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Roles of Ubiquitination and SUMOylation in the Regulation of Angiogenesis

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Abstract

The generation of new blood vessels from the existing vasculature is a dynamic and complex mechanism known as angiogenesis. Angiogenesis occurs during the entire lifespan of vertebrates and participates in many physiological processes. Furthermore, angiogenesis is also actively involved in many human diseases and disorders, including cancer, obesity and infections. Several inter-connected molecular pathways regulate angiogenesis, and post-translational modifications, such as phosphorylation, ubiquitination and SUMOylation, tightly regulate these mechanisms and play a key role in the control of the process. Here, we describe in detail the roles of ubiquitination and SUMOylation in the regulation of angiogenesis.

Introduction

The growth of new blood vessels from the existing vasculature is a process known as angiogenesis (Carmeliet, 2003; Ucuzian *et al.*, 2010). In vertebrates, angiogenesis occurs across the entire lifespan and participates in multiple physiological processes, such as pregnancy, embryonic development and wound healing. Moreover, many diseases can promote *de novo* angiogenesis, a process also known as pathological angiogenesis or neo-angiogenesis. In this regard, a well-known example is tumorigenesis-induced angiogenesis, during which hypoxic and starved cancer cells activate the molecular pathways involved in the formation of novel blood vessels, in order to supply nutrients and oxygen required for the tumour growth. Additionally, more than 70 different disorders have been associated to *de novo* angiogenesis including obesity, bacterial infections and AIDS (Carmeliet, 2003).

At the molecular level, angiogenesis relays on several pathways that cooperate in order to regulate in a precise spatial and temporal order the process. In this context, post-translational modifications (PTMs) play a central role in the regulation of these events, influencing the activation and stability of many growth factors, membrane receptors and downstream signalling effector molecules. Here, we will focus on the role of ubiquitination and SUMOylation in the regulation of angiogenesis.

Molecular basics of angiogenesis

Blood vessels arise from endothelial precursor cells, in a process known as vasculogenesis. Further stabilization of the new blood vessels, including their expansive growth and the formation of collateral bridges is known as angiogenesis (Carmeliet, 2003). During angiogenesis, a dynamic

and complex crosstalk occurs between endothelial cells and the extracellular matrix in a tightly regulated manner in order to promote endothelial cells proliferation and differentiation, cytoskeletal reorganization and cell migration, and the formation of novel vessels (Carmeliet, 2003; Huang and Bao, 2004; Muñoz-Chápuli *et al.*, 2004; Ucuzian *et al.*, 2010). Endothelial cells, fibroblasts, platelets, inflammatory cells and cancer cells (Ucuzian *et al.*, 2010) can all act as sources of angiogenic factors. Key pro-angiogenic factors are the Vascular Endothelial Growth Factors (VEGF1–5) and their receptors (VEGFR1, VEGFR2 and VEGFR3), the Placental Growth Factors (PlGFs), the Fibroblast Growth Factors (FGF1 and FGF2) and FGF receptors (FGFR1–4), the Transforming Growth Factor (TGF-β) family, the Tumour Necrosis Factor (TNF-α), the family of the Angiopoietins (ANG1 and ANG2) and the TIE-1 and -2 receptors, Ephrins and Leptins (Carmeliet, 2003; Huang and Bao, 2004; Ucuzian *et al.*, 2010). On the other hand, anti-angiogenic factors include the Thrombospondins (TSP1–4 and TSP-5/COMP), Angiostatins and Endostatins (Huang and Bao, 2004). Moreover, other players may differentially contribute to the control of angiogenesis, like the Matrix Metalloproteinases (MMPs), Integrins, and the extracellular matrix (ECM) (Kessenbrock *et al.*, 2010). These factors activate several downstream signalling pathways. For example, VEGF, and similarly FGF, mainly activate the ERK/MAPK pathway (Larsson *et al.*, 1999; Cross *et al.*, 2000; Wu *et al.*, 2000), leading to the transcription of master genes involved in cell proliferation, such as *MYC*, *ELK-1*, *FOS*, etc. (Muñoz-Chápuli *et al.*, 2004). On the other end, VEGF can also act independently of the ERK/MAPK cascade by activating other pathways such as the STAT signalling (Muñoz-Chápuli *et al.*, 2004). Interestingly, additional stimuli can cooperate with angiogenic factors. Accordingly, Nitric Oxide (NO) is able to potentiate the VEGF-dependent activation of the angiogenic pathways (Donnini and Ziche, 2002). VEGF also directly controls the migration of endothelial cells during angiogenesis activating the RHO GTPases RHO and RAC, which are required for cell motility and the formation of focal adhesions (Soga *et al.*, 2001a,b). Moreover, other factors, such as the Protein Kinase C (PKC) (Yamamura *et al.*, 1996), or the receptor NOTCH (Hellström *et al.*, 2007), can regulate cell migration in response to VEGF. On the other hand, anti-angiogenic factors such as the Endostatins are potent inhibitors of endothelial cells migration counteracting the formation of focal adhesions (Dixelius *et al.*, 2003; Eriksson *et al.*, 2003).

Typically, during endothelial cell migration, cell proliferation is enhanced, while apoptosis is repressed. Generally, the apoptotic signals that regulate angiogenesis and the fate of endothelial cells are mediated by TNF-α and TGF-β signalling (Polunovsky *et al.*, 1994; Choi and Ballermann, 1995). During angiogenesis, however, apoptotic pathways are inhibited by the crosstalk between Integrins, VEGF and FGF cascades that converge toward the activation of the AKT pathway (Gerber *et al.*, 1998). Other signalling pathways involved in angiogenesis include WNT signalling (Dufourcq *et al.*, 2002), and the pathways activated by cytokines, such as Pleiotrophin and Midkine (Stoica *et al.*, 2001, 2002), and oestrogens (Hyder *et al.*, 1996).

Because hypoxia is an important factor in angiogenesis, also Hypoxia-Inducible Factors (HIFs) play a fundamental role in neo-angiogenesis during tumour development (Pugh and Ratcliffe, 2003). HIF is a basic helix–loop–helix heterodimeric transcription factor that under hypoxic condition binds to hypoxic response elements (HREs) of the DNA inducing the transcription of a series of hypoxia-related genes, many of which are involved in angiogenesis (Semenza, 2000; Wenger, 2002). For example, *VEGF* transcription is directly up-regulated by HIF activity in hypoxic conditions (Levy *et al.*, 1998; Pugh and Ratcliffe, 2003; Zhang *et al.*, 2012; De Francesco *et al.*, 2013). Accordingly, *Hif-1α* knock out mice show abnormal vascular development and embryonic lethality (Maltepe *et al.*, 1997; Kotch *et al.*, 1999).

PTMs in angiogenesis

PTMs are a series of covalent modifications that occur following protein synthesis, and regulate protein activity, turnover and/or localization. The most common PTMs include phosphorylation, acetylation, glycosylation, ubiquitination and SUMOylation. Every PTM is strictly regulated by several molecular mechanisms and feedback loops. Interestingly, every single PTM described so far participates in the regulation and control

of angiogenesis (Rahimi and Costello, 2015). In this review, we will focus on ubiquitination and SUMOylation, and will describe how these PTMs work and impact angiogenesis.

Ubiquitination and SUMOylation in angiogenesis

Ubiquitination and SUMOylation are PTMs that regulate the activity and fate of a plethora of proteins (Clague *et al.*, 2015; Hendriks *et al.*, 2015). Both ubiquitination and SUMOylation consist of the covalent binding of a small protein modifier (ubiquitin, Ub hereafter, or Small Ubiquitin-like Modifier, SUMO hereafter) to one or multiple lysine (K) residues of a target protein. Both processes require three consecutive steps (Fig. 18.1), sequentially catalysed by E1, E2 and E3 ligases (Swatek and Komander, 2016; Rabellino *et al.*, 2017). While for ubiquitination, a variety of E1–3 ligases are known, for SUMOylation, only one E1 (SAE1/2) and one E2 (UBC9) are known, and only few classes of E3 ligases have been described so far, including RanBP2, PC2, TOPORS and the PIAS family (Rabellino *et al.*, 2017).

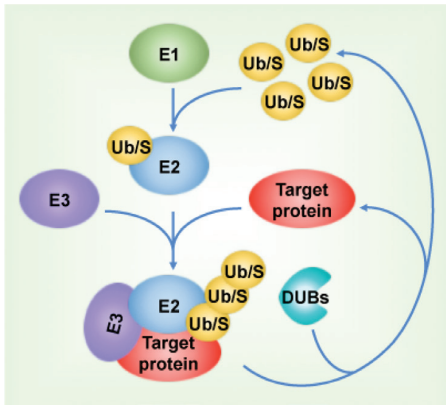

Figure 18.1 Ubiquitination and SUMOylation are reversible PTMs occurring through an E1, E2 and E3 enzymatic cascade. Ubiquitination and SUMOylation consist in the binding of either Ub or SUMO (Ub/S in the figure) modifiers to a final target protein. This process occurs trough a sequential enzymatic cascade involving E1, E2 and E3 ligases. The last step of the reaction is usually facilitated by an E3 ligase that promotes the interaction between the E2 ligase and the target protein to be modified. Both ubiquitination and SUMOylation are reversible processes: specific de-ubiquitinase and de-SUMOylase enzymes (DUBs) remove Ub/SUMO from the target protein.

Both ubiquitination and SUMOylation start with the attachment of a single Ub or SUMO to the target protein: these mono-ubiquitination/SUMOylation events have several repercussions on the fate of the target. Moreover, both Ub and SUMO often form complex branched chains, and the complexity and/or the length of the chains will determine the fate of the modified-target. For example, Ub contains seven K residues that can be ubiquitinated, thus participating to the formation of complex and branched Ub chains (Kim *et al.*, 2011; Wagner *et al.*, 2011).

Owing to the presence of multiple SUMO paralogs, the SUMOylation machinery is more complex than ubiquitination. In vertebrates, five different *SUMO* genes exist and they encode for 5 different SUMO proteins (SUMO1–5): SUMO1, SUMO2 and SUMO3 are ubiquitously expressed, while SUMO4 and SUMO5 are tissue specific and not well characterized yet (Guo *et al.*, 2004; Liang *et al.*, 2016). In particular, SUMO2 and SUMO3 are 97% alike, however, they share only 50% homology with SUMO1 (Saitoh and Hinchey, 2000). Moreover, SUMO1 cannot be SUMOylated due to the lack of an internal acceptor K. Therefore, SUMO1 is not able to form SUMO chains and it is considered a SUMO-chain terminator (Matic *et al.*, 2008).

Both ubiquitination and SUMOylation are reversible modifications, and specific de-ubiquitination and de-SUMOylation enzymes (DUBs) are able to cleave Ub and SUMO from a modified protein (Wing, 2003; Yeh, 2009) (Fig. 18.1).

Although they share a very similar enzymatic cascade, ubiquitination and SUMOylation play different roles in several cellular processes. The main function of ubiquitination is to target proteins for their proteasome-dependent degradation (Swatek and Komander, 2016). However, depending on the size and the level of complexity of the Ub chain(s), the outcome can be different: some evidences indicate that multiple short- or branched-chains are more prone to induce protein degradation, while a single chain or a mono-ubiquitination tags can have a major role in intracellular signalling. Ubiquitination has been linked to DNA damage repair, transcriptional regulation, autophagy, activation of kinases and signalling, and regulation of the endosomal compartments during their internalization

(Johnson, 2002; Sun and Chen, 2004; Grumati and Dikic, 2018). Similarly, SUMOylation has been associated to many important cellular functions, such as nuclear trafficking, DNA transcription, DNA damage repair, regulation of the cell cycle, and innate immunity (Flotho and Melchior, 2013).

Interestingly, ubiquitination and SUMOylation often cooperate. This is the case of DNA damage repair, where ubiquitination and SUMOylation tightly control the activity of the DNA damage repair machinery (Galanty *et al.*, 2009; Morris *et al.*, 2009). Alternatively, ubiquitination and SUMOylation cooperate to induce protein degradation, as in the case of PML and its oncogenic counterpart PML-RARA, where the SUMOylated PML is degraded after the specific ubiquitination of its SUMO chain (Lallemand-Breitenbach *et al.*, 2008; Rabellino *et al.*, 2012; Rabellino and Scaglioni, 2013). Finally, ubiquitination and SUMOylation can counteract each other's function, as for MYC, where its SUMOylation inhibits the interaction with the ubiquitination machinery (Rabellino *et al.*, 2016).

SUMO-1 and the regulation of endothelial cells

SUMO proteins are evolutionary conserved across the whole eukaryotic kingdom and play important role in every aspect of cell physiology, including angiogenesis (Flotho and Melchior, 2013). It has been shown that *SUMO1* expression in porcine aortic endothelial cells (PAECs) promotes cell proliferation, cell migration, and resistance to apoptosis, in a SUMO1-dose-dependent manner. Importantly, expression of SUMO1 improves the ability of the endothelial cells to form tubes and branching points, underlying its role in angiogenesis. Accordingly, similar observations were also obtained by studying the *SUMO1* knock in mouse model, which exhibits a higher neo-vasculogenesis capacity than the control counterpart (Yang *et al.*, 2013). Taken together, these data indicate that SUMO1 is directly involved in the regulation of endothelial cells during angiogenesis. It is worth noting that it has been established that SUMO2 and SUMO3 can compensate for SUMO1 functions (Evdokimov *et al.*, 2008). Based on these observations it will be interesting to determine whether SUMO2/SUMO3 can compensate for the role of SUMO1 in angiogenesis or whether SUMO1 is indispensable for this process.

The regulation of VEGFR by ubiquitination and SUMOylation

One of the most important factors involved in angiogenesis is VEGF and its associated receptors, VEGFRs. Particularly, VEGFR2 is a major key player in both physiological and pathological angiogenesis and it is massively regulated by PTMs, including phosphorylation, ubiquitination and SUMOylation. In particular, the binding of VEGF to VEGFR2 causes the activation of the receptor through multiple phosphorylation events, followed by its ubiquitination and internalization *via* clathrin-mediated/endosomal structures (Duval *et al.*, 2003; Ewan *et al.*, 2006; Bruns *et al.*, 2010). It has been shown that VEGFR2 ubiquitination is required of its internalization, and once internalized, the receptor can be degraded through the lysosomes or can be recycled back to the plasma membrane (Bruns *et al.*, 2010; Jopling *et al.*, 2014). Interestingly, it has been recently reported that VEGFR2 can be ubiquitinated and degraded also in a VEGF-independent manner: in this case, the E1 ubiquitin-activating enzyme UBA1 controls the basal levels of VEGFR2 as well as its activity (Smith *et al.*, 2017). These findings suggest that ubiquitination can independently regulate the availability of the VEGFR2 receptor during angiogenesis. Finally, the balance between the ubiquitinated and de-ubiquitinated status of VEGFR has also very important repercussions on endothelial cells during angiogenesis. Lately, it has been demonstrated the de-ubiquitinating enzyme USP8 plays a central role in the regulation of this balance. Accordingly, USP8 modulates the trafficking of VEGFR2 through the endosome and lysosome compartments regulating the degradation of the receptor (Smith *et al.*, 2016).

Based on the studies summarized here, it is clear that ubiquitination plays a major role in the regulation of VEGFR signalling and trafficking in angiogenesis. Interestingly, a study using a knock out mouse model of the de-SUMOylase *SENP1* has described that also SUMOylation regulates the intracellular trafficking of VEGFR (Zhou *et al.*, 2018). In particular, it has been demonstrated that SENP1 protein levels increase in

vascular endothelial cells in response to ischaemia. Further analyses have shown that *SENP1* knock down in endothelial cells leads to an increase of the SUMOylation levels of VEGFR2, and to an impaired VEGFR2-dependent angiogenic signalling. Specifically, the SUMOylation of residue K1270 in VEGFR2 causes the receptor to accumulate in the Golgi compartment reducing its localization on the cell membrane (Zhou *et al.*, 2018). Accordingly, analyses performed in diabetic mouse models, indicated that *SENP1* expression was drastically reduced leading to an increase of VEGFR2 SUMOylation and inhibition of its signalling. All together, these data indicate that SUMOylation inhibits VEGFR2-dependent angiogenesis (Fig. 18.2), suggesting that the balance between the SUMOylated and non-SUMOylated VEGFR2 dictates its activation during angiogenesis (Zhou *et al.*, 2018).

Interestingly, SUMOylation can also indirectly control VEGFR by regulating its gene expression. In this context, it has been reported that the master regulator of lymphangiogenesis PROX1 induces *VEGFR* expression in a SUMO-dependent manner (Pan *et al.*, 2009). Based on these evidences, we conclude that during angiogenesis, SUMOylation can positively control the activity of VEGFR by regulating its spatial localization and/or its gene transcription. Further analyses are needed to identify the SUMO E3 ligases that control these processes.

The regulation of NOTCH during angiogenesis by ubiquitination and SUMOylation

NOTCH proteins (NOTCH1–4) are transmembrane receptors that operate in many cell types and at various stages during development. After the binding of one of their ligands, NOTCH undergoes a catalytic cleavage that releases its intracellular domain. At this point, the NOTCH intracellular domain (NOTCH-ICD) translocates into the nucleus where it forms an active transcriptional complex by interacting with CSL/RBP-J and MAML (Siebel and Lendahl, 2017) (Fig. 18.2). Extensive analyses of the NOTCH signalling have underlined its pivotal role in development and angiogenesis. Accordingly, NOTCH signalling regulates the transcription of a series of genes involved in angiogenesis, including VEGFR and Ephrins (Siekmann and Lawson, 2007; Kofler *et al.*, 2011). These observations have been also validated in the *Notch1–4* knock out mouse models, which exhibit severe defects in angiogenesis and vascular remodelling (Krebs *et al.*, 2000).

Several PTMs regulates NOTCH signalling, including ubiquitination and SUMOylation. In particular, different ubiquitin E3 ligases have been associated to its degradation. However, it is not clear whether these ubiquitination processes directly impact or not on the angiogenic role of NOTCH (Lai, 2002). So far, the only ubiquitin E3 ligase that has been linked to the angiogenic activity of NOTCH is FBW7 (Tsunematsu *et al.*, 2004) (Fig. 18.2). Accordingly, it has been shown that the *Fbw7* knock out mouse model is embryonically lethal, and embryos die at early stage with massive abnormalities in the vascular development. Particularly, *Fbw7* knock out embryos show an impaired vascular remodelling in the yolk sac and brain, with the ablation of major veins formation. Molecular analyses revealed that this phenotype is caused by Notch4 accumulation in the embryos. The accumulation of Notch4 results in turn in the over-expression of Hey1, a transcriptional repressor directly regulated by Notch4 and involved in vascular development and angiogenesis. Taken together, these data highlight the role of the ubiquitin ligase FBW7 in the positive regulation of angiogenesis, by directly regulating the Notch4-Hey1 signalling pathway (Tsunematsu *et al.*, 2004).

Recently, it has also been shown that SUMOylation regulates angiogenesis by modulating NOTCH activity. For instance, in endothelial cells, the binding of the ligand DLL4 to NOTCH1 leads to *VEGF* transcriptional repression and to the inhibition of the VEGF signalling pathway (Fig. 18.2). This process impairs the angiogenic potential of endothelial cells (Benedito *et al.*, 2009). Both *in vitro* and *in vivo* evidence has shown that inactivation of the de-SUMOylase SENP1 reduces cell motility, spheroid sprouting and capillary formation. This phenotype was associated to an increase of NOTCH1 activity, linking the function of SENP1 to NOTCH1 during angiogenesis. Noteworthy, the C-terminal domain of NOTCH-ICD contains several SUMO-binding motifs, and biochemical analyses confirmed that NOTCH-ICD

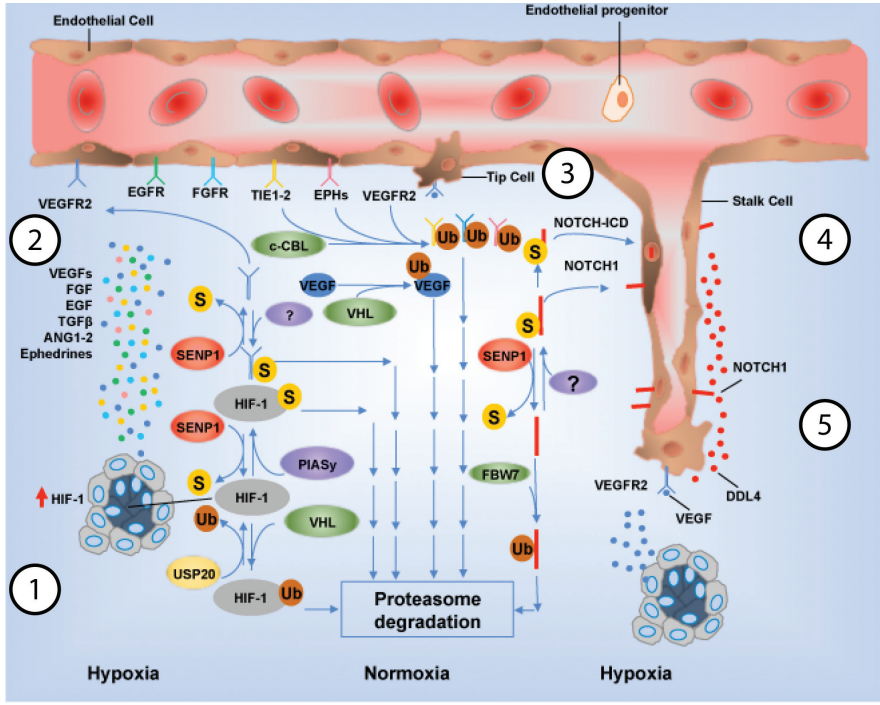

Figure 18.2 Ubiquitination and SUMOylation during angiogenesis. (1) Pro-angiogenic stimuli, such as a hypoxic environment, stimulate the expression of HIF-1 that in turn promotes the transcription of pro-angiogenic genes including *VEGF*. The level of HIF-1 depends on its ubiquitination/SUMOylation status: in normoxic or non-angiogenic conditions, the ubiquitin ligase VHL ubiquitinates HIF-1α directing it to proteasomal degradation. Moreover, SUMOylation PIASy-dependent leads to proteasome-dependent degradation of HIF-1α. On the other hand, both de-ubiquitination by ubiquitin-specific proteases (i.e. USP20) and de-SUMOylation by SENP1 are necessary to sustain HIF-1α stability and activity during angiogenesis. (2) HIF-1 leads to the transcription of genes encoding for pro-angiogenic factors such as *VEGF*, *FGF*, *TGF-β*, *ANG1–2*, and *Ephedrines*. These pro-angiogenic factors bind to the corresponding receptors exposed on the vascular endothelial cells (VEGFR2, FGFR, TIE1–2, EPHs). (3) The angiogenic factors gradient induces the migration of specialized endothelial cells (tip cells) that will begin the sprouting of new vessels. De-SUMOylation of VEGFR2 by SENP1 is required for angiogenesis, while SUMOylation of VEGFR2 promotes its degradation. However, the specific SUMO E3 ligase of VEGFR2 is still unknown. VEGFR, TIE1–2 and EPHs, are directed to degradation by c-CBL-dependent ubiquitination in non-angiogenic conditions. Under normoxic conditions also VEGF is targeted for degradation by VHL-dependent ubiquitination. (4) Endothelial progenitors differentiate into proliferative stalk cells that build up the main body of the new vessels. (5) To stop the process of sprouting and tube formation, VEGF induces the tip cells to secrete DLL4 ligand that will bind to NOTCH receptor on stalk cells. Activation of NOTCH, and its cleavage into NOTCH-ICD followed by its translocation into the nucleus, leads to *VEGFR2* transcriptional repression thereby suppressing endothelial proliferation. SUMOylation of NOTCH1 is required for its cleavage into NOTCH-ICD contributing to the anti-angiogenic activity of NOTCH. While SENP1 is responsible for the de-SUMOylation of NOTCH, its specific SUMO E3 ligase has not been identified yet. Ubiquitination of NOTCH by FBW7 causes its inhibition and degradation.

is indeed SUMOylated on three residues (K2049, K2150 and K2252). Moreover, it has been shown that in endothelial cells, NOTCH-ICD exists predominately in its SUMOylated form and that SUMOylation of NOTCH1 is necessary for the cleavage and the formation of NOTCH-ICD upon DLL4 activation. Furthermore, SUMOylation increases NOTCH-ICD transcriptional activity and half-life, potentiating its anti-angiogenic signal. These data indicate that SUMOylation is a fundamental step for the positive regulation of NOTCH1 during angiogenesis. According to this hypothesis, SENP1 interacts with NOTCH1 and regulates its level of SUMOylation, modulating

its anti-angiogenic activity (Zhu *et al.*, 2017). Interestingly, the SUMO E3 ligase involved in the SUMOylation of NOTCH1 has not been identified yet and further analyses are needed in order to address this topic.

Noteworthy, SENP1 activity has also been directly correlated to erythropoiesis, where SENP1-dependent de-SUMOylation of GATA1 is required during embryonic erythropoiesis (Yu *et al.*, 2010).

The VEGFR and NOTCH converging angiogenic signalling is regulated by ubiquitination

Recent studies have demonstrated that the homeostasis between pro-angiogenic and anti-angiogenic signalling in endothelial cells is maintained by the balance between VEGFR and NOTCH signalling (Hellström *et al.*, 2007; Lobov *et al.*, 2007; Suchting *et al.*, 2007; Benedito *et al.*, 2009; Sakaue *et al.*, 2017). In order to identify proteins involved in VEGFR activation that can also impact NOTCH signalling, human umbilical vein endothelial cells (HUVECs), a well-established cellular model used to study angiogenesis, were intensively screened in order to identify proteins that are up-regulated upon VEGFR activation but that can also impact NOTCH signalling. The findings revealed that when HUVEC cells are stimulated with VEGF, the zinc finger protein BAZF is up-regulated, leading to the induction of filopodia, cell elongation and the formation of a cellular network typical of angiogenesis. Accordingly, BAZF also negatively controls NOTCH signalling pathway, promoting VEGF-dependent angiogenesis. Mechanistically, BAZF interacts with the NOTCH signalling factor CBF1 in a VEGFR-dependent way. Indeed, BAZF binding suppresses the transcriptional activity of CBF1 by releasing it from the promoters of the target genes. In addition, BAZF induces the ubiquitination of CBF1, targeting it for cytoplasmic translocation and proteasomal degradation. Further analyses showed that BAZF mediates the formation of a complex between CBF1 and the ubiquitin E3 ligase CUL3, with this effect being triggered by VEGFR activation. This finding indicates that VEGF-dependent angiogenesis induces CUL3-dependent ubiquitination and degradation of CBF1 using BAZF as mediator. Accordingly, it has been shown that *Bazf* knock out mice suffer from angiogenic defects, up-regulation of the Notch signalling during development, and impaired wound healing during adulthood (Ohnuki *et al.*, 2012).

Taken together, these data demonstrate that the ubiquitination machinery is able to regulate simultaneously pro- and anti-angiogenic factors in order to guarantee a fine-tuning of a complex mechanism such as angiogenesis.

Hypoxia-induced angiogenesis

Hypoxia-induced angiogenesis is a well-established hallmark of cancer (Hanahan and Weinberg, 2011). Accordingly, HIF-1, the master regulator of hypoxia, is up-regulated in several human cancers, and it associates with poor prognosis (Semenza, 2012). Interestingly, also HIF-1 is massively regulated by PTMs including ubiquitination and SUMOylation.

HIF-1 is a heterodimeric protein composed by the HIF-1α and HIF-1β subunits. While HIF-1β is constitutively expressed, HIF-1α is tightly regulated by oxygen availability. Under hypoxic conditions, HIF-1α translocates from the cytosol to the nucleus where it interacts with HIF-1β promoting the transcription of hypoxic genes, including *VEGFR* (Eguchi *et al.*, 1997). It has been established that, in normoxic conditions, HIF-1α expression is usually kept at undetectable levels. Accordingly, oxygen induces HIF-1α poly-ubiquitination and degradation by the ubiquitin E3 ligase complex PHD/VHL/VBC (Masoud and Li, 2015) (Fig. 18.2). Even though other pathways contribute to regulate HIF-1α stability (for example by regulating its mRNA levels or its translation), HIF-1α ubiquitin-dependent degradation represents the major control mechanism. The mechanism of HIF-1α regulation by the ubiquitination machinery has been extensively elucidated. Briefly, in normoxic conditions, the proline residues P402 and P564 of HIF-1α are hydroxylated by the dioxygenases PHD1–3 in an oxygen-dependent way (Epstein *et al.*, 2001; Ivan *et al.*, 2001). In turn, this PTM activates the ubiquitination of HIF-1α by VHL, leading to its proteasome-dependent degradation (Maxwell *et al.*, 1999; Ohh *et al.*, 2000; Tanimoto *et al.*, 2000). Furthermore, HIF-1α levels can be regulated by mechanisms independent from the classic PHD/VHL machinery. For example, the chaperone protein HSP90 protects HIF-1α from degradation. It has been shown that RACK1 mediates the dissociation of HSP90 from HIF-1α, which is in turn recognized by the ubiquitin ligase Elongin-B/C and

degraded (Liu *et al.*, 2007). Alternatively, the kinase PLK3 regulates HIF-1α levels during hypoxia by phosphorylating the serine residues S576 and S657 thereby inducing the degradation of HIF-1α (Xu *et al.*, 2010a). Additionally, the transcription factor TAp3 is able to directly interact with HIF-1α, promoting the recruitment of the ubiquitin ligase MDM2, followed by its poly-ubiquitination and degradation in an oxygen-dependent manner (Amelio *et al.*, 2015).

Since ubiquitination is largely involved in the control of HIF-1α levels, de-ubiquitinating enzymes play an equally important role in maintaining the physiological level of HIF-1α. In this context, the de-ubiquitinating enzyme USP20 is able to interact with HIF-1α and to regulate the transcription of downstream genes of HIF-1α such as *VEGF* (Li *et al.*, 2005) (Fig. 18.2). Similarly, other de-ubiquitinating enzymes such as USP8 and UCHL1 were shown to modulate HIF-1α levels and stability (Troilo *et al.*, 2014; Goto *et al.*, 2015).

Owing to the major role that HIF-1 plays in tumour-induced angiogenesis, the development of drugs able to promote its degradation has gained a lot of interest. In this scenario, the small molecules SCH66336 and Apigenin disrupt the interaction of HSP90 with HIF-1α, therefore inducing HIF-1α degradation (Osada *et al.*, 2004; Han *et al.*, 2005; Melstrom *et al.*, 2011). Moreover, other small molecules were found able to activate or increase the activity of the ubiquitin ligase complex. This is the case of the small molecule LW6, which increases the expression of VHL with a mechanism that has not been clarified yet (Lee *et al.*, 2010).

Similar to ubiquitination, also SUMOylation has been suggested to regulate HIF-1α levels, however, it is not clear whether SUMOylation increases or decreases HIF-1α stability. HIF-1α SUMOylation on the residues K391 and K477 was described for the first time in 2004, when it was proposed that the binding of SUMO1 to HIF-1α promotes its stabilization and transcriptional activity (Bae *et al.*, 2004). Similarly, the protein RSUME can SUMOylate HIF-1α, increasing its stability. RSUME is upregulated on hypoxic stress and promotes SUMO conjugation by interacting with UBC9 (Carbia-Nagashima *et al.*, 2007). Furthermore, the SUMO E3 ligase CBX4 increases hypoxia-induced *VEGF* expression and angiogenesis by SUMOylating HIF-1α on the residues K391 and K477, increasing its transcriptional activity. These results were also corroborated by the observation that CBX4 expression positively correlates with the level of *VEGF* expression, angiogenesis and over-all survival in hepatocellular carcinoma patients (Li *et al.*, 2014).

Despite these observations, other results indicated that the binding of SUMO1–3 to HIF-1α negatively regulates its transcriptional activity without altering its half-life (Berta *et al.*, 2007). A different and complex scenario has been reported about the effects of SENP1-dependent de-SUMOylation of HIF-1α. It has been shown that the de-SUMOylation of HIF-1α by SENP1 inhibits the interaction between HIF-1α and VHL, resulting in the stabilization of HIF-1α, therefore suggesting that HIF-1α SUMOylation promotes its degradation (Cheng *et al.*, 2007). These data were confirmed by *Senp1* knock out mice, which showed a lower induction of HIF-1 signalling (Xu *et al.*, 2010b). Similarly, SENP1 stabilizes HIF-1α levels and downstream signalling during myocardial ischaemia/reperfusion injury (Gu *et al.*, 2014). Taken together, these results indicate that de-SUMOylation plays a pivotal role in maintaining HIF-1α levels during angiogenesis and suggesting that SUMOylation might directly signal for the ubiquitination/degradation of HIF-1α. However, this evidence contradicts the hypothesis that SUMOylation is required for maintaining the stability of HIF-1α, and additional work is needed to solve these inconsistencies.

Whether PIAS family members contribute to regulate HIF-1 activity is also controversial. PIAS proteins (PIAS1–3 and PIASy) are SUMO E3 ligases involved in the regulation of several cellular functions, including angiogenesis, and they have been also associated to human malignancies (Rabellino *et al.*, 2017). It has been reported that in hypoxic condition, PIASy interacts with HIF-1α triggering its SUMOylation thereby promoting its degradation (Kang *et al.*, 2010) (Fig. 18.2). Opposite results have been reported regarding the interaction of PIAS3 with HIF-1α. It has been shown that PIAS3 positively regulates HIF-1α transcriptional activity, however, this function is independent of the SUMO E3 ligase activity of PIAS3 (Nakagawa *et al.*, 2016). Taken together, these controversial observations suggest that different PIAS family members might have different roles in HIF-1α regulation and activity. These

controversies need to be addressed in more detail in the future.

Role of the SUMO E3 ligase PIAS1 in angiogenesis

The PIAS SUMO E3 ligases have been directly associated to angiogenesis independently of their ability to regulate HIF-1. In particular, the role of PIAS1 in angiogenesis has been recently characterized using the *Pias1* knock out mice model (Constanzo *et al.*, 2016). It has been demonstrated that ablation of *Pias1* in mice is embryonically lethal due to major defects in the vascular plexus of the yolk sac and thus in angiogenesis and erythropoiesis. Accordingly, *Pias1* null mice embryos showed a significant reduction in blood vessel size and branching, which correlates with a low expression of the endothelial activation markers Angp2 and Vcam-1 (Constanzo *et al.*, 2016). Interestingly, *Vegfr* levels were up-regulated in the yolk sac of *Pias1* null mice suggesting a compensatory mechanism required for the activation of angiogenesis. These data were confirmed by *in vitro* experiments performed in HUVEC cells. Accordingly, expression of *PIAS1* in this endothelial cellular model induces the expression of angiogenic markers and the down-regulation of anti-angiogenic genes, while *PIAS1* knock down reduces the ability of HUVECs to form *de novo* tubes and branching structures (Constanzo *et al.*, 2016). These data underline the role of PIAS1 in regulating angiogenesis during embryogenesis, however it has not been described whether its function relies on its SUMO E3 ligase activity.

This issue was investigated by a recent work in which PIAS1 was described as the SUMO E3 ligase of MYC (Rabellino *et al.*, 2016). The transcription factor MYC is a master transcription regulator involved in several cellular functions, including angiogenesis, and it is causally implicated in several human malignancies (Baudino *et al.*, 2002; Dang, 2012). It has been described that the PIAS1-dependent SUMOylation of MYC increases its half-life and its transcriptional activity. Accordingly, analyses of *Pias1* null mice recapitulate the characteristics of the *Myc* null mouse model, showing a developmentally delayed and hypoplastic yolk sac, lacking the characteristic microvillar structures of the vascular plexus (Rabellino *et al.*, 2016). Taken together, these data strongly suggest that PIAS1 plays a fundamental role in angiogenesis, and this activity is likely due to its SUMO E3 ligase activity. Further studies will shed more light on the role of this SUMO ligase.

PML in angiogenesis

The promyelocytic leukaemia gene *PML* was described for the first time as product of the chromosomal translocation t(15;17)(q24;q21) in acute promyelocytic leukaemia (APL) (Piazza *et al.*, 2001). Soon, it became clear that PML is involved in the positive regulation of several tumour suppressive functions and other cellular processes, including angiogenesis (Salomoni and Pandolfi, 2002; Rabellino and Scaglioni, 2013). PML is massively regulated by several PTMs, including ubiquitination and SUMOylation, which influence its activity, functions and regulation, including the formation of the functional units of PML, known as PML Nuclear Bodies (PML-NBs) (Bernardi and Pandolfi, 2007; Rabellino and Scaglioni, 2013). It has been shown that PML negatively controls angiogenesis through the inhibition of *HIF-1a* translation by repressing mTOR activity (Bernardi *et al.*, 2006). These findings elegantly described a new role of PML in controlling angiogenesis, however, whether PML ubiquitination or SUMOylation take part of this process is not clear.

In most recent years, however, a new layer of complexity regarding how PML regulates the mTOR/HIF-1α pathway has been added. PML degradation is tightly regulated by a series of PTMs, including phosphorylation, SUMOylation and ubiquitination that occur in a precise spatial and temporal order (Scaglioni *et al.*, 2006; Yuan *et al.*, 2011; Rabellino and Scaglioni, 2013). It has been shown that under hypoxia conditions, the ubiquitin E3 ligase CUL3 substrate KLHL20 co-operates with CDK1/2 and with the isomerase PIN1 in order to induce PML ubiquitination and degradation in a HIF-1 dependent way. In this scenario, it has been also demonstrated that the KLHL20-dependent PML degradation promotes neo-angiogenesis (Yuan *et al.*, 2011), pointing toward anti-angiogenic properties of PML. Furthermore, it has been shown that this mechanism is counteracted by SCP phosphatases, which de-phosphorylate PML blocking the KLHL20-dependent degradation, which in turn will inhibit HIF-1 signalling in a mTOR-dependent way (Lin *et al.*, 2014). Interestingly, it has also been shown that PML-NBs are the site of the interaction

between CUL3 and CBF1 during the regulation of the VEGF-dependent NOTCH signalling (Ohnuki *et al.*, 2012), suggesting that PML and PML-NBs might regulate angiogenesis through several pathways and mechanisms. Despite the fact that SUMOylation has not been directly implicated in this process, based on the data available to date, we speculate that SUMOylation might be critical for the correct outcome of the process.

Finally, the role of PML in the inhibition of angiogenesis has been also demonstrated by its role in the positive-regulation of the anti-angiogenic factor Interferon-α (INF-α). Degradation of PML massively reduces the angiostatic effects of INF-α. Interestingly, INF-α stimulation leads to the induction of PML, which in turn activates STAT1 and STAT2 anti-angiogenic activity by promoting STAT1/2 ISGylation (Hsu *et al.*, 2017), an ubiquitin-like modification which functions and regulation are still largely unknown (Villarroya-Beltri *et al.*, 2017).

The regulation of TIE2 and FGFR by c-CBL ubiquitination

The TIE2 receptor belongs to the family of the tyrosine kinase receptor (RTK) and is predominantly expressed on the surface of endothelial and hematopoietic cells (Dumont *et al.*, 1992). The binding of TIE2 to its ligand ANG1 activates a downstream signalling cascade that positively regulates angiogenesis (Jones *et al.*, 2001). Accordingly, *Tie2* null mice die at early embryonic stage due to the lack of the formation of the capillary plexus and severe heart defects (Dumont *et al.*, 1994). It has been shown that ubiquitination regulates the turnover of TIE2 in a ligand-specific fashion. Indeed, the binding of ANG1 to TIE2 is sufficient to induce the activation of the receptor and its subsequent ubiquitination by the Ub E3 ligase c-CBL (Wehrle *et al.*, 2009).

FGFR is another RTK that plays an essential role in angiogenesis (Yang *et al.*, 2015). Similar to TIE2, ubiquitination regulates the turnover of FGFR and modulates its downstream signalling. Also, in this case, the ubiquitin E3 ligase involved in the ubiquitination of FGFR is c-CBL (Wong *et al.*, 2002; Haugsten *et al.*, 2008) (Fig. 18.2).

While these data indicate that ubiquitination of TIE2 and FGFR is necessary to regulate them during angiogenesis, it is not clear whether SUMOylation may modulate the activity and the turnover of these receptors.

Ubiquitination and de-ubiquitination of the WNT signalling in angiogenesis

The WNT signalling pathways control a wide spectrum of cellular functions, including cell proliferation and migration. WNT pathways can be classified in canonical/β-catenin-dependent and non-canonical pathways, and they both regulate and control angiogenesis. Accordingly, both *in vitro* and *in vivo* studies demonstrated that WNT and its Frizzled receptors regulate the migration of endothelial cells during angiogenesis (Zerlin *et al.*, 2008). In the canonical pathway, WNT up-regulates the level of cytosolic β-catenin by inhibiting its ubiquitin-dependent degradation (Li *et al.*, 1999). The WNT-dependent accumulation of β-catenin promotes the nuclear translocation of β-catenin where it activates the transcription of genes involved in cell growth regulation and pro-angiogenic genes, such as *VEGF* and *IL-8* (Tetsu and McCormick, 1999; You *et al.*, 2002; MacDonald *et al.*, 2009). Hence, the regulation of the β-catenin is critical, and ubiquitination plays a central role. It has been shown that c-CBL induces the ubiquitination of nuclear β-catenin thereby promoting its degradation, therefore negatively regulating angiogenesis. Interestingly, the re-localization of c-CBL from the cytoplasm to the nucleus it is induced by WNT (Chitalia *et al.*, 2013), suggesting the activation of a feedback mechanism that controls this pathway. The re-localization of c-CBL is induced by the WNT-dependent phosphorylation of c-CBL on the tyrosine Y731, which promotes c-CBL dimerization, binding to the β-catenin and the nuclear re-localization (Shivanna *et al.*, 2015).

Another ubiquitination-dependent regulation of the WNT signalling during angiogenesis has been described for the de-ubiquitinase Gumby. Noteworthy, the *Gumby* mouse mutants show severe angiogenic impairment during embryogenesis (Rivkin *et al.*, 2013). Accordingly, *Gumby* knock out embryos die at early stage due to the insufficient development of the branching of the vascular system. It was previously reported that Gumby interacts with DVL2, which also plays an important role in WNT signalling (Rual *et al.*, 2005). Further analyses performed in both *in vitro* and *in vivo* setting, indicated that Gumby negatively

regulates WNT activity in endothelial cells, compromising their angiogenic potential (Rivkin *et al.*, 2013).

Studies reported that SUMOylation of the co-repressors TBL1 and TBLR1 led to the activation of the WNT signalling in a β-catenin-dependent manner. Conversely, the SENP-1-dependent de-SUMOylation of TBL1 and TBLR1 inhibits this mechanism (Choi *et al.*, 2011). Similarly, it has been demonstrated that the SUMO E3 ligase PIASy SUMOylates the WNT downstream effector TCF4, enhancing the β-catenin-dependent transcriptional activity of TCF4 (Yamamoto *et al.*, 2003). These findings suggest that SUMOylation may play a role in the regulation of the WNT signalling, however, its role in this context has not been elucidated yet.

Ephrins regulation during angiogenesis

The binding of the membrane ligand Ephrins to their receptors initiates a series of downstream signalling that regulate the fate of endothelial cells during angiogenesis. Ephrins receptors are RTKs subdivided in two subclasses, EPHA and EPHB, activated by the ligands EphrinA and EphrinB, respectively. In vertebrates, a total of ten EPHA and six EPHB are expressed, and several *in vitro* and *in vivo* studies have underlined the role of EPH receptors and their ligands in the regulation of angiogenesis (Pasquale, 2005). Because Ephrin ligands are anchored to the cell membrane, the interaction with their receptors requires a cell-to-cell interaction. Once the activated receptor induces downstream signalling cascade, the stimulus is extinguished through a process that includes the receptor internalization and its degradation *via* ubiquitination (Litterst *et al.*, 2007). Similar to other RTKs involved in angiogenesis, it has been shown that c-CBL is the ubiquitin E3 ligase responsible for EPHB receptor ubiquitination (Fasen *et al.*, 2008) (Fig. 18.2). To date, however, there are no evidences that SUMOylation is involved in the regulation of Ephrins.

The role of extracellular Ub in the regulation of angiogenesis

Extracellular Ub regulates immune responses during inflammation, organ injuries and fibrosis, and elevated plasma levels of Ub correlate with several human pathologies (Asseman *et al.*, 1994; Takagi *et al.*, 1999; Majetschak *et al.*, 2005; Sujashvili, 2016). It has been also shown that the extracellular Ub promotes angiogenesis. Accordingly, using cardiac micro-vascular endothelial cells (CMECs), which is the major cell type involved in cardiac angiogenesis, it has been demonstrated that extracellular Ub promotes the expression of *VEGFR*, thereby triggering cytoskeletal rearrangement, cell migration and tube formation (Steagall *et al.*, 2014). These observations raise several questions regarding the molecular mechanisms by which extracellular Ub activates angiogenesis. Such aspects should be of potential interest in view of future therapeutic applications of this discovery.

Conclusions and remarks

Ubiquitination and SUMOylation are PTMs that play fundamental roles in every aspect of human physiology. Here we have summarized their major roles in angiogenesis known so far. Because of the extreme significance of angiogenesis in tumour development and in other human diseases, both ubiquitination and SUMOylation might represent valuable candidate targets for the generation of new, more effective drugs for the treatment of these pathologies.

In particular, even though SUMOylation has being known for more than two decades (Geiss-Friedlander and Melchior, 2007), it is still a fairly unknown process, and its involvement in angiogenesis regulation remains largely uncharacterized. More efforts should be made in order to shed light on this important PTM and its contribution to angiogenesis.

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