne, R.D., and Zerial, M. (1999). The Rab5 effector EEA1 is a core component of endosome docking. Nature *397*, 621-625.

Chung, A.S., Lee, J., and Ferrara, N. (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. Nature reviews Cancer *10*, 505-514.

Cipolat, S., O., M.d.B., B., D.Z., and Scorrano, L. (2004). OPA1 requires mitofusin 1 to promote mitochondrial fusion. ProcNatlAcadSciUSA *101*, 15927-15932.

Cudmore, M.J., Ramma, W., Cai, M., Fujisawa, T., Ahmad, S., Al-Ani, B., and Ahmed, A. (2012). Resveratrol inhibits the release of soluble fms-like tyrosine kinase (sFlt-1) from human placenta. Am J Obstet Gynecol *206*, 253 e210-255.

De Smet, F., Segura, I., De Bock, K., Hohensinner, P.J., and Carmeliet, P. (2009). Mechanisms of vessel branching: filopodia on endothelial tip cells lead the way. Arteriosclerosis, thrombosis, and vascular biology *29*, 639-649.

Demory, M.L., Boerner, J.L., Davidson, R., Faust, W., Miyake, T., Lee, I., Huttemann, M., Douglas, R., Haddad, G., and Parsons, S.J. (2009). Epidermal growth factor receptor translocation to the mitochondria: regulation and effect. J Biol Chem *284*, 36592-36604.

Ehling, M., Adams, S., Benedito, R., and Adams, R.H. (2013). Notch controls retinal blood vessel maturation and quiescence. Development *140*, 3051-3061.

Eichmann, A., and Simons, M. (2012). VEGF signaling inside vascular endothelial cells and beyond. Curr Opin Cell Biol *24*, 188-193.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., and Moore, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*, 439-442.

Fong, G.H., Rossant, J., Gertsenstein, M., and Breitman, M.L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature *376*, 66-70.

Francy, C.A., Alvarez, F.J., Zhou, L., Ramachandran, R., and Mears, J.A. (2015). The mechanoenzymatic core of dynamin-related protein 1 comprises the minimal machinery required for membrane constriction. J Biol Chem *290*, 11692-11703.

Friedman, J.R., Lackner, L.L., West, M., DiBenedetto, J.R., Nunnari, J., and Voeltz, G.K. (2011). ER Tubules Mark Sites of Mitochondrial Division. Science *334*, 358-362.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D.*, et al.* (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. The Journal of cell biology *161*, 1163-1177.

Giedt, R.J., Yang, C., Zweier, J.L., Matzavinos, A., and Alevriadou, B.R. (2012). Mitochondrial fission in endothelial cells after simulated ischemia/reperfusion: role of nitric oxide and reactive oxygen species. Free Radic Biol Med *52*, 348-356.

Greenberg, J.I., Shields, D.J., Barillas, S.G., Acevedo, L.M., Murphy, E., Huang, J., Scheppke, L., Stockmann, C., Johnson, R.S., Angle, N.*, et al.* (2008). A role for VEGF as a negative regulator of pericyte function and vessel maturation. Nature *456*, 809-813.

Hatch, A.L., Ji, W.K., Merrill, R.A., Strack, S., and Higgs, H.N. (2016). Actin filaments as dynamic reservoirs for Drp1 recruitment. Molecular biology of the cell *27*, 3109-3121.

Hellstrom, M., Phng, L.K., Hofmann, J.J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A.K., Karlsson, L., Gaiano, N.*, et al.* (2007). Dll4 signaling through Notch1 regulates formation of tip cells during angiogenesis. Nature *445*, 776-780.

Hong, C.C., Peterson, Q.P., Hong, J.Y., and Peterson, R.T. (2006). Artery/vein specification is governed by opposing phosphatidylinositol-3 kinase and MAP kinase/ERK signaling. Curr Biol *16*, 1366-1372.

Ishihara, N., Eura, Y., and Mihara, K. (2004). Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. Journal of cell science *117*, 6535-6546.

Ishihara, N., Nomura, M., Jofuku, A., Kato, H., Suzuki, S.O., Masuda, K., Otera, H., Nakanishi, Y., Nonaka, I., Goto, Y.*, et al.* (2009). Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nat Cell Biol *11*, 958-966.

Kasahara, A., Cipolat, S., Chen, Y., Dorn, G.W., and Scorrano, L. (2013). Mitochondrial fusion directs cardiomyocyte differentiation via calcineurin and Notch signaling. Science *342*, 734-737.

Kashatus, J.A., Nascimento, A., Myers, L.J., Sher, A., Byrne, F.L., Hoehn, K.L., Counter, C.M., and Kashatus, D.F. (2015). Erk2 phosphorylation of Drp1 promotes mitochondrial fission and MAPK-driven tumor growth. Molecular cell *57*, 537-551.

Kerr, B.A., West, X.Z., Kim, Y.W., Zhao, Y., Tischenko, M., Cull, R.M., Phares, T.W., Peng, X.D., Bernier-Latmani, J., Petrova, T.V.*, et al.* (2016). Stability and function of adult vasculature is sustained by Akt/Jagged1 signaling axis in endothelium. Nature communications *7*, 10960.

Kisanuki, Y.Y., Hammer, R.E., Miyazaki, J., Williams, S.C., Richardson, J.A., and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev Biol *230*, 230-242.

Korn, C., and Augustin, H.G. (2015). Mechanisms of Vessel Pruning and Regression. Developmental cell *34*, 5-17.

Korobova, F., Gauvin, T.J., and Higgs, H.N. (2014). A role for myosin II in mammalian mitochondrial fission. Curr Biol *24*, 409-414.

Lampugnani, M.G., Orsenigo, F., Gagliani, M.C., Tacchetti, C., and Dejana, E. (2006). Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. The Journal of cell biology *174*, 593-604.

Lanahan, A., Zhang, X., Fantin, A., Zhuang, Z., Rivera-Molina, F., Speichinger, K., Prahst, C., Zhang, J., Wang, Y., Davis, G.*, et al.* (2013). The neuropilin 1 cytoplasmic domain is required for VEGF-A-dependent arteriogenesis. Developmental cell *25*, 156-168.

Lanahan, A.A., Hermans, K., Claes, F., Kerley-Hamilton, J.S., Zhuang, Z.W., Giordano, F.J., Carmeliet, P., and Simons, M. (2010). VEGF receptor 2 endocytic trafficking regulates arterial morphogenesis. Developmental cell *18*, 713-724.

Li, X., and Gould, S.J. (2003). The dynamin-like GTPase DLP1 is essential for peroxisome division and is recruited to peroxisomes in part by PEX11. JBiolChem *278*, 17012-17020.

Lin, J.R., Shen, W.L., Yan, C., and Gao, P.J. (2015). Downregulation of dynamin-related protein 1 contributes to impaired autophagic flux and angiogenic function in senescent endothelial cells. Arteriosclerosis, thrombosis, and vascular biology *35*, 1413-1422.

Macdonald, P.J., Stepanyants, N., Mehrotra, N., Mears, J.A., Qi, X., Sesaki, H., and Ramachandran, R. (2014). A dimeric equilibrium intermediate nucleates Drp1 reassembly on mitochondrial membranes for fission. Molecular biology of the cell *25*, 1905-1915.

Marsboom, G., Toth, P.T., Ryan, J.J., Hong, Z., Wu, X., Fang, Y.H., Thenappan, T., Piao, L., Zhang, H.J., Pogoriler, J.*, et al.* (2012). Dynamin-related protein 1-mediated mitochondrial mitotic fission permits hyperproliferation of vascular smooth muscle cells and offers a novel therapeutic target in pulmonary hypertension. Circulation research *110*, 1484-1497.

Matsumoto, T., Bohman, S., Dixelius, J., Berge, T., Dimberg, A., Magnusson, P., Wang, L., Wikner, C., Qi, J.H., Wernstedt, C.*, et al.* (2005). VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. The EMBO journal *24*, 2342-2353.

Monvoisin, A., Alva, J.A., Hofmann, J.J., Zovein, A.C., Lane, T.F., and Iruela-Arispe, M.L. (2006). VE-cadherin-CreERT2 transgenic mouse: a model for inducible recombination in the endothelium. Developmental dynamics : an official publication of the American Association of Anatomists *235*, 3413-3422.

Nielsen, E., Christoforidis, S., Uttenweiler-Joseph, S., Miaczynska, M., Dewitte, F., Wilm, M., Hoflack, B., and Zerial, M. (2000). Rabenosyn-5, a novel Rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE finger domain. The Journal of cell biology *151*, 601-612.

Ortiz-Sandoval, C.G., Hughes, S.C., Dacks, J.B., and Simmen, T. (2014). Interaction with the effector dynamin-related protein 1 (Drp1) is an ancient function of Rab32 subfamily proteins. Cellular logistics *4*, e986399.

Pernas, L., and Scorrano, L. (2016). Mito-Morphosis: Mitochondrial Fusion, Fission, and Cristae Remodeling as Key Mediators of Cellular Function. Annu Rev Physiol *78*, 505-531.

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. Cell *146*, 873-887.

Rubino, M., Miaczynska, M., Lippe, R., and Zerial, M. (2000). Selective membrane recruitment of EEA1 suggests a role in directional transport of clathrin-coated vesicles to early endosomes. J Biol Chem *275*, 3745-3748.

Shah, A.V., Birdsey, G.M., Peghaire, C., Pitulescu, M.E., Dufton, N.P., Yang, Y., Weinberg, I., Osuna Almagro, L., Payne, L., Mason, J.C.*, et al.* (2017). The endothelial transcription factor ERG mediates Angiopoietin-1-dependent control of Notch signaling and vascular stability. Nature communications *8*, 16002.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L., and Schuh, A.C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature *376*, 62-66.

Simons, M. (2012). An inside view: VEGF receptor trafficking and signaling. Physiology *27*, 213-222.

Simons, M., Gordon, E., and Claesson-Welsh, L. (2016). Mechanisms and regulation of endothelial VEGF receptor signaling. Nature reviews Molecular cell biology *17*, 611-625.

Smirnova, E., Griparic, L., Shurland, D.L., and van der Bliek, A.M. (2001). Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. MolBiolCell *12*, 2245-2256.

Soderberg, O., Leuchowius, K.J., Gullberg, M., Jarvius, M., Weibrecht, I., Larsson, L.G., and Landegren, U. (2008). Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay. Methods *45*, 227-232.

Stahl, A., Connor, K.M., Sapieha, P., Chen, J., Dennison, R.J., Krah, N.M., Seaward, M.R., Willett, K.L., Aderman, C.M., Guerin, K.I.*, et al.* (2010). The mouse retina as an angiogenesis model. Invest Ophthalmol Vis Sci *51*, 2813-2826.

Villasenor, R., Nonaka, H., Del Conte-Zerial, P., Kalaidzidis, Y., and Zerial, M. (2015). Regulation of EGFR signal transduction by analogue-to-digital conversion in endosomes. eLife *4*.

Wakabayashi, J., Zhang, Z., Wakabayashi, N., Tamura, Y., Fukaya, M., Kensler, T.W., Iijima, M., and Sesaki, H. (2009). The dynamin-related GTPase Drp1 is required for embryonic and brain development in mice. J Cell Biol *186*, 805-816.

Wang, W., Wang, Y., Long, J., Wang, J., Haudek, S.B., Overbeek, P., Chang, B.H., Schumacker, P.T., and Danesh, F.R. (2012). Mitochondrial fission triggered by hyperglycemia is mediated by ROCK1 activation in podocytes and endothelial cells. Cell Metab *15*, 186-200.

Waterham, H.R., Koster, J., van Roermund, C.W., Mooyer, P.A., Wanders, R.J., and Leonard, J.V. (2007). A lethal defect of mitochondrial and peroxisomal fission. NEnglJMed *356*, 1736-1741.

Westermann, B. (2010). Mitochondrial fusion and fission in cell life and death. Nat RevMol Cell Biol *11*, 872-884.

Yoon, Y., Pitts, K.R., Dahan, S., and McNiven, M.A. (1998). A novel dynamin-like protein associates with cytoplasmic vesicles and tubules of the endoplasmic reticulum in mammalian cells. JCell Biol *140*, 779-793.

General conclusions

Dynamin-related protein 1 (Drp1) is a multidomain GTPase with a complex function, regulated at multiple levels including gene expression, alternative splicing, post-translational modifications, organelle adaptor proteins, membrane lipid composition, intra and intermolecular interactions and GTPase activity. Its functionality is mainly studied in the context of mitochondrial fission, however an increasing number of studies are reporting fission-independent functions for Drp1 (Cho et al., 2013).

In this work we provided evidence that Drp1 is involved in endocytic trafficking during angiogenic signaling in endothelial cells. Upon VEGF stimulation Drp1 interacts with Vascular endothelial growth factor receptor (VEGFR2) and early endosomal marker Rab5. Lack of Drp1 stimulates VEGFR2-Rab5 interaction, leading to an upregulation of VEGFR2 activation and downstream ERK1/2 signaling. This induces angiogenesis *in vitro* and *in vivo*. Our findings indicate that Drp1-dependent negative regulation of VEGFR2 signaling is required for normal vascular development and maturation of blood vessels.

The new function of Drp1 we described could be investigated in variety of physiological conditions and cell types, known to be dependent on vesicular trafficking and tyrosine kinase signaling events. However, the precise mechanism of Drp1 action in VEGFR2-Rab5 communication is not clear.

Early endosomes (EE) are the first endocytic compartment to accept incoming cargo internalized from the plasma membrane (PM). In the case of VEGFR2 it is directly involved in mediation of downstream angiogenic properties as well as driving the receptor towards recycling to the PM or lysosomal degradation. To fulfill this complex function EEs, much like the mitochondria, are highly dynamic structures easily adapting to the signaling context by undergoing processes of membrane tethering, fusion, motility and fission (Gruenberg et al., 1989). These processes are orchestrated by the Ras-associated binding (Rab) proteins. Rabs are small GTP-binding proteins that cycle between a GTP-bound active state and a GDP-bound inactive state and their functionality is regulated by specific recruitment of effector proteins (Zerial and McBride, 2001b)

The nature of the early endosomal compartment and Drp1 functionality suggests many possible interactions and regulation pathways. Being a GTPase, Drp1 itself could act as an effector of Rab5, modulating its activity though the energy conversion of GTP hydrolysis.
The initial description of Drp1 immunofluorescence staining pattern as vesicle-like structures likely to be linked to the cytoskeleton as well as the interaction with motor proteins Myosin II and Dynein (Korobova et al., 2014; Varadi et al., 2004) and its role in mitochondrial motility (Verstreken et al., 2005), suggest that Drp1 could act as an adaptor protein of EE to the cytoskeletal motor proteins during cytosolic movement of the vesicle organelles.
Being a compartment of decision-making and sorting of internalized molecules, EEs are composed of many different regions at which specific subsets of trafficking markers accumulate. Separation of these membrane subcompartments designed for either recycling, degradation or further early-endosomal signaling events requires membrane fission events. Drp1 has not yet been described to participate in this process, however the possibility could not be excluded given its master role in mitochondrial fission. Moreover, organelle contact sites between ER and mitochondria are established during fission (Friedman et al., 2011). The ER is also reported to mark fission sites of early endosomes (Rowland et al., 2014).

Finally, it is essential to determine the post-translation modification and adaptor machinery involved in Drp1-Rab5 communication. It is likely that classical pathways regulating Drp1's activity such as PKA and calcineurin as well as OMM anchoring proteins such as Fis1 of Mff are involved in the process we described (Cho et al., 2013).

Even though not completely understood, Drp1's organelle translocation and membrane constriction activity is also regulated by the lipid composition. Interestingly, unsaturated phospholipids like CL stimulate mitochondrial fission whereas unsaturated such as PA are blocking it. Membrane fluidity depends on the composition of saturated versus unsaturated phospholipids. Lower fluidity is relative to more saturated lipids whereas high fluidity corresponds to a higher concentration of unsaturated lipids. Membrane fluidity is an important factor in ligand/receptor-mediated signaling and in membrane trafficking events such as endocytic sorting. Thus, it is interesting to speculate that membrane fluidity state could be a regulation step for Drp1 activity in general.

In conclusion, our work not only describes a new mechanism of action for Drp1 but also gives important insights into the precise regulation of VEGFR2 signaling by the endocytic trafficking. Better understanding of the molecular mechanisms regulating blood vessel development and maturation is a crucial step in targeting vascular-related pathologies.

Bibliography

Acin-Perez, R., Fernandez-Silva, P., Peleato, M.L., Perez-Martos, A., and Enriquez, J.A. (2008). Respiratory active mitochondrial supercomplexes. MolCell *32*, 529-539.

Adachi, Y., Itoh, K., Yamada, T., Cerveny, K.L., Suzuki, T.L., Macdonald, P., Frohman, M.A., Ramachandran, R., Iijima, M., and Sesaki, H. (2016). Coincident Phosphatidic Acid Interaction Restrains Drp1 in Mitochondrial Division. Molecular cell *63*, 1034-1043.

Afolayan, A.J., Eis, A., Alexander, M., Michalkiewicz, T., Teng, R.J., Lakshminrusimha, S., and Konduri, G.G. (2016). Decreased endothelial nitric oxide synthase expression and function contribute to impaired mitochondrial biogenesis and oxidative stress in fetal lambs with persistent pulmonary hypertension. American journal of physiology Lung cellular and molecular physiology *310*, L40-49.

Al-Mehdi, A.B., Pastukh, V.M., Swiger, B.M., Reed, D.J., Patel, M.R., Bardwell, G.C., Pastukh, V.V., Alexeyev, M.F., and Gillespie, M.N. (2012). Perinuclear mitochondrial clustering creates an oxidant-rich nuclear domain required for hypoxia-induced transcription. Science signaling *5*, ra47.

Al-Shabrawey, M., Bartoli, M., El-Remessy, A.B., Platt, D.H., Matragoon, S., Behzadian, M.A., Caldwell, R.W., and Caldwell, R.B. (2005). Inhibition of NAD(P)H oxidase activity blocks vascular endothelial growth factor overexpression and neovascularization during ischemic retinopathy. The American journal of pathology *167*, 599-607.

Alderton, W.K., Cooper, C.E., and Knowles, R.G. (2001). Nitric oxide synthases: structure, function and inhibition. Biochem J *357*, 593-615.

Alexander, C., Votruba, M., Pesch, U.E., Thiselton, D.L., Mayer, S., Moore, A., Rodriguez, M., Kellner, U., Leo-Kottler, B., Auburger, G.*, et al.* (2000). OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. NatGenet *26*, 211-215.

Alexander, R.A., Prager, G.W., Mihaly-Bison, J., Uhrin, P., Sunzenauer, S., Binder, B.R., Schutz, G.J., Freissmuth, M., and Breuss, J.M. (2012). VEGF-induced endothelial cell migration requires urokinase receptor (uPAR)-dependent integrin redistribution. Cardiovascular research *94*, 125-135.

Anderson, J.F., Siller, E., and Barral, J.M. (2010). The sacsin repeating region (SRR): a novel Hsp90-related supra-domain associated with neurodegeneration. J Mol Biol *400*, 665-674.

Anisimov, A., Leppanen, V.M., Tvorogov, D., Zarkada, G., Jeltsch, M., Holopainen, T., Kaijalainen, S., and Alitalo, K. (2013). The basis for the distinct biological activities of vascular endothelial growth factor receptor-1 ligands. Science signaling *6*, ra52.

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell *118*, 149-161.

Arasaki, K., Shimizu, H., Mogari, H., Nishida, N., Hirota, N., Furuno, A., Kudo, Y., Baba, M., Baba, N., Cheng, J.*, et al.* (2015). A role for the ancient SNARE syntaxin 17 in regulating mitochondrial division. Developmental cell *32*, 304-317.

Archer, S.L., Weir, E.K., and Wilkins, M.R. (2010). Basic science of pulmonary arterial hypertension for clinicians: new concepts and experimental therapies. Circulation *121*, 2045-2066.

Ardail, D., Privat, J.P., Egret-Charlier, M., Levrat, C., Lerme, F., and Louisot, P. (1990). Mitochondrial contact sites. Lipid composition and dynamics. J Biol Chem *265*, 18797-18802.

Artwohl, M., Brunmair, B., Furnsinn, C., Holzenbein, T., Rainer, G., Freudenthaler, A., Porod, E.M., Huttary, N., and Baumgartner-Parzer, S.M. (2007). Insulin does not regulate glucose transport and metabolism in human endothelium. European journal of clinical investigation *37*, 643-650.

Augustin, H.G., Koh, G.Y., Thurston, G., and Alitalo, K. (2009). Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. Nature reviews Molecular cell biology *10*, 165-177.

Avraamides, C.J., Garmy-Susini, B., and Varner, J.A. (2008). Integrins in angiogenesis and lymphangiogenesis. Nature reviews Cancer *8*, 604-617.

Ballmer-Hofer, K., Andersson, A.E., Ratcliffe, L.E., and Berger, P. (2011). Neuropilin-1 promotes VEGFR-2 trafficking through Rab11 vesicles thereby specifying signal output. Blood *118*, 816-826.

Ban, T., Heymann, J.A., Song, Z., Hinshaw, J.E., and Chan, D.C. (2010). OPA1 disease alleles causing dominant optic atrophy have defects in cardiolipin-stimulated GTP hydrolysis and membrane tubulation. Hum Mol Genet *19*, 2113-2122.

Ban, T., Ishihara, T., Kohno, H., Saita, S., Ichimura, A., Maenaka, K., Oka, T., Mihara, K., and Ishihara, N. (2017). Molecular basis of selective mitochondrial fusion by heterotypic action between OPA1 and cardiolipin. Nature cell biology *19*, 856-863.

Bartlett, K., and Eaton, S. (2004). Mitochondrial beta-oxidation. Eur J Biochem *271*, 462-469.

Bates, D.O., Hillman, N.J., Williams, B., Neal, C.R., and Pocock, T.M. (2002). Regulation of microvascular permeability by vascular endothelial growth factors. Journal of anatomy *200*, 581-597.

Baumer, S., Keller, L., Holtmann, A., Funke, R., August, B., Gamp, A., Wolburg, H., Wolburg-Buchholz, K., Deutsch, U., and Vestweber, D. (2006). Vascular endothelial cell-specific phosphotyrosine phosphatase (VE-PTP) activity is required for blood vessel development. Blood *107*, 4754-4762.

Bazan, S., Mileykovskaya, E., Mallampalli, V.K., Heacock, P., Sparagna, G.C., and Dowhan, W. (2013). Cardiolipin-dependent reconstitution of respiratory supercomplexes from purified Saccharomyces cerevisiae complexes III and IV. J Biol Chem *288*, 401-411.

Benedito, R., Roca, C., Sorensen, I., Adams, S., Gossler, A., Fruttiger, M., and Adams, R.H. (2009). The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. Cell *137*, 1124-1135.

Bhuin, T., and Roy, J.K. (2014). Rab proteins: the key regulators of intracellular vesicle transport. Experimental cell research *328*, 1-19.

Bierhansl, L., Conradi, L.C., Treps, L., Dewerchin, M., and Carmeliet, P. (2017). Central Role of Metabolism in Endothelial Cell Function and Vascular Disease. Physiology *32*, 126-140.

Blanco, R., and Gerhardt, H. (2013). VEGF and Notch in tip and stalk cell selection. Cold Spring Harbor perspectives in medicine *3*, a006569.

Bleazard, W., McCaffery, J.M., King, E.J., Bale, S., Mozdy, A., Tieu, Q., Nunnari, J., and Shaw, J.M. (1999). The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. NatCell Biol *1*, 298-304.

Bogenhagen, D.F. (2012). Mitochondrial DNA nucleoid structure. Biochim Biophys Acta *1819*, 914-920.

Bogenhagen, D.F., Rousseau, D., and Burke, S. (2008). The layered structure of human mitochondrial DNA nucleoids. J Biol Chem *283*, 3665-3675.

Bonnefont, J.P., Djouadi, F., Prip-Buus, C., Gobin, S., Munnich, A., and Bastin, J. (2004). Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. Molecular aspects of medicine *25*, 495-520.

Bonnet, S., Michelakis, E.D., Porter, C.J., Andrade-Navarro, M.A., Thebaud, B., Bonnet, S., Haromy, A., Harry, G., Moudgil, R., McMurtry, M.S*., et al.* (2006). An abnormal mitochondrial-hypoxia inducible factor-1alpha-Kv channel pathway disrupts oxygen sensing and triggers pulmonary arterial hypertension in fawn hooded rats: similarities to human pulmonary arterial hypertension. Circulation *113*, 2630-2641.

Bornhovd, C., Vogel, F., Neupert, W., and Reichert, A.S. (2006). Mitochondrial membrane potential is dependent on the oligomeric state of F1F0-ATP synthase supracomplexes. JBiolChem *281*, 13990-13998.

Bottinger, L., Horvath, S.E., Kleinschroth, T., Hunte, C., Daum, G., Pfanner, N., and Becker, T. (2012). Phosphatidylethanolamine and cardiolipin differentially affect the stability of mitochondrial respiratory chain supercomplexes. J Mol Biol *423*, 677-686.

Braschi, E., Zunino, R., and McBride, H.M. (2009). MAPL is a new mitochondrial SUMO E3 ligase that regulates mitochondrial fission. EMBORep *10*, 748-754.

Brdiczka, D.G., Zorov, D.B., and Sheu, S.S. (2006). Mitochondrial contact sites: their role in energy metabolism and apoptosis. Biochim Biophys Acta *1762*, 148-163.

Breckenridge, D.G., Kang, B.H., Kokel, D., Mitani, S., Staehelin, L.A., and Xue, D. (2008). Caenorhabditis elegans drp-1 and fis-2 regulate distinct cell-death execution pathways downstream of ced-3 and independent of ced-9. MolCell *31*, 586-597.

Brooks, C., Wei, Q., Feng, L., Dong, G., Tao, Y., Mei, L., Xie, Z.J., and Dong, Z. (2007). Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins. ProcNatlAcadSciUSA *104*, 11649-11654.

Bui, M., Gilady, S.Y., Fitzsimmons, R.E., Benson, M.D., Lynes, E.M., Gesson, K., Alto, N.M., Strack, S., Scott, J.D., and Simmen, T. (2010). Rab32 modulates apoptosis onset and mitochondria-associated membrane (MAM) properties. J BiolChem *285*, 31590-31602.

Bustillo-Zabalbeitia, I., Montessuit, S., Raemy, E., Basanez, G., Terrones, O., and Martinou, J.C. (2014). Specific interaction with cardiolipin triggers functional activation of Dynamin-Related Protein 1. PloS one *9*, e102738.

Byzova, T.V., Goldman, C.K., Pampori, N., Thomas, K.A., Bett, A., Shattil, S.J., and Plow, E.F. (2000). A mechanism for modulation of cellular responses to VEGF: activation of the integrins. Molecular cell *6*, 851-860.

Camici, G.G., Schiavoni, M., Francia, P., Bachschmid, M., Martin-Padura, I., Hersberger, M., Tanner, F.C., Pelicci, P., Volpe, M., Anversa, P.*, et al.* (2007). Genetic deletion of p66(Shc) adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress. Proceedings of the National Academy of Sciences of the United States of America *104*, 5217-5222.

Campello, S., Lacalle, R.A., Bettella, M., Manes, S., Scorrano, L., and Viola, A. (2006). Orchestration of lymphocyte chemotaxis by mitochondrial dynamics. JExpMed *203*, 2879-2886.

Camus, S., Quevedo, C., Menendez, S., Paramonov, I., Stouten, P.F., Janssen, R.A., Rueb, S., He, S., Snaar-Jagalska, B.E., Laricchia-Robbio, L.*, et al.* (2012). Identification of phosphorylase kinase as a novel therapeutic target through high-throughput screening for anti-angiogenesis compounds in zebrafish. Oncogene *31*, 4333-4342.

Cantelmo, A.R., Brajic, A., and Carmeliet, P. (2015). Endothelial Metabolism Driving Angiogenesis: Emerging Concepts and Principles. Cancer journal *21*, 244-249.

Cantelmo, A.R., Conradi, L.C., Brajic, A., Goveia, J., Kalucka, J., Pircher, A., Chaturvedi, P., Hol, J., Thienpont, B., Teuwen, L.A.*, et al.* (2016). Inhibition of the Glycolytic Activator PFKFB3 in Endothelium Induces Tumor Vessel Normalization, Impairs Metastasis, and Improves Chemotherapy. Cancer Cell *30*, 968-985.

Carden, D.L., and Granger, D.N. (2000). Pathophysiology of ischaemia-reperfusion injury. The Journal of pathology *190*, 255-266.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. In Nature medicine, pp. 389-395.

Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. Nature *438*, 932-936.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C.*, et al.* (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*, 435-439.

Carmeliet, P., Lampugnani, M.G., Moons, L., Breviario, F., Compernolle, V., Bono, F., Balconi, G., Spagnuolo, R., Oosthuyse, B., Dewerchin, M.*, et al.* (1999). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell *98*, 147-157.

Casanovas, O., Hicklin, D.J., Bergers, G., and Hanahan, D. (2005). Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell *8*, 299-309.

Caswell, P.T., Vadrevu, S., and Norman, J.C. (2009). Integrins: masters and slaves of endocytic transport. Nature reviews Molecular cell biology *10*, 843-853.

Cereghetti, G.M., Stangherlin, A., Martins de, B.O., Chang, C.R., Blackstone, C., Bernardi, P., and Scorrano, L. (2008). Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. ProcNatlAcadSciUSA *105*, 15803-15808.

Chang, C.R., and Blackstone, C. (2010). Dynamic regulation of mitochondrial fission through modification of the dynamin-related protein Drp1. AnnNYAcadSci *1201*, 34-39.

Chang, C.R., Manlandro, C.M., Arnoult, D., Stadler, J., Posey, A.E., Hill, R.B., and Blackstone, C. (2010). A lethal de novo mutation in the middle domain of the dynamin-related GTPase Drp1 impairs higher order assembly and mitochondrial division. J BiolChem *285*, 32494-32503.

Chen, H., Chedotal, A., He, Z., Goodman, C.S., and Tessier-Lavigne, M. (1997). Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron *19*, 547-559.

Chen, H., Chomyn, A., and Chan, D.C. (2005a). Disruption of fusion results in mitochondrial heterogeneity and dysfunction. J Biol Chem *280*, 26185-26192.

Chen, H., Detmer, S.A., Ewald, A.J., Griffin, E.E., Fraser, S.E., and Chan, D.C. (2003). Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. The Journal of cell biology *160*, 189-200.

Chen, H., McCaffery, J.M., and Chan, D.C. (2007). Mitochondrial fusion protects against neurodegeneration in the cerebellum. Cell *130*, 548-562.

Chen, H., Ren, S., Clish, C., Jain, M., Mootha, V., McCaffery, J.M., and Chan, D.C. (2015). Titration of mitochondrial fusion rescues Mff-deficient cardiomyopathy. The Journal of cell biology *211*, 795-805.

Chen, J., Somanath, P.R., Razorenova, O., Chen, W.S., Hay, N., Bornstein, P., and Byzova, T.V. (2005b). Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. Nature medicine *11*, 1188-1196.

Chen, K.H., Dasgupta, A., Ding, J., Indig, F.E., Ghosh, P., and Longo, D.L. (2014). Role of mitofusin 2 (Mfn2) in controlling cellular proliferation. FASEB J *28*, 382-394.

Chen, T.T., Luque, A., Lee, S., Anderson, S.M., Segura, T., and Iruela-Arispe, M.L. (2010). Anchorage of VEGF to the extracellular matrix conveys differential signaling responses to endothelial cells. The Journal of cell biology *188*, 595-609.

Chen, X., Gays, D., Milia, C., and Santoro, M.M. (2017). Cilia Control Vascular Mural Cell Recruitment in Vertebrates. Cell reports *18*, 1033-1047.

Chen, X.L., Nam, J.O., Jean, C., Lawson, C., Walsh, C.T., Goka, E., Lim, S.T., Tomar, A., Tancioni, I., Uryu, S*., et al.* (2012). VEGF-induced vascular permeability is mediated by FAK. Developmental cell *22*, 146-157.

Chittenden, T.W., Claes, F., Lanahan, A.A., Autiero, M., Palac, R.T., Tkachenko, E.V., Elfenbein, A., Ruiz de Almodovar, C., Dedkov, E., Tomanek, R*., et al.* (2006). Selective regulation of arterial branching morphogenesis by synectin. Developmental cell *10*, 783-795.

Cho, B., Choi, S.Y., Cho, H.M., Kim, H.J., and Sun, W. (2013). Physiological and pathological significance of dynamin-related protein 1 (drp1)-dependent mitochondrial fission in the nervous system. Experimental neurobiology *22*, 149-157.

Choi, S.Y., Huang, P., Jenkins, G.M., Chan, D.C., Schiller, J., and Frohman, M.A. (2006). A common lipid links Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. NatCellBiol *8*, 1255-1262.

Christoforidis, S., McBride, H.M., Burgoyne, R.D., and Zerial, M. (1999). The Rab5 effector EEA1 is a core component of endosome docking. Nature *397*, 621-625.

Chung, A.S., Lee, J., and Ferrara, N. (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. Nature reviews Cancer *10*, 505-514.

Cipolat, S., O., M.d.B., B., D.Z., and Scorrano, L. (2004). OPA1 requires mitofusin 1 to promote mitochondrial fusion. ProcNatlAcadSciUSA *101*, 15927-15932.

Cipolat, S., Rudka, T., Hartmann, D., Costa, V., Serneels, L., Craessaerts, K., Metzger, K., Frezza, C., Annaert, W., D'Adamio, L*., et al.* (2006). Mitochondrial Rhomboid PARL Regulates Cytochrome c Release during Apoptosis via OPA1-Dependent Cristae Remodeling. Cell *126*, 163-175.

Civiletto, G., Varanita, T., Cerutti, R., Gorletta, T., Barbaro, S., Marchet, S., Lamperti, C., Viscomi, C., Scorrano, L., and Zeviani, M. (2015). Opa1 overexpression ameliorates the phenotype of two mitochondrial disease mouse models. Cell Metab *21*, 845-854.

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. Cell *127*, 469-480.

Cogliati, S., Enriquez, J.A., and Scorrano, L. (2016). Mitochondrial Cristae: Where Beauty Meets Functionality. Trends Biochem Sci *41*, 261-273.

Cogliati, S., Frezza, C., Soriano, M.-á., Varanita, T., Quintana-Cabrera, R., Corrado, M., Cipolat, S., Costa, V., Casarin, A., Gomes, L.-á*., et al.* (2013a). Mitochondrial Cristae Shape Determines Respiratory Chain Supercomplexes Assembly and Respiratory Efficiency. Cell *155*, 160-171.

Cogliati, S., Frezza, C., Soriano, M.E., Varanita, T., Quintana-Cabrera, R., Corrado, M., Cipolat, S., Costa, V., Casarin, A., Gomes, L.C*., et al.* (2013b). Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. Cell *155*, 160-171.

Coon, B.G., Baeyens, N., Han, J., Budatha, M., Ross, T.D., Fang, J.S., Yun, S., Thomas, J.L., and Schwartz, M.A. (2015). Intramembrane binding of VE-cadherin to VEGFR2 and VEGFR3 assembles the endothelial mechanosensory complex. The Journal of cell biology *208*, 975-986.

Corada, M., Nyqvist, D., Orsenigo, F., Caprini, A., Giampietro, C., Taketo, M.M., Iruela-Arispe, M.L., Adams, R.H., and Dejana, E. (2010). The Wnt/beta-catenin pathway modulates vascular remodeling and specification by upregulating Dll4/Notch signaling. Developmental cell *18*, 938-949.

Costa, V., Giacomello, M., Hudec, R., Lopreiato, R., Ermak, G., Lim, D., Malorni, W., Davies, K.J., Carafoli, E., and Scorrano, L. (2010). Mitochondrial fission and cristae disruption increase the response of cell models of Huntington's disease to apoptotic stimuli. EMBO Mol Med *2*, 490-503.

Costa, V., and Scorrano, L. (2012). Shaping the role of mitochondria in the pathogenesis of Huntington's disease. The EMBO journal *31*, 1853-1864.

Coussens, L.M., Raymond, W.W., Bergers, G., Laig-Webster, M., Behrendtsen, O., Werb, Z., Caughey, G.H., and Hanahan, D. (1999). Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. Genes & development *13*, 1382-1397.

Cribbs, J.T., and Strack, S. (2007). Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. EMBO Rep *8*, 939-944.

Croci, D.O., Cerliani, J.P., Pinto, N.A., Morosi, L.G., and Rabinovich, G.A. (2014). Regulatory role of glycans in the control of hypoxia-driven angiogenesis and sensitivity to anti-angiogenic treatment. Glycobiology *24*, 1283-1290.

Cudmore, M.J., Ramma, W., Cai, M., Fujisawa, T., Ahmad, S., Al-Ani, B., and Ahmed, A. (2012). Resveratrol inhibits the release of soluble fms-like tyrosine kinase (sFlt-1) from human placenta. Am J Obstet Gynecol *206*, 253 e210-255.

Damke, H., Baba, T., Warnock, D.E., and Schmid, S.L. (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. The Journal of cell biology *127*, 915-934.

Davies, V.J., Hollins, A.J., Piechota, M.J., Yip, W., Davies, J.R., White, K.E., Nicols, P.P., Boulton, M.E., and Votruba, M. (2007). Opa1 deficiency in a mouse model of autosomal dominant optic atrophy impairs mitochondrial morphology, optic nerve structure and visual function. HumMolGenet *16*, 1307-1318.

Davis, S., Aldrich, T.H., Jones, P.F., Acheson, A., Compton, D.L., Jain, V., Ryan, T.E., Bruno, J., Radziejewski, C., Maisonpierre, P.C*., et al.* (1996). Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell *87*, 1161-1169.

De Bock, K., Georgiadou, M., Schoors, S., Kuchnio, A., Wong, B.W., Cantelmo, A.R., Quaegebeur, A., Ghesquiere, B., Cauwenberghs, S., Eelen, G*., et al.* (2013). Role of PFKFB3-driven glycolysis in vessel sprouting. Cell *154*, 651-663.

de Brito, O.M., and Scorrano, L. (2008a). Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature *456*, 605-610.

de Brito, O.M., and Scorrano, L. (2008b). Mitofusin 2: a mitochondria-shaping protein with signaling roles beyond fusion. AntioxidRedoxSignal *10*, 621-633.

de Kroon, A.I., Dolis, D., Mayer, A., Lill, R., and de Kruijff, B. (1997). Phospholipid composition of highly purified mitochondrial outer membranes of rat liver and Neurospora crassa. Is cardiolipin present in the mitochondrial outer membrane? Biochim Biophys Acta *1325*, 108-116.

De Smet, F., Segura, I., De Bock, K., Hohensinner, P.J., and Carmeliet, P. (2009). Mechanisms of vessel branching: filopodia on endothelial tip cells lead the way. Arteriosclerosis, thrombosis, and vascular biology *29*, 639-649.

Debattisti, V., Pendin, D., Ziviani, E., Daga, A., and Scorrano, L. (2014). Reduction of endoplasmic reticulum stress attenuates the defects caused by Drosophila mitofusin depletion. The Journal of cell biology *204*, 303-312.

Dejana, E., Bazzoni, G., and Lampugnani, M.G. (1999). The role of endothelial cell-to-cell junctions in vascular morphogenesis. Thrombosis and haemostasis *82*, 755-761.

Dejana, E., Orsenigo, F., and Lampugnani, M.G. (2008). The role of adherens junctions and VE-cadherin in the control of vascular permeability. Journal of cell science *121*, 2115-2122.

Delettre, C., Lenaers, G., Griffoin, J.M., Gigarel, N., Lorenzo, C., Belenguer, P., Pelloquin, L., Grosgeorge, J., Turc-Carel, C., Perret, E.*, et al.* (2000). Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. NatGenet *26*, 207-210.

Demory, M.L., Boerner, J.L., Davidson, R., Faust, W., Miyake, T., Lee, I., Huttemann, M., Douglas, R., Haddad, G., and Parsons, S.J. (2009). Epidermal growth factor receptor translocation to the mitochondria: regulation and effect. J Biol Chem *284*, 36592-36604.

Desai, S.P., Bhatia, S.N., Toner, M., and Irimia, D. (2013). Mitochondrial localization and the persistent migration of epithelial cancer cells. Biophys J *104*, 2077-2088.

Detmer, S.A., and Chan, D.C. (2007). Functions and dysfunctions of mitochondrial dynamics. Nature reviews Molecular cell biology *8*, 870-879.

Diakos, N.A., Navankasattusas, S., Abel, E.D., Rutter, J., McCreath, L., Ferrin, P., McKellar, S.H., Miller, D.V., Park, S.Y., Richardson, R.S.*, et al.* (2016). Evidence of Glycolysis Up-Regulation and Pyruvate Mitochondrial Oxidation Mismatch During Mechanical Unloading of the Failing Human Heart: Implications for Cardiac Reloading and Conditioning. JACC Basic to translational science *1*, 432-444.

Dickson, M.C., Martin, J.S., Cousins, F.M., Kulkarni, A.B., Karlsson, S., and Akhurst, R.J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development *121*, 1845-1854.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A.M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature *399*, 601-605.

Dimmeler, S., and Zeiher, A.M. (1999). Nitric oxide-an endothelial cell survival factor. Cell Death Differ *6*, 964-968.

Dixit, R., Ross, J.L., Goldman, Y.E., and Holzbaur, E.L. (2008). Differential regulation of dynein and kinesin motor proteins by tau. Science *319*, 1086-1089.

Doddaballapur, A., Michalik, K.M., Manavski, Y., Lucas, T., Houtkooper, R.H., You, X., Chen, W., Zeiher, A.M., Potente, M., Dimmeler, S.*, et al.* (2015). Laminar shear stress inhibits endothelial cell metabolism via KLF2-mediated repression of PFKFB3. Arteriosclerosis, thrombosis, and vascular biology *35*, 137-145.

Doughan, A.K., Harrison, D.G., and Dikalov, S.I. (2008). Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. Circulation research *102*, 488-496.

DuBoff, B., Gotz, J., and Feany, M.B. (2012). Tau promotes neurodegeneration via DRP1 mislocalization in vivo. Neuron *75*, 618-632.

Dudkina, N.V., Heinemeyer, J., Keegstra, W., Boekema, E.J., and Braun, H.P. (2005). Structure of dimeric ATP synthase from mitochondria: an angular association of monomers induces the strong curvature of the inner membrane. FEBSLett *579*, 5769-5772.

Dumont, D.J., Gradwohl, G., Fong, G.H., Puri, M.C., Gertsenstein, M., Auerbach, A., and Breitman, M.L. (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes & development *8*, 1897-1909.

Dumont, D.J., Yamaguchi, T.P., Conlon, R.A., Rossant, J., and Breitman, M.L. (1992). tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. Oncogene *7*, 1471-1480.

Ebneth, A., Godemann, R., Stamer, K., Illenberger, S., Trinczek, B., and Mandelkow, E. (1998). Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implications for Alzheimer's disease. The Journal of cell biology *143*, 777-794.

Ehling, M., Adams, S., Benedito, R., and Adams, R.H. (2013). Notch controls retinal blood vessel maturation and quiescence. Development *140*, 3051-3061.

Ehses, S., Raschke, I., Mancuso, G., Bernacchia, A., Geimer, S., Tondera, D., Martinou, J.C., Westermann, B., Rugarli, E.I., and Langer, T. (2009). Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1. The Journal of cell biology *187*, 1023-1036.

Eichmann, A., and Simons, M. (2012). VEGF signaling inside vascular endothelial cells and beyond. Curr Opin Cell Biol *24*, 188-193.

Elgass, K., Pakay, J., Ryan, M.T., and Palmer, C.S. Recent advances into the understanding of mitochondrial fission. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.

Ewan, L.C., Jopling, H.M., Jia, H., Mittar, S., Bagherzadeh, A., Howell, G.J., Walker, J.H., Zachary, I.C., and Ponnambalam, S. (2006). Intrinsic tyrosine kinase activity is required for vascular endothelial growth factor receptor 2 ubiquitination, sorting and degradation in endothelial cells. Traffic *7*, 1270-1282.

Fannjiang, Y., Cheng, W.C., Lee, S.J., Qi, B., Pevsner, J., McCaffery, J.M., Hill, R.B., Basanez, G., and Hardwick, J.M. (2004). Mitochondrial fission proteins regulate programmed cell death in yeast. Genes Dev *18*, 2785-2797.

Farmer, T., Reinecke, J.B., Xie, S., Bahl, K., Naslavsky, N., and Caplan, S. (2017). Control of mitochondrial homeostasis by endocytic regulatory proteins. Journal of cell science *130*, 2359-2370.

Ferrando, I.M., Chaerkady, R., Zhong, J., Molina, H., Jacob, H.K., Herbst-Robinson, K., Dancy, B.M., Katju, V., Bose, R., Zhang, J.*, et al.* (2012). Identification of targets of c-Src tyrosine kinase by chemical complementation and phosphoproteomics. Mol Cell Proteomics *11*, 355-369.

Ferrara, N. (2010). Pathways mediating VEGF-independent tumor angiogenesis. Cytokine & growth factor reviews *21*, 21-26.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., and Moore, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*, 439-442.

Ferrara, N., Hillan, K.J., Gerber, H.P., and Novotny, W. (2004). Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nature reviews Drug discovery *3*, 391-400.

Ferreira-da-Silva, A., Valacca, C., Rios, E., Populo, H., Soares, P., Sobrinho-Simoes, M., Scorrano, L., Maximo, V., and Campello, S. (2015). Mitochondrial dynamics protein Drp1 is overexpressed in oncocytic thyroid tumors and regulates cancer cell migration. PLoS One *10*, e0122308.

Figueroa-Romero, C., I+¦iguez-Lluh+¡, J.A., Stadler, J., Chang, C.R., Arnoult, D., Keller, P.J., Hong, Y., Blackstone, C., and Feldman, E.L. (2009). SUMOylation of the mitochondrial fission protein Drp1 occurs at multiple nonconsensus sites within the B domain and is linked to its activity cycle. The FASEB Journal *23*, 3917-3927.

Filadi, R., Greotti, E., Turacchio, G., Luini, A., Pozzan, T., and Pizzo, P. (2015). Mitofusin 2 ablation increases endoplasmic reticulum-mitochondria coupling. Proceedings of the National Academy of Sciences of the United States of America *112*, E2174-2181.

Fong, G.H., Rossant, J., Gertsenstein, M., and Breitman, M.L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature *376*, 66-70.

Forstermann, U., and Munzel, T. (2006). Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation *113*, 1708-1714.

Forstermann, U., and Sessa, W.C. (2012). Nitric oxide synthases: regulation and function. European heart journal *33*, 829-837, 837a-837d.

Fraisl, P. (2013). Crosstalk between oxygen- and nitric oxide-dependent signaling pathways in angiogenesis. Experimental cell research *319*, 1331-1339.

Franco, C.A., Liebner, S., and Gerhardt, H. (2009). Vascular morphogenesis: a Wnt for every vessel? Current opinion in genetics & development *19*, 476-483.

Francy, C.A., Alvarez, F.J., Zhou, L., Ramachandran, R., and Mears, J.A. (2015). The mechanoenzymatic core of dynamin-related protein 1 comprises the minimal machinery required for membrane constriction. J Biol Chem *290*, 11692-11703.

Frank, S., Gaume, B., Bergmann-Leitner, E.S., Leitner, W.W., Robert, E.G., Catez, F., Smith, C.L., and Youle, R.J. (2001). The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. DevCell *1*, 515-525.

Frey, T.G., and Mannella, C.A. (2000). The internal structure of mitochondria. Trends BiochemSci *25*, 319-324.

Frey, T.G., Renken, C.W., and Perkins, G.A. (2002). Insight into mitochondrial structure and function from electron tomography. BiochimBiophysActa *1555*, 196-203.

Frezza, C., Cipolat, S., Martins, d.B., Micaroni, M., Beznoussenko, G.V., Rudka, T., Bartoli, D., Polishuck, R.S., Danial, N.N., De Strooper, B.*, et al.* (2006). OPA1 Controls Apoptotic Cristae Remodeling Independently from Mitochondrial Fusion. Cell *126*, 177-189.

Friedman, J.R., Lackner, L.L., West, M., DiBenedetto, J.R., Nunnari, J., and Voeltz, G.K. (2011). ER Tubules Mark Sites of Mitochondrial Division. Science *334*, 358-362.

Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A., and Sessa, W.C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature *399*, 597-601.

Gaengel, K., Genove, G., Armulik, A., and Betsholtz, C. (2009). Endothelial-mural cell signaling in vascular development and angiogenesis. Arteriosclerosis, thrombosis, and vascular biology *29*, 630-638.

Gale, N.W., Thurston, G., Hackett, S.F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M.H., Jackson, D.*, et al.* (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Developmental cell *3*, 411-423.

Gampel, A., Moss, L., Jones, M.C., Brunton, V., Norman, J.C., and Mellor, H. (2006). VEGF regulates the mobilization of VEGFR2/KDR from an intracellular endothelial storage compartment. Blood *108*, 2624-2631.

Gandre-Babbe, S., and van der Bliek, A.M. (2008). The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. MolBiolCell *19*, 2402-2412.

Gawlowski, T., Suarez, J., Scott, B., Torres-Gonzalez, M., Wang, H., Schwappacher, R., Han, X., Yates, J.R., Hoshijima, M., and Dillmann, W. (2012). Modulation of Dynamin-related Protein 1 (DRP1) Function by Increased O-linked-+¦-N-acetylglucosamine Modification (O-GlcNAc) in Cardiac Myocytes. JOURNAL OF BIOLOGICAL CHEMISTRY *287*, 30024-30034.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D.*, et al.* (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. The Journal of cell biology *161*, 1163-1177.

Giacco, F., and Brownlee, M. (2010). Oxidative stress and diabetic complications. Circulation research *107*, 1058-1070.

Giedt, R.J., Yang, C., Zweier, J.L., Matzavinos, A., and Alevriadou, B.R. (2012). Mitochondrial fission in endothelial cells after simulated ischemia/reperfusion: role of nitric oxide and reactive oxygen species. Free Radic Biol Med *52*, 348-356.

Gieffers, C., Korioth, F., Heimann, P., Ungermann, C., and Frey, J. (1997). Mitofilin is a transmembrane protein of the inner mitochondrial membrane expressed as two isoforms. ExpCell Res *232*, 395-399.

Gilkerson, R.W., Selker, J.M., and Capaldi, R.A. (2003). The cristal membrane of mitochondria is the principal site of oxidative phosphorylation. FEBS Lett *546*, 355-358.

Girard, M., Lariviere, R., Parfitt, D.A., Deane, E.C., Gaudet, R., Nossova, N., Blondeau, F., Prenosil, G., Vermeulen, E.G., Duchen, M.R*., et al.* (2012). Mitochondrial dysfunction and Purkinje cell loss in autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS). Proceedings of the National Academy of Sciences of the United States of America *109*, 1661-1666.

Glytsou, C., Calvo, E., Cogliati, S., Mehrotra, A., Anastasia, I., Rigoni, G., Raimondi, A., Shintani, N., Loureiro, M., Vazquez, J*., et al.* (2016). Optic Atrophy 1 Is Epistatic to the Core MICOS Component MIC60 in Mitochondrial Cristae Shape Control. Cell reports *17*, 3024-3034.

Gomes, L.C., Di, B.G., and Scorrano, L. (2011). During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. NatCell Biol *13*, 589-598.

Gomes, L.C., and Scorrano, L. (2008). High levels of Fis1, a pro-fission mitochondrial protein, trigger autophagy. BiochimBiophysActa *1777*, 860-866.

Goodwin, A.M., Kitajewski, J., and D'Amore, P.A. (2007). Wnt1 and Wnt5a affect endothelial proliferation and capillary length; Wnt2 does not. Growth factors *25*, 25-32.

Gourlaouen, M., Welti, J.C., Vasudev, N.S., and Reynolds, A.R. (2013). Essential role for endocytosis in the growth factor-stimulated activation of ERK1/2 in endothelial cells. J Biol Chem *288*, 7467-7480.

Goyal, G., Fell, B., Sarin, A., Youle, R.J., and Sriram, V. (2007). Role of mitochondrial remodeling in programmed cell death in Drosophila melanogaster. DevCell *12*, 807-816.

Graham, D.A., and Rush, J.W. (2004). Exercise training improves aortic endothelium-dependent vasorelaxation and determinants of nitric oxide bioavailability in spontaneously hypertensive rats. Journal of applied physiology *96*, 2088-2096.

Grassart, A., Cheng, A.T., Hong, S.H., Zhang, F., Zenzer, N., Feng, Y., Briner, D.M., Davis, G.D., Malkov, D., and Drubin, D.G. (2014). Actin and dynamin2 dynamics and interplay during clathrin-mediated endocytosis. The Journal of cell biology *205*, 721-735.

Greenberg, J.I., Shields, D.J., Barillas, S.G., Acevedo, L.M., Murphy, E., Huang, J., Scheppke, L., Stockmann, C., Johnson, R.S., Angle, N*., et al.* (2008). A role for VEGF as a negative regulator of pericyte function and vessel maturation. Nature *456*, 809-813.

Gridley, T. (2010). Notch signaling in the vasculature. Current topics in developmental biology *92*, 277-309.

Griparic, L., Kanazawa, T., and van der Bliek, A.M. (2007). Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage. JCell Biol *178*, 757-764.

Griparic, L., van der Wel, N.N., Orozco, I.J., Peters, P.J., and van der Bliek, A.M. (2004). Loss of the intermembrane space protein Mgm1/OPA1 induces swelling and localized constrictions along the lengths of mitochondria. JBiolChem *279*, 18792-18798.

Gruenberg, J., Griffiths, G., and Howell, K.E. (1989). Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro. The Journal of cell biology *108*, 1301-1316.

Gu, C., Yaddanapudi, S., Weins, A., Osborn, T., Reiser, J., Pollak, M., Hartwig, J., and Sever, S. (2010). Direct dynamin-actin interactions regulate the actin cytoskeleton. The EMBO journal *29*, 3593-3606.

Guarani, V., Deflorian, G., Franco, C.A., Kruger, M., Phng, L.K., Bentley, K., Toussaint, L., Dequiedt, F., Mostoslavsky, R., Schmidt, M.H.*, et al.* (2011). Acetylation-dependent regulation of endothelial Notch signalling by the SIRT1 deacetylase. Nature *473*, 234-238.

Guo, X., Macleod, G.T., Wellington, A., Hu, F., Panchumarthi, S., Schoenfield, M., Marin, L., Charlton, M.P., Atwood, H.L., and Zinsmaier, K.E. (2005). The GTPase dMiro is required for axonal transport of mitochondria to Drosophila synapses. Neuron *47*, 379-393.

Hackenbrock, C.R., Chazotte, B., and Gupte, S.S. (1986). The random collision model and a critical assessment of diffusion and collision in mitochondrial electron transport. J Bioenerg Biomembr *18*, 331-368.

Hackett, S.F., Wiegand, S., Yancopoulos, G., and Campochiaro, P.A. (2002). Angiopoietin-2 plays an important role in retinal angiogenesis. Journal of cellular physiology *192*, 182-187.

Hales, K.G., and Fuller, M.T. (1997). Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. Cell *90*, 121-129.

Hamasaki, M., Furuta, N., Matsuda, A., Nezu, A., Yamamoto, A., Fujita, N., Oomori, H., Noda, T., Haraguchi, T., Hiraoka, Y.*, et al.* (2013). Autophagosomes form at ER-mitochondria contact sites. Nature *495*, 389-393.

Han, X.J., Lu, Y.F., Li, S.A., Kaitsuka, T., Sato, Y., Tomizawa, K., Nairn, A.C., Takei, K., Matsui, H., and Matsushita, M. (2008). CaM kinase I alpha-induced phosphorylation of Drp1 regulates mitochondrial morphology. The Journal of cell biology *182*, 573-585.

Harfouche, R., Malak, N.A., Brandes, R.P., Karsan, A., Irani, K., and Hussain, S.N. (2005). Roles of reactive oxygen species in angiopoietin-1/tie-2 receptor signaling. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *19*, 1728-1730.

Hatch, A.L., Ji, W.K., Merrill, R.A., Strack, S., and Higgs, H.N. (2016). Actin filaments as dynamic reservoirs for Drp1 recruitment. Molecular biology of the cell *27*, 3109-3121.

Haun, F., Nakamura, T., Shiu, A.D., Cho, D.H., Tsunemi, T., Holland, E.A., La Spada, A.R., and Lipton, S.A. (2013). S-nitrosylation of dynamin-related protein 1 mediates mutant huntingtin-induced mitochondrial fragmentation and neuronal injury in Huntington's disease. Antioxidants & redox signaling *19*, 1173-1184.

He, M., Siow, R.C., Sugden, D., Gao, L., Cheng, X., and Mann, G.E. (2011). Induction of HO-1 and redox signaling in endothelial cells by advanced glycation end products: a role for Nrf2 in vascular protection in diabetes. Nutrition, metabolism, and cardiovascular diseases : NMCD *21*, 277-285.

He, Z., and Tessier-Lavigne, M. (1997). Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell *90*, 739-751.

Heitzer, T., Krohn, K., Albers, S., and Meinertz, T. (2000). Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with Type II diabetes mellitus. Diabetologia *43*, 1435-1438.

Hellstrom, M., Phng, L.K., Hofmann, J.J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A.K., Karlsson, L., Gaiano, N.*, et al.* (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature *445*, 776-780.

Hendrix, M.J., Seftor, E.A., Hess, A.R., and Seftor, R.E. (2003). Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. Nature reviews Cancer *3*, 411-421.

Herbert, S.P., and Stainier, D.Y. (2011). Molecular control of endothelial cell behaviour during blood vessel morphogenesis. Nature reviews Molecular cell biology *12*, 551-564.

Herkenne, S., Paques, C., Nivelles, O., Lion, M., Bajou, K., Pollenus, T., Fontaine, M., Carmeliet, P., Martial, J.A., Nguyen, N.Q.*, et al.* (2015). The interaction of uPAR with VEGFR2 promotes VEGF-induced angiogenesis. Science signaling *8*, ra117.

Hermann, G.J., Thatcher, J.W., Mills, J.P., Hales, K.G., Fuller, M.T., Nunnari, J., and Shaw, J.M. (1998). Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. JCell Biol *143*, 359-373.

Herren, B., Levkau, B., Raines, E.W., and Ross, R. (1998). Cleavage of beta-catenin and plakoglobin and shedding of VE-cadherin during endothelial apoptosis: evidence for a role for caspases and metalloproteinases. Molecular biology of the cell *9*, 1589-1601.

Holmes, D.I., and Zachary, I.C. (2008). Vascular endothelial growth factor regulates stanniocalcin-1 expression via neuropilin-1-dependent regulation of KDR and synergism with fibroblast growth factor-2. Cell Signal *20*, 569-579.

Holthuis, J.C., and Ungermann, C. (2013). Cellular microcompartments constitute general suborganellar functional units in cells. Biological chemistry *394*, 151-161.

Hong, C.C., Peterson, Q.P., Hong, J.Y., and Peterson, R.T. (2006). Artery/vein specification is governed by opposing phosphatidylinositol-3 kinase and MAP kinase/ERK signaling. Curr Biol *16*, 1366-1372.

Hoppins, S., Collins, S.R., Cassidy-Stone, A., Hummel, E., DeVay, R.M., Lackner, L.L., Westermann, B., Schuldiner, M., Weissman, J.S., and Nunnari, J. (2011a). A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. The Journal of cell biology *195*, 323-340.

Hoppins, S., Edlich, F., Cleland, M.M., Banerjee, S., McCaffery, J.M., Youle, R.J., and Nunnari, J. (2011b). The soluble form of Bax regulates mitochondrial fusion via MFN2 homotypic complexes. Molecular cell *41*, 150-160.

Houten, S.M., and Wanders, R.J. (2010). A general introduction to the biochemistry of mitochondrial fatty acid beta-oxidation. Journal of inherited metabolic disease *33*, 469-477.

Huang, H., Vandekeere, S., Kalucka, J., Bierhansl, L., Zecchin, A., Bruning, U., Visnagri, A., Yuldasheva, N., Goveia, J., Cruys, B.*, et al.* (2017). Role of glutamine and interlinked asparagine metabolism in vessel formation. The EMBO journal *36*, 2334-2352.

Hynes, R.O. (2002). Integrins: bidirectional, allosteric signaling machines. Cell *110*, 673-687.

Ikeda, S., Ushio-Fukai, M., Zuo, L., Tojo, T., Dikalov, S., Patrushev, N.A., and Alexander, R.W. (2005). Novel role of ARF6 in vascular endothelial growth factor-induced signaling and angiogenesis. Circulation research *96*, 467-475.

Ikeda, Y., Shirakabe, A., Maejima, Y., Zhai, P., Sciarretta, S., Toli, J., Nomura, M., Mihara, K., Egashira, K., Ohishi, M.*, et al.* (2015). Endogenous Drp1 mediates mitochondrial autophagy and protects the heart against energy stress. Circulation research *116*, 264-278.

Iruela-Arispe, M.L., and Davis, G.E. (2009). Cellular and molecular mechanisms of vascular lumen formation. Developmental cell *16*, 222-231.

Ishihara, N., Eura, Y., and Mihara, K. (2004). Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. Journal of cell science *117*, 6535-6546.

Ishihara, N., Nomura, M., Jofuku, A., Kato, H., Suzuki, S.O., Masuda, K., Otera, H., Nakanishi, Y., Nonaka, I., Goto, Y.*, et al.* (2009). Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nat Cell Biol *11*, 958-966.

Ishikawa, T., Tamai, Y., Zorn, A.M., Yoshida, H., Seldin, M.F., Nishikawa, S., and Taketo, M.M. (2001). Mouse Wnt receptor gene Fzd5 is essential for yolk sac and placental angiogenesis. Development *128*, 25-33.

Ittner, L.M., and Gotz, J. (2011). Amyloid-beta and tau--a toxic pas de deux in Alzheimer's disease. Nat Rev Neurosci *12*, 65-72.

Iwama, A., Hamaguchi, I., Hashiyama, M., Murayama, Y., Yasunaga, K., and Suda, T. (1993). Molecular cloning and characterization of mouse TIE and TEK receptor tyrosine kinase genes and their expression in hematopoietic stem cells. Biochemical and biophysical research communications *195*, 301-309.

Iwasawa, R., Mahul-Mellier, A.L., Datler, C., Pazarentzos, E., and Grimm, S. (2011). Fis1 and Bap31 bridge the mitochondria-ER interface to establish a platform for apoptosis induction. EMBO J *30*, 556-568.

Jagasia, R., Grote, P., Westermann, B., and Conradt, B. (2005). DRP-1-mediated mitochondrial fragmentation during EGL-1-induced cell death in C. elegans. Nature *433*, 754-760.

Jakobsson, L., Franco, C.A., Bentley, K., Collins, R.T., Ponsioen, B., Aspalter, I.M., Rosewell, I., Busse, M., Thurston, G., Medvinsky, A.*, et al.* (2010). Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. Nature cell biology *12*, 943-953.

James, D.I., Parone, P.A., Mattenberger, Y., and Martinou, J.C. (2003). hFis1, a novel component of the mammalian mitochondrial fission machinery. JBiolChem *278*, 36373-36379.

Janer, A., Prudent, J., Paupe, V., Fahiminiya, S., Majewski, J., Sgarioto, N., Des Rosiers, C., Forest, A., Lin, Z.Y., Gingras, A.C.*, et al.* (2016). SLC25A46 is required for mitochondrial lipid homeostasis and cristae maintenance and is responsible for Leigh syndrome. EMBO Mol Med *8*, 1019-1038.

Ji, W.K., Hatch, A.L., Merrill, R.A., Strack, S., and Higgs, H.N. (2015). Actin filaments target the oligomeric maturation of the dynamin GTPase Drp1 to mitochondrial fission sites. eLife *4*, e11553.

Jin, F., Hagemann, N., Brockmeier, U., Schafer, S.T., Zechariah, A., and Hermann, D.M. (2013). LDL attenuates VEGF-induced angiogenesis via mechanisms involving VEGFR2 internalization and degradation following endosome-trans-Golgi network trafficking. Angiogenesis *16*, 625-637.

Jo, Y., Cho, H.M., Sun, W., and Ryu, J.R. (2017). Localization of dynamin-related protein 1 and its potential role in lamellipodia formation. Histochemistry and cell biology *148*, 13-20.

John, G.B., Shang, Y., Li, L., Renken, C., Mannella, C.A., Selker, J.M., Rangell, L., Bennett, M.J., and Zha, J. (2005). The mitochondrial inner membrane protein mitofilin controls cristae morphology. MolBiolCell *16*, 1543-1554.

Jopling, H.M., Odell, A.F., Hooper, N.M., Zachary, I.C., Walker, J.H., and Ponnambalam, S. (2009). Rab GTPase regulation of VEGFR2 trafficking and signaling in endothelial cells. Arteriosclerosis, thrombosis, and vascular biology *29*, 1119-1124.

Kageyama, Y., Zhang, Z., Roda, R., Fukaya, M., Wakabayashi, J., Wakabayashi, N., Kensler, T.W., Reddy, P.H., Iijima, M., and Sesaki, H. (2012). Mitochondrial division ensures the survival of postmitotic neurons by suppressing oxidative damage. JCell Biol *197*, 535-551.

Kamei, M., Saunders, W.B., Bayless, K.J., Dye, L., Davis, G.E., and Weinstein, B.M. (2006). Endothelial tubes assemble from intracellular vacuoles in vivo. Nature *442*, 453-456.

Karbowski, M., Jeong, S.Y., and Youle, R.J. (2004). Endophilin B1 is required for the maintenance of mitochondrial morphology. JCell Biol *166*, 1027-1039.

Karbowski, M., Lee, Y.J., Gaume, B., Jeong, S.Y., Frank, S., Nechushtan, A., Santel, A., Fuller, M., Smith, C.L., and Youle, R.J. (2002). Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. JCell Biol *159*, 931-938.

Karbowski, M., Neutzner, A., and Youle, R.J. (2007). The mitochondrial E3 ubiquitin ligase MARCH5 is required for Drp1 dependent mitochondrial division. JCell Biol *178*, 71-84.

Karbowski, M., Norris, K.L., Cleland, M.M., Jeong, S.Y., and Youle, R.J. (2006). Role of Bax and Bak in mitochondrial morphogenesis. Nature *443*, 658-662.

Karkkainen, M.J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T.V., Jeltsch, M., Jackson, D.G., Talikka, M., Rauvala, H.*, et al.* (2004). Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nature immunology *5*, 74-80.

Kasahara, A., Cipolat, S., Chen, Y., Dorn, G.W., and Scorrano, L. (2013). Mitochondrial fusion directs cardiomyocyte differentiation via calcineurin and Notch signaling. Science *342*, 734-737.

Kasamatsu, H., and Vinograd, J. (1974). Replication of circular DNA in eukaryotic cells. Annu Rev Biochem *43*, 695-719.

Kasashima, K., Ohta, E., Kagawa, Y., and Endo, H. (2006). Mitochondrial functions and estrogen receptor-dependent nuclear translocation of pleiotropic human prohibitin 2. J Biol Chem *281*, 36401-36410.

Kashatus, D.F., Lim, K.H., Brady, D.C., Pershing, N.L., Cox, A.D., and Counter, C.M. (2011). RALA and RALBP1 regulate mitochondrial fission at mitosis. Nature cell biology *13*, 1108-1115.

Kashatus, J.A., Nascimento, A., Myers, L.J., Sher, A., Byrne, F.L., Hoehn, K.L., Counter, C.M., and Kashatus, D.F. (2015). Erk2 phosphorylation of Drp1 promotes mitochondrial fission and MAPK-driven tumor growth. Molecular cell *57*, 537-551.

Kerr, B.A., West, X.Z., Kim, Y.W., Zhao, Y., Tischenko, M., Cull, R.M., Phares, T.W., Peng, X.D., Bernier-Latmani, J., Petrova, T.V.*, et al.* (2016). Stability and function of adult vasculature is sustained by Akt/Jagged1 signalling axis in endothelium. Nature communications *7*, 10960.

Kietadisorn, R., Juni, R.P., and Moens, A.L. (2012). Tackling endothelial dysfunction by modulating NOS uncoupling: new insights into its pathogenesis and therapeutic possibilities. Am J Physiol Endocrinol Metab *302*, E481-495.

Kisanuki, Y.Y., Hammer, R.E., Miyazaki, J., Williams, S.C., Richardson, J.A., and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev Biol *230*, 230-242.

Kobayashi, T., Oishi, K., Hayashi, Y., Matsumoto, T., and Kamata, K. (2006). Changes in aortic endothelial gene expressions and relaxation responses following chronic short-term insulin treatment in diabetic rats. Atherosclerosis *185*, 47-57.

Koch, S., and Claesson-Welsh, L. (2012). Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harbor perspectives in medicine *2*, a006502.

Koch, S., van Meeteren, L.A., Morin, E., Testini, C., Westrom, S., Bjorkelund, H., Le Jan, S., Adler, J., Berger, P., and Claesson-Welsh, L. (2014). NRP1 presented in trans to the endothelium arrests VEGFR2 endocytosis, preventing angiogenic signaling and tumor initiation. Developmental cell *28*, 633-646.

Korn, C., and Augustin, H.G. (2015). Mechanisms of Vessel Pruning and Regression. Developmental cell *34*, 5-17.

Korobova, F., Gauvin, T.J., and Higgs, H.N. (2014). A role for myosin II in mammalian mitochondrial fission. Curr Biol *24*, 409-414.

Korobova, F., Ramabhadran, V., and Higgs, H.N. (2013). An actin-dependent step in mitochondrial fission mediated by the ER-associated formin INF2. Science *339*, 464-467.

Koshiba, T., Detmer, S.A., Kaiser, J.T., Chen, H., McCaffery, J.M., and Chan, D.C. (2004). Structural basis of mitochondrial tethering by mitofusin complexes. Science *305*, 858-862.

Labrecque, L., Royal, I., Surprenant, D.S., Patterson, C., Gingras, D., and Beliveau, R. (2003). Regulation of vascular endothelial growth factor receptor-2 activity by caveolin-1 and plasma membrane cholesterol. Molecular biology of the cell *14*, 334-347.

Labrousse, A.M., Zappaterra, M.D., Rube, D.A., and van der Bliek, A.M. (1999). C. elegans dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane. MolCell *4*, 815-826.

Lampugnani, M.G., Dejana, E., and Giampietro, C. (2017). Vascular Endothelial (VE)-Cadherin, Endothelial Adherens Junctions, and Vascular Disease. Cold Spring Harbor perspectives in biology.

Lampugnani, M.G., Orsenigo, F., Gagliani, M.C., Tacchetti, C., and Dejana, E. (2006). Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. The Journal of cell biology *174*, 593-604.

Lampugnani, M.G., Orsenigo, F., Rudini, N., Maddaluno, L., Boulday, G., Chapon, F., and Dejana, E. (2010). CCM1 regulates vascular-lumen organization by inducing endothelial polarity. Journal of cell science *123*, 1073-1080.

Lanahan, A., Zhang, X., Fantin, A., Zhuang, Z., Rivera-Molina, F., Speichinger, K., Prahst, C., Zhang, J., Wang, Y., Davis, G*., et al.* (2013). The neuropilin 1 cytoplasmic domain is required for VEGF-A-dependent arteriogenesis. Developmental cell *25*, 156-168.

Lanahan, A.A., Hermans, K., Claes, F., Kerley-Hamilton, J.S., Zhuang, Z.W., Giordano, F.J., Carmeliet, P., and Simons, M. (2010). VEGF receptor 2 endocytic trafficking regulates arterial morphogenesis. Developmental cell *18*, 713-724.

Landmesser, U., Dikalov, S., Price, S.R., McCann, L., Fukai, T., Holland, S.M., Mitch, W.E., and Harrison, D.G. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. The Journal of clinical investigation *111*, 1201-1209.

Lawson, V.H., Graham, B.V., and Flanigan, K.M. (2005). Clinical and electrophysiologic features of CMT2A with mutations in the mitofusin 2 gene. Neurology *65*, 197-204.

Lee, J.E., Westrate, L.M., Wu, H., Page, C., and Voeltz, G.K. (2016). Multiple dynamin family members collaborate to drive mitochondrial division. Nature *540*, 139-143.

Lee, M.Y., Luciano, A.K., Ackah, E., Rodriguez-Vita, J., Bancroft, T.A., Eichmann, A., Simons, M., Kyriakides, T.R., Morales-Ruiz, M., and Sessa, W.C. (2014a). Endothelial Akt1 mediates angiogenesis by phosphorylating multiple angiogenic substrates. Proceedings of the National Academy of Sciences of the United States of America *111*, 12865-12870.

Lee, M.Y., Skoura, A., Park, E.J., Landskroner-Eiger, S., Jozsef, L., Luciano, A.K., Murata, T., Pasula, S., Dong, Y., Bouaouina, M.*, et al.* (2014b). Dynamin 2 regulation of integrin endocytosis, but not VEGF signaling, is crucial for developmental angiogenesis. Development *141*, 1465-1472.

Lee, P.C., Salyapongse, A.N., Bragdon, G.A., Shears, L.L., 2nd, Watkins, S.C., Edington, H.D., and Billiar, T.R. (1999). Impaired wound healing and angiogenesis in eNOS-deficient mice. The American journal of physiology *277*, H1600-1608.

Lee, S., Chen, T.T., Barber, C.L., Jordan, M.C., Murdock, J., Desai, S., Ferrara, N., Nagy, A., Roos, K.P., and Iruela-Arispe, M.L. (2007). Autocrine VEGF signaling is required for vascular homeostasis. Cell *130*, 691-703.

Legros, F., Malka, F., Frachon, P., Lombes, A., and Rojo, M. (2004). Organization and dynamics of human mitochondrial DNA. JCellSci *117*, 2653-2662.

Lemmon, M.A., and Schlessinger, J. (2010). Cell signaling by receptor tyrosine kinases. Cell *141*, 1117-1134.

Leone, T.C., Lehman, J.J., Finck, B.N., Schaeffer, P.J., Wende, A.R., Boudina, S., Courtois, M., Wozniak, D.F., Sambandam, N., Bernal-Mizrachi, C.*, et al.* (2005). PGC-1alpha deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. PLoS Biol *3*, e101.

Leopold, J.A., Dam, A., Maron, B.A., Scribner, A.W., Liao, R., Handy, D.E., Stanton, R.C., Pitt, B., and Loscalzo, J. (2007). Aldosterone impairs vascular reactivity by decreasing glucose-6-phosphate dehydrogenase activity. Nature medicine *13*, 189-197.

Leopold, J.A., Walker, J., Scribner, A.W., Voetsch, B., Zhang, Y.Y., Loscalzo, A.J., Stanton, R.C., and Loscalzo, J. (2003). Glucose-6-phosphate dehydrogenase modulates vascular endothelial growth factor-mediated angiogenesis. J Biol Chem *278*, 32100-32106.

Li, C., Ruan, L., Sood, S.G., Papapetropoulos, A., Fulton, D., and Venema, R.C. (2007). Role of eNOS phosphorylation at Ser-116 in regulation of eNOS activity in endothelial cells. Vascular pharmacology *47*, 257-264.

Li, H., Alavian, K.N., Lazrove, E., Mehta, N., Jones, A., Zhang, P., Licznerski, P., Graham, M., Uo, T., Guo, J.*, et al.* (2013). A Bcl-xL-Drp1 complex regulates synaptic vesicle membrane dynamics during endocytosis. Nature cell biology *15*, 773-785.

Li, X., and Gould, S.J. (2003). The dynamin-like GTPase DLP1 is essential for peroxisome division and is recruited to peroxisomes in part by PEX11. JBiolChem *278*, 17012-17020.

Li, X., Padhan, N., Sjostrom, E.O., Roche, F.P., Testini, C., Honkura, N., Sainz-Jaspeado, M., Gordon, E., Bentley, K., Philippides, A.*, et al.* (2016). VEGFR2 pY949 signalling regulates adherens junction integrity and metastatic spread. Nature communications *7*, 11017.

Li, Y., Song, Y.H., Li, F., Yang, T., Lu, Y.W., and Geng, Y.J. (2009). MicroRNA-221 regulates high glucose-induced endothelial dysfunction. Biochemical and biophysical research communications *381*, 81-83.

Li, Z., Okamoto, K., Hayashi, Y., and Sheng, M. (2004). The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. Cell *119*, 873-887.

Lin, J.R., Shen, W.L., Yan, C., and Gao, P.J. (2015). Downregulation of dynamin-related protein 1 contributes to impaired autophagic flux and angiogenic function in senescent endothelial cells. Arteriosclerosis, thrombosis, and vascular biology *35*, 1413-1422.

Liu, Y., Li, H., Bubolz, A.H., Zhang, D.X., and Gutterman, D.D. (2008). Endothelial cytoskeletal elements are critical for flow-mediated dilation in human coronary arterioles. Medical & biological engineering & computing *46*, 469-478.

Liu, Y., and Senger, D.R. (2004). Matrix-specific activation of Src and Rho initiates capillary morphogenesis of endothelial cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *18*, 457-468.

Loges, S., Mazzone, M., Hohensinner, P., and Carmeliet, P. (2009). Silencing or fueling metastasis with VEGF inhibitors: antiangiogenesis revisited. Cancer Cell *15*, 167-170.

Lu, C., Bonome, T., Li, Y., Kamat, A.A., Han, L.Y., Schmandt, R., Coleman, R.L., Gershenson, D.M., Jaffe, R.B., Birrer, M.J*., et al.* (2007). Gene alterations identified by expression profiling in tumor-associated endothelial cells from invasive ovarian carcinoma. Cancer Res *67*, 1757-1768.

Lucke, S., and Levkau, B. (2010). Endothelial functions of sphingosine-1-phosphate. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology *26*, 87-96.

Lugus, J.J., Ngoh, G.A., Bachschmid, M.M., and Walsh, K. (2011). Mitofusins are required for angiogenic function and modulate different signaling pathways in cultured endothelial cells. Journal of molecular and cellular cardiology *51*, 885-893.

MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. Developmental cell *17*, 9-26.

Macdonald, P.J., Stepanyants, N., Mehrotra, N., Mears, J.A., Qi, X., Sesaki, H., and Ramachandran, R. (2014). A dimeric equilibrium intermediate nucleates Drp1 reassembly on mitochondrial membranes for fission. Molecular biology of the cell *25*, 1905-1915.

Maharaj, A.S., Saint-Geniez, M., Maldonado, A.E., and D'Amore, P.A. (2006). Vascular endothelial growth factor localization in the adult. The American journal of pathology *168*, 639-648.

Mai, S., Klinkenberg, M., Auburger, G., Bereiter-Hahn, J., and Jendrach, M. (2010). Decreased expression of Drp1 and Fis1 mediates mitochondrial elongation in senescent cells and enhances resistance to oxidative stress through PINK1. Journal of cell science *123*, 917-926.

Maisonpierre, P.C., Goldfarb, M., Yancopoulos, G.D., and Gao, G. (1993). Distinct rat genes with related profiles of expression define a TIE receptor tyrosine kinase family. Oncogene *8*, 1631-1637.

Maisonpierre, P.C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S.J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T.H., Papadopoulos, N.*, et al.* (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science *277*, 55-60.

Makino, A., Scott, B.T., and Dillmann, W.H. (2010). Mitochondrial fragmentation and superoxide anion production in coronary endothelial cells from a mouse model of type 1 diabetes. Diabetologia *53*, 1783-1794.

Malka, F., Guillery, O., Cifuentes-Diaz, C., Guillou, E., Belenguer, P., Lombes, A., and Rojo, M. (2005). Separate fusion of outer and inner mitochondrial membranes. EMBO Rep *6*, 853-859.

Manczak, M., Sesaki, H., Kageyama, Y., and Reddy, P.H. (2012). Dynamin-related protein 1 heterozygote knockout mice do not have synaptic and mitochondrial deficiencies. Biochim Biophys Acta *1822*, 862-874.

Manor, U., Bartholomew, S., Golani, G., Christenson, E., Kozlov, M., Higgs, H., Spudich, J., and Lippincott-Schwartz, J. (2015). A mitochondria-anchored isoform of the actin-nucleating spire protein regulates mitochondrial division. eLife *4*.

Mapanga, R.F., and Essop, M.F. (2016). Damaging effects of hyperglycemia on cardiovascular function: spotlight on glucose metabolic pathways. American journal of physiology Heart and circulatory physiology *310*, H153-173.

Marsboom, G., Toth, P.T., Ryan, J.J., Hong, Z., Wu, X., Fang, Y.H., Thenappan, T., Piao, L., Zhang, H.J., Pogoriler, J.*, et al.* (2012). Dynamin-related protein 1-mediated mitochondrial mitotic fission permits hyperproliferation of vascular smooth muscle cells and offers a novel therapeutic target in pulmonary hypertension. Circulation research *110*, 1484-1497.

Masckauchan, T.N., Agalliu, D., Vorontchikhina, M., Ahn, A., Parmalee, N.L., Li, C.M., Khoo, A., Tycko, B., Brown, A.M., and Kitajewski, J. (2006). Wnt5a signaling induces proliferation and survival of endothelial cells in vitro and expression of MMP-1 and Tie-2. Molecular biology of the cell *17*, 5163-5172.

Matsumoto, T., Bohman, S., Dixelius, J., Berge, T., Dimberg, A., Magnusson, P., Wang, L., Wikner, C., Qi, J.H., Wernstedt, C.*, et al.* (2005). VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. The EMBO journal *24*, 2342-2353.

McCarthy, M.J., Crowther, M., Bell, P.R., and Brindle, N.P. (1998). The endothelial receptor tyrosine kinase tie-1 is upregulated by hypoxia and vascular endothelial growth factor. FEBS letters *423*, 334-338.

Mears, J.A., Lackner, L.L., Fang, S., Ingerman, E., Nunnari, J., and Hinshaw, J.E. (2011). Conformational changes in Dnm1 support a contractile mechanism for mitochondrial fission. NatStructMolBiol *18*, 20-26.

Meeusen, S., DeVay, R., Block, J., Cassidy-Stone, A., Wayson, S., McCaffery, J.M., and Nunnari, J. (2006). Mitochondrial inner-membrane fusion and crista maintenance requires the dynamin-related GTPase Mgm1. Cell *127*, 383-395.

Merkwirth, C., Dargazanli, S., Tatsuta, T., Geimer, S., Lower, B., Wunderlich, F.T., von Kleist-Retzow, J.C., Waisman, A., Westermann, B., and Langer, T. (2008). Prohibitins control cell proliferation and apoptosis by regulating OPA1-dependent cristae morphogenesis in mitochondria. Genes Dev *22*, 476-488.

Meyer, R.D., Dayanir, V., Majnoun, F., and Rahimi, N. (2002). The presence of a single tyrosine residue at the carboxyl domain of vascular endothelial growth factor receptor-2/FLK-1 regulates its autophosphorylation and activation of signaling molecules. J Biol Chem *277*, 27081-27087.

Minauro-Sanmiguel, F., Wilkens, S., and Garcia, J.J. (2005). Structure of dimeric mitochondrial ATP synthase: novel F0 bridging features and the structural basis of mitochondrial cristae biogenesis. ProcNatlAcadSciUSA *102*, 12356-12358.

Misaka, T., Miyashita, T., and Kubo, Y. (2002). Primary structure of a dynamin-related mouse mitochondrial GTPase and its distribution in brain, subcellular localization, and effect on mitochondrial morphology. JBiolChem *277*, 15834-15842.

Mitra, K., Rikhy, R., Lilly, M., and Lippincott-Schwartz, J. (2012). DRP1-dependent mitochondrial fission initiates follicle cell differentiation during Drosophila oogenesis. JCell Biol *197*, 487-497.

Moncada, S., and Higgs, A. (1993). The L-arginine-nitric oxide pathway. N Engl J Med *329*, 2002-2012.

Montessuit, S., Somasekharan, S.P., Terrones, O., Lucken-Ardjomande, S., Herzig, S., Schwarzenbacher, R., Manstein, D.J., Bossy-Wetzel, E., Basanez, G., Meda, P.*, et al.* (2010). Membrane remodeling induced by the dynamin-related protein Drp1 stimulates Bax oligomerization. Cell *142*, 889-901.

Montoya, J., Ojala, D., and Attardi, G. (1981). Distinctive features of the 5'-terminal sequences of the human mitochondrial mRNAs. Nature *290*, 465-470.

Monvoisin, A., Alva, J.A., Hofmann, J.J., Zovein, A.C., Lane, T.F., and Iruela-Arispe, M.L. (2006). VE-cadherin-CreERT2 transgenic mouse: a model for inducible recombination in the endothelium. Developmental dynamics : an official publication of the American Association of Anatomists *235*, 3413-3422.

Munoz, J.P., Ivanova, S., Sanchez-Wandelmer, J., Martinez-Cristobal, P., Noguera, E., Sancho, A., Diaz-Ramos, A., Hernandez-Alvarez, M.I., Sebastian, D., Mauvezin, C.*, et al.* (2013). Mfn2 modulates the UPR and mitochondrial function via repression of PERK. The EMBO journal *32*, 2348-2361.

Murley, A., Lackner, L.L., Osman, C., West, M., Voeltz, G.K., Walter, P., and Nunnari, J. (2013). ER-associated mitochondrial division links the distribution of mitochondria and mitochondrial DNA in yeast. Elife *2*, e00422.

Muster, B., Kohl, W., Wittig, I., Strecker, V., Joos, F., Haase, W., Bereiter-Hahn, J., and Busch, K. (2010). Respiratory chain complexes in dynamic mitochondria display a patchy distribution in life cells. PloS one *5*, e11910.

Nakamura, N., Kimura, Y., Tokuda, M., Honda, S., and Hirose, S. (2006). MARCH-V is a novel mitofusin 2- and Drp1-binding protein able to change mitochondrial morphology. EMBORep *7*, 1019-1022.

Nakayama, M., Nakayama, A., van Lessen, M., Yamamoto, H., Hoffmann, S., Drexler, H.C., Itoh, N., Hirose, T., Breier, G., Vestweber, D*., et al.* (2013). Spatial regulation of VEGF receptor endocytosis in angiogenesis. Nature cell biology *15*, 249-260.

Naon, D., Zaninello, M., Giacomello, M., Varanita, T., Grespi, F., Lakshminaranayan, S., Serafini, A., Semenzato, M., Herkenne, S., Hernandez-Alvarez, M.I*., et al.* (2016). Critical reappraisal confirms that Mitofusin 2 is an endoplasmic reticulum-mitochondria tether. Proceedings of the National Academy of Sciences of the United States of America *113*, 11249-11254.

Ngoh, G.A., Facundo, H.T., Zafir, A., and Jones, S.P. (2010). O-GlcNAc signaling in the cardiovascular system. Circulation research *107*, 171-185.

Ngoh, G.A., Papanicolaou, K.N., and Walsh, K. (2012). Loss of mitofusin 2 promotes endoplasmic reticulum stress. JBiolChem *287*, 20321-20332.

Nielsen, E., Christoforidis, S., Uttenweiler-Joseph, S., Miaczynska, M., Dewitte, F., Wilm, M., Hoflack, B., and Zerial, M. (2000). Rabenosyn-5, a novel Rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE finger domain. The Journal of cell biology *151*, 601-612.

Niemann, A., Ruegg, M., La, P., V, Schenone, A., and Suter, U. (2005). Ganglioside-induced differentiation associated protein 1 is a regulator of the mitochondrial network: new implications for Charcot-Marie-Tooth disease. JCell Biol *170*, 1067-1078.

Niu, J., Yu, M., Wang, C., and Xu, Z. (2012). Leucine-rich repeat kinase 2 disturbs mitochondrial dynamics via Dynamin-like protein. J Neurochem *122*, 650-658.

Odgren, P.R., Toukatly, G., Bangs, P.L., Gilmore, R., and Fey, E.G. (1996). Molecular characterization of mitofilin (HMP), a mitochondria-associated protein with predicted coiled coil and intermembrane space targeting domains. JCell Sci *109 (Pt 9)*, 2253-2264.

Oettinghaus, B., D'Alonzo, D., Barbieri, E., Restelli, L.M., Savoia, C., Licci, M., Tolnay, M., Frank, S., and Scorrano, L. (2016). DRP1-dependent apoptotic mitochondrial fission occurs independently of BAX, BAK and APAF1 to amplify cell death by BID and oxidative stress. Biochim Biophys Acta *1857*, 1267-1276.

Ojala, D., Montoya, J., and Attardi, G. (1981). tRNA punctuation model of RNA processing in human mitochondria. Nature *290*, 470-474.

Okabe, K., Kobayashi, S., Yamada, T., Kurihara, T., Tai-Nagara, I., Miyamoto, T., Mukouyama, Y.S., Sato, T.N., Suda, T., Ema, M*., et al.* (2014). Neurons limit angiogenesis by titrating VEGF in retina. Cell *159*, 584-596.

Olichon, A., Baricault, L., Gas, N., Guillou, E., Valette, A., Belenguer, P., and Lenaers, G. (2003). Loss of OPA1 perturbates the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis. JBiolChem *278*, 7743-7746.

Ortiz-Sandoval, C.G., Hughes, S.C., Dacks, J.B., and Simmen, T. (2014). Interaction with the effector dynamin-related protein 1 (Drp1) is an ancient function of Rab32 subfamily proteins. Cellular logistics *4*, e986399.

Otera, H., Miyata, N., Kuge, O., and Mihara, K. (2016). Drp1-dependent mitochondrial fission via MiD49/51 is essential for apoptotic cristae remodeling. The Journal of cell biology *212*, 531-544.

Otera, H., Wang, C., Cleland, M.M., Setoguchi, K., Yokota, S., Youle, R.J., and Mihara, K. (2010). Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J Cell Biol *191*, 1141-1158.

Pagliuso, A., Tham, T.N., Stevens, J.K., Lagache, T., Persson, R., Salles, A., Olivo-Marin, J.C., Oddos, S., Spang, A., Cossart, P*., et al.* (2016). A role for septin 2 in Drp1-mediated mitochondrial fission. EMBO Rep *17*, 858-873.

Palmer, C.S., Osellame, L.D., Laine, D., Koutsopoulos, O.S., Frazier, A.E., and Ryan, M.T. (2011). MiD49 and MiD51, new components of the mitochondrial fission machinery. EMBO Rep *12*, 565-573.

Palmer, R.M., Ashton, D.S., and Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature *333*, 664-666.

Paltauf-Doburzynska, J., Malli, R., and Graier, W.F. (2004). Hyperglycemic conditions affect shape and Ca2+ homeostasis of mitochondria in endothelial cells. Journal of cardiovascular pharmacology *44*, 423-436.

Paneni, F., and Cosentino, F. (2012). p66 Shc as the engine of vascular aging. Current vascular pharmacology *10*, 697-699.

Paneni, F., Mocharla, P., Akhmedov, A., Costantino, S., Osto, E., Volpe, M., Luscher, T.F., and Cosentino, F. (2012). Gene silencing of the mitochondrial adaptor p66(Shc) suppresses vascular hyperglycemic memory in diabetes. Circulation research *111*, 278-289.

Paravicini, T.M., and Touyz, R.M. (2008). NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. Diabetes care *31 Suppl 2*, S170-180.

Pardali, E., Goumans, M.J., and ten Dijke, P. (2010). Signaling by members of the TGF-beta family in vascular morphogenesis and disease. Trends in cell biology *20*, 556-567.

Parker, D.J., Iyer, A., Shah, S., Moran, A., Hjelmeland, A.B., Basu, M.K., Liu, R., and Mitra, K. (2015). A new mitochondrial pool of cyclin E, regulated by Drp1, is linked to cell-density-dependent cell proliferation. Journal of cell science *128*, 4171-4182.

Parker, M.W., Xu, P., Li, X., and Vander Kooi, C.W. (2012). Structural basis for selective vascular endothelial growth factor-A (VEGF-A) binding to neuropilin-1. J Biol Chem *287*, 11082-11089.

Partanen, J., Armstrong, E., Makela, T.P., Korhonen, J., Sandberg, M., Renkonen, R., Knuutila, S., Huebner, K., and Alitalo, K. (1992). A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains. Molecular and cellular biology *12*, 1698-1707.

Patan, S. (1998). TIE1 and TIE2 receptor tyrosine kinases inversely regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular growth. Microvascular research *56*, 1-21.

Patella, F., Schug, Z.T., Persi, E., Neilson, L.J., Erami, Z., Avanzato, D., Maione, F., Hernandez-Fernaud, J.R., Mackay, G., Zheng, L*., et al.* (2015). Proteomics-based metabolic modeling reveals that fatty acid oxidation (FAO) controls endothelial cell (EC) permeability. Mol Cell Proteomics *14*, 621-634.

Patten, I.S., Rana, S., Shahul, S., Rowe, G.C., Jang, C., Liu, L., Hacker, M.R., Rhee, J.S., Mitchell, J., Mahmood, F*., et al.* (2012). Cardiac angiogenic imbalance leads to peripartum cardiomyopathy. Nature *485*, 333-338.

Paumard, P., Vaillier, J., Coulary, B., Schaeffer, J., Soubannier, V., Mueller, D.M., Brethes, D., di Rago, J.P., and Velours, J. (2002). The ATP synthase is involved in generating mitochondrial cristae morphology. EMBO J *21*, 221-230.

Pedrola, L., Espert, A., Wu, X., Claramunt, R., Shy, M.E., and Palau, F. (2005). GDAP1, the protein causing Charcot-Marie-Tooth disease type 4A, is expressed in neurons and is associated with mitochondria. Hum Mol Genet *14*, 1087-1094.

Perciavalle, R.M., Stewart, D.P., Koss, B., Lynch, J., Milasta, S., Bathina, M., Temirov, J., Cleland, M.M., Pelletier, S., Schuetz, J.D*., et al.* (2012). Anti-apoptotic MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration. Nature cell biology *14*, 575-583.

Perkins, G., Renken, C., Martone, M.E., Young, S.J., Ellisman, M., and Frey, T. (1997). Electron tomography of neuronal mitochondria: three-dimensional structure and organization of cristae and membrane contacts. JStructBiol *119*, 260-272.

Pernas, L., and Scorrano, L. (2016). Mito-Morphosis: Mitochondrial Fusion, Fission, and Cristae Remodeling as Key Mediators of Cellular Function. Annu Rev Physiol *78*, 505-531.

Pfanner, N., van der Laan, M., Amati, P., Capaldi, R.A., Caudy, A.A., Chacinska, A., Darshi, M., Deckers, M., Hoppins, S., Icho, T*., et al.* (2014). Uniform nomenclature for the mitochondrial contact site and cristae organizing system. The Journal of cell biology *204*, 1083-1086.

Phng, L.K., Potente, M., Leslie, J.D., Babbage, J., Nyqvist, D., Lobov, I., Ondr, J.K., Rao, S., Lang, R.A., Thurston, G*., et al.* (2009). Nrarp coordinates endothelial Notch and Wnt signaling to control vessel density in angiogenesis. Developmental cell *16*, 70-82.

Pineau, B., Bourge, M., Marion, J., Mauve, C., Gilard, F., Maneta-Peyret, L., Moreau, P., Satiat-Jeunemaitre, B., Brown, S.C., De Paepe, R*., et al.* (2013). The importance of cardiolipin synthase for mitochondrial ultrastructure, respiratory function, plant development, and stress responses in Arabidopsis. Plant Cell *25*, 4195-4208.

Pitulescu, M.E., Schmidt, I., Giaimo, B.D., Antoine, T., Berkenfeld, F., Ferrante, F., Park, H., Ehling, M., Biljes, D., Rocha, S.F*., et al.* (2017). Dll4 and Notch signalling couples sprouting angiogenesis and artery formation. Nature cell biology *19*, 915-927.

Plein, A., Fantin, A., and Ruhrberg, C. (2014). Neuropilin regulation of angiogenesis, arteriogenesis, and vascular permeability. Microcirculation *21*, 315-323.

Porat, R.M., Grunewald, M., Globerman, A., Itin, A., Barshtein, G., Alhonen, L., Alitalo, K., and Keshet, E. (2004). Specific induction of tie1 promoter by disturbed flow in atherosclerosis-prone vascular niches and flow-obstructing pathologies. Circulation research *94*, 394-401.

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. Cell *146*, 873-887.

Prager, G.W., Breuss, J.M., Steurer, S., Mihaly, J., and Binder, B.R. (2004a). Vascular endothelial growth factor (VEGF) induces rapid prourokinase (pro-uPA) activation on the surface of endothelial cells. Blood *103*, 955-962.

Prager, G.W., Breuss, J.M., Steurer, S., Olcaydu, D., Mihaly, J., Brunner, P.M., Stockinger, H., and Binder, B.R. (2004b). Vascular endothelial growth factor receptor-2-induced initial endothelial cell migration depends on the presence of the urokinase receptor. Circulation research *94*, 1562-1570.

Prudent, J., Zunino, R., Sugiura, A., Mattie, S., Shore, G.C., and McBride, H.M. (2015). MAPL SUMOylation of Drp1 Stabilizes an ER/Mitochondrial Platform Required for Cell Death. Molecular cell *59*, 941-955.

Puri, M.C., Rossant, J., Alitalo, K., Bernstein, A., and Partanen, J. (1995). The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. The EMBO journal *14*, 5884-5891.

Pyakurel, A., Savoia, C., Hess, D., and Scorrano, L. (2015). Extracellular Regulated Kinase Phosphorylates Mitofusin 1 to Control Mitochondrial Morphology and Apoptosis. Molecular cell.

Rabl, R., Soubannier, V., Scholz, R., Vogel, F., Mendl, N., Vasiljev-Neumeyer, A., Korner, C., Jagasia, R., Keil, T., Baumeister, W*., et al.* (2009). Formation of cristae and crista junctions in mitochondria depends on antagonism between Fcj1 and Su e/g. JCell Biol *185*, 1047-1063.

Rahimi, N., and Costello, C.E. (2015). Emerging roles of post-translational modifications in signal transduction and angiogenesis. Proteomics *15*, 300-309.

Rambold, A.S., Kostelecky, B., Elia, N., and Lippincott-Schwartz, J. (2011). Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. ProcNatlAcadSciUSA *108*, 10190-10195.

Rapaport, D., Brunner, M., Neupert, W., and Westermann, B. (1998). Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in Saccharomyces cerevisiae. JBiolChem *273*, 20150-20155.

Raturi, A., and Simmen, T. (2012). Where the endoplasmic reticulum and the mitochondrion tie the knot: The mitochondria-associated membrane (MAM). BiochimBiophysActa.

Reichert, A., and Neupert, W. (2002). Contact sites between the outer and inner membrane of mitochondria-role in protein transport. Biochim Biophys Acta *1592*.

Rinaldo, P., Matern, D., and Bennett, M.J. (2002). Fatty acid oxidation disorders. Annu Rev Physiol *64*, 477-502.

Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993). Microdomains with high Ca2+ close to IP3-sensitive channels that are sensed by neighboring mitochondria. Science *262*, 744-747.

Rizzuto, R., De, S.D., Raffaello, A., and Mammucari, C. (2012). Mitochondria as sensors and regulators of calcium signalling. NatRevMolCell Biol *13*, 566-578.

Roca, C., and Adams, R.H. (2007). Regulation of vascular morphogenesis by Notch signaling. Genes & development *21*, 2511-2524.

Rodewald, H.R., and Sato, T.N. (1996). Tie1, a receptor tyrosine kinase essential for vascular endothelial cell integrity, is not critical for the development of hematopoietic cells. Oncogene *12*, 397-404.

Rojo, M., Legros, F., Chateau, D., and Lombes, A. (2002). Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. JCell Sci *115*, 1663-1674.

Rowland, A.A., Chitwood, P.J., Phillips, M.J., and Voeltz, G.K. (2014). ER contact sites define the position and timing of endosome fission. Cell *159*, 1027-1041.

Rube, D.A., and van der Bliek, A.M. (2004). Mitochondrial morphology is dynamic and varied. MolCell Biochem *256-257*, 331-339.

Rubino, M., Miaczynska, M., Lippe, R., and Zerial, M. (2000). Selective membrane recruitment of EEA1 suggests a role in directional transport of clathrin-coated vesicles to early endosomes. J Biol Chem *275*, 3745-3748.

Safi, S.Z., Batumalaie, K., Mansor, M., Chinna, K., Mohan, S., Karimian, H., Qvist, R., Ashraf, M.A., and Yan, G.O. (2015). Glutamine treatment attenuates hyperglycemia-induced mitochondrial stress and apoptosis in umbilical vein endothelial cells. Clinics *70*, 569-576.

Saharinen, P., Eklund, L., Miettinen, J., Wirkkala, R., Anisimov, A., Winderlich, M., Nottebaum, A., Vestweber, D., Deutsch, U., Koh, G.Y.*, et al.* (2008). Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. Nature cell biology *10*, 527-537.

Saharinen, P., Kerkela, K., Ekman, N., Marron, M., Brindle, N., Lee, G.M., Augustin, H., Koh, G.Y., and Alitalo, K. (2005). Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2. The Journal of cell biology *169*, 239-243.

Sakuma, I., Stuehr, D.J., Gross, S.S., Nathan, C., and Levi, R. (1988). Identification of arginine as a precursor of endothelium-derived relaxing factor. Proceedings of the National Academy of Sciences of the United States of America *85*, 8664-8667.

Sakurai, Y., Ohgimoto, K., Kataoka, Y., Yoshida, N., and Shibuya, M. (2005). Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. Proceedings of the National Academy of Sciences of the United States of America *102*, 1076-1081.

Santel, A., Frank, S., Gaume, B., Herrler, M., Youle, R.J., and Fuller, M.T. (2003). Mitofusin-1 protein is a generally expressed mediator of mitochondrial fusion in mammalian cells. JCell Sci *116*, 2763-2774.

Santel, A., and Fuller, M.T. (2001). Control of mitochondrial morphology by a human mitofusin. JCell Sci *114*, 867-874.

Santillo, M., Colantuoni, A., Mondola, P., Guida, B., and Damiano, S. (2015). NOX signaling in molecular cardiovascular mechanisms involved in the blood pressure homeostasis. Front Physiol *6*, 194.

Sato, T.N., Qin, Y., Kozak, C.A., and Audus, K.L. (1993). Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. Proceedings of the National Academy of Sciences of the United States of America *90*, 9355-9358.

Sato, T.N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature *376*, 70-74.

Savant, S., La Porta, S., Budnik, A., Busch, K., Hu, J., Tisch, N., Korn, C., Valls, A.F., Benest, A.V., Terhardt, D*., et al.* (2015). The Orphan Receptor Tie1 Controls Angiogenesis and Vascular Remodeling by Differentially Regulating Tie2 in Tip and Stalk Cells. Cell reports *12*, 1761-1773.

Sawamiphak, S., Seidel, S., Essmann, C.L., Wilkinson, G.A., Pitulescu, M.E., Acker, T., and Acker-Palmer, A. (2010). Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. Nature *465*, 487-491.

Scarabelli, T.M., Stephanou, A., Pasini, E., Comini, L., Raddino, R., Knight, R.A., and Latchman, D.S. (2002). Different signaling pathways induce apoptosis in endothelial cells and cardiac myocytes during ischemia/reperfusion injury. Circulation research *90*, 745-748.

Scarpulla, R.C. (2008). Nuclear control of respiratory chain expression by nuclear respiratory factors and PGC-1-related coactivator. Annals of the New York Academy of Sciences *1147*, 321-334.

Scarpulla, R.C. (2012). Nucleus-encoded regulators of mitochondrial function: integration of respiratory chain expression, nutrient sensing and metabolic stress. Biochim Biophys Acta *1819*, 1088-1097.

Schagger, H. (1995). Native electrophoresis for isolation of mitochondrial oxidative phosphorylation protein complexes. Methods Enzymol *260*, 190-202.

Scheubel, R.J., Tostlebe, M., Simm, A., Rohrbach, S., Prondzinsky, R., Gellerich, F.N., Silber, R.E., and Holtz, J. (2002). Dysfunction of mitochondrial respiratory chain complex I in human failing myocardium is not due to disturbed mitochondrial gene expression. JAmCollCardiol *40*, 2174-2181.

Schleicher, M., Shepherd, B.R., Suarez, Y., Fernandez-Hernando, C., Yu, J., Pan, Y., Acevedo, L.M., Shadel, G.S., and Sessa, W.C. (2008). Prohibitin-1 maintains the angiogenic capacity of endothelial cells by regulating mitochondrial function and senescence. The Journal of cell biology *180*, 101-112.

Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A.V., Witke, W., Huttner, W.B., and Soling, H.D. (1999). Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. Nature *401*, 133-141.

Schmidt, H.H., Nau, H., Wittfoht, W., Gerlach, J., Prescher, K.E., Klein, M.M., Niroomand, F., and Bohme, E. (1988). Arginine is a physiological precursor of endothelium-derived nitric oxide. European journal of pharmacology *154*, 213-216.

Schnurch, H., and Risau, W. (1993). Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage. Development *119*, 957-968.

Schoors, S., Bruning, U., Missiaen, R., Queiroz, K.C., Borgers, G., Elia, I., Zecchin, A., Cantelmo, A.R., Christen, S., Goveia, J*., et al.* (2015). Corrigendum: Fatty acid carbon is essential for dNTP synthesis in endothelial cells. Nature *526*, 144.

Schoors, S., De Bock, K., Cantelmo, A.R., Georgiadou, M., Ghesquiere, B., Cauwenberghs, S., Kuchnio, A., Wong, B.W., Quaegebeur, A., Goveia, J*., et al.* (2014). Partial and transient reduction of glycolysis by PFKFB3 blockade reduces pathological angiogenesis. Cell Metab *19*, 37-48.

Scorrano, L. (2003). Divide et impera: Ca^{2+} signals, mitochondrial fission and sensitization to apoptosis. Cell DeathDiffer *10*, 1287-1289.

Scorrano, L., Ashiya, M., Buttle, K., Weiler, S., Oakes, S.A., Mannella, C.A., and Korsmeyer, S.J. (2002). A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. DevCell *2*, 55-67.

Seegar, T.C., Eller, B., Tzvetkova-Robev, D., Kolev, M.V., Henderson, S.C., Nikolov, D.B., and Barton, W.A. (2010). Tie1-Tie2 interactions mediate functional differences between angiopoietin ligands. Molecular cell *37*, 643-655.

Senger, D.R., Asch, B.B., Smith, B.D., Perruzzi, C.A., and Dvorak, H.F. (1983). A secreted phosphoprotein marker for neoplastic transformation of both epithelial and fibroblastic cells. Nature *302*, 714-715.

Senger, D.R., Perruzzi, C.A., Feder, J., and Dvorak, H.F. (1986). A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. Cancer Res *46*, 5629-5632.

Sesaki, H., and Jensen, R.E. (1999). Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. JCell Biol *147*, 699-706.

Sesaki, H., Southard, S.M., Yaffe, M.P., and Jensen, R.E. (2003). Mgm1p, a dynamin-related GTPase, is essential for fusion of the mitochondrial outer membrane. MolBiolCell *14*, 2342-2356.

Shah, A.V., Birdsey, G.M., Peghaire, C., Pitulescu, M.E., Dufton, N.P., Yang, Y., Weinberg, I., Osuna Almagro, L., Payne, L., Mason, J.C.*, et al.* (2017). The endothelial transcription factor ERG mediates Angiopoietin-1-dependent control of Notch signalling and vascular stability. Nature communications *8*, 16002.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L., and Schuh, A.C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature *376*, 62-66.

Shen, Q., Yamano, K., Head, B.P., Kawajiri, S., Cheung, J.T., Wang, C., Cho, J.H., Hattori, N., Youle, R.J., and van der Bliek, A.M. (2014). Mutations in Fis1 disrupt orderly disposal of defective mitochondria. Molecular biology of the cell *25*, 145-159.

Shenouda, S.M., Widlansky, M.E., Chen, K., Xu, G., Holbrook, M., Tabit, C.E., Hamburg, N.M., Frame, A.A., Caiano, T.L., Kluge, M.A.*, et al.* (2011). Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus. Circulation *124*, 444-453.

Simons, M. (2012). An inside view: VEGF receptor trafficking and signaling. Physiology *27*, 213-222.

Simons, M., and Eichmann, A. (2015). Molecular controls of arterial morphogenesis. Circulation research *116*, 1712-1724.

Simons, M., Gordon, E., and Claesson-Welsh, L. (2016). Mechanisms and regulation of endothelial VEGF receptor signalling. Nature reviews Molecular cell biology *17*, 611-625.

Singh, A.J., Meyer, R.D., Band, H., and Rahimi, N. (2005). The carboxyl terminus of VEGFR-2 is required for PKC-mediated down-regulation. Molecular biology of the cell *16*, 2106-2118.

Skidd, P.M., Lessell, S., and Cestari, D.M. (2013). Autosomal dominant hereditary optic neuropathy (ADOA): a review of the genetics and clinical manifestations of ADOA and ADOA+. Seminars in ophthalmology *28*, 422-426.

Smirnova, E., Griparic, L., Shurland, D.L., and van der Bliek, A.M. (2001). Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. MolBiolCell *12*, 2245-2256.

Smirnova, E., Shurland, D.-L., Ryazantsev, S.N., and van der Bliek, A.M. (1998). A human dynamin-related protein controls the distribution of mitochondria. JCell Biol *143*.

Smith, G.A., Fearnley, G.W., Tomlinson, D.C., Harrison, M.A., and Ponnambalam, S. (2015). The cellular response to vascular endothelial growth factors requires co-ordinated signal transduction, trafficking and proteolysis. Bioscience reports *35*.

Soderberg, O., Leuchowius, K.J., Gullberg, M., Jarvius, M., Weibrecht, I., Larsson, L.G., and Landegren, U. (2008). Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay. Methods *45*, 227-232.

Song, M., Gong, G., Burelle, Y., Gustafsson, A.B., Kitsis, R.N., Matkovich, S.J., and Dorn, G.W., 2nd (2015). Interdependence of Parkin-Mediated Mitophagy and Mitochondrial Fission in Adult Mouse Hearts. Circulation research *117*, 346-351.

Song, W., Chen, J., Petrilli, A., Liot, G., Klinglmayr, E., Zhou, Y., Poquiz, P., Tjong, J., Pouladi, M.A., Hayden, M.R.*, et al.* (2011). Mutant huntingtin binds the mitochondrial fission GTPase dynamin-related protein-1 and increases its enzymatic activity. Nature medicine *17*, 377-382.

Song, Z., Chen, H., Fiket, M., Alexander, C., and Chan, D.C. (2007). OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. JCell Biol *178*, 749-755.

Song, Z., Ghochani, M., McCaffery, J.M., Frey, T.G., and Chan, D.C. (2009). Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. Molecular biology of the cell *20*, 3525-3532.

Stahl, A., Connor, K.M., Sapieha, P., Chen, J., Dennison, R.J., Krah, N.M., Seaward, M.R., Willett, K.L., Aderman, C.M., Guerin, K.I.*, et al.* (2010). The mouse retina as an angiogenesis model. Invest Ophthalmol Vis Sci *51*, 2813-2826.

Stanton, R.C. (2012). Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. IUBMB Life *64*, 362-369.

Steglich, G., Neupert, W., and Langer, T. (1999). Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria. Molecular and cellular biology *19*, 3435-3442.

Stepanyants, N., Macdonald, P.J., Francy, C.A., Mears, J.A., Qi, X., and Ramachandran, R. (2015). Cardiolipin's propensity for phase transition and its reorganization by dynamin-related protein 1 form a basis for mitochondrial membrane fission. Molecular biology of the cell *26*, 3104-3116.

Stepniak, E., Radice, G.L., and Vasioukhin, V. (2009). Adhesive and signaling functions of cadherins and catenins in vertebrate development. Cold Spring Harbor perspectives in biology *1*, a002949.

Stojanovski, D., Koutsopoulos, O.S., Okamoto, K., and Ryan, M.T. (2004). Levels of human Fis1 at the mitochondrial outer membrane regulate mitochondrial morphology. JCell Sci *117*, 1201-1210.

Stowers, R.S., Megeath, L.J., Gorska-Andrzejak, J., Meinertzhagen, I.A., and Schwarz, T.L. (2002). Axonal transport of mitochondria to synapses depends on milton, a novel Drosophila protein. Neuron *36*, 1063-1077.

Strack, S., Wilson, T.J., and Cribbs, J.T. (2013). Cyclin-dependent kinases regulate splice-specific targeting of dynamin-related protein 1 to microtubules. The Journal of cell biology *201*, 1037-1051.

Strilic, B., Kucera, T., Eglinger, J., Hughes, M.R., McNagny, K.M., Tsukita, S., Dejana, E., Ferrara, N., and Lammert, E. (2009). The molecular basis of vascular lumen formation in the developing mouse aorta. Developmental cell *17*, 505-515.

Stroh, A., Anderka, O., Pfeiffer, K., Yagi, T., Finel, M., Ludwig, B., and Schagger, H. (2004). Assembly of respiratory complexes I, III, and IV into NADH oxidase supercomplex stabilizes complex I in Paracoccus denitrificans. J Biol Chem *279*, 5000-5007.

Sugiura, A., Yonashiro, R., Fukuda, T., Matsushita, N., Nagashima, S., Inatome, R., and Yanagi, S. (2011). A mitochondrial ubiquitin ligase MITOL controls cell toxicity of polyglutamine-expanded protein. Mitochondrion *11*, 139-146.

Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N., and Yancopoulos, G.D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell *87*, 1171-1180.

Taguchi, N., Ishihara, N., Jofuku, A., Oka, T., and Mihara, K. (2007). Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. JBiol Chem *282*, 11521-11529.

Takakura, N., Huang, X.L., Naruse, T., Hamaguchi, I., Dumont, D.J., Yancopoulos, G.D., and Suda, T. (1998). Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. Immunity *9*, 677-686.

Tatsuta, T., Scharwey, M., and Langer, T. (2014). Mitochondrial lipid trafficking. Trends in cell biology *24*, 44-52.

Taylor, R.W., and Turnbull, D.M. (2005). Mitochondrial DNA mutations in human disease. Nat Rev Genet *6*, 389-402.

Tkachuk, V.A., Plekhanova, O.S., and Parfyonova, Y.V. (2009). Regulation of arterial remodeling and angiogenesis by urokinase-type plasminogen activator. Canadian journal of physiology and pharmacology *87*, 231-251.

Tondera, D., Czauderna, F., Paulick, K., Schwarzer, R., Kaufmann, J., and Santel, A. (2005). The mitochondrial protein MTP18 contributes to mitochondrial fission in mammalian cells. Journal of cell science *118*, 3049-3059.

Tondera, D., Grandemange, S., Jourdain, A., Karbowski, M., Mattenberger, Y., Herzig, S., Da Cruz, S., Clerc, P., Raschke, I., Merkwirth, C*., et al.* (2009). SLP-2 is required for stress-induced mitochondrial hyperfusion. EMBOJ *28*, 1589-1600.

Tondera, D., Santel, A., Schwarzer, R., Dames, S., Giese, K., Klippel, A., and Kaufmann, J. (2004). Knockdown of MTP18, a novel phosphatidylinositol 3-kinase-dependent protein, affects mitochondrial morphology and induces apoptosis. J Biol Chem *279*, 31544-31555.

Touvier, T., De Palma, C., Rigamonti, E., Scagliola, A., Incerti, E., Mazelin, L., Thomas, J.L., D'Antonio, M., Politi, L., Schaeffer, L*., et al.* (2015). Muscle-specific Drp1 overexpression impairs skeletal muscle growth via translational attenuation. Cell death & disease *6*, e1663.

Trudeau, K., Molina, A.J., Guo, W., and Roy, S. (2010). High glucose disrupts mitochondrial morphology in retinal endothelial cells: implications for diabetic retinopathy. The American journal of pathology *177*, 447-455.

Tzima, E., Irani-Tehrani, M., Kiosses, W.B., Dejana, E., Schultz, D.A., Engelhardt, B., Cao, G., DeLisser, H., and Schwartz, M.A. (2005). A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. Nature *437*, 426-431.

Udagawa, O., Ishihara, T., Maeda, M., Matsunaga, Y., Tsukamoto, S., Kawano, N., Miyado, K., Shitara, H., Yokota, S., Nomura, M.*, et al.* (2014). Mitochondrial fission factor Drp1 maintains oocyte quality via dynamic rearrangement of multiple organelles. Curr Biol *24*, 2451-2458.

Ugarte-Uribe, B., Muller, H.M., Otsuki, M., Nickel, W., and Garcia-Saez, A.J. (2014). Dynamin-related protein 1 (Drp1) promotes structural intermediates of membrane division. J Biol Chem *289*, 30645-30656.

Unterluggauer, H., Mazurek, S., Lener, B., Hutter, E., Eigenbrodt, E., Zwerschke, W., and Jansen-Durr, P. (2008). Premature senescence of human endothelial cells induced by inhibition of glutaminase. Biogerontology *9*, 247-259.

Uo, T., Dworzak, J., Kinoshita, C., Inman, D.M., Kinoshita, Y., Horner, P.J., and Morrison, R.S. (2009). Drp1 levels constitutively regulate mitochondrial dynamics and cell survival in cortical neurons. Experimental neurology *218*, 274-285.

Ushio-Fukai, M., and Alexander, R.W. (2004). Reactive oxygen species as mediators of angiogenesis signaling: role of NAD(P)H oxidase. Mol Cell Biochem *264*, 85-97.

Ushio-Fukai, M., Tang, Y., Fukai, T., Dikalov, S.I., Ma, Y., Fujimoto, M., Quinn, M.T., Pagano, P.J., Johnson, C., and Alexander, R.W. (2002). Novel role of gp91(phox)-containing NAD(P)H oxidase in vascular endothelial growth factor-induced signaling and angiogenesis. Circulation research *91*, 1160-1167.

Valle, I., Alvarez-Barrientos, A., Arza, E., Lamas, S., and Monsalve, M. (2005). PGC-1alpha regulates the mitochondrial antioxidant defense system in vascular endothelial cells. Cardiovascular research *66*, 562-573.

Varadi, A., Johnson-Cadwell, L.I., Cirulli, V., Yoon, Y., Allan, V.J., and Rutter, G.A. (2004). Cytoplasmic dynein regulates the subcellular distribution of mitochondria by controlling the recruitment of the fission factor dynamin-related protein-1. JCell Sci *117*, 4389-4400.

Varanita, T., Soriano, M.E., Romanello, V., Zaglia, T., Quintana-Cabrera, R., Semenzato, M., Menabo, R., Costa, V., Civiletto, G., Pesce, P.*, et al.* (2015). The OPA1-dependent mitochondrial cristae remodeling pathway controls atrophic, apoptotic, and ischemic tissue damage. Cell Metab *21*, 834-844.

Ventura-Clapier, R., Garnier, A., and Veksler, V. (2008). Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. Cardiovascular research *79*, 208-217.

Verstreken, P., Ly, C.V., Venken, K.J., Koh, T.W., Zhou, Y., and Bellen, H.J. (2005). Synaptic mitochondria are critical for mobilization of reserve pool vesicles at Drosophila neuromuscular junctions. Neuron *47*, 365-378.

Villena, J.A. (2015). New insights into PGC-1 coactivators: redefining their role in the regulation of mitochondrial function and beyond. FEBS J *282*, 647-672.

Vizan, P., Alcarraz-Vizan, G., Diaz-Moralli, S., Solovjeva, O.N., Frederiks, W.M., and Cascante, M. (2009a). Modulation of pentose phosphate pathway during cell cycle progression in human colon adenocarcinoma cell line HT29. Int J Cancer *124*, 2789-2796.

Vizan, P., Sanchez-Tena, S., Alcarraz-Vizan, G., Soler, M., Messeguer, R., Pujol, M.D., Lee, W.N., and Cascante, M. (2009b). Characterization of the metabolic changes underlying growth factor angiogenic activation: identification of new potential therapeutic targets. Carcinogenesis *30*, 946-952.

Vogel, F., Bornhovd, C., Neupert, W., and Reichert, A.S. (2006). Dynamic subcompartmentalization of the mitochondrial inner membrane. JCell Biol *175*, 237-247.

von der, M.K., Muller, J.M., Bohnert, M., Oeljeklaus, S., Kwiatkowska, P., Becker, T., Loniewska-Lwowska, A., Wiese, S., Rao, S., Milenkovic, D*., et al.* (2011). Dual role of mitofilin in mitochondrial membrane organization and protein biogenesis. DevCell *21*, 694-707.

Voss, C., Lahiri, S., Young, B.P., Loewen, C.J., and Prinz, W.A. (2012). ER-shaping proteins facilitate lipid exchange between the ER and mitochondria in S. cerevisiae. Journal of cell science *125*, 4791-4799.

Wakabayashi, J., Zhang, Z., Wakabayashi, N., Tamura, Y., Fukaya, M., Kensler, T.W., Iijima, M., and Sesaki, H. (2009a). The dynamin-related GTPase Drp1 is required for embryonic and brain development in mice. J Cell Biol *186*, 805-816.

Wakabayashi, J., Zhang, Z., Wakabayashi, N., Tamura, Y., Fukaya, M., Kensler, T.W., Iijima, M., and Sesaki, H. (2009b). The dynamin-related GTPase Drp1 is required for embryonic and brain development in mice. J Cell Biol *186*, 805-816.

Wang, H., Song, P., Du, L., Tian, W., Yue, W., Liu, M., Li, D., Wang, B., Zhu, Y., Cao, C*., et al.* (2011). Parkin Ubiquitinates Drp1 for Proteasome-dependent Degradation: IMPLICATION OF DYSREGULATED MITOCHONDRIAL DYNAMICS IN PARKINSON DISEASE. JOURNAL OF BIOLOGICAL CHEMISTRY *286*, 11649-11658.

Wang, J.L., Zhang, J.L., Chen, W., Xu, X.F., Gao, N., Fan, D.Y., and An, J. (2010). Roles of small GTPase Rac1 in the regulation of actin cytoskeleton during dengue virus infection. PLoS neglected tropical diseases *4*.

Wang, J.Z., and Liu, F. (2008). Microtubule-associated protein tau in development, degeneration and protection of neurons. Progress in neurobiology *85*, 148-175.

Wang, W., Wang, Y., Long, J., Wang, J., Haudek, S.B., Overbeek, P., Chang, B.H., Schumacker, P.T., and Danesh, F.R. (2012a). Mitochondrial fission triggered by hyperglycemia is mediated by ROCK1 activation in podocytes and endothelial cells. Cell Metab *15*, 186-200.

Wang, W., Xie, Q., Zhou, X., Yao, J., Zhu, X., Huang, P., Zhang, L., Wei, J., Xie, H., Zhou, L*., et al.* (2015). Mitofusin-2 triggers mitochondria Ca2+ influx from the endoplasmic reticulum to induce apoptosis in hepatocellular carcinoma cells. Cancer Lett *358*, 47-58.

Wang, X., Su, B., Zheng, L., Perry, G., Smith, M.A., and Zhu, X. (2009). The role of abnormal mitochondrial dynamics in the pathogenesis of Alzheimer's disease. J Neurochem *109 Suppl 1*, 153-159.

Wang, X., Yan, M.H., Fujioka, H., Liu, J., Wilson-Delfosse, A., Chen, S.G., Perry, G., Casadesus, G., and Zhu, X. (2012b). LRRK2 regulates mitochondrial dynamics and function through direct interaction with DLP1. Hum Mol Genet *21*, 1931-1944.

Wasiak, S., Zunino, R., and McBride, H.M. (2007). Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death. JCell Biol *177*, 439-450.

Waterham, H.R., Koster, J., van Roermund, C.W., Mooyer, P.A., Wanders, R.J., and Leonard, J.V. (2007). A lethal defect of mitochondrial and peroxisomal fission. NEnglJMed *356*, 1736-1741.

Weis, S., Cui, J., Barnes, L., and Cheresh, D. (2004). Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. The Journal of cell biology *167*, 223-229.

Werner, E.R., Gorren, A.C., Heller, R., Werner-Felmayer, G., and Mayer, B. (2003). Tetrahydrobiopterin and nitric oxide: mechanistic and pharmacological aspects. Experimental biology and medicine *228*, 1291-1302.

Westermann, B. (2010). Mitochondrial fusion and fission in cell life and death. Nat RevMol Cell Biol *11*, 872-884.

Westhoff, M.A., Serrels, B., Fincham, V.J., Frame, M.C., and Carragher, N.O. (2004). SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. Molecular and cellular biology *24*, 8113-8133.

Wilhelm, K., Happel, K., Eelen, G., Schoors, S., Oellerich, M.F., Lim, R., Zimmermann, B., Aspalter, I.M., Franco, C.A., Boettger, T.*, et al.* (2016). FOXO1 couples metabolic activity and growth state in the vascular endothelium. Nature *529*, 216-220.

Willems, A.P., van Engelen, B.G., and Lefeber, D.J. (2016). Genetic defects in the hexosamine and sialic acid biosynthesis pathway. Biochim Biophys Acta *1860*, 1640-1654.

Winter, A., Kamarainen, O., and Hofmann, A. (2007). Molecular modeling of prohibitin domains. Proteins *68*, 353-362.

Wong, B.W., Marsch, E., Treps, L., Baes, M., and Carmeliet, P. (2017). Endothelial cell metabolism in health and disease: impact of hypoxia. The EMBO journal *36*, 2187-2203.

Woo, K.V., and Baldwin, H.S. (2011). Role of Tie1 in shear stress and atherosclerosis. Trends in cardiovascular medicine *21*, 118-123.

Wright, G.L., Maroulakou, I.G., Eldridge, J., Liby, T.L., Sridharan, V., Tsichlis, P.N., and Muise-Helmericks, R.C. (2008). VEGF stimulation of mitochondrial biogenesis: requirement of AKT3 kinase. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *22*, 3264-3275.

Wu, G., and Meininger, C.J. (2000). Arginine nutrition and cardiovascular function. The Journal of nutrition *130*, 2626-2629.

Xu, Y., An, X., Guo, X., Habtetsion, T.G., Wang, Y., Xu, X., Kandala, S., Li, Q., Li, H., Zhang, C.*, et al.* (2014). Endothelial PFKFB3 plays a critical role in angiogenesis. Arteriosclerosis, thrombosis, and vascular biology *34*, 1231-1239.

Yamamoto, H., Ehling, M., Kato, K., Kanai, K., van Lessen, M., Frye, M., Zeuschner, D., Nakayama, M., Vestweber, D., and Adams, R.H. (2015). Integrin beta1 controls VE-cadherin localization and blood vessel stability. Nature communications *6*, 6429.

Yamaoka-Tojo, M., Ushio-Fukai, M., Hilenski, L., Dikalov, S.I., Chen, Y.E., Tojo, T., Fukai, T., Fujimoto, M., Patrushev, N.A., Wang, N.*, et al.* (2004). IQGAP1, a novel vascular endothelial growth factor receptor binding protein, is involved in reactive oxygen species--dependent endothelial migration and proliferation. Circulation research *95*, 276-283.

Ye, X., Wang, Y., Cahill, H., Yu, M., Badea, T.C., Smallwood, P.M., Peachey, N.S., and Nathans, J. (2009). Norrin, frizzled-4, and Lrp5 signaling in endothelial cells controls a genetic program for retinal vascularization. Cell *139*, 285-298.

Yonashiro, R., Ishido, S., Kyo, S., Fukuda, T., Goto, E., Matsuki, Y., Ohmura-Hoshino, M., Sada, K., Hotta, H., Yamamura, H.*, et al.* (2006). A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. EMBO J *25*, 3618-3626.

Yonashiro, R., Sugiura, A., Miyachi, M., Fukuda, T., Matsushita, N., Inatome, R., Ogata, Y., Suzuki, T., Dohmae, N., and Yanagi, S. (2009). Mitochondrial ubiquitin ligase MITOL ubiquitinates mutant SOD1 and attenuates mutant SOD1-induced reactive oxygen species generation. MolBiol Cell *20*, 4524-4530.

Yoon, Y., Krueger, E.W., Oswald, B.J., and McNiven, M.A. (2003). The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. MolCell Biol *23*, 5409-5420.

Yoon, Y., Pitts, K.R., Dahan, S., and McNiven, M.A. (1998). A novel dynamin-like protein associates with cytoplasmic vesicles and tubules of the endoplasmic reticulum in mammalian cells. JCell Biol *140*, 779-793.

Yoon, Y., Pitts, K.R., and McNiven, M.A. (2001). Mammalian dynamin-like protein DLP1 tubulates membranes. Molecular biology of the cell *12*, 2894-2905.

Yoon, Y.G., Koob, M.D., and Yoo, Y.H. (2010). Re-engineering the mitochondrial genomes in mammalian cells. Anatomy & cell biology *43*, 97-109.

Yu, T., Robotham, J.L., and Yoon, Y. (2006). Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. ProcNatlAcadSciUSA *103*, 2653-2658.

Yu, T., Sheu, S.S., Robotham, J.L., and Yoon, Y. (2008). Mitochondrial fission mediates high glucose-induced cell death through elevated production of reactive oxygen species. CardiovascRes *79*, 341-351.

Zerbes, R.M., van der Klei, I.J., Veenhuis, M., Pfanner, N., van der Laan, M., and Bohnert, M. (2012). Mitofilin complexes: conserved organizers of mitochondrial membrane architecture. Biol Chem *393*, 1247-1261.

Zerial, M., and McBride, H. (2001a). Rab proteins as membrane organizers. Nature reviews Molecular cell biology *2*, 107-117.

Zerial, M., and McBride, H. (2001b). Rab proteins as membrane organizers. Nature reviews Molecular cell biology *2*.

Zerlin, M., Julius, M.A., and Kitajewski, J. (2008). Wnt/Frizzled signaling in angiogenesis. Angiogenesis *11*, 63-69.

Zhang, X., and Simons, M. (2014). Receptor tyrosine kinases endocytosis in endothelium: biology and signaling. Arteriosclerosis, thrombosis, and vascular biology *34*, 1831-1837.

Zhang, Y., and Chan, D.C. (2007). Structural basis for recruitment of mitochondrial fission complexes by Fis1. Proceedings of the National Academy of Sciences of the United States of America *104*, 18526-18530.

Zhang, Y., Gao, X., and Garavito, R.M. (2011). Biochemical characterization of human dynamin-like protein 1. J Biochem *150*, 627-633.

Zhang, Z., Apse, K., Pang, J., and Stanton, R.C. (2000). High glucose inhibits glucose-6-phosphate dehydrogenase via cAMP in aortic endothelial cells. J Biol Chem *275*, 40042-40047.

Zhao, J., Liu, T., Jin, S., Wang, X., Qu, M., Uhlen, P., Tomilin, N., Shupliakov, O., Lendahl, U., and Nister, M. (2011). Human MIEF1 recruits Drp1 to mitochondrial outer membranes and promotes mitochondrial fusion rather than fission. The EMBO journal *30*, 2762-2778.

Zhao, J., Zhang, J., Yu, M., Xie, Y., Huang, Y., Wolff, D.W., Abel, P.W., and Tu, Y. (2013). Mitochondrial dynamics regulates migration and invasion of breast cancer cells. Oncogene *32*, 4814-4824.

Zhu, P.P., Patterson, A., Stadler, J., Seeburg, D.P., Sheng, M., and Blackstone, C. (2004). Intra- and intermolecular domain interactions of the C-terminal GTPase effector domain of the multimeric dynamin-like GTPase Drp1. JBiolChem *279*, 35967-35974.

Zick, M., Rabl, R., and Reichert, A.S. (2009). Cristae formation-linking ultrastructure and function of mitochondria. Biochim Biophys Acta *1793*, 5-19.

Zinser, E., Sperka-Gottlieb, C.D., Fasch, E.V., Kohlwein, S.D., Paltauf, F., and Daum, G. (1991). Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. Journal of bacteriology *173*, 2026-2034.

Zorov, D.B., Juhaszova, M., and Sollott, S.J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol Rev *94*, 909-950.

Zou, M.H., Shi, C., and Cohen, R.A. (2002). Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. The Journal of clinical investigation *109*, 817-826.

Zovein, A.C., Luque, A., Turlo, K.A., Hofmann, J.J., Yee, K.M., Becker, M.S., Fassler, R., Mellman, I., Lane, T.F., and Iruela-Arispe, M.L. (2010). Beta1 integrin establishes endothelial cell polarity

and arteriolar lumen formation via a Par3-dependent mechanism. Developmental cell *18*, 39-51.

Zunino, R., Braschi, E., Xu, L., and McBride, H.M. (2009). Translocation of SenP5 from the nucleoli to the mitochondria modulates DRP1-dependent fission during mitosis. JBiolChem *284*, 17783-17795.

Zunino, R., Schauss, A., Rippstein, P., ndrade-Navarro, M., and McBride, H.M. (2007). The SUMO protease SENP5 is required to maintain mitochondrial morphology and function. JCell Sci *120*, 1178-1188.