



# Positive and negative feedback mechanisms controlling tip/stalk cell identity during sprouting angiogenesis

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## Abstract

Vascular endothelial growth factor-A (VEGF-A/VEGF) interaction with VEGF receptor 2 (VEGFR2) is key for sprouting angiogenesis in health and disease. VEGF/VEGFR2 signaling promotes endothelial proliferation and migration, as well as the hierarchical organization into leader (tip) and follower (stalk) cells via a dynamic interplay with Notch. Recent studies reveal novel molecular mechanisms to fine-tune VEGF/Notch signaling and tip/stalk cell function during sprouting angiogenesis.

**Keywords** Dll4 · Notch · ERK · Sprouting angiogenesis · Tip cells · VEGF signaling

## Abbreviations

aPKC	Atypical PKC
DAG	Diacylglycerol
Dll4	Delta-like ligand 4
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FOXO1	Forkhead box protein O1
IP3	Inositoltrisphosphate
Mst1	Mammalian sterile 20-like kinase 1
ROS	Reactive oxygen species
PI3-K	Phosphoinositide 3-kinase
PKC	Protein kinase C
TMEM33	Transmembrane protein 33
VEGF	Vascular endothelial growth factor
VEGFR2	VEGF receptor 2

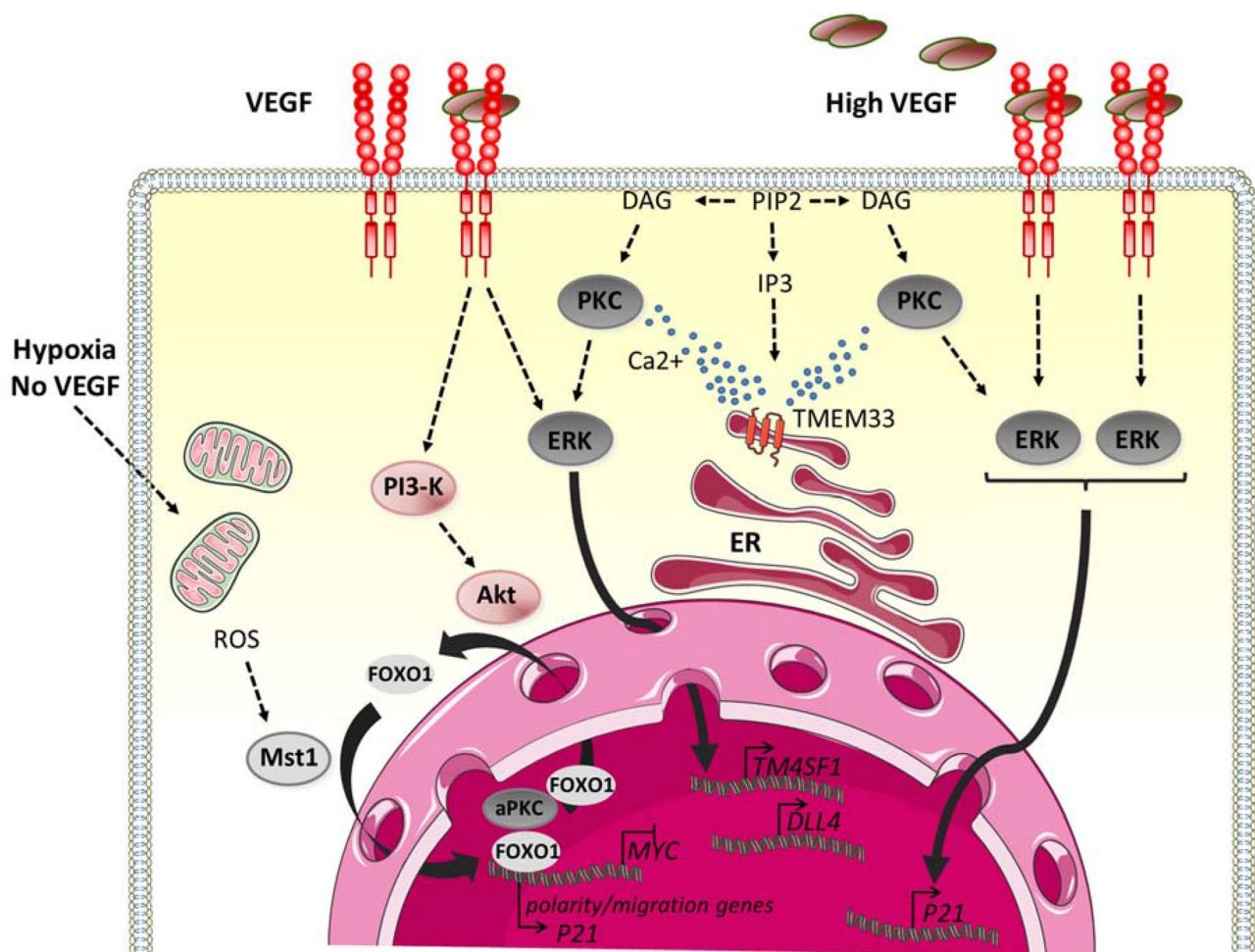
VEGF/VEGFR2 interaction promotes endothelial cell proliferation, migration, and survival, largely via activation of the phosphoinositide 3-kinase (PI3-K)/Akt and extracellular signal-regulated kinase (ERK) pathways [1]. In addition, VEGF-induced kinase signaling promotes the development of tip cells, which have a specific transcriptional signature and express a variety of proteins that mark their identity [2, 3]. Some of these proteins, such as Delta-like 4 (Dll4), suppress the development of tip cell properties in adjacent stalk

cells, by *trans*-activation of the Notch pathway [4]. Although tip cells encounter highest levels of VEGF, they are generally less proliferative than stalk cells and mainly stimulate sprout migration. Conversely, stalk cells are actively proliferating despite high Notch signaling, which is thought to inhibit proliferation. Thus, anti- and pro-proliferative cues resulting from VEGF/Notch signaling need to be continuously fine-tuned in a spatiotemporal manner, to achieve appropriate cellular responses in angiogenic sprouts. Recent data illuminate novel mechanisms by which such spatiotemporal control is exerted by kinases.

ERK activation occurs via the canonical Ras/Raf pathway but is further enhanced by protein kinase C (PKC), in a manner dependent on diacylglycerol and inositoltrisphosphate-triggered release of Ca<sup>2+</sup> from the endoplasmic reticulum (ER) (Fig. 1). In a recent study, it was found that the ER-resident protein transmembrane protein 33 (TMEM33) is required for VEGF-induced Ca<sup>2+</sup> release from the ER, and that cytosolic Ca<sup>2+</sup> oscillations in tip cells under the control of TMEM33 promote filopodia formation and migration of tip cells [5]. Furthermore, Ca<sup>2+</sup> influx potentiates ERK activation and downstream Dll4/Notch signaling, and the knockdown of Tmem33 in zebrafish impaired VEGF-dependent ERK phosphorylation, downstream Notch signaling, and vascular morphogenesis during embryonic development [5]. In another recent report, zebrafish endothelial cells were examined for genes that are transcriptionally activated by VEGF and repressed by Notch, to identify positive-feedback regulators of VEGF/VEGFR2 signaling. A transcript was identified encoding the atypical tetraspanin Tm4sf18 (TM4SF1 in humans). Subsequent studies using zebrafish

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**Fig. 1** Regulatory mechanisms controlling tip/stalk cell biology. VEGF/VEGFR2 signaling activates the PI3-K/Akt and ERK pathways important for proliferation, migration, and the expression of tip cell genes including *DLL4*. ERK activation is enhanced by PKC, which requires TMEM33-dependent release of Ca<sup>2+</sup> from the ER and is further promoted by the positive-feedback regulator TM4SF1. However, excessive ERK activation in tip cells induces expression of

the cell cycle inhibitor *P21*, leading to cell cycle arrest. *P21* expression is regulated by FOXO1, a transcription factor that stimulates tip cell polarity and migration while preventing proliferation. While hypoxia promotes the nuclear retention and DNA-binding activity of FOXO1 via Mst1, these events are opposed by PI3-K/Akt and aPKC $\lambda$  in response to VEGF

mutants and knockdown experiments in human endothelial cells revealed that Tm4sf18/TM4SF1 amplifies VEGF/VEGFR2 signaling to ERK and stimulates the rapid selection of tip cells [6]. In the absence of this amplification loop, lower numbers of branching cells and reduced cellularity of newly formed vessels were observed [6]. Since tetraspansins associate with cell-surface proteins including integrins and receptor tyrosine kinases, it is possible that Tm4sf18/TM4SF1 modulates VEGFR2 signaling output directly from the plasma membrane.

The PI3-K/Akt pathway leads to phosphorylation of Forkhead box protein O1 (FOXO1), a member of the FOXO family of transcription factors that are highly responsive to cellular stress and locate in the nucleus when environmental growth factors are low. In such conditions, FOXO1

maintains endothelial quiescence by reducing glycolysis and mitochondrial respiration and by blocking cell proliferation. The cell cycle arrest imposed by FOXO1 is due to expression of cell cycle inhibitors including p21 and p27, as well as the simultaneous suppression of cyclins and c-Myc. FOXO1 phosphorylation in response to growth factor-induced PI3-K/Akt signaling triggers its nuclear export, thus inhibiting FOXO1-dependent gene regulation and alleviating the cell cycle block (Fig. 1). A recent study now shows that the effects of the PI3-K/Akt pathway on FOXO1 are opposed by Mammalian sterile 20-like kinase 1 (Mst1), which phosphorylates FOXO1 at a site different from that used by Akt. Mst1 is activated by cellular stress such as growth factor/nutrient deprivation or hypoxia, which leads to accumulation of reactive oxygen species (ROS) produced by mitochondria

(Fig. 1). Mst1-dependent phosphorylation enhances the nuclear import of FOXO1, and augments the transcription of tip cell-, polarity-, and migration-associated genes, which is induced by FOXO1 in response to hypoxia [7, 8]. In this way, the Mst1-FOXO1 cascade promotes polarized, directional migration of tip cells towards hypoxic regions, and endothelial-specific deletion of either Mst1 or FOXO1 in mice inhibits tip cell polarization and sprouting angiogenesis [7, 8]. In addition to this mechanism, another recent study unveiled that atypical PKC $\lambda$  (aPKC $\lambda$ ) also phosphorylates FOXO1, at a site close to but distinct from that recognized by Mst1. Intriguingly, this phosphorylation does not affect the nuclear localization of FOXO1 but impairs its binding to DNA and therefore stimulates c-Myc expression and proliferation [9]. Consequently, endothelial deletion of aPKC $\lambda$  in mice impairs c-Myc expression and proliferation, both during developmental angiogenesis and in malignant endothelial neoplasms [9]. Thus, multiple kinases jointly regulate FOXO1 function at several levels and therefore shape the balance between cell cycle arrest, migration, and proliferation in response to various environmental stimuli.

Intriguingly, it was recently found that endothelial cell cycle exit imposed by high p21 levels can also result from excessive VEGF stimulation and ERK signaling. Tip cells in sprouting retinal vessels encounter highest concentrations of VEGF and as a result have high levels of ERK signaling, but withdraw from the cell cycle due to the induction of p21 [10]. The cell cycle arrest enhances endothelial sprouting and migration, but eventually blocks angiogenesis because of insufficient proliferation. The cause for this unexpected finding is a bell-shaped dose/response curve to VEGF-induced ERK signaling; either very low or very high levels of active ERK trigger p21-dependent cell cycle arrest. This mechanism explains why tip cells are generally less proliferative despite being exposed to high levels of VEGF, while stalk cells are actively cycling at lower VEGF concentrations and lower ERK activation levels [10].

Together, these studies emphasize the context dependency of VEGF/Notch signaling, the importance of local VEGF fluctuations, and the intricate way in which endothelial cells spatiotemporally control pro- and anti-angiogenic functions of the same stimuli. It will be very important to take these mechanisms into account when considering future VEGF/Notch-targeted therapies for the inhibition or stimulation of angiogenesis in disease settings.

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