Post-translational modifications and molecular interactions regulating VEGFR2 activity

PhD Thesis

Scuola Normale Superiore

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SYNOPSIS

The work described in this thesis has been mainly focused on the study of a key molecule involved in blood vessel formation, the tyrosine kinase receptor VEGFR2.

Considering that VEGFR2 biology should be tighly regulated to allow proper blood vessel formation and maintenance, we investigated two different mechanims influencing VEGFR2 activity: post translational modification and receptor complex formation.

Since VEGFR2 biology is governed through protein modication, mainly phosphorylation, we decided to investigate the possible role of acetylation in VEGFR2 activity. Combining biochemical and proteomic studies, we showed that VEGFR2 is modified by acetylation. Starting from this observation, we further investigated the impact of VEGFR2 acetylation on protein stability and phosphorylation in response to ligand. These findings are of particular interest, since, to our knowledge, this is the first report that a tyrosine kinase receptor might be regulated by acetylation.

Additionally, we decided to elucidate the interaction of VEGFR2 with its coreceptor Neuropilin1, with particular attention to the Neuropilin1 molecule, by taking advantage of the FRET imaging technique. Collectively, our work characterizes VEGFR2-Neuropilin1 and Neuropilin1-Neuropilin1 complex formation in response to VEGFs and SEMA3A. Altough we do not provide direct evidence for Neuropilin1 direct signalling, our data suggest that Neuropilin1 oligomer formation might be a key step in Neuropilin1 biology.

Chapter 1

INTRODUCTION

INTRODUCTION

Blood vessel development

The blood vessel network supplies oxygen and metabolites, as well as immune cells, to all organs and tissues of our body. Establishing a functional vascular system is a complex event, governed by a perfect orchestration of cellular and molecular players.

Vessel development could be reduced to different processes, often overlapping in nature: formation, branching, remodelling-pruning, stabilization, and specialization (Jain, 2003).

De novo vessel formation could be explained by at least two mechanisms, vasculogenesis and angiogenesis (Figure 1.1) (Folkman, 2006). During embryonic development, haemangioblasts migrate and aggregate to give rise to blood islands (vasculogenesis), characterized by endothelial cell (EC) progenitors in the outer layer and haematopoietic progenitors in the inner layer (Choi et al., 1998; Huber et al., 2004; Risau, 1991; Vogeli et al., 2006). Cells of the primary plexus stain positive for specific markers, such as CD34, Tie2, Sca-1 and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) (Coultas et al., 2005; Faloon et al., 2000; Lancrin et al., 2009). The primitive vascular plexus undergoes remodelling through angiogenesis; this process combines sprouting, bridging and branching that convert existing primitive vessels in a mature circulation. Vascular remodelling requires proliferation, survival, migration, and differentiation of ECs (Carmeliet, 2003; Cleaver and Melton, 2003; Hanahan, 1997; Risau, 1991). Accordingly, several molecules are involved in these processes, and more than 30 genes, such as Vascular Endothelial Growth Factor (VEGF), Tie 2 and Transforming Growth Factor-β (TGFβ), are essential for vascular development (Carmeliet et al., 1996; Dickson et al., 1995; Dumont et al., 1994; Ferrara et al., 1996). A newly formed vessel network requires

stabilization, provided mainly by the recruitment of mural cells (MC) and the generation of extracellular matrix (ECM) (Cleaver and Melton, 2003; Jain, 2003). At least four different pathways contribute and control this process, essentially providing communication between endothelial and mural cells. They are activated by a ligand/receptor pair interaction: PDGF/PDGFR-β, SIP1/EGD1, Ang-Tie and TGF-β/TGF-βR (reviewed in (Armulik et al., 2005)).

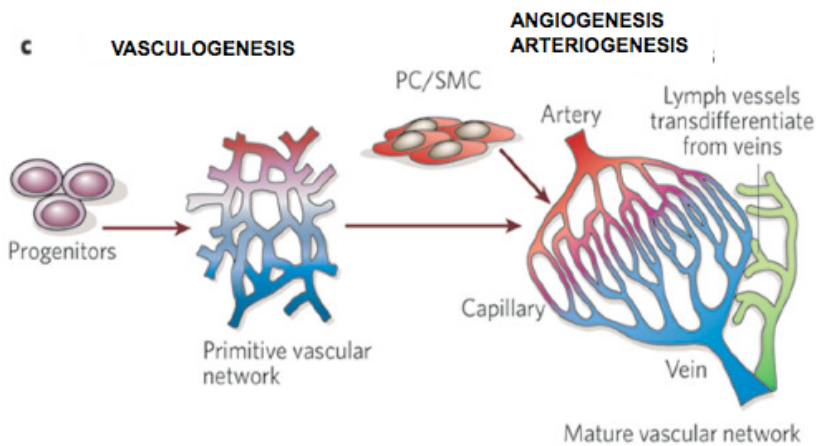

Figure 1.1. Mechanisms of vessel development. A primitive vascular network is formed by endothelial cell progenitors in a process defined as vasculogenesis; blood vessel remodelling, stabilization and specialization during angiogenesis give rise to a mature vessel network. PC: pericytes, SMC: smooth muscle cells. *Adapted from Carmeliet, Nature 2005.*

An additional step in blood vessel formation is vessel specialization, a still poorly characterized tissue- and organ-specific process, which includes the formation of functional junctions between ECs and MCs, capillaries diversification as well as arterious-venous specification (Ruoslahti, 2002). In particular VEGF-signalling promotes arterial specification upstream the Notch pathway (Lawson et al., 2001; Lawson et al., 2002), while the

transcription factor COUP-TFII regulates differentiation towards the venous phenotype, inhibiting Neuropilin1 and Notch pathways (You et al., 2005).
Interestingly, a mature blood vessel network displays a striking parallel with a nerve fiber network, suggesting the existence, even at a molecular level, of common pathways during development. Actually, compelling evidence establishes a growing role for axon guidance molecules even in vessel guidance, complementing and cooperating with canonical pathways; among them, the Semaphorin-Neuropilin1-Plexin and Netrin pathways (Carmeliet and Tessier-Lavigne, 2005).

Pathological and physiological angiogenesis

Blood vessels do not undergo major modifications in healthy adult organism, and angiogenesis is restricted to cycling ovary and placenta during pregnancy. Nonetheless, even though quiescent, ECs and therefore vessels are still angiogenesis-competent, as they can respond to hypoxic and inflammatory stimuli, as it happens during wound healing.
Imbalance of the angiogenic stimulus, both towards excessive and insufficient angiogenesis, is characteristic of several pathologies; as a consenquence, optimal regulation of angiogenesis represents a crucial issue in human physiology and pathology (Figure 1.2).
Excessive angiogenesis accompanies inflammatory and ocular disorders, in addition to tumor growth (Carmeliet, 2005a). The observation that tumor growth relies on blood supply led to the hypothesis that tumor angiogenesis inhibition could provide a successful strategy to treat cancer (Folkman, 1971). Consequently, given the key role in angiogenesis for the VEGF pathway (Ferrara et al., 2003), several VEGF-targeting strategies are exploited in cancer treatment, including neutralizing anti-VEGF and anti-VEGFR antibodies, soluble VEGFRs and tyrosine kinase inhibitors (Ferrara and Kerbel, 2005). Even though several clinical trials are currently exploiting more than 20 anti-VEGF agents, many unresolved issues, such

as development of drug resistance, still limit the efficacy of this therapy (Ellis and Hicklin, 2008; Ellis and Reardon, 2009).

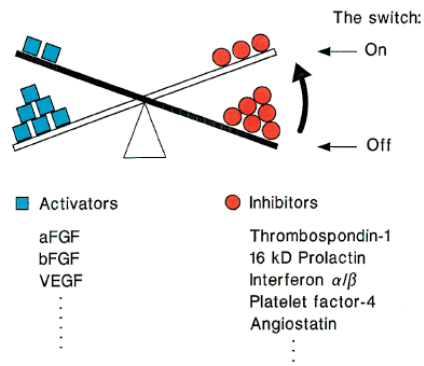

Figure 1.2. The angiogenetic switch. Several molecules are involved in fine balancing angiogenesis in human physiology and pathology, functioning both as positive and negative regulators. *Adapted from Hanahan and Folkman, Cell 1996.*

To overcome these difficulties, while initial anti-angiogenesis strategies exploited mainly EC-targeting agents, more recent findings encourage the targeting of different cell types that indirectly influence tumor angiogenesis, such as cells of the immune system (Carmeliet, 2005a; Ferrara and Kerbel, 2005). In addition, the lack of success of many anti-angiogenic therapies is influenced by the complex interaction between cellular and molecular players that characterizes vessel biology.

Conversely, insufficient angiogenesis characterizes many ischemic diseases, such as limb and heart ischemia, leading to inadequate vessel formation. Therefore, induction of revascularization by delivering pro-angiogenic molecules represents an appealing therapy for patients with ischemic diseases (Baumgartner and Isner, 2001; Isner et al., 2001). Results obtained from several clinical trials, that exploited recombinant protein delivery as well as gene therapy with pro-angiogenic factors,

indicate that the best therapeutical strategy is the combination of pro-angiogenic agents (Rissanen and Yla-Herttuala, 2007). Adeno Associated Virus-based vectors represent, for their tropism and low inflammatory potential, a good candidate for cardiovascular gene therapy (Giacca, 2007). Concluding, several clinical trials are currently ongoing for vessel imbalance-related diseases, and again, due to its role as a master switch in the angiogenic process, VEGF has been extensively studied.
The most interesting features of VEGF and VEGFRs, with particular attention to VEGFR2, will be discussed in the following paragraphs.

Vascular Endothelial Growth Factors

Currently, the VEGF family comprises seven members, namely PlGF (Placenta Growth Factor), VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and sv-VEGF (snake venom VEGF). They act mainly as inducers and modulators of vasculogenesis, angiogenesis and vascular remodelling *in vivo*, even if their influence is not restricted to endothelial cells (Carmeliet and Storkebaum, 2002; Carmeliet and Tessier-Lavigne, 2005; Ferrara et al., 2003; Matsumoto and Claesson-Welsh, 2001) (Figure 1.4).
Active, secreted forms of VEGF members form homo and hetero-dimers, thus increasing signalling diversity; their biological effects are mainly mediated by their receptors VEGFR1, VEGFR2 and VEGFR3, in association with coreceptors, such as those belonging to the Neuropilin, Heparan Sulfate Proteoglycan (HSPG) and integrin families (Jakobsson et al., 2006; Soker et al., 1998; Soldi et al., 1999).

VEGF-A

VEGF-A, also referred as VEGF, was discovered as an essential player in angiogenesis (Ferrara and Henzel, 1989). Remarkably, VEGF-A is required *in vivo* for proper development of the cardiovascular system; even the quantity of VEGF-A is critical in order to obtain normal vessel development,

since VEGF-A is haploinsufficient (Carmeliet et al., 1996; Ferrara et al., 1996). Recent data pointed out the importance of VEGF also in vessel maintenance: VEGF produced by endothelial cells is crucial for vascular homeostasis, through a cell autonomous VEGF signalling (Lee et al., 2007); this observation confirms the pleiotropic role of VEGF in vessel biology.

Importantly, VEGF-A has been originally described as Vascular Permeability Factor (VPF), due to its ability to increase vascular permeability, disrupting vascular barrier integrity (reviewed in (Weis and Cheresh, 2005)).

In accordance with its key role in vessel formation, VEGF availability is controlled at many levels, including transcription, mRNA stability and translation, post translational modification and binding affinity.

At the transcriptional level, many stimuli, including growth factors, p53 mutation, nitric oxide (NO), hormones, cytokines and cellular stress control VEGF expression (Takahashi and Shibuya, 2005). In particular, hypoxia, via Hypoxia Inducible Factor 1 (HIF-1) is the major positive regulator of VEGF expression. Hypoxic conditions induce accumulation of the highly instable α subunit of HIF-1, leading to the formation of an active transcriptional activator that binds to the Hypoxia Responsive Elements (HRE) in the 5' flanking region of the VEGF promoter (Pages and Pouyssegur, 2005).

Hypoxia is a key factor for VEGF stabilization also at mRNA level, controlling mRNA stability by binding of regulatory proteins to the 3' Untranslated Region (UTR), as well as mRNA translation via IRES sequences present in the 5' UTR (Stein et al., 1998).

The observation that Dicer protein mutant mice are characterized by severely compromised blood vessel formation, displaying reduced levels of VEGF-A mRNA, suggest that also miRNA have a role in controlling VEGF levels at the post transcriptional level (Yang et al., 2005).

Human VEGF-A gene, located on chromosome 6, is about 25 Kb long and comprises 8 exons (Tischer et al., 1991). VEGF mRNA undergoes alternative splicing of exons 6 and 7, which encode for binding motifs to heparin and similar molecules. In humans, alternative splicing give rise to at least nine isoforms: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$ (Figure 1.3). In mice all isoforms are one aminoacid shorter than their human counterparts. Another isoform, $VEGF_{165b}$, is generated by exon 8 distal splice site selection, and differs from $VEGF_{165}$ only in the carboxy-terminal six amino acids, thus resulting in a change of the aminoacidic sequence form CDKPRR to SLTRKD (Harper and Bates, 2008).

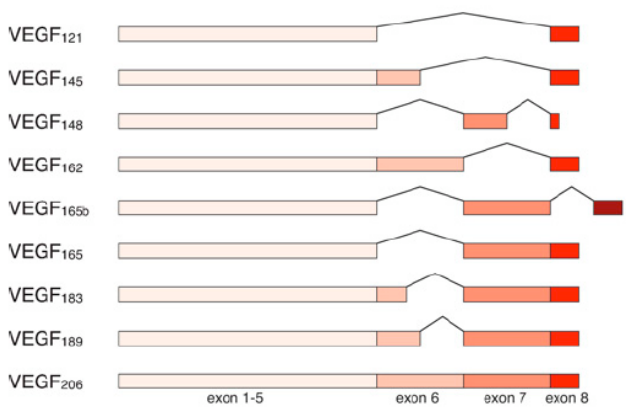

Figure 1.3. VEGF-A splicing. Alternative splicing of VEGF-A mRNA gives rise to almost nine VEGF isoforms, characterized by different length (small numbers) and therefore different binding properties. A different distal splice site selection in exon 8 originates $VEGF_{165b}$. *Adapted from Takahashi and Shibuya, Clinical Science 2005.*

Remarkably, even if $VEGF_{165b}$ seems to bind to VEGFR2 with the same affinity as $VEGF_{165}$, it lacks angiogenic properties, and is therefore defined as anti-angiogenic (Woolard et al., 2004). The molecular bases of $VEGF_{165b}$ properties were recently clarified: $VEGF_{165b}$ is not able to engage Neuropilin1 in the VEGFR2 signalling complex, and is a weak inducer of

VEGFR2 phosphorylation at tyrosines 1054-1059 in the activation loop (Kawamura et al., 2008b).

The $VEGF_{165}$ isoform is the one preferentially expressed, followed by $VEGF_{121}$ and $VEGF_{189}$; how differential splicing of these isoforms is regulated is however largely unknown.

$VEGF_{165}$, a secreted homodimer with moderate affinity for heparin, is a powerful inducer of endothelial cell migration, proliferation, survival and vascular permeability (Leung et al., 1989; Senger et al., 1983). The importance of $VEGF_{165}$ in vessel development is highlighted by the observation that $VEGF_{164}$ mice are normal and healthy, while $VEGF_{120}$ puppies exhibit serious vascular remodelling defects, including defective branching (Stalmans et al., 2002). Altogether, the use of transgenic animals expressing selectively VEGF isoforms indicates that they play distinct roles in vascular morphogenesis and arterial development, a feature depending on their diffusibility and differential interaction with VEGF coreceptors, as discussed later (Carmeliet et al., 1999; Ruhrberg et al., 2002; Stalmans et al., 2002).

In conclusion, VEGF is a key regulator of angiogenesis in health ad disease, stimulating endothelial cell migration and division, and is critical for vascular development. Therefore gene expression regulation, alternative splicing, in addition to other mechanism, contribute to the tight regulation of VEGF activity, crucial for the formation and maintenance of a proper blood vessel network.

It is noteworthy to mention that recent studies also indicate that VEGF might exert its effect on a variety of cell types, besides ECs. For instance VEGF appeared to be critical to prevent motor neuron degeneration, also exerting a direct action on neurons (Zacchigna et al., 2008a). A potent effect of VEGF in promoting survival and regeneration of skeletal muscle cells was also reported (Arsic et al., 2004; Germani et al., 2003). Finally, we

observed an effect of $VEGF_{165}$ on bone marrow (BM) derived $CD11b^+$ cells, as these cells expressed VEGF receptors. In particular we observed the induction of VEGF-dependent BM cells migration, proliferation and secretion of cytokines able to trigger smooth muscle cell recruitment (Zacchigna et al., 2008c).

VEGF has a role also in tissue homeostasis, for instance in liver biology (LeCouter et al., 2003).

PlGF

PlGF is a secreted factor, and like VEGF-A, can undergo differential splicing, giving rise to at least four different isoforms, PlGF-1, PlGF-2, PlGF-3 and PlGF-4 (Yang et al., 2003).

Several gene inactivation studies have revealed that PlGF deficient mice, differently from VEGF-A, are viable and healthy, suggesting that endogenous PlGF is not necessary for vascular development and physiological vessel maintenance in healthy adult organisms (Carmeliet et al., 2001).

At a cellular level, PlGF acts as a pleiotropic factor, stimulating angiogenesis directly targeting endothelial and mural cells, and indirectly through the recruitment of pro-angiogenic cell (Clauss et al., 1996; Hattori et al., 2002; Luttun et al., 2002). Its pleiotropic activity is reflected by the observation that PlGF stimulates EC growth, migration and survival, while is mainly a chemoattractant for BM progenitors (Cao, 2009). PlGF-activity on these cells is mediated by binding to VEGFR1, but not to VEGFR2, and probably to Neuropilin1 and Neuropilin2, two PlGF coreceptors; downstream intracellular signalling switches on a series of pro-angiogenic genes (Autiero et al., 2003b).

Interestingly, animal knock out studies have revealed that PlGF activity is fundamental for the angiogenic and inflammatory switch in various pathologies, such as tumor growth and ischemia, raising the possibility that

PlGF might be a disease-specific angiogenic factor (Carmeliet et al., 2001). Additionally, PlGF levels are undetectable in most organs in healthy conditions, while many cell types, including ECs, SMCs, and BM progenitors express PlGF in pathological conditions (Fischer et al., 2008). Taken together these results suggest that PlGF targeting might reduce pathological angiogenesis without affecting healthy blood vessels. A monoclonal blocking antibody recognizing mPlGF (αPlGF) inhibits the growth and metastasis in more than 10 tumor models in mice. Inhibition of tumor growth depends on the pleiotropic effect of this antibody: i) αPlGF not only inhibits tumor vessel growth, but also causes regression of pre-existing tumor vessels; ii) it is anti-inflammatory, inhibiting recruitment of Tumor Associated Macropharges (TAM); iii) it impairs tumor lymphangiogenesis, mainly through macrophage inhibition (Fischer et al., 2007).

Therefore genetic and pharmacological studies have identified PlGF as a possible therapeutic target for anticancer therapy, particularly in combination with VEGF-VEGFR inhibitors.

VEGF-B

The VEGF-B gene generates, through alternative splicing, two protein isoforms, a heparin-binding isoform of 167 amino acids and a diffusible isoform of 186 amino acids, differing at their C-terminus (Li et al., 2001; Olofsson et al., 1996a; Olofsson et al., 1996b). In addition to VEGFR1, and similar to the VEGFA isoforms containing exon 7 of the VEGFA gene (including VEGF-A_{165}) and to PlGF, both $VEGFB_{167}$ and $VEGFB_{186}$ also bind NP1 and NP2, expressed on endothelial as well as on other cell types (Li et al., 2001).

The VEGFB gene displays a unique expression pattern with prominent expression in the heart during embryonic development (Lagercrantz et al., 1998). Mice knock out for this factor develop normally, however display a mild cardiac phenotype, characterized by dysfunctional coronary

vasculature, impaired recovery from cardiac ischemia, and, most notably, decreased heart size (Bellomo et al., 2000). In another mouse strain, the VEGFB knock out specifically generates atrial conduction abnormalities (Aase et al., 2001). In addition, recent work reassessing the role of $VEGFB_{167}$ during ischemia has indicated that this factor, delivered either as a recombinant protein, or using adenoviral vectors or through the implantation of transfected myoblasts, significantly increased revascularization of the infarcted myocardium, however failed to enhance vascular growth in the skin or ischemic limb (Li et al., 2008).

VEGF-B overexpression has been proven to induce myocardium specific angiogenesis and arteriogenesis in rabbits and pigs upon acute infarction (Lahteenvuo et al., 2009).

Heart and skeletal muscle are the tissues characterized by the highest VEGF-B expression, even if many different tissues, including ECs and brain, usually express VEGF-B (Li et al., 2001); conversely, the role of VEGF-B in tumor growth remains elusive. At the cellular level, VEGF-B receptors are VEGFR1 and NP1, and VEGF-B stimulates directly endothelial cell migration and growth (Cao, 2009).

VEGF-C and VEGF-D

VEGF-C and VEGF-D share peculiar characteristics: they present unique N and C terminal extensions different from the other VEGF members, they bind and activate VEGFR3 in addition to VEGFR2 and their affinity for this receptor is increased upon their proteolitic cleavage (Lohela et al., 2009).

VEGF-C gene is composed by seven exons, and this protein is expressed in the heart, small intestine, placenta, ovary and the thyroid gland in adults (Roy et al., 2006). VEGF-C is essential for lymphangiogenesis, as lack of VEGF-C in mice leads to a complete absence of lymph vessels and embryonic lethality, while blood vasculature grows normally (Karkkainen et al., 2004).

VEGF-D is 48% identical to VEGF-C, and it is expressed in many adult tissues including the vascular endothelium, heart, skeletal muscle, lung, and bowel (Roy et al., 2006).
VEGF-D has been shown to be largely dispensable for the development of lymphatic system, and its physiological role needs to be further clarified (Baldwin et al., 2005), even if *in vitro* this factor is able to stimulate migration and proliferation of endothelial cells; additionally, this factor seems to have a role in tumor angiogenesis and lymphangiogenesis (Achen et al., 2001; Stacker et al., 2001).
Recently, data obtained using double KO mice for VEGF-C and VEGF-D, showed that both VEGF-C and VEGF-D are displensable for embryonic angiogenesis; additionally, it has been shown that VEGF-C/D KO does not phenocopy VEGFR3 KO (Haiko et al., 2008).

The discovery of VEGF homologues in the genome of parapoxvirus Orf (VEGF-E) and in snake venom (svVEGF) finally confirms the pleiotropic role of VEGF family members (Shibuya, 2003).

VEGF Receptors

A key element in the complex regulation of VEGF activity is represented by the VEGF-receptors (VEGFR): VEGFR1 (or Flt1), VEGFR2 (or KDR, Flk1) and VEGFR3 (Flt4), expressed by several cell types (Figure 1.4). VEGFRs belong to the Tyrosine Kinase Receptor (TKR) superfamily. These consist of an extracellular domain composed by seven Immunoglobulin (Ig) like domains (Ig), a short transmembrane and a juxtamembrane segment, and are characterized by a split intracellular tyrosine kinase domain interrupted by a 70 aa long kinase insert domain (Carmeliet, 2005b; Matsumoto and Claesson-Welsh, 2001; McTigue et al., 1999; Olsson et al., 2006; Roskoski, 2008; Shibuya and Claesson-Welsh, 2006).

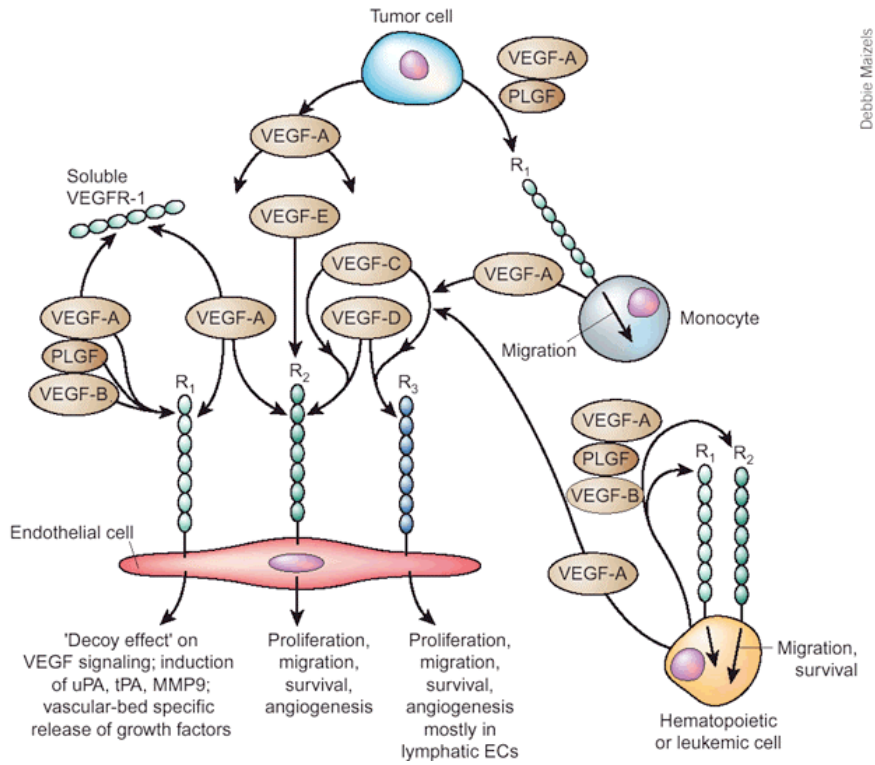

Figure 1.4. Interaction between VEGFs and VEGFRs. VEGFs can selectively bind different VEGFRs, expressed on the cell surface of endothelial cells, monocytes and tumor cells. The three VEGF tyrosine kinase receptors can form homo and hetero-dimers as a consequence of ligand binding, eventually leading to intracellular signalling. *Adapted from Ferrara et al, Nature Medicine 2003.*

VEGF binding to VEGFRs is the initial step for signal transduction, accompanied by receptor homo and hetero-dimerization. Therefore, the characterization of receptor dimer dynamics represents a crucial step in understanding receptor activation. Tyrosine kinase activation parallels

dimer formation and leads to autophosphorylation; finally, phosphorylated receptors recruit intracellular partners.

VEGFR1

VEGFR1/Flt1, together with VEGFR2, has a primary role in angiogenesis, playing a complex regulatory role (Shibuya, 2006). Even if VEGFR1 and VEGFR2 are structurally similar, VEGFR1 function is different and multifaceted, functioning both as a negative and positive regulator of angiogenesis (Olsson et al., 2006). Interestingly, VEGFR1 has ten fold higher affinity for VEGF compared to VEGFR2 (Waltenberger et al., 1994).
VEGFR1 activity results from its binding to three different gene products belonging to the VEGF family, namely VEGF-A, VEGF-B and PlGF.
The concept that VEGFR1 has a negative regulatory role during early embryogenesis was suggested by the observation that VEGFR1 null mice die at embryonic day 8.5-9.0, displaying a disorganized vascular endothelium and overgrowth of endothelial cells (Fong et al., 1995). Interestingly, VEGFR1 kinase activity seems to be dispensable during embryogenesis, since mice expressing a VEGFR1 mutant that lacks the tyrosine kinase domain develop an essentially normal vasculature (Hiratsuka et al., 1998). Therefore, VEGFR1 has been proposed to act as a VEGF-A trap, thus preventing excessive VEGFR2 activation during embryonic development (Shibuya, 2001). A physiological role of this endogenous VEGF-A trap was demonstrated in adult life; a soluble VEGFR1, known as sFlt1 and expressed also by human placenta (Shibuya et al., 1990), is essential in order to preserve corneal avascularity (Ambati et al., 2006; Ambati et al., 2007).
Nonetheless, VEGFR1 has a functional role in active transduction of VEGF signalling, well clarified in cells lacking other VEGFRs. Monocytes/macrophages migration in response to VEGF-A is mediated by VEGFR1, also trough interaction with Neuropilin1 (Barleon et al., 1996;

Zacchigna et al., 2008c). Additionally, VEGFR1-mediated migration of haematopoietic bone marrow progenitors initiates the pre-metastatic niche in mouse models (Kaplan et al., 2005), even if this finding has been recently discussed (Dawson et al., 2009a; Dawson et al., 2009b).

VEGFR1 signalling.

Despite its ability to bind VEGF-A with more then 10-fold higher affinity than VEGFR2, VEGFR1 undergoes only a weak phosphorylation, even if all kinase motifs are conserved (Waltenberger et al., 1994). Relevant to this issue, VEGFR1 and VEGFR2 share a 43% of overall homology, lower in the extracellular domain (33%) and higher in the kinase domains (70%). Nevertheless, the mechanism responsible for VEGFR1 kinase-impaired activity is still debated. On one hand the juxtamembrane domain, probably by forming an intracellular structure inhibiting accessibility of regulatory sequences, has been implicated in kinase repression (Gille et al., 2000). On the other hand, it has been proposed that substitution of a single aminoacid from aspartic acid at position 1050 (conserved among many RTKs) to asparagine (in VEGFR1) is linked with its decoy activity, in particular by inhibiting phosphorylation in the activation loop (Meyer et al., 2006). Additionally, taking advantage of overexpression studies to overcome weak signals, several VEGFR1 tyrosine residues were identified as phosphorylated, together with their interacting partners (among others SH2, p38/PI3K, Grb2 and Nkc) (Olsson et al., 2006; Shibuya, 2006). Further observations indicate that different VEGFR1 ligands, VEGF-A and PlGF, induce different phosphorylation patterns (Autiero et al., 2003a). Nevertheless, the complexity of VEGFR1 downstream signalling is far from being clarified.

An additional regulation level is provided by the interplay between different VEGFRs, usually coexpressed by endothelial cells. In particular, VEGFR1 has the ability to form heterodimers with other VEGFRs, thus modulating

their response both in a negative and positive manner. In support of its inhibitory role it has been shown that Flt1 can suppress VEGFR2 mediated proliferation but not migration of endothelial cells (Zeng et al., 2001). Conversely, it has been demonstrated that PlGF-driven VEGFR1 activation leads to amplification of VEGFR2-mediated angiogenesis, through intermolecular trans-phosphorylation of Flk1; this observation suggests an inter- and intra-molecular crosstalk between VEGFR1 and VEGFR2 (Autiero et al., 2003b).

VEGFR1, together with Neuropilin1 and Neuropilin2, constitutes the functional receptor for PlGF in cells where it is primarily expressed, such as tumor cells. For this reason, an anti-PlGF blocking antibody, inhibiting binding to VEGFR1 and formation of VEGFR1-Neuropilin1 complexes, inhibits also the growth and metastasis of various tumors (Fischer et al., 2007). These results strengthen the functional role of VEGFR1 in pathological conditions such as tumor growth.

Thus, the role and the mechanism by which VEGFR1 supports angiogenesis are complicated and likely involve several different mechanisms, including VEGFR1 decoy activity, formation of complexes with other receptors as well as direct signalling.

In order to further clarify VEGFR1 biology, it will be particularly interesting to study *in vivo* interaction between VEGFR1 and VEGFR2, and how this interaction might give rise to different biological effects.

VEGFR2

The experimental evidence that VEGFR2 signalling is required for cardiovascular development (Shalaby et al., 1995) and that has a major role in neovascularization in both physiological and pathological conditions has stimulated a general interest in understanding VEGFR2 biology. Therefore, due to its key role in vessel biology, several aspects regulating VEGFR2 activity have been studied and will be discussed (Figure 1.5).

Expression

During development, VEGFR2 is detectable from E7.5 in mesodermal cells of the tail region; VEGFR2 positive cells migrate and differentiate into primitive endothelial cells (Shalaby et al., 1995). During adult life VEGFR2 is expressed mostly in vascular and lymphatic ECs. Even if lower levels of VEGFR2 are detected in haematopoietic stem cells, neurons, osteoblasts as well as megakaryocites (Matsumoto and Claesson-Welsh, 2001), VEGFR2 biology has been so far analyzed mainly in EC. The 5' non-coding region and the 3' region of the first intron are required to properly regulate VEGFR2 expression in ECs (Shibuya and Claesson-Welsh, 2006). Recent data show that Extra Cellular Matrix (ECM) is able to control VEGFR2 expression *in vitro* and *in vivo*, and that this action is mediated by p190RhoGAP (Mammoto et al., 2009).

In accordance to its major regulatory role in angiogenesis, VEGFR2 is upregulated during pathological angiogenesis, such as in tumors, and VEGF-A has a positive effect on VEGFR2 expression by means of a positive feedback mechanism (Shibuya and Claesson-Welsh, 2006).

Ligand binding and dimerization

In addition to VEGF-A (K_d=75-760 pM), VEGFR2 is able to bind VEGF-E, sv-VEGF as well as processed VEGF-C and VEGF-D. Thereby, VEGFR-2 represents the major mediator of VEGF-A-induced proliferation, migration and permeability in EC.

Since the observation that VEGFR2-VEGF binding requires Ig domain 2 and 3 of the receptor and is likely to occur at the ligand dimer interface, many crystallography studies have focused on the VEGF-VEGFR2 interaction in order to develop anti-angiogenic drugs (Roskoski, 2008).

Ligand binding is accompanied, as in many RTK, by receptor dimerization, the first step toward receptor activation.

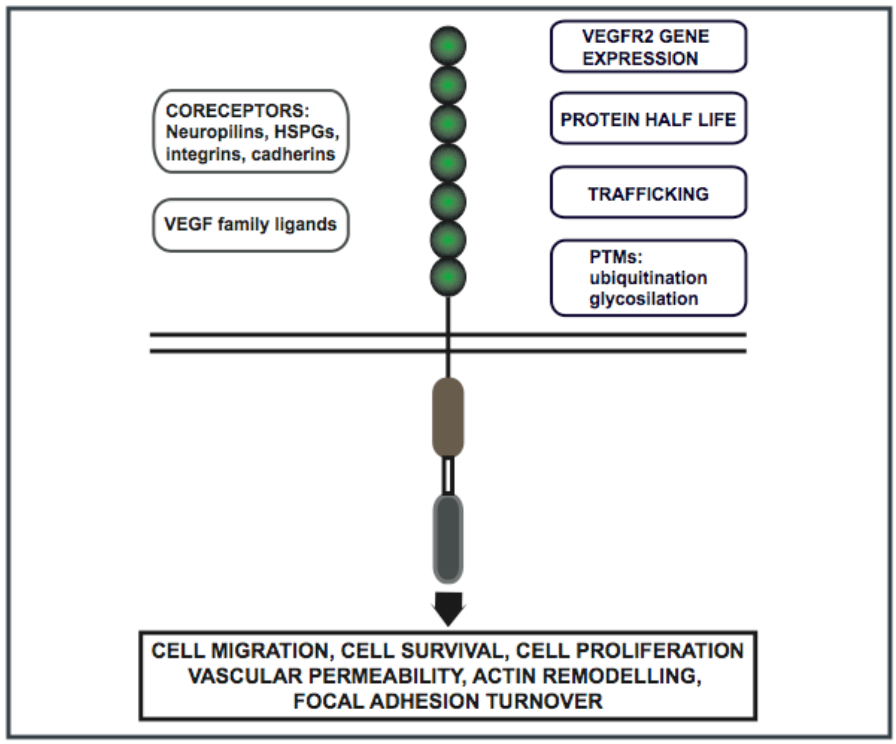

Figure 1.5. VEGFR2 activity regulation. VEGFs induce VEGFR2 phosphorylation, and in addition receptor activation is tightly regulated by coreceptors expressed even in trans. Additional cellular mechanisms contribute to fine tune VEGFR2 signalling.

In particular, Ruch and coworkers, based on Electro Microscopy observations of soluble molecules, propose that VEGF induces transition of VEGFR-2 extracellular domains from an highly flexible conformation to a more stable, rigid arrangement, which is stabilized by homotypic interactions of membrane-proximal and membrane-distal immunoglobulin-like domains (in particular involving the Ig domain 7). In the full-length protein, the rigid arrangement of two receptor monomers is probably required for the exact positioning of the intracellular kinase domains (Ruch

et al., 2007).

Nonetheless, the precise mechanism linking VEGF binding and receptor homo and hetero-dimerization is still far from being clarified.

Trafficking

Since protein trafficking controls the relative amount of receptor available for VEGF binding at the plasma membrane, this aspect is crucial in fine-tuning VEGF activity at the cellular level. Nonetheless, many issues regarding VEGFR2 trafficking are still debated, such as internalization through caveolin-1 vesicles or clathrin coated pits (Mukherjee et al., 2006).

Surprisingly, in HUVE cells more than 40% of VEGFR2 protein was found to be localized in an internal vesicular pool positive for early endosomal compartment markers (EEA1, Rab4); VEGF stimulation not only induces VEGFR2 downregulation, as expected for a TKR, but also redistribution to a late endosomal compartment and finally recycling of the receptor (Gampel et al., 2006). Apart from intrinsic tyrosine kinase activity, required for VEGFR2 trafficking and degradation (Ewan et al., 2006), the stimuli controlling the endocytic itinerary of VEGFR2 are still undisclosed.

Moreover, it is still not clear whether VEGFR2 internalization and degradation depend on c-Cbl mediated ubiquitination or on PKC-mediated VEGFR2 C-tail serine phosphorylation (Duval et al., 2003; Singh et al., 2005).

The complexity of VEGFR2 trafficking is emphasized by the observation that internalized receptors are still able to induce intracellular signalling, and therefore molecules such as Vascular Endothellial (VE)-cadherin, controlling VEGFR2 internalization, might finally control VEGF signalling (Lampugnani et al., 2006).(Jakobsson et al., 2006)

Coreceptors

An additional mechanism that regulates VEGFR2 downstream signalling is represented by membrane associated VEGFR2 coreceptors, such as

Neuropilins (Neufeld et al., 2002), Heparan Sulfate Proteoglycans (HSPGs), and VE-cadherin (Carmeliet and Collen, 2000). Interestingly, the interaction between VEGFR2 and some coreceptors can occur *in trans*: it has been shown that $VEGF_{165}$-VEGFR2 signalling is increased in EC, when HSPG are expressed in trans in a co-culture system (Jakobsson et al., 2006).

Moreover, VEGFR2 was found to be part of a mechano-sensory complex triggered by fluid shear stress and comprising PECAM-1 and VE-cadherin, that leads to the conformational activation of integrins (Tzima et al., 2005). VE-cadherin, a specific component of endothelial adherent junctions, has been found to limit VEGFR2 mediated mitogenic signal (Grazia Lampugnani et al., 2003). This VE-cadherin induced inactivation, mediated by decreased receptor phosphorylation, can be interrupted by receptor internalization (Lampugnani et al., 2006).

Finally, integrins have been found to associate with VEGFR2 in EC, and play a crucial role in balancing VEGFR2 activity. For instance, the αvβ3 integrin, upon binding to its ligand vitronectin, enhances VEGFR-2 phosphorylation, PI 3-kinase activity, focal adhesion dynamics as well as proliferation and migration of ECs triggered by VEGF-A (Napione et al., 2007; Soldi et al., 1999). In contrast to the αvβ3/vitronectin (or fibrin) pair, collagen I, the ligand of α1β3 and α2β1 integrins, exerts an inhibitory action reducing VEGF-A-induced VEGFR-2 autophosphorylation by recruiting the tyrosine phosphatase SHP2 (Mitola et al., 2006).

Structure and kinase activity

Relatively few tyrosine (Tyr) residues were identified as phosphorylation sites in VEGFR2 including human Tyr951 (949 in the mouse sequence) and 996 (994) in the kinase insert domain, Tyr1054 (1052) and 1059 (1057) in the kinase domain, and Tyr1175 and 1214 in the C-terminal tail. Proteomic studies identified three additional tyrosines as phosphorylated at a low stoichiometry in the C-terminal tail of hVEGFR2: Tyr1305, 1309 and 1319

(Matsumoto et al., 2005). On the other hand, Tyr1175 and 1214 were identified as two major VEGF-A dependent autophosphorylation residues (Takahashi and Shibuya, 2005) (Figure 1.6).

Since VEGFR2 is a tyrosine kinase enzyme, its phosphorylation is linked to its structure, as VEGF-A binding leads to receptor dimerization, protein kinase activation and trans-autophosphorylation. Therefore, comprehension of VEGFR2 structure is functional in order to understand its activity.

From a structural point of view, human VEGFR2 kinase domain can be subdivided in:

- a proximal kinase domain (residues 827-931)
- a kinase insert domain (residues 932-998); this segment, although not directly involved in catalysis, is important in signal transduction as a docking site for cellular proteins
- a distal kinase domain (residues 999-1158), containing both the catalytic and the activation loop.

Two lobes, similarly to other TKRs such as EGFR, characterize the catalytic core of VEGFR2, with the active site located in the cleft between the N-terminal and the C-terminal lobe. The smaller N-lobe is predominantly formed by antiparallel β-sheets and contains the glycin-rich ATP-Phosphate binding loop, while the larger C-lobe is characterized by α-helices and includes the activation and catalytic loops (McTigue et al., 1999). Conformational changes at the level of these lobes are linked to inter-conversion of the kinase from inactive to active state and finally to catalysis. In particular, when VEGFR2 is an inactive state, the activation loop that comprises two tyrosines (1054 and 1059) is in a “closed” conformation that prevents substrate binding. Phosphorylation in the activation loop stabilizes it in its active, “opened” conformation (Roskoski, 2008) (see http://www.ebi.ac.uk/pdbsum/2oh4 for VEGFR2 crystal structure).

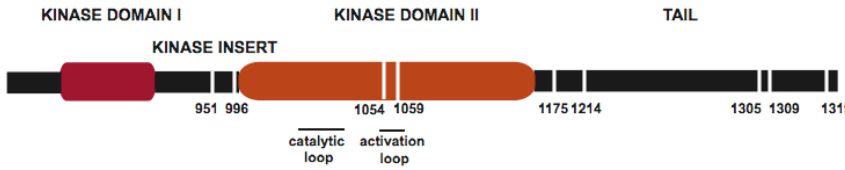

Figure 1.6. VEGFR2 intracellular part. Human VEGFR2 intracellular domains are highlighted, while white bars and residue numbers indicates the main phosphorylated tyrosines. In addition, position of the catalytic and activation loops is shown.

An interesting issue is how VEGFR2 dephosphorylation is regulated. To our knowledge, SHP2 and VE-PTP are the only tyrosine phosphatases that have been demonstrated to interact and dephosphorylate VEGFR2 (Mellberg et al., 2009; Mitola et al., 2006); in particular SHP2 favours VEGFR2 internalization, and might be responsible for collagen I negative regulation of VEGFR2 signalling (Mitola et al., 2006).

Downstream signalling

The multiplicity of cellular functions controlled by the VEGF-VEGFR2 axis is reflected by the diversity of signalling cascades that are activated upon ligand binding. Accordingly, a complex network of signal transduction leads to cell migration, cell survival, cell proliferation, vascular permeability, actin remodelling and focal adhesion turnover.

The characteristics of VEGFR2 downstream signalling are additionally modulated by the formation of different membrane signalling complexes, in addition to association with other VEGFRs and coreceptors such as Neuropilins, as discussed later.

PLCγ. Endothelial cell culture experiments demonstrated that phosphorylated Tyr1175 represents the single major site for PLCγ binding to VEGFR2, and has a key role in the activation of the PLCγ-PKC-MAPK pathway (Takahashi et al., 2001). Later on, the same group demonstrated

the relevance of Tyr1175 (1173 in mice) and its downstream signalling *in vivo*. Knock-in mice with a single aminoacid substitution (Tyrosine 1173 to phenylalanine) died at E8.5-9.5 as a consequence of endothelial and haematopoietic cells defects, such as blood vessel deficiency, comparable to VEGFR2 null mice. Conversely, substitution of the highly phosphorylated, but not required for PLCγ signalling, Tyr 1212 (1214 in human) with phenylalanine, gave rise to viable and fertile mice (Sakurai et al., 2005).

Even PLCγ activity is fine tuned by VEGFR2 signalling: c-Cbl, recruited and phosphorylated by VEGFR2, promotes PLCγ ubiquitination, inhibiting signal transduction (Singh et al., 2007).

Recently, VEGF induced PLCγ-PKC pathway activation was linked to VEGF -target genes regulation, through phosphorylation of HDAC7 and its nuclear export, thus providing a direct connection between VEGF stimulation and histone acetylation (Ha et al., 2008).

***PI3K*.** Another event that strictly relies on Tyr1175 phosphorylation is the activation of the PI3K-Akt-PKB pathway for cell survival and migration, as inferred form studies in endothelial cells (Dayanir et al., 2001; Gerber et al., 1998). Shb is one of the adaptor molecules binding to pTyr1175 and mediating PI3K-dependent endothelial cell migration (Holmqvist et al., 2004). Nonetheless, since VEGFR2 induced PI3K activation is not so strong, activation of an additional pathway to sustain VEGF induce migration is likely to occur (Takahashi and Shibuya, 2005). One single specific PI3K isoform, namely 110α, is the preferential mediator of VEGF-A dependent migration of endothelial cells *in vitro*, and has been linked with developmental angiogenesis *in vivo* (Graupera et al., 2008).

Recent studies highlighted the role of Akt and its substrate Gridin in neovascularization during adult life, as mediators of VEGF-triggered vascular remodelling (Kitamura et al., 2008).

Ras. Even if VEGF is not a powerful mitogen for EC, Ras dependent and independent Raf-MEK-MAPK pathway activation seems to occur depending on cell types (Olsson et al., 2006).

Gab and Grb2. Grb2, Gab1 and Gab2 are scaffolding adaptors involved in many TKR signalling such as EGFR. Gab1 and Grb2 were shown to be involved in VEGFR2 downstream signalling, and a model inferred from *in vitro* data includes a direct interaction between VEGFR and Grb2, the latter able to bind Gab1. This system has been linked to PI3K activation, endothelial cell migration and capillary formation (Laramee et al., 2007).

TSAd/VRAP. Tyr951 (mouse 949), an additional VEGFR2 phosphorylation site, has been identified as the binding site for the T-cell specific adaptor (TSAd, or VEGFR-associated protein VRAP) (Matsumoto et al., 2005). The same group demonstrated that VEGFR2-TSAd coupling is responsible of actin reorganization and therefore endothelial cell migration, but seems not to be linked to cell proliferation; additionally, TSAd is probably involved in tumor angiogenesis.

Cdc42 p38 MAPK. EC migration downstream to VEGFR2 is also regulated by phosphorylation of Tyr1214 (1212), which triggers sequential activation of Cdc42 and SAPK2/p38, finally driving the SAPK2/p38-mediated actin remodelling of stress fibers in endothelial cells exposed to VEGF (Lamalice et al., 2004). Additional molecules, such as IQGAP1, are able to bind VEGFR2, and induce both VEGF induced migration and proliferation (Meyer et al., 2008)

FAK-paxillin. VEGF promotes cell migration through a RhoA-ROCK dependent mechanism, activating Focal Adesion Kinase (FAK) and finally paxillin (Le Boeuf et al., 2006).

VEGFR2 post-translational modifications

As post translational modifications (PTMs) represent a fine mechanism to control protein function, it is not surprising that VEGFR2 can be targeted by

different PTMs in addition to tyrosine phosphorylation, crucial for receptor activation.

Glycosilation is important in VEGFR2 protein maturation; the VEGFR2 pool present at the plasma membrane is N-glycosilated (Takahashi and Shibuya, 1997).

Poly-ubiquitination is one of the signals involved in receptor down-regulation upon VEGF stimulation, even if the details of this modification are still unknown (Duval et al., 2003).

VEGFR3

VEGFR3/Flt4 constitutes the receptor for the VEGF-C and VEGF-D family members. The observation that, in adults, VEGFR3 expression is restricted to the lymphatic endothelium and the use of genetic models linked this receptor mainly to lymphatics development and maintenance (Kaipainen et al., 1995; Veikkola et al., 2001). In particular, VEGFR3 signalling is required for lymphatic endothelial cells sprouting as well as lymphatic vessel maintenance through the inhibition of apoptosis (Alitalo et al., 2005). Nonetheless, VEGFR3 gene targeted mice exhibit defects in arterial-venous remodelling of the primary vascular plexus, leading to embryonic lethality from day E9.5 (Dumont et al., 1998). Therefore, during embryonic development, VEGFR3 activity is not restricted to lymphatics, but has an important function in blood vessel development. Recent data extended the role of VEGFR3 in angiogenesis also during adult life; VEGFR3 was found to be highly expressed in angiogenic sprouts, while targeting of VEGFR3 signalling resulted in decreased sprouting, vascular density, vessel branching and endothelial cell proliferation in mouse angiogenesis models (Tammela et al., 2008). VEGFR3 is also found to be upregulated in tumor microvasculature, thus opening the possibility to exploit VEGFR3 targeting agents to inhibit tumor growth (Saharinen et al., 2004).

The extension of VEGFR3 role not only to lymphatic biology, but also to pathological and embryonic development, warrants further studies to define the molecular mediators of these diverse activities, still poorly explored.

VEGFR3 signalling

Two conserved tyrosine residues in the kinase domain of VEGFR3 are probably responsible for its kinase activity, and additional tyrosine phosphorylation sites have been identified in the VEGFR3 C-terminal tail (Dixelius et al., 2003). Signal transduction downstream to VEGFR3 has been only partially characterized: Shc2/Grb2 interact directly with the receptor, while downstream intracellular mediators identified until now comprise ERK1/2, PI3F-Akt, STAT3 and STAT5 transcription factors (Olsson et al., 2006).

As already mentioned in the case of VEGFR1, VEGFR3 signalling can be modulated by the interaction with VEGFR2 and other co-receptors, in this case Neuropilin2. The formation of these complexes is biologically relevant and, for example, VEGFR2-VEGFR3 hetero-dimers might form in vivo both in lymphatic cells and subtypes of endothelial cells, resulting in differential phosphorylation sites and finally differential signalling (Dixelius et al., 2003). All these observation suggest that VEGFR3, besides its fundamental role in lymphatic vessel development and maintenance, also acts as a regulator of vascular network formation. In this respect VEGFR3 may constitute an additional target of anti-angiogenic therapies.

Semaphorins and plexins in vessel biology

Initially described as axon guidance molecules, semaphorins are also implicated in the regulation of neural development and organ morphogenesis, together with angiogenesis and invasive tumor growth (Larrivee et al., 2009; Serini et al., 2009). For instance, as semaphorins and their receptors plexins are expressed by tumor and endothelial cells, they are now emerging as important factors in tumor angiogenesis (Neufeld and

Kessler, 2008). This family of membrane-bound and secreted proteins influences cytoskeletal remodelling, integrin-dependent adhesion, cell proliferation, apoptosis and differentiation (Kruger et al., 2005). All semaphorins contain an amino terminal sema domain, required for signalling, and were subdivided into eight groups: invertebrate semaphorins in group 1 and 2, vertebrate semaphorins in groups 3 to 7, and viral semaphorins in group 8. Interestingly, class3 semaphorins are the only secreted vertebrate semaphorins and, with one exception, require Neuropilin binding to signal through class A plexins.

Plexins represent the main functional family of semaphorin receptors. In mammals, nine plexins have been identified so far, subdivided in four subfamilies based on homology: PlexinA1 to A4, Plexin B1 to B3, Plexin C1 and PlexinD1 (Franco and Tamagnone, 2008). The extracellular domains of these single pass transmembrane receptors are distinguished by the presence of sema, PSI and IPT domains. While semaphorins belonging to classes 4-7 as well as SEMA3E bind directly to specific plexins and activate plexin-mediated signal transduction, other class 3 semaphorins binds to Neuropilins, while Plexins (PlexinAs and PlexinD1) serve as signal transduction elements.

Plexins are characterized by a unique, but highly conserved, cytoplasmic region, which has been associated to multiple signal transducers (Kruger et al., 2005). For instance, plexin intracellular domain contains GTPase-activating protein (GAP)-like motifs, able to interact with G-protein R-Ras, and activates Rho-GTPases. Additionally, it has been shown that plexins become tyrosine phosphorylated, even if the regulatory role of this posttranslational modification need to be further investigated (Franco and Tamagnone, 2008).

Neuropilins

Neuropilins (Neuropilin1/NP1 and Neuropilin2/NP2), initially identified as receptors for several class 3 semaphorins, in association with plexin family receptors mediate repulsive axon guidance in the developing nervous system (Fujisawa, 2004).

Even if Neuropilin1 and Neuropilin2 share only 44% homology, they have similar structural features: the extracellular part of these single pass transmembrane receptors has two Complement binding domains (CUB, or a1 and a2), two coagulation factor V/VII homology domains (b1 and b2) ad a single meprin domain (MAM or c). Notably, the intracellular domain of neuropilins is only 40-aminoacid long, displays poor homology with other proteins and lacks enzymatic activity. Despite structural homologies, Neuropilin1 and Neuropilin2 differ for binding and signal properties (Neufeld et al., 2002). In particular, SEMA3A binds only to NP1, SEMA3F and SEMA3G interact only with NP2, while SEMA3B, SEMA3C and SEMA3D bind both receptors. Additionally, Neuropilin1 and Neuropilin2 also display specific and mutually selective binding to factors not belonging to the SEMA family, such as VEGF-A, VEGF-B, VEGF-C, PlGF, PDGF-bb, FGF2, TGFβ, HGF and galectin (Figure 1.7).

Moreover, Neuropilins differ in VEGF-A isoform binding, as NP2 binds to $VEGF_{165}$ and $VEGF_{145}$, while NP1 binds $VEGF_{165}$ and possibly $VEGF_{121}$, as discussed afterwards. The biological difference between NP1 and NP2 is enforced by their non-redundant role in development, as NP1 deficient mice die during mid-gestation with defects in the heart, vasculature, and nerve projection (Kawasaki et al., 1999), while NP2 KO mice are viable and display only defects in nerve projection (Chen et al., 2000; Giger et al., 2000). Interestingly, the double NP1/NP2 knockout mouse had a more severe abnormal vascular phenotype than either NP1 or NP2 single

knockouts, resembling the phenotypes of VEGF and VEGFR-2 knockouts (Takashima et al., 2002).

Neuropilin1

Neuropilin1 is a functional, transmembrane receptor, able to mediate signalling from structurally distinct ligands during nervous system, heart and vascular development, as shown in Figure 1.7. In particular Neuropilin1 expression in EC is required for cardiovascular development (Gu et al., 2003), and is important for the formation of the capillary plexus, partially independently from blood flow (Jones et al., 2008).

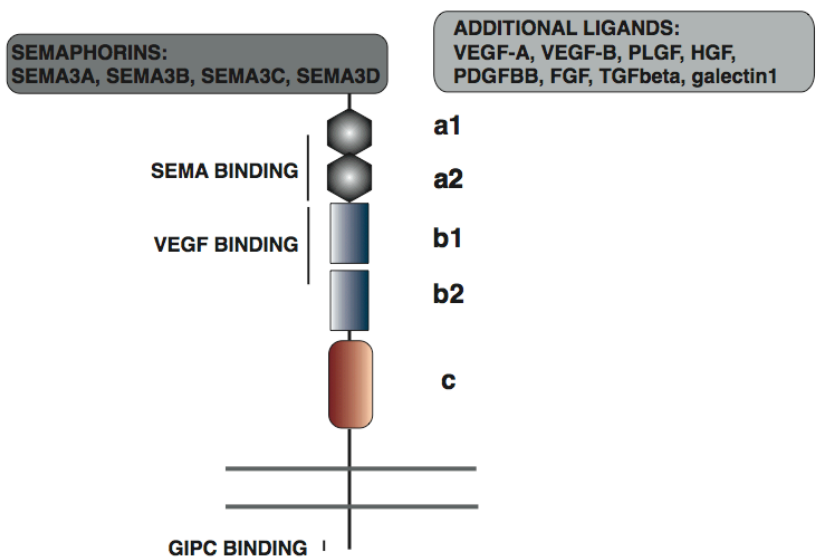

Figure 1.7. Interaction of Neuropilin1 with different ligands. NP1 ligands belonging to the semaphorin and other families are shown. Predicted binding sites for SEMA, VEGF and GIPC are indicated by bars. Additionally, the structural domains of Neuropilin1 CUB (a1, a2), FV/FVIII (b1/b2) and MAM (c) are shown.

Neuropilin1 was shown to bind to VEGF-A isoforms containing exon 7 (the heparin binding domain) and therefore common usage of the NP1 receptor by factors, such as $VEGF_{165}$ and SEMA3A, raised the possibility of a competition or at least a cross regulation.

VEGF and SEMA3A bind to two distinct, even if partially overlapping, domains on NP1 extracellular part (Gu et al., 2002), and structural studies show that VEGF and SEMA3A do not compete directly for NP1 binding (Appleton et al., 2007; Vander Kooi et al., 2007). Nonetheless, several data show how SEMA3A and $VEGF_{165}$ can have opposite effect on cell migration and survival (for a review see (Kruger et al., 2005)); recent findings open the possibility that SEMA3A and VEGF can act by promoting independent signals downstream to NP1 (Acevedo et al., 2008; Guttmann-Raviv et al., 2007).

Even if Neuropilin1 was initially proposed to simply act by favouring $VEGF_{165}$, but not $VEGF_{121}$, binding to VEGFR2, thus enhancing VEGF-induced chemotaxis in cultured endothelial cells (Miao et al., 1999; Soker et al., 1996; Soker et al., 2002; Soker et al., 1998; Whitaker et al., 2001), recent data clearly suggest a more complicated picture for the Neuropilin1 – VEGFR2 system activity in EC. This model implies that $VEGF_{121}$, which does not have the Neuropilin1 binding region coded by exon 7, is a weaker VEGFR2 activator as compared to $VEGF_{165}$. Nonetheless, one group showed that both Neuropilin1 and 2 are able to enhance $VEGF_{121}$-induced signal transduction by VEGFR2 in PAE and HUVE cells (Shraga-Heled et al., 2007). Another group provided evidence that $VEGF_{121}$ is also able to directly bind Neuropilin1 by means of the tail region instead of exon 7, but without inducing the formation of VEGFR2-Neuropilin1 complexes (Pan et al., 2007b). More recently, formation of stable $VEGF_{165}$-NP1-VEGFR2 complexes has been linked to sprouting angiogenesis both *in vitro* and *in*

vivo through p38 signalling (Kawamura et al., 2008a). In conclusion, $VEGF_{165}$, and not $VEGF_{121}$, induces the formation of VEGFR2-Neuropilin1 complexes, even if the dynamics of these functional angiogenic receptor clusters have never been studied in detail (Soker et al., 2002; Whitaker et al., 2001).

Genetic and biochemical studies generated strong evidence for a role of Neuropilin1 in vascular morphogenesis; therefore is not surprising that even NP1 has been exploited as a target for anti-cancer therapy. In particular, two antibodies, blocking respectively VEGF and SEMA binding to NP1, synergize with anti-VEGF in reducing tumor growth, even diminishing vascular density and EC-pericyte association. (Liang et al., 2007; Pan et al., 2007a).

Neuropilin1 signalling

Due to its short and catalytically inactive intracellular tail, for a long time Neuropilin1 has been considered only able to enhance VEGF-induced signal through VEGFR2 interaction (Figure 1.8). Recent evidence suggests that Neuropilin1 mediates HUVEC adhesion to ECM (Murga et al., 2005) as well as increases VEGF induced EC survival (Wang et al., 2007), independently from VEGFR2.

Notably, a protein called RGS-GAIP-interacting protein (GIPC or synectin), involved in vesicle trafficking, was found to interact with Neuropilin1 (Cai and Reed, 1999). The PDZ-binding domain of Neuropilin1, involved in GIPC1 interaction, seems to be required for VEGFR2-NP1 complex formation *in vitro* (Prahst et al., 2008), as well as to promote integrin internalization and indirectly EC adhesion (Valdembri et al., 2009). This interaction is important *in vivo* for proper angiogenesis in zebrafish (Wang et al., 2006) while it has possibly a role in branching morphogenesis in mice (Chittenden et al., 2006).

How Neuropilin1 might initiate an intracellular signalling cascade in response to an extracellular stimulus is still unresolved. It has been shown that NP1 is differentially glycosaminoglycan-modified in EC and SMC (Shintani et al., 2006), but no other PTMs has been characterized so far, suggesting a signalling mechanism different from post translational modifications.

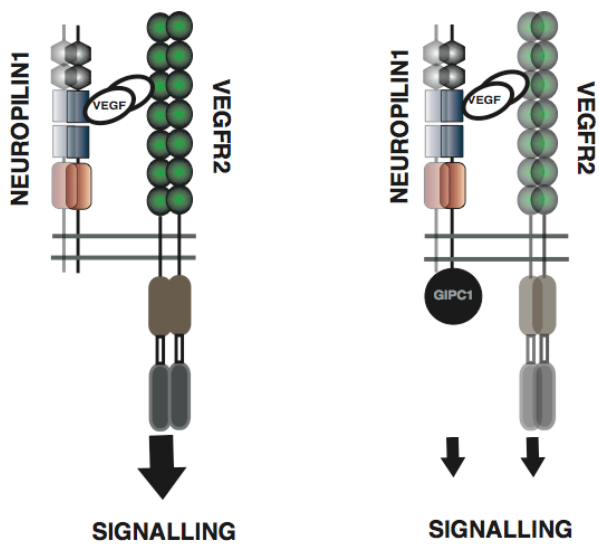

Figure 1.8. Models for Neuropilin1 activity in enhancing VEGF signalling. In the first model (left), enhanced signalling is due to enhanced VEGF binding to VEGFR2, while in the second model (right) Neuropilin1 contributes directly to signalling, independently from VEGFR2, and even trough GIPC1. The role of Neuropilin1 homo-complexes formation in signalling is still debated.

One possibility is that ligand binding might induce a structural modification and therefore triggers NP1 downstream pathways. Neuropilin1 is able to

form dimers, probably not only through the MAM domain as initially supposed (Nakamura et al., 1998), but also through the transmembrane domain, and the formation complexes seems to be involved in Neuropilin1 signalling (Roth et al., 2008).

Concluding, Neuropilin1 seems to act in regulating EC functions by almost two different mechanisms: by enhancing VEGF binding to VEGFR2 and by a VEGFR2 independent signalling, but how these mechanisms are regulated is still an unresolved question.

Protein function regulation by acetylation

Proteins are naturally modified by several mechanisms, including phosphorylation, ubiquitination, sumoylation, neddylation, acetylation, methylation and isomerization. In addition, the combination of PTMs generates a great potential for cross-regulation. In particular lysine can be the target of multiple, mutually exclusive modifications, as shown in Figure 1.9.

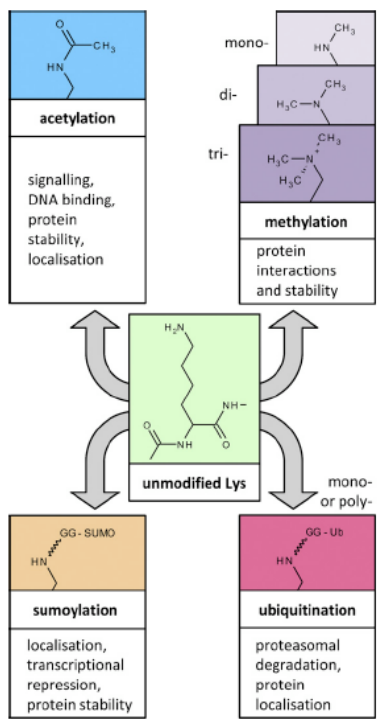

Figure 1.9. Lysine modifications. The ε-amino group of lysine can be modified by acetylation and several covalent modifications, resulting in different biological outcomes. *Adapted from Spange, International Journal of Biochemistry and Cell Biology, 2009.*

Lysine acetylation is a reversible process that entails transfer of an acetyl group from Acetyl coenzyme A to the ε-amino group of a lysine residue, thus removing the positive charge from the lysine. Both protein acetylation and deacetylation requires dedicated enzymes, defined as lysine acetyltransferases (KATs) and deacetylases (KDACs), respectively (Allis et al., 2007).

Even if acetylation has been initially defined as a histone-specific modification, recent reports demonstrate that more than 100 proteins, both nuclear and cytoplasmic, are acetylated in one or more residues (Glozak et al., 2005; Kim et al., 2006; Vidali et al., 1968) (see Table 1).

Some representative examples will be provided to illustrate the complex role of acetylation in regulating protein function.

PROTEIN CATEGORY	SELECTED EXAMPLES
CORE HISTONES	H2A, H2B, H3, H4
NON-HISTONE CHROMATIN PROTEINS	HMGB
KAT	p300/CBP, PCAF
TRANSCRIPTION FACTORS	p53, STAT3, c-Myc, MyoD, E2F, Rp, NF_kB, HIF
CYTOSKELETAL PROTEINS	ALPHA-TUBULIN, cortactin
CHAPERONES	Hsp90
MITOCHONDRIAL PROTEINS	ACETYLCoA synthase, thioredoxin
VIRAL PROTEINS	Adenoviral E1A, HIV Tat and Integrase
TRANSMEMBRANE RECEPTORS	Type I INFR

Table 1. Selected acetylated proteins. Acetylated proteins are listed, and cytoplasmic acetylated proteins are highlighted. References: Histones (Roth et al., 2001); HMGB (Sterner et al., 1979); p300, PCAF (Santos-Rosa et al., 2003; Thompson et al., 2004); transcription factors (Galbiati et al., 2005; Gu and Roeder, 1997; Jeong et al., 2002; Marzio et al., 2000; Polesskaya et al., 2000; Sakaguchi et al., 1998; Sartorelli et al., 1999); cytiskeletal proteins (Hubbert et al., 2002; Zhang et al., 2007); chaperones (Kovacs et al., 2005); mitochondrial proteins (Starai et al., 2002); viral proteins (Cereseto et al., 2005; Kiernan et al., 1999; Madison et al., 2002; Marzio et al., 1998; Ott et al., 1999; Zhang et al., 2000); INFR (Tang et al., 2007).

Lysine acetyltransferases (KATs)

Several proteins have been found to have intrinsic lysine acetyltransferase activity, while no acetylation consensus site on target proteins has been characterized until now. Nonetheless, acetylases modify very few lysines within a given protein, thus indicating that some specificity exists; moreover, proteomic studies suggest that acetylation is favoured in the context of specific secondary structural characteristics (Kim et al., 2006). According to sequence similarity, KATs can be subdivided into three major groups.

The Gcn5-related N-acetyltransferases (GNATs) family includes GCN5, PCAF, Elp3, Hat1, Hpa2 and Nut1. GCN5 and PCAF are characterized by a Histone Acetyl Transferase (HAT) domain and by a bromodomain, that possesses specific acetyl-lysine recognizing ability; these proteins are co-transcriptional activators able to acetylate histones, but they can acetylate non-histone protein as well (Yang, 2004). Interestingly, the Elp3 subunit of the elongator complex possesses acetyltransferase activity (Wittschieben et al., 1999) and has been recently identified as the enzyme responsible for α-tubulin lysine modification (Creppe et al., 2009).

The p300/CBP family has been extensively characterized for its pleiotropic role in cell biology (Goodman and Smolik, 2000). Both p300 and CBP, two proteins with a high homology, work as transcriptional co-activators, and are able to catalyze acetylation of non-histones substrates.

The third major group is constituted by the MYST family: human MOZ (monocytic leukaemia zinc finger protein), yeast Ybf2, yeast Sas2 and mammalian Tip60 (Avvakumov and Cote, 2007). This large and diverse family has been poorly characterized in comparison with GNATs and p300/CBP.

In accordance with their important role in nuclear, as well as cytoplasmic, protein functions, KAT enzymatic activity can be regulated by multiple mechanisms.

- Most KATs actually exist as part of multi-subunit complexes *in vivo*; and their function and the specificity of the catalytic subunit depend on the nature of the complex (Lee and Workman, 2007).
- Some KATS, such as p300 and CBP are auto-acetylated (Karanam et al., 2006), and acetylation seems to be linked to enzyme activation.
- In addition to acetylation, KATs are regulated by several PTMs: ubiquitination, phosphorylation and sumoylation.
- Enzymatic activities of PCAF and p300/CBP are regulated by their interaction with transcription factors as well as viral proteins.
- Sub cellular compartmentalization has been shown to be an important mechanism for KAT and KDAC function, for example in response to interferon (Tang et al., 2007).

Lysines deacetylases (KDACs)

Known eukaryotic deacetylases are divided in two families: the HDACs (Histone Deacetylase) and Sirtuins. These two major groups display a different cofactor requirement: HDACs, strictly related to the yeast Hda1/Rpd3 proteins, require a divalent Zinc cation for deacetylation, while Silent information regulator 2 (SIR2)-related enzymes rely on NAD^+ for their catalytic activity (Imai et al., 2000). Despite structural and functional diversity, both families not only contribute to histone deacetylation, but also participate in other cellular processes by targeting non-histone substrates as well.

HDACs have a major role in controlling negatively gene expression, through chromatin compaction that favours transcriptional repression. Nonetheless, it is now clear how they have also more specific functions, especially in the regulation of key transcriptional factors, such as HIF1-α

(Haberland et al., 2009; Jeong et al., 2002). In addition, *in vitro* and *in vivo* studies revealed highly specific functions for the 11 mammalian HDAC isoforms, which differ in structure, enzymatic functions, sub cellular localization and expression patterns.

Class I is constituted by HDAC1, 2, 3 and 8. These Rpd3-related molecules are characterized by a high enzymatic activity towards histones, are ubiquitously expressed and are usually located into the nucleus (Taunton et al., 1996).

HDAC4, 5, 7 and 9 belong to Class IIa family. These proteins have restricted expression patterns: HDAC7 for example is enriched in ECs (Chang et al., 2006), while HDAC 5 and 9 are highly expressed in heart tissue (Chang et al., 2004). Interestingly, phosphorylation of these HDACs has been linked to nuclear-cytoplasm shuttling (McKinsey et al., 2000). Finally, Class II HDACs repress transcription by a not completely elucidated mechanism, since they are characterized by a low, if any, enzymatic activity (Haberland et al., 2009).

Class IIb has two members: HDAC6 and HDAC10. Interestingly, HDAC6 represents the main cytoplasmic mammalian deacetylase (Zhang et al., 2008). Additionally, it has been demonstrated that this enzyme can directly deacetylate cytoplasmic proteins, such as α-tubulin, cortactin, as well as IFNRα (Hubbert et al., 2002; Tang et al., 2007; Zhang et al., 2008).

HDAC 11 is the only Class IV member; this enzyme is enriched in some tissues, such as brain and heart, but has been poorly characterized from a functional point of view (Gao et al., 2002).

Intriguingly, many HDAC inhibitors (HDACi) are actually under investigation, even in human clinical trials, for their effectiveness in the treatment of a variety of disorders (Buchwald et al., 2009).

Within the sirtuin family, sometimes defined as Class III KDACs, there are seven mammalian homologues, SIRT1 to 7, initially identified for their role

in cell metabolism (Guarente, 2006). Nowadays it is clear that sirtuins are complex regulators of several cell functions, such as DNA repair, as well as vascular endothelial homeostasis (Finkel et al., 2009; Potente and Dimmeler, 2008).

Concluding, the growing number of identified acetylated non-histone proteins raises the question whether data obtained upon HDAC inhibition have to be completely ascribed to chromatin modification, or might equally be obtained as a consequence of non-histone protein acetylation (Haberland et al., 2009).

Crosstalk between acetylation and other PTMs in regulating protein function

The effects of acetylation in protein function are multiple, primarily depending on protein activity and on the nature of the acetylated residues. For instance, even in a single protein such as p53, acetylation has been linked to: i) enhancement of specific DNA binding (Brooks and Gu, 2003; Prives and Manley, 2001); ii) increase protein-protein interaction (Li et al., 2007); iii) control of protein stability (Brooks and Gu, 2003; Le Cam et al., 2006); iv) crosstalk with other PTMs, similar to histones (Kurash et al., 2008), and therefore regulation of additional functions.

In order to simplify, acetylation events can be classified into three categories. First, acetylation occurring at one or a few lysines can act as a simple on/off switch; acetylation can lead to both “gain of function” and “loss of function” effects. One example is acetylation of nitric oxide synthase (eNOS), which inactivates the enzyme in EC (Mattagajasingh et al., 2007). Second, acetylation can occur at a lysine cluster, which constitute positively charged patches; changes in the charge of these patches influences cortactin binding (Zhang et al., 2007) and p300 enzymatic activity (Thompson et al., 2004). Finally, acetylation might

crosstalk with diverse PTMs, such as phosphorylation, leading to site-specific effects, which act in a combinatorial way (Figure 1.10).

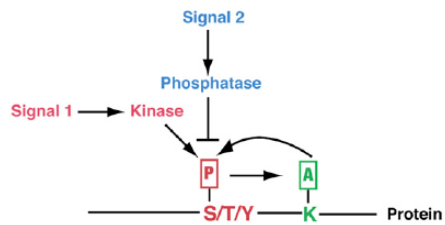

Figure 1.10. Crosstalk between phosphorylation and acetylation. Phosphorylation of diverse residues can influence acetylation, and acetylation might also regulate phosphorylation. Lysines and phosphorylation sites can be also far away from each other. *Adapted from Yang, Molecular Cell 2008.*

Crosstalk between different PTMs can be either positive or negative in nature. In positive crosstalk, acetylation constitutes a signal for addition or removal of other PTMs, or indirectly creates a recognition site for a binding protein carrying a second PTM. Even negative crosstalk can be direct or indirect; in the first case we can find direct competition for lysine modification, such as between acetylation, ubiquitination and methylation, while in the second case, one modification inhibits further modifications by masking their recognition site (Hunter, 2007).

In the effort to dissect the multifaceted role of combination of PTMs in protein function, researchers are looking for a posttranslational code, as discussed for example in the histone code hypothesis (Strahl and Allis, 2000) and in the “post translation code” model for p53 (Appella and Anderson, 2000).

Therefore, PTMs form a dynamic layer of molecular information, beyond amino acid sequences, where acetylation has been proposed to be a rival

of phosphorylation (Kouzarides, 2000). A recent proteomic study revealed that almost 200 proteins are acetylated in HeLa cells, disclosing the role of acetylation in the regulation and diversification of signalling networks even out of the nucleus (Kim et al., 2006).

Acetylation of non-nuclear proteins

The role of acetylation in non-nuclear protein function has been discovered only recently, nonetheless its relevance is continually growing. For instance several enzymes, such as nitric oxide synthase, acetyl-CoA synthase, glutamate dehydrogenase and metabolic enzymes, are regulated by acetylation, usually by an on/off switch. Following is a description of a few examples of how acetylation might crosstalk with other PTMs to modulate cytoplasmic protein function.

α-tubulin. Despite the fact that α-tubulin acetylation has been discovered more than 20 years ago (L'Hernault and Rosenbaum, 1985) the exact role of tubulin acetylation in cell biology has not been fully elucidated. Interestingly, interaction of tubulin with other proteins is influenced by many PTMs, including phosphorylation and palmitoylation (Westermann and Weber, 2003). The α-tubulin acetylation site, lysine 40, is located in the lumen of microtubules, and this modification has been correlated to binding of cargo proteins, and therefore to vesicular transport (Dompierre et al., 2007; Reed et al., 2006). HDAC6 is the major responsible for tubulin de-acetylation (Hubbert et al., 2002). HDAC6 KO mice are viable even if they show tubulin hyper-acetylation (Hubbert et al., 2002; Zhang et al., 2008). Interestingly, the enzyme responsible for tubulin acetylation has been recently identified as a component of the Elongator complex. This protein, Elp3, has been classified as a member of the GNAT family (Svejstrup, 2007; Wittschieben et al., 1999). This complex, possibly by acetylating α-

tubulin, is able to control cortical neuron differentiation and migration (Creppe et al., 2009).

Cortactin. This protein, involved in cell motility, has been detected as acetylated on almost 10 residues; acetylation of a single lysine is not important, rather the overall number of modified lysines critically influences F1-actin binding and cell motility (Zhang et al., 2007). Cortactin can be modified by PCAF and p300, and its deacetylases are both HDAC6 and SIRT1 (Zhang et al., 2007; Zhang et al., 2009). Interestingly, cortactin is phosphorylated on tyrosines and 3D structure analysis suggests that phosphorylation of Tyr466 can crosstalk with acetylation (Yang and Seto, 2008).

INFαR. Interferon α (INFα) triggers INFα receptor 1 and 2 oligomerization and their tyrosine phosphorylation; at the intracellular level INFα antiviral response is mainly mediated by STAT1 and 2 proteins (Schindler et al., 2007). A recent study has revealed how acetylation is involved in many steps of type I INF signalling pathway (Tang et al., 2007). In particular it was shown that: i) INF treatment induce CBP nuclear export; ii) CBP/p300 associate with INFRs, and this association depends on two phosphorylated serines; iii) INFαR2 is acetylated in cells on Lys399 upon INFα treatment; iv) Acetyl lysine 399 provide the docking site for interferon regulatory factor 9 (IRF9); v) IRF9 acetylation influences dimer formation and DNA binding; vi) STAT1 and STAT2 interaction is regulated by acetylation (Figure 1.11).

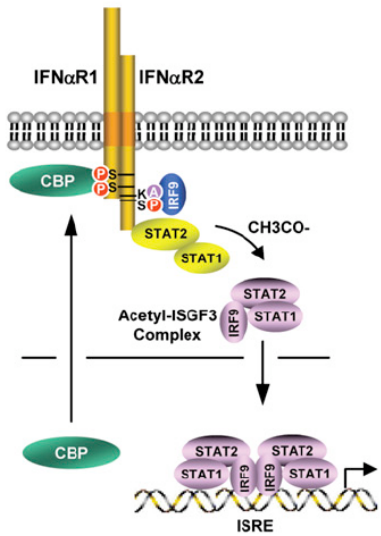

Figure 1.11. INFα receptor acetylation. The model outline how CBP-mediated acetylation influences INFα signalling pathway at different steps. *Adapted from Tang, Cell 2007*.

This study suggests acetylation as a key regulator in signal transduction pathway and more importantly is the first indication that a transmembrane receptor can undergo acetylation.

Hsp90. Hsp90 chaperon activity is important for the regulation of several signalling proteins, and recently its activity has been linked to acetylation. In particular two groups demonstrated HDAC6-Hsp90 binding and deacetylation, and showed how protein hyper acetylation compromises Hsp90 binding to client proteins (Bali et al., 2005; Kovacs et al., 2005). Since Hsp90 can undergo different PTMs and is required for maturation of many kinases, its acetylation could affect multiple pathways.

Acetylation and blood vessel biology

Lysine acetylation and deacetylation, also of non-histone proteins, have been linked to many physiological as well as pathological processes, as recently reviewed (Finkel et al., 2009; Haberland et al., 2009; Spange et al.,

2009). Interestingly, growing body of evidence indicates that lysine acetylation has a major role in endothelial cell biology; as already discussed, blood vessel growth is a complex mechanism that requires the coordination of several molecular players, including several transcription factors and co-factors. In this perspective, it is not surprising that lysine acetylation of nuclear proteins, as discussed later, can influence blood vessel growth ad homeostasis (Potente and Dimmeler, 2008), as outlined in Figure 1.12.

The important angiogenesis regulator HIF1α and its function are directly and indirectly controlled by acetylation, and this issue has been extensively studied (Ellis et al., 2009). For instance HIF1α acetylation has been linked to its degradation (Jeong et al., 2002), and HIF1α forms complexes with CBP/p300 transcriptional activator (Freedman et al., 2002). Recent data show also how SIRT selectively stimulates activity of the transcription factor HIF2α during hypoxia. Importantly, the effect of SIRT1 on HIF2α required direct interaction between the two the proteins and intact deacetylase activity of SIRT1 (Dioum et al., 2009).

Additionally, some KDACs have been linked to vessel biology and will be discussed in detail.

SIRT1. SIRT1 is required during embryonic and early post-natal development for heart and eye formation, and maintains its regulatory role during adulthood (Cheng et al., 2003; McBurney et al., 2003). Recent data identified SIRT1 as a key mediator of sprouting angiogenesis during vascular growth (Potente et al., 2007). SIRT1, which is expressed by EC both *in vitro* and *in vivo*, was shown to regulate angiogenic sprouting and VEGF-induced cell migration *in vitro.* Moreover, SIRT1 deficient zebrafish displayed vascular patterning defects, and endothelial specific SIRT1 KO mice, which develop normally, have impaired ability to form new vessel in ischemic tissues. SIRT1 activity in endothelial cells, as a stress-responsive

angiogenesis regulator, is in part mediated by its interaction with Foxo1, a crucial transcription factor in ECs (Dejana et al., 2007). Nonetheless, given also the multitude of SIRT1 targets described until now (Haigis and Guarente, 2006), SIRT1 probably has additional partners and targets in EC. In addition to sprouting angiogenesis, SIRT1 is able to regulate vascular tone, by directly acting on eNOS synthase (Mattagajasingh et al., 2007). In particular SIRT1 targets eNOs synthase for deacetylation leading to enhanced NO production.

Sirtuins are not the only deacetylases to be involved in vessel biology: even the HDAC family members have a role in blood vessel formation. For instance, Trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA), two HDAC inhibitors (HDACi), suppress spontaneous and VEGF-induced angiogenesis in *in vitro* models; in addition, HDACi block VEGF-induced VEGFR2, VEGFR1 and Neuropilin1 overexpression at the mRNA level (Deroanne et al., 2002).

HDAC5. Since siRNA-mediated downregulation of HDAC5 increased angiogenic sprouting and stimulated EC migration, this enzyme is probably a negative regulator of angiogenesis (Urbich et al., 2009). Another study showed that VEGF-induced angiogenesis depends on PKD mediated HDAC5 nuclear export, thus strengthening the hypothesis that HDAC5 acts to alter gene expression, and in particular that HDAC5 nuclear export relieves an inhibitory signal (Ha et al., 2008).

HDAC7. Specific expression of HDAC7 in endothelial cells during early mouse development indicates that this protein might have a role in vessel development (Chang et al., 2006). HDAC7 genetic deletion is embryonic lethal and is accompanied by loss of EC interactions and MMP10 upregulation. Therefore, it hes been proposed that HDAC7 represses MMP10 gene transcription by associating with myocyte enhancer factor-2 (MEF2), a direct activator of MMP10 transcription and an essential

regulator of blood vessel development (Chang et al., 2006). More recently, the same group demonstrated that HDAC7 regulates VEGF-induced EC migration and proliferation (Wang et al., 2008). In particular they discovered how VEGF-A induces HDAC7 rapid phosphorylation and nuclear export, thus relieving the inhibition of VEGF responsive genes. Similarly to HDAC5, nuclear export is mediated by PKD phosphorylation (Wang et al., 2008).

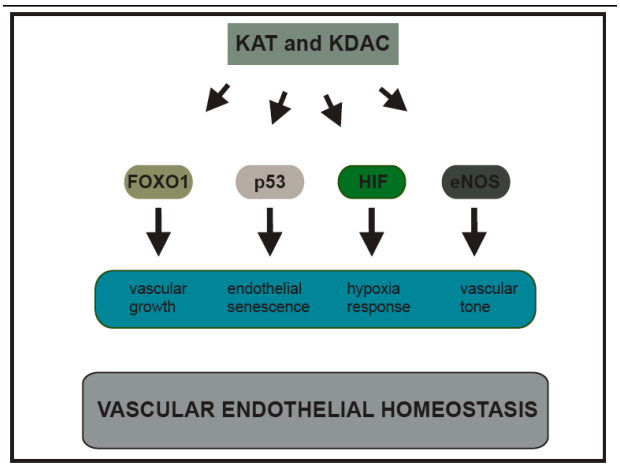

Figure 1.12. Aceylation is involved in vascular biology. KAT and KDAC are both positive and negative regulators of vascular homeostasis, and they act almost trough four factors, Foxo1, p53, HIF and eNOS.

Recent discovery of the role of acetylation in angiogenesis led to the hypothesis that targeting HDAC offers a new strategy in anti-cancer therapy, trough tumor angiogenesis inhibition; currently, pre-clinical and clinical studies suggest HDACi might improve the anti-angiogenic action of current anti-VEGF therapies (Buchwald et al., 2009; Ellis et al., 2009).

Visualization of protein interactions using fluorescent proteins

In the effort to identify the molecular basis of the green bioluminescence of living *Aequorea victoria* jellyfish, Shimomura and colleagues first isolated a protein displaying a strong green fluorescence as functional partner of the chemiluminescent protein aequorin (Johnson et al., 1962; Shimomura et al., 1962). They also demonstrated that the newly isolated protein, therefore defined Green Fluorescent Protein (GFP), is characterized by an emission spectra peak at 508 nm (Figure 1.13), close to the one emitted by living *Aequorea* tissues. Since the chemiluminescence of pure aequorin is blue, with a peak around 470 nm, close to the GFP excitation peaks, they hypothesized that GFP converted the blue emission of aequorin to the green glow of intact jellyfish.

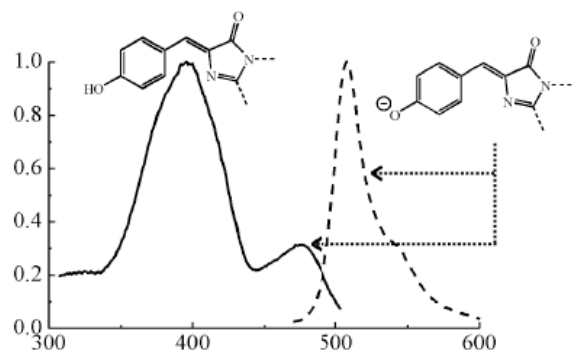

Figure 1.13. Spectral properties of GFP. The excitation (full-line curve) and the emission (dashed curve) spectra of wild type GFP protein from *Aequoera victoria* are shown, in association with the cromophore structures. X axis: wavelength in nm; Y axis: spectral amplitude normalized to a maximum of 1. *Adapted from Tsien, Ann.Rev. Biochem., 1998.*

This hypothesis was supported by data obtained upon GFP purification and crystallization: GFP is the acceptor, while aequorin is the donor in an

energy transfer reaction of Fluorescence (or Foster) Resonance Energy Transfer (FRET) type (Morise et al., 1974).

The cloning of the complete ORF of GFP, coding for a protein of 238 aa, from a cDNA library of *Aequoera victoria* together with protein characterization, constituted an additional step towards the application of GFP in biology (Prasher et al., 1992). Nonetheless, the critical question whether the GFP-chromofore forms spontaneously or whether its maturation requires an auxiliary enzyme expressed by jellyfish was still unresolved.

The findings that fluorescent GFP could be expressed from the GFP gene, in *E.coli*, *C. elegans*, *S. cerevisiae* and *D. melanogaster*, discloses the potential of GFP as a universal genetic tag in biological research (Chalfie et al., 1994; Wang and Hazelrigg, 1994). Therefore, the GFP gene contains all the information required for posttranslational synthesis of the chromophore, and no jellyfish specific enzymes are needed (Tsien, 1998).

GFP: Structure, folding and fluorescence properties

Since the structure of the chromophore seems to be *per se* responsible for fluorescence, several efforts helped to clarify this peculiar property. Interestingly, the 238 aa of GFP are folded into a unique 11 β-sheet barrel structure with a diameter of about 2,4 nm and a height of 4,2 nm. An α-helix runs diagonally trough the can formed by the β-sheets, and the chromophore is locate almost perfectly in the centre of the cylinder, attached to the α-helix (Ormo et al., 1996; Yang et al., 1996) (Figure 1.14). The GFP structure confers stability to the protein and protects the chromophore from quenching. The core of the protein, the chromophore, is a p-hydroxybenzylideneimidazolinone, which forms spontaneously from intramolecular cyclization of three aminoacid residues, namely Serine 65,

Tyrosine 66 and Glycine 67. The GFP folding into its peculiar structure is probably the crucial event for the formation of an active chromophore.

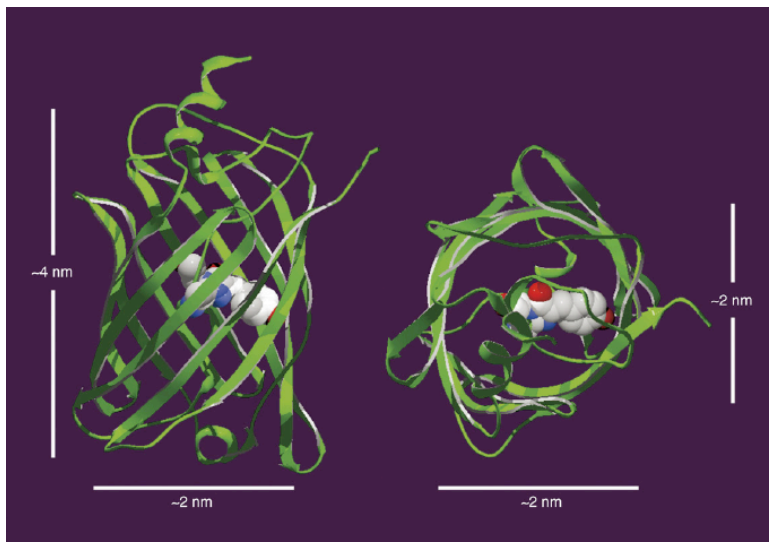

Figure 1.14. GFP structure. 11 β-sheets constitute the β-barrel that characterizes native GFP structure. The chromophore responsible for GFP fluorescence is buried in the can (spheres). *Adapted from Piston and Kremers, TRENDS in Biochemical Sciences, 2007.*

Upon GFP folding, Glycine 67 (conserved among all GFP variants that retain fluorescence) and Serine 65 form an imidazolone. Dehydratation, conjugation with Tyrosine 66 and more importantly dehydrogenation by oxygen are fundamental steps required to acquire fluorescence (Tsien, 1998). The 27 kDa GFP protein can undergo reversible denaturation, and fluorescence lost in the denatured GFP is regained when the β-barrel is reformed. Interestingly, non-fluorescent fragments coding for the N terminus and the C-terminus parts of GFP can undergo complementation in cultured cells, producing a fluorescent complex resembling the native protein (Hu et al., 2002). Finally, it has been demonstrated that GFP can form dimers (Yang et al., 1996).

GFP optimization and spectral variants

Even if many features rendered *Aequorea victoria* wild type GFP a useful tool for biological application, in order to improve its use the original sequence has been optimized by mutations, using several different strategies (Zacharias and Tsien, 2006).

Two main objectives characterize GFP mutations: optimization of the protein for biological studies and creation of new, different spectral variants.

Regarding protein optimization, GFP engineering has improved brightness and photo stability, two important features for the experimental use of fluorescent proteins (FPs). In addition, it has been shown that several mutations improve GFP folding, in particular allowing faster and more efficient maturation of the protein even at 37 °C (Zacharias and Tsien, 2006). Finally, the GFP sequence was modified to improve expression in mammalian cells: more than 190 silent mutations were inserted to optimize codon usage, together with a Kozak sequence for translation initiation. Taken together, all these modifications contributed to obtain high expression levels of mature, fluorescent GFP in mammalian systems.

Using similar mutational techniques, several different spectral variants were originated form GFP by aminoacidic substitutions, mainly in the three residues involved in chromophore formation; these proteins exhibit emission spectra ranging from blue to yellow (Table 2). The palette of these FPs has been exploited by several biological applications (Nguyen and Daugherty, 2005). They can be subdivided into seven classes according to the components of the chromophores:

1st) WILD TYPE GFP. Characterized by a complex excitation peak.

2nd) Enhanced GFPs. Represent the more commonly used variants, due to enhanced brightness, simple excitation as a consequence of substitution on Ser65.

3rd) Sapphire. The single mutation from Thr 203 to Ile gives rise to these mutants characterized by a large Stokes shift.

4th) Yellow Fluorescent Proteins (YFPs). Substitution of Thr 203 with aromatic residues results in YFPs. YFPs are characterized by a red shift in both excitation and emission spectra, resulting in an emission peak around 529 nm, the longest known for GFP mutants.

5th) Cyan Fluorescent Proteins (CFPs). Tyr 66 to Trp modification in the GFP produces a different, new chromophore. These proteins are characterized by double excitation and emission peaks, and give rise to blue-green to cyan emissions.

6th) Blue Fluorescent Proteins (BFPs). Again, substitution of Tyr 66, in this case with His, leads to a blue fluorescent protein, not extensively used due to low quantum yield and easy bleaching.

7th) Phenyl in chromophore derived from Tyr66Phe. This protein is characterized by the shortest wavelengths among GFP derivates, and has therefore been poorly investigated.

Class	Protein	Source laboratory (references)	Excitation[c] (nm)	Emission[d] (nm)	Brightness[e]	Photostability[f]	pKa	Oligomerization
Far-red	mPlum[g]	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry[g]	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato[g]	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry[g]	Tsien (4)	574	596	26	15	<4.5	Monomer
	J-Red[h]	Evrogen	584	610	8.8*	13	5.0	Dimer
	DsRed-monomer[h]	Clontech	556	586	3.5	16	4.5	Monomer
Orange	mOrange[g]	Tsien (4)	548	562	49	9.0	6.5	Monomer
	mKO	MBL Intl. (10)	548	559	31*	122	5.0	Monomer
Yellow-green	mCitrine[i]	Tsien (16,23)	516	529	59	49	5.7	Monomer
	Venus	Miyawaki (1)	515	528	53*	15	6.0	Weak dimer[j]
	YPet[g]	Daugherty (2)	517	530	80*	49	5.6	Weak dimer[j]
	EYFP	Invitrogen (18)	514	527	51	60	6.9	Weak dimer[j]
Green	Emerald[g]	Invitrogen (18)	487	509	39	0.69[k]	6.0	Weak dimer[j]
	EGFP	Clontech[i]	488	507	34	174	6.0	Weak dimer[j]
Cyan	CyPet	Daugherty (2)	435	477	18*	59	5.0	Weak dimer[j]
	mCFPm[m]	Tsien (23)	433	475	13	64	4.7	Monomer
	Cerulean[g]	Piston (3)	433	475	27*	36	4.7	Weak dimer[j]
UV-excitable green	T-Sapphire[g]	Griesbeck (6)	399	511	25*	25	4.9	Weak dimer[j]

Table 2. Characteristics of the principal fluorescent proteins. Name and spectral characteristics are outlined. *Adapted from Shaner, Nature Methods 2005.*

Other fluorescent proteins

Discovery, and subsequent optimization, of novel GFP-like proteins from Anthozoa significantly expanded the colour palette available for cell biology experiments. Of particular interest for future applications, in particular FRET, are proteins presenting emission spectra characterized by peaks in red and far-red wavelengths (Red Fluorescent Proteins, RFPs). For instance, red, orange and yellow proteins were obtained modifying a red fluorescent protein of *Discosoma* species (Shaner et al., 2004).

Fluorescent proteins as a powerful tool in biology

Many aspects of GFP and its derivatives make them a versatile and powerful tool in bioscience. Among these: i) brightness; ii) photo-stability of the molecule; iii) existence of protein with spectral properties covering the whole visible region; iv) fast and efficient folding of the molecules in cells; v) rapid and autonomous maturation of the chromophore upon protein folding; vi) predominant monomeric protein configuration.

Therefore, GFPs have been successfully employed to investigate cell and protein functions.

- PROTEIN EXPRESSION AND LOCALIZATION. The first application of the GFP was as a reporter gene (Chalfie et al., 1994), and this tool has been extensively used to study gene expression during development. The combinatorial expression of fluorescent proteins in the nervous system of Brainbow mice further confirmed the potential of this tool (Livet et al., 2007).
- PROTEIN DIFFUSION AND TRAFFICKING. Protein localization and fate can be analyzed using a chimera between the protein of interest

and GFP variants. GFP has been targeted successfully to almost every major organelle in the cell, including plasma membrane, nucleus, endoplasmic reticulum, Golgi apparatus, vesicles and mitochondria (Tsien, 1998). Several microscopy techniques have been developed in order to unravel protein dynamics. Fluorescence Recovery After Photobleaching (FRAP), exploiting fluorophore bleaching, allows measurement of bi-dimensional and three-dimensional mobility of fluorescent particles.

- CONFORMATIONAL CHANGES. FRET based sensors, i.e. fusion of a protein with a FRET pair, have been widely used to study dynamic conformational changes (Giepmans et al., 2006). For instance a Src reporter that allows imaging and quantification of spatio-temporal Src activation in EC has been optimized (Wang et al., 2005).
- PROTEIN-PROTEIN INTERACTIONS. Intermolecular FRET can detect dynamic protein-protein interaction in living cells, provided that the proteins are fluorescently tagged, as discussed later in this section. Another method to detect protein-protein interaction takes advantage of the property of two GFP fragments to form a fluorescent molecule, if the two fragments are in proximity (Kerppola, 2006). This technique has been defined as bimolecular fluorescence complementation (BIFC).
- PROTEIN SYNTHESIS AND TURNOVER. Photoactivation, photoconversion, and photobleaching can be used to highlight different protein populations, in combination with time-lapse imaging.
- SENSORS. Many FPs display a pH-dependent fluorescent behaviour, and have been widely used to monitor pH in vivo, and a series of pH sensitive GFP (pHfluorins) have been developed. Additionally, GFP-based Ca^{2+} indicators, called cameleons, are commonly used.

Fluorescence (Foster) Resonance Energy Transfer (FRET)

Combination of different fluorescent proteins and FRET methods provides high spatial resolution assays of protein-protein interaction in living cells. FRET is a physical phenomenon occurring between two fluorescent molecules that are sufficiently close to each other. The energy is transferred form one molecule (the donor) to another molecule (the acceptor), through a non-radiative mechanism (resonance energy transfer). For FRET to occur, almost two conditions must be fulfilled: a) the emission spectra of the donor molecule and the absorption spectra of the acceptor must overlap (see Figure 1.15); b) since the energy transfer rate varies inversely with the 6^{th} power of donor-acceptor distance, the two molecules must reside within 10nm.

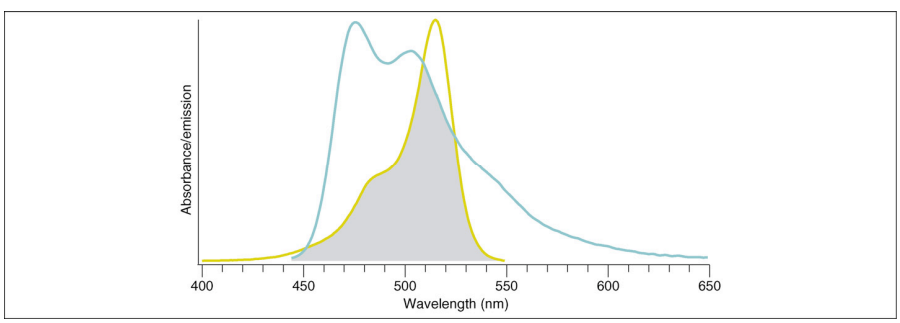

Figure 1.15. Spectral overlap between of CFP/YFP pair. The emission spectrum of CFP (cyan line) shows a good overlap with the absorption spectrum of YFP (yellow line), a requisite for FRET. *Adapted from Piston and Kremers, TRENDS in Biochemical Sciences, 2007.*

Other properties of the FPs, such as relative orientation of the two fluorophores, quantum yield of the donor and brightness, can affect resonance energy transfer efficiency, therefore limiting FRET technique application (Jares-Erijman and Jovin, 2003). Even if genetic labelling with FPs has led to increased interest in FRET technique, the choice of suitable

fluorophore pair (the FRET pair) is crucial (Tsien, 1998). Different GFP variant are good candidate ad have been already used for FRET experiments, among them the GFP/BFP and CFP/YFP couples (Piston and Kremers, 2007). At the moment, CFP and YFP remain the best FRET pair, despite the significant crosstalk in both the excitation and emission spectra of these two FPs (Nguyen and Daugherty, 2005).

Imaging FRET for cell biology applications

Even if several methods have been developed to detect FRET signals, five approaches have been proved particularly useful in cell biology applications.

1) Sensitized emission. With this method, fluorescence emitted by the donor and the acceptor is collected separately upon exclusive excitation of the donor with a specific wavelength. FRET quantification using this method is not simple, and this kind of assay can be applied only to experiments characterized by a strong signal (Gordon et al., 1998).
2) Spectral imaging. Similarly to sensitized emission, this methodology entails excitation only of the donor, followed by collection of the whole excitation spectra from both the donor and the acceptor. Positive FRET can be detected by spectral modifications.
3) Acceptor photobleaching. Acceptor photobleaching, or donor dequencing, relies on the principle that energy transfer from the donor is reduced or eliminated when acceptor is quenched (or bleached), thereby inducing an increase in donor fluorescence. This method is straightforward, quantitative and less prone to false positive results than other methods (Karpova et al., 2003), and has been used to study nuclear dynamics (Cereseto et al., 2005).

4) Fluorescence Lifetime Imaging (FLIM). Since the donor fluorescence is naturally quenched by FRET, FLIM measures directly the shortening of the fluorescence decay time of the donor in presence of FRET. This method, even if it is rigorous and avoids cross-talk artefacts, is technically demanding (Levitt et al., 2009).
5) Polarization anisotropy imaging. Fluorescence polarization can be used to discriminate between the presence and the absence of FRET, even if is not a good approach for subtle FRET quantifications (Mattheyses et al., 2004).

Intermolecular FRET

Intermolecular FRET, occurring when the donor and acceptor molecules are on different proteins, has been widely and successfully used to analyze protein-protein interactions (Giepmans et al., 2006). FRET presents several advantages for protein-protein interaction detection, in comparison with other techniques. First, positive FRET signal indicates protein-protein interaction at a distance of a few nanometres (1-8 nm) for the vast majority of fluorophores couples. Second, protein tagging with GFP variants allows visualization in real time and in living cells, and this is particularly useful to detect the effect of external stimuli on protein complexes formation. Third, FRET permits to detect complex formation in defined cell compartments, or even sub-compartments (Marcello et al., 2001). This is of particular value, given the increasing role attributed to compartmentalization in different biological processes, for example at the level of the plasma membrane.

The previous limitation of FRET technique, that allowed detection of only a proteins pair, has been recently overcame. Ternary complexes have been analyzed in cells combining BIFC and FRET: in this assays two proteins (A and B) are fused to two non-fluorescent FPs fragments, whereas the third protein (C) is fused to a full-length FP. Interactions between A and B

reconstructs one fluorophore, and if A, B and C are in proximity, FRET can occur (Shyu et al., 2008a, b) (Figure 1.16).

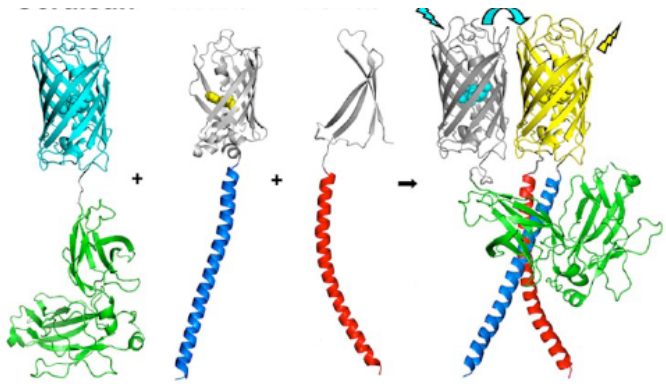

Figure 1.16. BIFC based FRET. Visualization of ternary complexes can be obtained combining FRET with BIFC; two proteins (blue and red helices) are fused to non-fluorescent fragments of a YFP variant, while the third protein (in green) is fused to a fluorescent CFP. FRET is detectable upon ternary complex reconstruction. *Adapted from Shyu, PNAS 2008.*

FRET applied to transmembrane receptor biology

FRET has been extensively exploited to study protein-protein interactions in different compartments, including the cell nucleus, cytoplasm and plasma membrane. Of particular interest is the application of FRET to elucidate and image the mechanisms underlying transmembrane receptor activation, complex formation and signal transduction.

For instance, G Protein Coupled Receptor (GPCR) biology has been analyzed using FRET. A FRET methodology recently optimized allows the analysis of oligomeric assembly of GPCRs at the cell surface and of the stoichiometry of class C GPCR oligomers in living cells (Maurel et al., 2008). Additionally, the FRET technique has been used to analyze in details the formation of GPCRs heterodimers composed by CCR5 and

CXCR4, on the plasma membrane of living cells and in response to ligand binding (Isik et al., 2008).

FRET contributed to the comprehension of Epidermal Growth Factor Receptor (EGFR) biology, belonging to the tyrosine kinase receptors family. Several FRET quantification methods were successfully applied to analyze EGFR oligomers formation on the plasma membrane, conformational changes and interactions with second messengers (Hayes et al., 2004). FRET between PDGF-R and FGF-R1 occurring in PAE cells treated with FGF-2/PDGF-BB mixture was detected, further supporting the finding that FGF-R1 and PDGF-R directly interact forming heterodimers (Faraone et al., 2006).

FRET can be used even in homodimers analysis. For instance Neuropilin1-derived peptide dimerization was actually measured in solution using sensitized emission (Roth et al., 2008).

Finally, FRET was applied to detect the interaction between VEGFR2 and the tyrosine phosphatase SHP2 upon $VEGF_{165}$ stimulation in PAE cells (Mitola et al., 2006).

Chapter 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Plasmids

pCDNA3-mouseVEGFR2, pEGFP-N1-Neuropilin1 and pCMV-PlexinA1 were kindly provided by Professor Federico Bussolino. From pCDNA3-mouseVEGFR2 we constructed a pCDNA3-VEGFR2-Flag with the Flag tag fused to the C-terminal part of VEGFR2. The following VEGFR2 mutants were cloned by PCR amplification of mouse VEGFR2 with primers specific for the mutated version: K1053R, K929R-K937R-K939R, K929R-K937R-K939R-K947R and K929R-K937R-K939R-K947R-K1053.

pCDNA3-p300 has been previously described (Marzio et al., 1998).

The pCMV-p300-DY-myc mutant was kindly provided by T.P.Yao (Durham).

pCDNA3-HA-GCN5 was prepared by A. Sabo' as previously reported (Sabo et al., 2008).

pCMV-Flag-PCAF was kindly provided by Professor E. Verdin.

pEGFP-N1, pECFP-N1 and pEYFP-N1 plasmids were purchased from Clontech (Mountain view, CA).

pECFP-EYFP was obtained by PCR cloning of ECFP cDNA in pEYFP-N, spaced by a sequence coding for a seven aminoacid-long linker.

pEGFP-Nterminal-NP1 and pEGFP-Cterminal-NP1 were cloned by PCR, subcloning the GFP N-terminus (residues 1-154) and C-terminus (155-238) respectively, at the C terminus of mouse Neuropilin1, as previously reported (Hu et al., 2002).

pCDNA3-VEGFR2-EGFP (or EYFP) was obtained by PCR subcloning of EGFP (or EYFP) cDNA at the C-terminus of VEGFR2.

pCMV-GIPC was purchased from Origine (Rockville, MD).

pEGFP (EYFP, ECFP)-GIPC was produced by PCR amplification of GIPC cDNA and cloning in pEGFP (EYFP, ECFP)-N1 and -C1.

Cell culture, transfection and treatment

HEK293T, HeLa and COS-7 cells were maintained in Dulbecco modified Eagle Medium with 10 % FBS and 0,1 mg/ml gentamicin unless differently indicated. PAE and PAE KDR cells, kindly provided by Professor Lena Claesson Welsh, were grown in HAM's F12 medium supplemented with 10% FBS. HUVE cells, purchased from ATTC, were maintained in EBM supplemented medium (Clonetics, Lonza). In the case of protein complementation assay (BIFC) experiments, cells were maintained at 20 °C overnight to improve complemented EYFP folding. Transfections were performed either by the standard calcium phosphate co-precipitation procedure or by Effectene (Qiagen, Germany) or Lipofectamine (Invitrogen, CA).

Cells were treated with TSA for 6h, 500 nM for HUVEC cells and 1 μM for HEK HEK293T cells.

Recombinant $VEGF_{165}$ was produced and purified by our laboratory using a baculovirus expression system, and used at a final concentration of 50 ng/ml, unless differently indicated.

Recombinant $VEGF_{121m}$ (corresponding to $VEGF_{112}$) was purchased from R&D (Minneapolis, MN), while full length $VEGF_{121}$ was purchased from PeproTech (Princeton, NJ); $VEGF_{121}$ was used at 50 ng/ml.

Recombinant SEMA3A was purchased from R&D (Minneapolis, MN), and used at 100 ng/ml, unless differently indicated.

Growth Factor Reduced Matrigel was purchased from BD, and PAE cells were transfected using Lipofectamine, and 24 hours upon transfection seeded onto Matrigel (100000 cells/slide), and treated with recombinant

factors as indicated. Upon O/N treatment, slides were fixed and mounted for FRET analysis.

Inhibition of protein synthesis was achieved by cell treatment with 30 mg/ml cycloheximide for the indicated time points.

The cellular treatment with Lys-CoA (synthesized at the ICGEB Peptide Synthesis Core Facility) was performed as in (Bandyopadhyay et al., 2002) with minor modifications. Briefly, 16x 10^6 exponentially growing HEKHEK293T, transfected or not with pCDNA3-VEGFR2-Flag cells were incubated with the ICB solution (10 mM HEPES, pH 7.0, 0.14 M KCl, 0.01 M NaCl, and 2.4 mM MgCl2) containing Lys-CoA (0.75 mM) and SPC (1.2 mg/ml) at 37°C for 20 min. Upon LysCoA treatment, cells were plated for additional 24 hours and than lysed as described.

Antibodies

For FRET-related experiments the following antibodies were used: anti-Neuropilin1 (Santa Cruz, CA), anti-Neuropilin1 (R&D, MN), anti-GFP (Santa Cruz, CA), anti-VEGFR2 (Santa Cruz, CA), anti-GIPC (Santa Cruz).

For Acetylation-related experiments the following antibodies were used: anti-VEGFR2 either free or conjugated with agarose beads, anti-HA, both from Santa Cruz; anti-Flag, either free or conjugated with agarose beads, and anti-tubulin, both from SIGMA; anti-total acetyl-lysine, anti phospho-VEGFR2 1173 are from Cell Signaling Technology (MA); anti-p300 from BD; anti-total phospho tyrosine clone 4G10 (Upstate).

Horseradish peroxidase conjugated secondary antibodies for western blotting detection were purchased from DAKO. Fluorescently tagged secondary antibodies were obtained from Invitrogen.

Anti-NP1A and anti-NP1B antibodies were obtaneid from Genentech (South San Francisco, CA), and their optimization has been described (Liang et al.,

2007; Pan et al., 2007a). For FRET experiments, antibodies were added two hours before $VEGF_{165}$ or SEMA3A treatment.

Anti-PlGF antibody was provided by Professor Peter Carmeliet group, together with control IgG_1 as a control. Antibodies are described in (Fischer et al., 2007). As in the case of anti-NP1 antobodies, anti-PlGF and control antibody in FRET exxpreiments were added 2 hours before VEGF treatment.

Western blot and immunoprecipitation

Cell lysis was performed using modified RIPA (150 mM NaCl, 50 mM TrisHCl pH 7.4, 1 mM EDTA, 1% NP-40, 0,25% Sodium-deoxycolate) or (Tris HCl 50 mM, NaCl 150 mM, pH 7,5, 1% Triton X-100), supplemented with protease inhibitors tablets (Roche), Sodium Fluoride 1mM, Sodium Butirrate 10mM and Sodium Orthovanadate 1mM (all form SIGMA). Protein lysate concentration was determined by Bradford Assay (BIORAD). Immunoprecipitation was carried out either with anti-Flag beads (SIGMA), or anti VEGFR2 beads or anti-GFP antibody coupled to agarose beads (both from Santa Cruz). Cells extracts where incubated for 4 hours or overnight with antibodies and/or beads, and then washed in lysis buffer. IPs were loaded onto SDS page and analyzed by western blotting, proteomics or silver stain.

For silver staining, gels upon fixation (50% Methanol, 12% acetic acid, 0.05% formalin) were sensitized (0,02% $Na_2S_2O_3$). Staining was performed with a 0,2% AgNo3, 0,076% formalin solution for 20'. Staining was developed (6% Na_2CO_3, 0,05% formalin and 0,0004% $Na_2S_2O_3$) and subsequently stopped with a 50% Methanol, 12% acetic acid solution.

Immunofluorescence

Following paraformaldehyde (or Zinc) fixation, cells were washed with 100 mM glycine and permeabilized with 0.1% TritonX-100, or 0.1 % saponin for 5'. Primary and secondary antibodies were incubated at 37 °C for 1h in an humidified chamber in phosphate-buffered saline (PBS) with the addition of 1% bovine serum albumin or 5% horse serum, and 0.1% Tween 20. Nuclei were counterstained using either 4',6-diamidino-2-phenylindole (DAPI) or Toto-3 (invitrogen). Images were acquired using either a Leica DMLB or Zeiss LSM510 META.

Wheat Germ Agglutinin (WGA)-TRITC conjugated (SIGMA) was used to detect glycosylated proteins by IF. Upon fixation and permeabilization, cells were incubated for 1hour with 5 μg/ml WGA in PBS, then washed three times in PBS and stained for microscopy analysis.

Proteomic analysis

For proteomic analysis, anti-Flag IPs were loaded on a SDS page gel and stained with E-Zinc Stain kit (Pierce), following the related protocol.

The band corresponding to VEGFR2 was excised from the gel and washed with 100 mM EDTA for 10 minutes, then subjected to reduction and alkylation using DTT and Iodoacetamide. The band was washed 3 times with 50 mM Triethylammonium Bicarbonate in 50% Acetonitrile for 30 minutes each. The band was rehydrated with water for 10 minutes and then incubated with Deuterated Acetic Anhydride in Methanol (1 part Anhydride: 2 parts Methanol) for 1 hour at RT. The reaction was quenched by addition of 1 M Ammonium Acetate, and then the band was washed 3 additional times using 50 mM Triethylammonium Bicarbonate in 50% Acetonitrile for 30 minutes each. The band was dehydrated for 10 minutes using 100% Acetonitrile and then subjected to trypsinization over night. The supernatant was harvested and the gel extracted with 1 M

Triethylammonium Bicarbonate and the extract was combined with the original supernatant.

The resulting peptide mixture was acidified with Acetic Acid to 1 M and desalted using STAGE-tips. The tips were eluted with a gradient from 10%-65% Acetonitrile (in 25 ul) and spotted directly onto MALDI targets. Mass spectrometry was performed on a 4800 MALDI-TOF/TOF mass spectrometer and MS/MS spectra were searched against the human and mouse databases using X!tandem.

FRET

For FRET experiments, we transfected PAE cells with different expression plasmids coding for fluorescent-tagged proteins of interest, using Lipofectamine (Invitrogen). Cells were serum starved overnight 24 hours upon transfection and treated with recombinant factors as detailed. Upon treatment, cells were fixed and 4% paraformaldehyde and mounted for FRET analysis with Vectashield mounting medium (Vector).

FRET analysis was performed using a LSM510 META microscope (ZEISS). Optimized FRET channels, as described in the result section, were used in these experiments to acquire CFP and YFP channel images. Two images were acquired before bleaching, each in the CFP and YFP channels separately; acceptor photobleaching was then achieved by using 100% power of the 514 nm laser line in the region of interest (ROI), located at the level of the plasma membrane. We considered for further FRET analysis only images in which we obtained, upon acceptor photobleaching, almost 90% drop in the acceptor fluorescence intensity in the ROI. Upon acceptor photobleaching, two additional images were acquired for each channel.

Following this procedure, we measured pixel-by-pixel fluorescence in the ROI of each image using the LSM5 examiner software. Additionally, we used a not bleached ROI as an internal control for fluorescence fluctuations

due to image acquisition. FRET efficiency in the ROI, upon normalization due to fluorescence variations, was measured according to the following formula:

FRET efficiency %= {[(Cyan intensity upon bleaching)- (Cyan intensity before bleaching)]/(cyan intensity upon bleaching)}x100.

Additionally, images were analyzed using the ImageJ software to obtain the FRET subtracted image of the CFP channel. Briefly, image obtained before acceptor photobleaching was subtracted from the image obtained upon acceptor photobleaching using ImageJ, and the resulting image is showing net increment in the CFP channel due to FRET.

Statistical analysis

Pair wise comparison between groups was performed using the Student's t test. $p < 0,05$ was considered statistically significant. Statistical analysis was performed using the Statview software.

Structural modelling

VEGFR2 activation loop modelling was performed in collaboration with Doctor Sergio Pantano, Biomolecular Simulations Group, Institut Pasteur of Montevideo, Uruguay, as described in (Pantano and Carafoli, 2007), with minor modifications. The starting coordinates for VEGFR2 structure were obtained from the following published structures (PDB codes): 2QU5, 2QU6, 1YWN, 2P2H and 2P2I, 2OH4. Three systems were simulated: i) the VEGFR2 activation loop without any post translational modification; ii) the VEGFR2 activation loop with tyr 1052 and 1057 phoshorylated; iii) the VEGFR2 activation looop with lys 1054 acetylated.

Chapter 3

RESULTS

RESULTS

During my PhD course I got interested in the molecular players of angiogenesis, in particular in the VEGF/VEGFR proteins.

The work described in this thesis has been mainly focused on the dissection of some of the molecular mechanism underlining VEGFR2 activity.

Bearing in mind the key role of VEGFR2 in angiogenesis, we decided to investigate by which mechanisms VEGFR2 activation might be fine-tuned.

As described in RESULTS PART I, we first analyzed how acetylation might directly affect VEGFR2 and its activity.

Additionally, as detailed in RESULTS PART II, we investigated the formation of VEGFR2-Neuropilin1 complexes in endothelial cells by taking advantage of the FRET technique.

RESULTS PART I

VEGFR2 is acetylated by p300

VEGFR2 is acetylated in PAE cells

In addition to tyrosine phosphorylation, a crucial event in receptor activation, PTMs can modulate VEGFR2 activity, such as glycosylation (Takahashi and Shibuya, 1997) and ubiquitination (Duval et al., 2003; Ewan et al., 2006). Lysine ε-acetylation has been extensively studied until now as a PTM modulating the activity of nuclear proteins, in particular histones; only recently a growing number of cytoplasmic proteins have been recently reported to be acetylated (Kim et al., 2006; Tang et al., 2007; Zhang et al., 2007)-see Introduction-. Additionally, regulation of protein activity by acetylation has been proposed to rival phosphorylation in intracellular signalling (Kouzarides, 2000).

Therefore, we wondered whether a transmembrane receptor such as VEGFR2 might be modified by acetylation *in vivo.* As a cellular model we used Porcine Aortic Endothelial cells stably expressing human VEGFR2/KDR (PAE KDR) (Waltenberger et al., 1994). We immunoprecipitated VEGFR2 from PAE KDR whole cell lysates and probed the immunoprecipitated protein with an antibody that specifically recognizes acetyl-modified lysines, irrespectively of their protein context. A specific band was detected as acetylated, corresponding to the higher band of the VEGFR2 doublet (Figure 3.1). Cell treatment with trichostatin A (TSA), a potent inhibitor of HDAC family member activity (Yoshida et al., 1995), increased the signal corresponding to acetylated VEGFR2 (Figure 3.1, compare lane 1 and 2), without affecting protein level.

This observation clearly indicates that VEGFR2 is acetylated *in vivo*.

p300 increases VEGFR2 acetylation in vivo

In order to identify the lysine acetyl transferase (KAT) responsible for VEGFR2 acetylation *in vivo*, we tested the ability of p300, GCN5 and pCAF enzymes, belonging to two different well characterized KAT families, to induce VEGFR2 acetylation in overexpression experiments. We performed the *in vivo* acetylation assay by cotransfecting HEK293T with VEGFR2 in combination with p300, GCN5-HA and PCAF-FLAG.

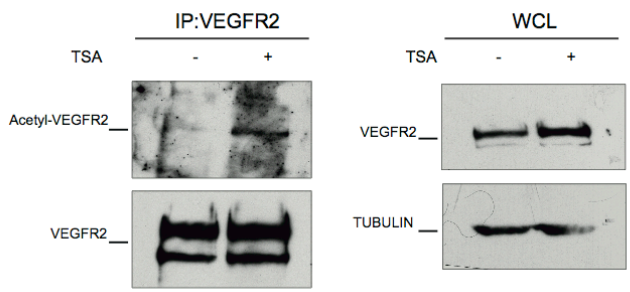

Figure 3.1. VEGFR2 acetylation in PAE KDR. PAE KDR whole cell lysates (WCL) were immunoprecipitated using an anti-VEGFR2 antibody, and then immunoblotted with an anti-acetyl lysine antibody. Upon stripping the same membrane was immunoblotted with an anti-VEGFR2 antibody (lower left). The right panels show WCL blotting with anti-VEGFR2 and anti-tubulin as an input control. Cells were treated for 6 hrs with 500 nM trichostatin A (TSA) or DMSO as control, as indicate at the top of each lane.

As shown in Figure 3.2, only in presence of over expressed p300 we were able to detect VEGFR2 acetylation upon VEGFR2 immunoprecipitation from WCL. We were also able to co-immunoprecipitate p300 together with VEGFR2, while in the same experimental conditions neither PCAF nor GCN5 co-immunoprecipitated with VEGFR2 (Figure 3.2, second panel).
Additionally, as INF-γ treatment has been demonstrated to be able to partially induce p300/CBP relocalization from the nucleus to the cytoplasm, thus increasing INFR type I acetylation, a p300/CBP target (Tang et al.,

2007), we wanted to test the effect of short term VEGF stimulation on VEGFR2 acetylation. Therefore in the same experiment we kept cells in 10% fetal calf serum, serum-starved or treated them with 50 ng/ml of rhVEGF165 for 7 min after serum starvation, as indicated on top of Figure 3.2. In our experimental conditions, short time treatment with VEGF did not modify VEGFR2 acetylation levels.

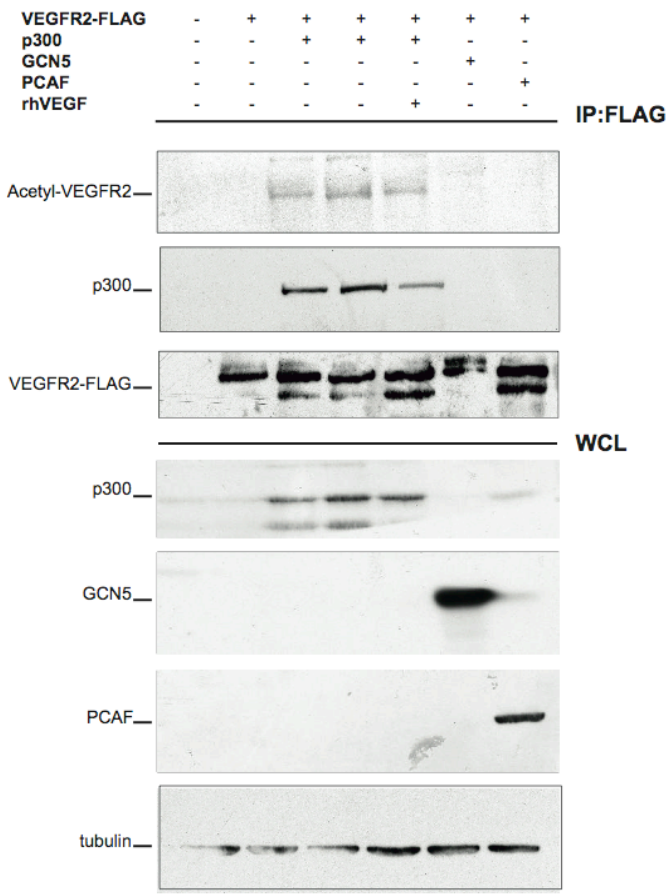

Figure 3.2. VEGFR2 acetylation is increased by p300. VEGFR2 was transiently transfected in combination with p300, GCN5 or PCAF. Additionally, cells were treated or not with VEGF165 as indicated. Anti-

VEGFR2 immunoprecipitates were immunoblotted with an anti-acetyla lysine, anti-VEGFR2 and anti p300. WCL were probed with anti-p300, anti-PCAF, anti-GCN5 and anti tubulin.

To further confirm this observation, we used LysCoA, an inhibitor of p300 enzymatic activity (Cereseto et al., 2005; Lau et al., 2000). HEK293T cell lysates, transfected with VEGFR2-Flag, were immunoprecipitated with an anti-Flag antibody upon LysCoA or vehicle treatment, as indicated in Figure 3.3. LysCoA treatment induced a dramatic decrease of the signal corresponding to acetylated VEGFR2, as visible comparing lanes 1 and 2 in Figure 3.3.

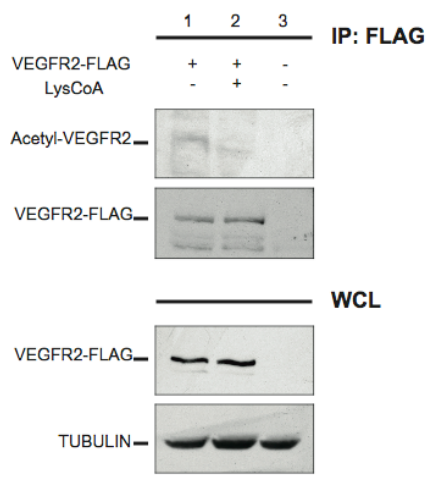

Figure 3.3. LysCoA treatment reduces VEGFR2 acetylation. HEK293T cells overexpressing VEGFR2 were treated with LysCoA or vector alone. Anti-VEGFR2-Flag immunoprecipitates were blotted with an anti total acetylated lysine antibody.

To distinguish between the role of p300 as a transcriptional activator and as an acetyl transferase in inducing VEGFR2 acetylation, we used a p300

enzymatically inactive mutant, p300 DY (Ito et al., 2001; Sabo et al., 2008). We contransfected HEK293T cells with VEGFR2-Flag in combination with wt p300, the DY mutant or an empty vector. While wt, active p300 strongly induced VEGFR2 acetylation as shown in Figure 3.4, upper panel, lane 1 and 2, the enzymatically inactive DY mutant was indistinguishable from controls in inducing VEGFR2 acetylation (compare lanes 3, 4, 5 and 6). Nonetheless, VEGFR2 acetylation even in presence of only endogenous active p300 became visible at higher exposure times (second panel from top).

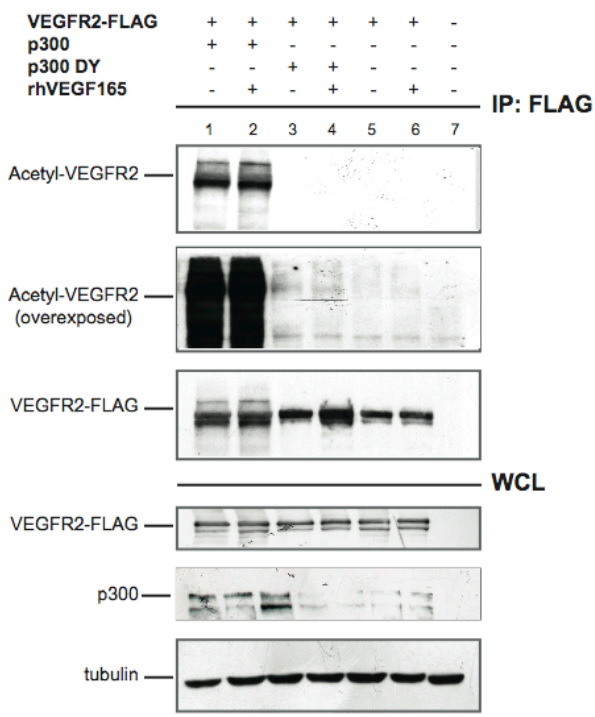

Figure 3.4. VEGFR2 acetylation parallels p300 activity. VEGFR2 was immunoprecipitated from HEK293T cells expressing the receptor in combination with p300 wt, p300 DY or with an empty vector. For each

experimental point cell were treated with or without rhVEGF$_{165}$ (50 ng/ml for 7 min). Receptor acetylation was detected using a total anti-acetyl lysine antibody.

Again, upon treatment with rhVEGF165 (50 ng/ml for 7 min), we were not able to detect any variation in VEGFR2 acetylation levels, as it is evident comparing lanes 1 and 2.

Finally, p300 activity, until now, has been extensively characterized for its ability to acetylate nuclear proteins, such as histones and transcriptional activators (Goodman and Smolik, 2000); on the other hand VEGFR2 has been widely characterized as a transmembrane protein, with few evidences indicating its possible nuclear translocation, for instance during shear stress (Feng et al., 1999; Shay-Salit et al., 2002).

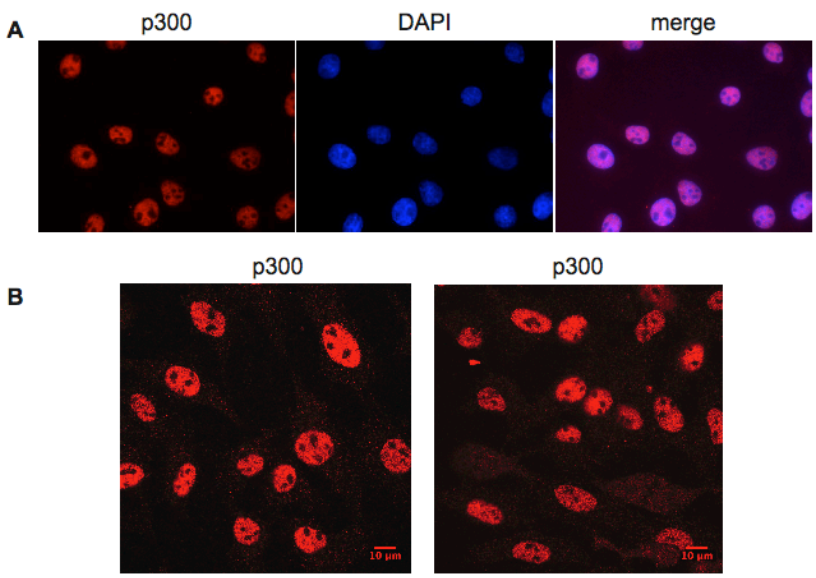

Figure 3.5. p300 subcellular localization in HUVEC. A) Images of HUVEC stained with anti-P300 and DAPI as indicated. B) Confocal images of cells stained with anti-p300.

We were not able to detect VEGFR2 nuclear localization nor in HEK293T overexpression experiments nor endogenous VEGFR2 in EC (data not shown). To further characterize p300 distribution in ECs we performed immunofluorescence experiments on HUVEC. As shown in Figure 3.5, we detected p300 only in the nuclear compartment (upper panels), while using confocal microscopy, we were able to detect p300 staining even in the cytoplasm, as visible from the lower panels in Figure 3.5.

Taken together, the above results clearly indicate that p300 is an acetyl transferase responsible *in vivo* for VEGFR2 acetylation; the results of the p300 localization experiments are consistent with this conclusion.

Identification of VEGFR2 acetylation sites

At least five VEGFR2 residues are acetylated

The mouse VEGFR2 sequence carries 79 lysines, the vast majority of which are species-conserved. On the other hand a p300 acetylation consensus site has not been identified until now. Therefore, all VEGFR2 lysines can be *bona-fide* acetylation sites.

In order to identify without ambiguities the VEGFR2 lysine(s) that are acetylated *in vivo*, we exploited proteomic analysis. We transfected HEK293T cells with VEGFR2-Flag or a pCDNA3-Flag plasmid as a control and immunoprecipitated the two proteins with an anti-Flag antibody. One tenth of the total IP was loaded on a gel and silver stained. As shown in Figure 3.6, we immunoprecipitated a band of approsimately 220 kDa corresponding to the size of fully glycosylated VEGFR2 (VEGFR2-A). In addition, to elucidate the IP content, we decided to analyze by mass spectrometry an additional band of smaller mass identified in the silver

stain (VEGFR2-B). Upon tryptic digestion and mass spectrometry analysis, we found that the upper band corresponded to acetylated and phosphorylated VEGFR2, while the lower band was again the same receptor, however in an unmodified state.

In three independent proteomic experiments we identified five VEGFR2 lysine sites that were detected as acetylated in almost two experiments. Interestingly, all these five sites are located in the intracellular sequence of the receptor.

Lysines we identified as aceylated in the mouse VEGFR2 protein are: Lys929, Lys937, Lys939, Lys347 and Lys1053 (corresponding to Lys931, 939, 941, 949 and 1055 of human VEGFR2, respectively). The position of the modified lysines n the VEGFR2 protein sequence is reported in Figure 3.7.

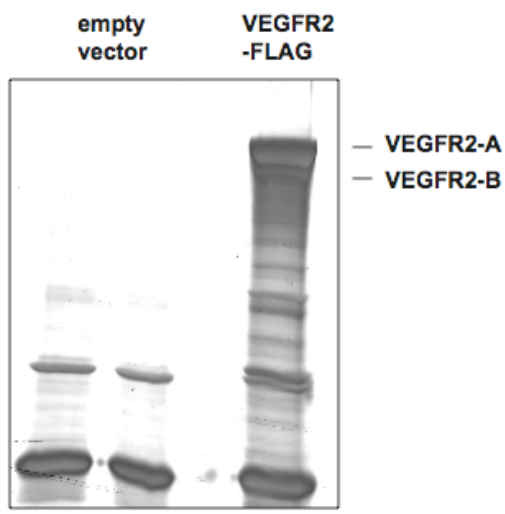

Figure 3.6. VEGFR2-Flag silver staining. Anti-flag immunoprecipitates upon VEGFR2-Flag or empty vector transient transfection were loaded on a gel and silver stained. Two bands corresponding to VEGFR2 were identified and underwent proteomic analysis.

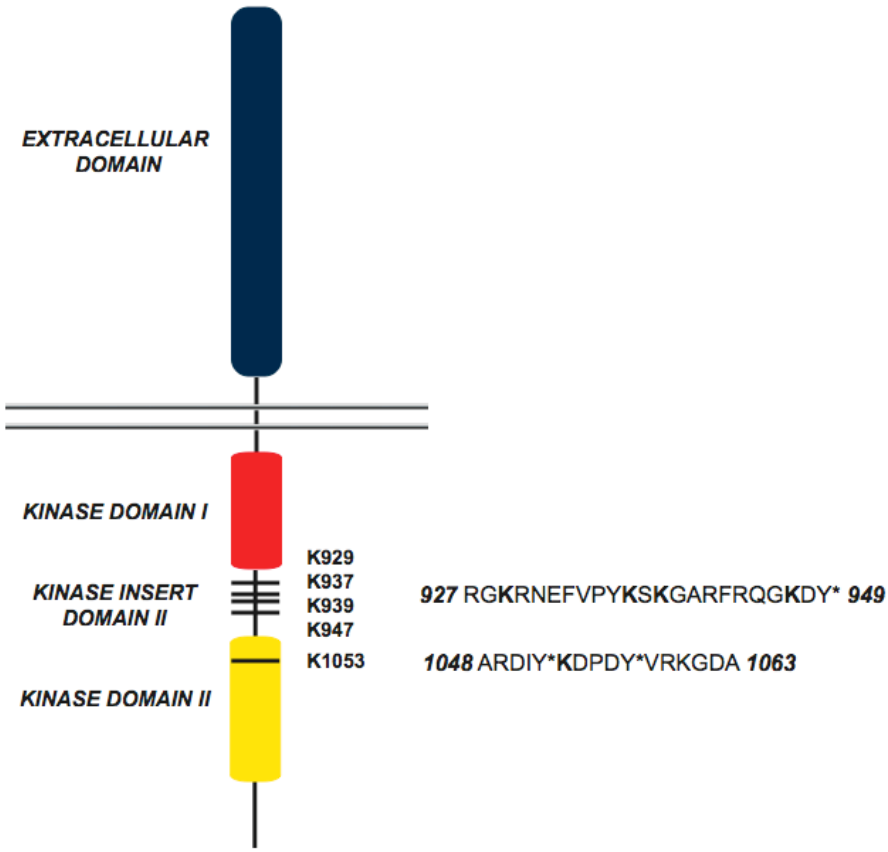

Figure 3.7. Schematic representation acetylated lysine distribution in mouse VEGFR2 sequence. The aminoacidic sequences surrounding acetylated lysine sites, detected by MS/MS, are reported. Acetylated lysines are in bold, phosphorylated tyrosines (according literature) are highlighted by an asterisk.

Four lysines (Lys929, Lys937, Lys939 and Lys947) are located in the insert kinase domain, characteristic of VEGFRs, and actually form a dense cluster; they are adjacent to a well-characterized tyrosine, Tyr949 (Tyr951), which was shown to be phosphorylated and to mediate TSAd dependent cell signalling (Matsumoto et al., 2005). Additionally, by proteomic analysis

we found that Lys929 can be modified by ubiquitination, and that this modification was alternative to acetylation.

Even more interestingly, the fifth site we characterized as acetylated was Lys1053: it is located in the second kinase domain, and it is placed in the activation loop of VEGFR2, following the first of the two phosphorylated tyrosines of the activation loop (Tyr1052 and Tyr1057 in the mouse sequence), required for maximal VEGFR2 kinase activity (Dougher and Terman, 1999). Intriguingly, Lys1053 is conserved not only among mouse, human and rat VEGFR2 sequences, but also in mouse VEGFR1 and VEGFR3, as shown by the protein alignment in Figure 3.8, panel B. This raises the possibility that Lys1053 has a conserved role in VEGFR2 activity. Among the other acetylated residues, only Lys929 seems to be conserved among different VEGFRs, while all other lysines are maintained in VEGFR2 of mouse, human and rat origin, but not in VEGFR1 and VEGFR3 (Figure 3.8, panel A).

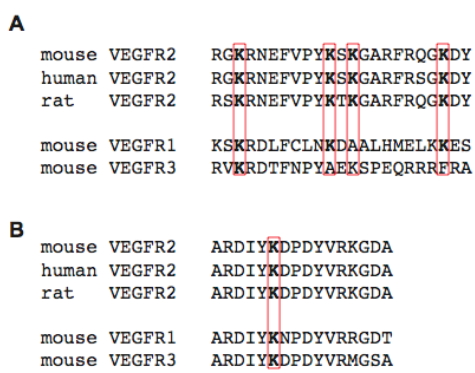

Figure 3.8. Comparison of VEGFRs sequences flanking acetylated lysines.

In conclusion, five lysine located in the cytoplasmic tail of VEGFR2 are modified by acetylation, and that these residues are highly conserved among species.

Substitution of lysine with arginine strongly reduces VEGFR2 acetylation

To estimate the role of lysines 929, 937, 939, 947 and 1053 in overall VEGFR2 acetylation, we constructed VEGFR2 with these five lysines mutated to arginines. Lysine to arginine substitution allows conservation of the positive charge, characteristic of the lysine residue, but impairs modification by acetylation. HEK293T cells were transfected with constructs coding for wt VEGFR2 or three different mutants: MUT A (Lys929Arg, Lys937Arg, Lys939Arg), MUT B (Lys947Arg) and MUT C (Lys1053Arg). WCL lysates were immunoprecipitated with an anti-Flag antibody, since all constructs were Flag-tagged, and the IPs were probed with an anti-acetyl lysine antibody to detect VEGFR2 acetylation. As shown in Figure 3.9, all three mutants showed a dramatic reduction in VEGFR2 acetylation compared to the wt protein; nonetheless we cannot exclude that VEGFR2 is still acetylated, even if at lower levels. Surprisingly for a single point mutant, a drop of protein abundance was especially observed upon MUT A transfection, as it is evident from the second panel from top of Figure 3.9 (compare lanes 1 and 2).

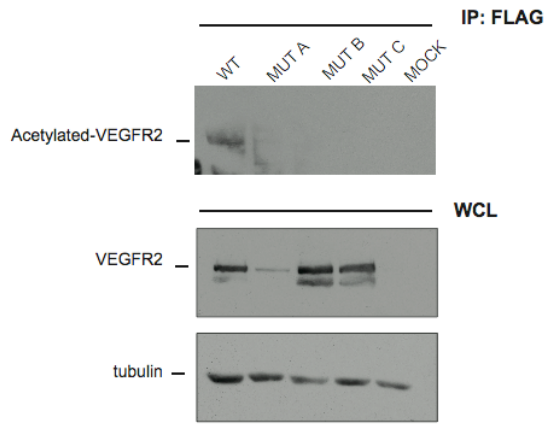

Figure 3.9. Lysine substitution largely abolishes VEGFR2 acetylation. HEK293T cells were transfected with different Flag-tagged constructs, coding for: WT VEGFR2, VEGFR2 MUT A (K929R, K937R and K939 R), VEGFR2 MUT B (K947R) or VEGFR2 MUT C (K1053R). Whole cell lysates were immunoprecipated using an anti-Flag antibody and probed with a total anti-acetyl-lysine antibody to detect acetylated VEGFR2.

VEGFR2 acetylation influences receptor phosphorylation and stability

The well-established role of the acetylation-phosphorylation crosstalk in regulating protein function (reviewed in (Yang and Seto, 2008)) together with the observation that VEGFR2 tyrosine phosphorylation is one of the first steps in VEGF-triggered intracellular signalling, raises the possibility that VEGFR2 acetylation and tyrosine phosphorylation might influence each other.

Additionally, since VEGFR2 has been shown to be ubiquitinated (Duval et al., 2003; Ewan et al., 2006) and ubiquitination and acetylation are mutually exclusive lysine posttranslational modifications, it is possible that VEGFR2

acetylation might influence protein levels, as also suggested by the results on total whole cell lysates shown in Figure 3.9.

VEGFR2 acetylation increases VEGF-induced tyrosine phosphorylation

As a first step to explore the consequences of VEGFR2 acetylation in signal transduction, we analyzed VEGFR2 phosphorylation on tyrosine residues in conditions in which the receptor was over-acetylated. In particular, we transfected VEGFR2 together with wt p300, the p300 DY mutant or an empty plasmid. As shown in Figure 3.10, p300 over-expression induced a dramatic increase in VEGFR2 tyrosine phosphorylation; this effect depended on p300 catalytic activity. We quantified VEGFR2 phosphorylation levels, upon normalization with tubulin and total VEGFR2 quantities. As plotted in the graph in Figure 3.10, p300 co-expression induced almost a four-fold increase in receptor tyrosine phosphorylation.
This observation, in combination with our previous findings suggesting that p300 is the HAT that acetylates VEGFR2 *in vivo*, indicates that VEGFR2 acetylation can have a role in modulating VEGF-mediated VEGFR2 phosphorylation of tyrosine residues.

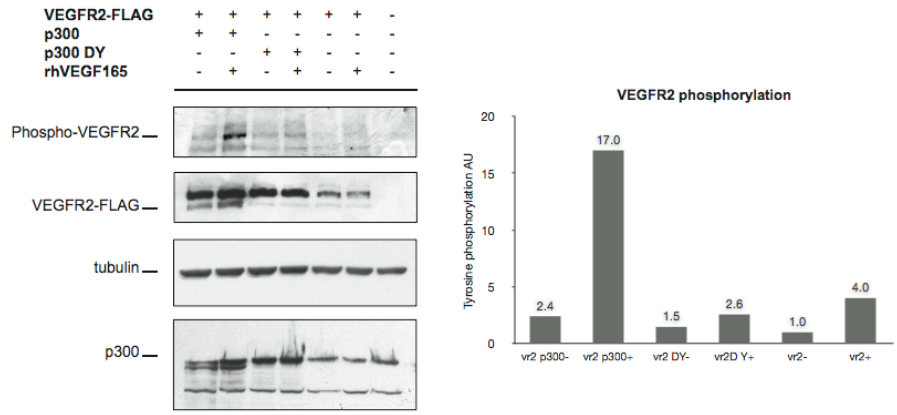

Figure 3.10 VEGFR2 phosphorylation is enhanced by p300 expression. HEK293T cells were transfected with VEGFR2 in combination with wt p300, p300 DY or an empty vector. Upon overnight serum starvation, cells were treated with 50 ng/ml of rhVEGF165 for 7 min as indicated. Whole cell lysates were blotted with an anti-total phospho tyrosine antibody to detect VEGFR2 phosphorylation in response to VEGF. In parallel, VEGFR2 levels were detected with an anti-Flag antibody. Quantification of protein phosphorylation was performed using ImageJ to quantify band intensity. Tyrosine phosphorylation values (in arbitrary units, AU) were obtained upon normalization with total VEGFR2 and tubulin. +: VEGF treated, - no VEGF treatment.

Mutation of lysine 1053 to arginine impairs VEGFR2 tyrosine phosphorylation but has no effect on protein stability

In an effort to dissect the possible role of different lysine residues in VEGFR2 phosphorylation and stability we started analyzing the single mutant Lys1053Arg. For this purpose we transfected PAE cells, lacking wild type VEGFR2 and the Neuropilin1 co-receptor, with wt VEGFR2 or the K1053R mutant; cells were treated with rhVEGF$_{165}$, as indicated, to induce VEGFR2 phosphorylation. Interestingly, lysine 1053 substitution with

arginine greatly reduced VEGFR2 phosphorylation at tyrosine 1173, a residue essential for VEGF-induced signal transduction (Holmqvist et al., 2004; Sakurai et al., 2005), as shown in Figure 3.11 panel A. Since we observed a small reduction in VEGFR2 total level (compare lanes 2 and 3 in panel 2), we asked whether the lower phosphorylation of the VEGFR2 mutant could be partially due to diminished protein stability. In order to address this question, we compared wt and mutant VEGFR2 half-life in HEK293T cells upon *de novo* protein synthesis inhibition with cycloheximide (CHX). As shown by panel B in Figure 3.11, mutant (red squares) and wt (blue rhomb) VEGFR2 stability curves over time were super imposable.

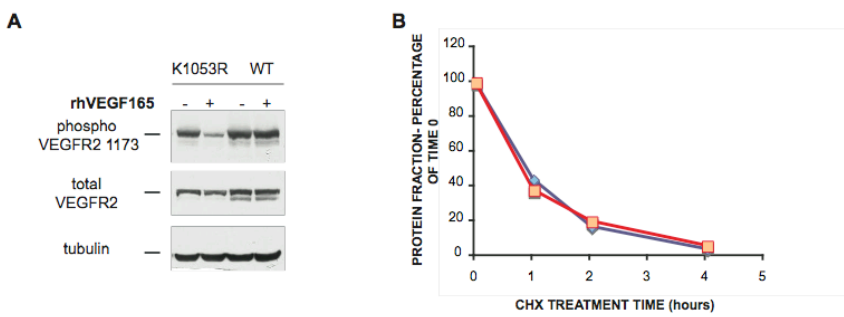

Figure 3.11. K1053R VEGFR2 mutant phosphorylation and stability. A) PAE were transfected with wt VEGFR2 or K1053R mutant, and treated as indicated with 50 ng/ml rhVEGF165 for 5 min. The membrane was blotted with anti-phosphoTyr1173(1175 in the human sequence), stripped and blotted with anti-VEGFR2. Anti-tubulin was used as a loading control. B) VEGFR2 wt (blue rhomb) or mutant (red square) protein levels are plotted over time. HEK293T cells transfected with wt or mutant VEGFR2, were treated for the indicated hours with cycloheximide (CHX) to inhibit protein synthesis. Protein decay over time was quantified with ImageJ from blots using an anti-VEGFR2 antibody and tubulin to normalize protein levels. Protein decay is expressed as percentage of the protein at time 0 (without CHX treatment).

Lysine 1053 is located in the activation loop of VEGFR2. Transition of the activation loop from a closed to an open conformation has been proposed to accompany phosphorylation of Tyr1052 and Tyr1057, two key residues involved in VEGFR2 activation (McTigue et al., 1999; Roskoski, 2008). To investigate the role of lys 1053 acetylation in the activation loop conformation, in collaboration with Doctor Sergio Pantano, Biomolecular Simulations Group, Institut Pasteur of Montevideo (Uruguay) we performed structural modelling. Our computational modelling, as shown in Figure 3.12, revealed that lysine 1053 (blue ribbon) is buried inside the middle of the activation loop, flanked by tyrosines 1052 and 1057 (blue rings). More interestingly, our modelling suggests that lysine 1053 modification by acetylation, by quenching the positive charge carried by the lysine amino group, contributes to the transition of the activation loop from the closed and inactive state (panel A), to the open, active and tyrosine phosphorylated state shown in panel B.

Overall, our data on the Lys1053Arg VEGFR2 mutant phosphorylation, stability and modelling indicate that this residue has a role in VEGFR2 phosphorylation almost at the level of tyrosine 1173, while has no effect on protein stability.

Substitution of five lysines with arginines reduces VEGFR2 stability

In order to clarify the role of Lys929, Lys937, Lys939 and Lys947 in protein stability, we transfected HEK293T cells with VEGFR2 wt or the mutant (with four lysines mutated to arginine), and assessed protein levels upon a time course CHX treatment. As shown in Figure 3.13, panel A, the four lysine mutant was not significantly less stable compared to the wt protein, similar to the Lys1053Arg mutant shown in Figure 3.11.

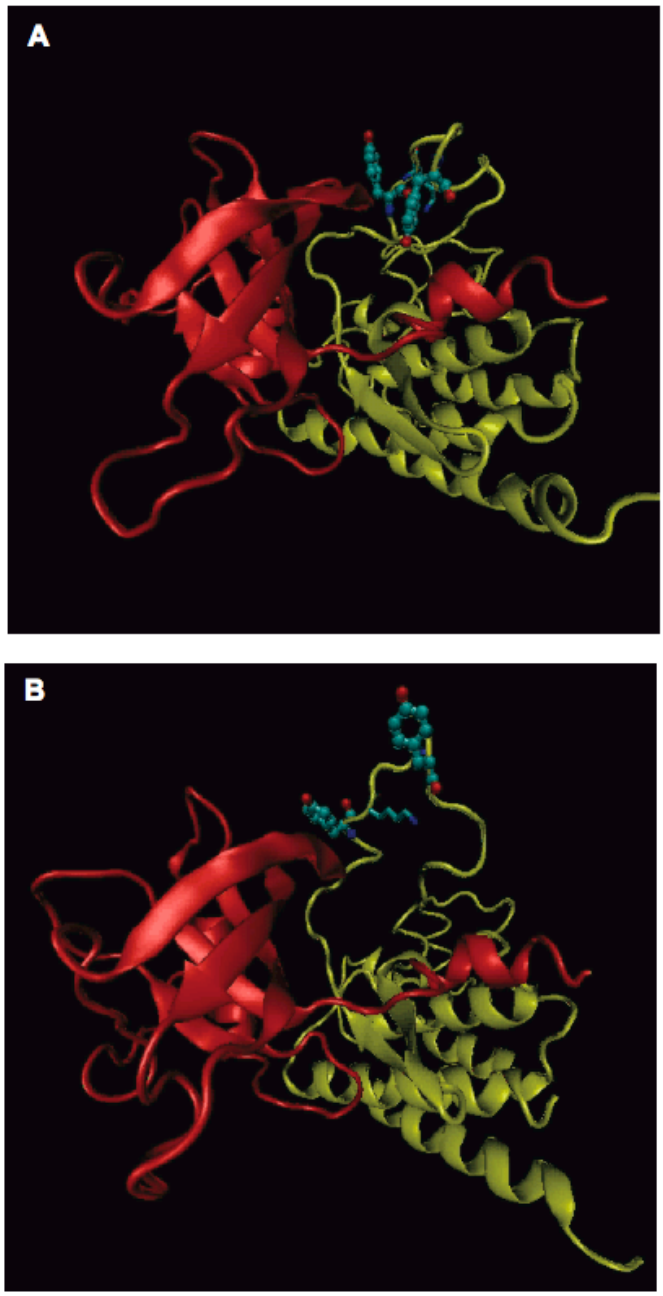

Figure 3.12. Modelling of VEGFR2 activation loop. The inactive and closed state of the activation loop is shown in A, while in B the open, active

conformation characterized by tyrosine residues phosphorylation is visible. Tyrosines 1052 and 1057 (blue rings) and lysine 1053 (blue ribbon) are shown in an expanded conformation.

Surprisingly, a VEGFR2 mutant in which we substituted all the five lysines we identified as acetylated with arginine, was less stable then the wt protein, and a difference in protein stability could be detected as early as after 1 hour of CHX treatment (Figure 3.13 panel C).

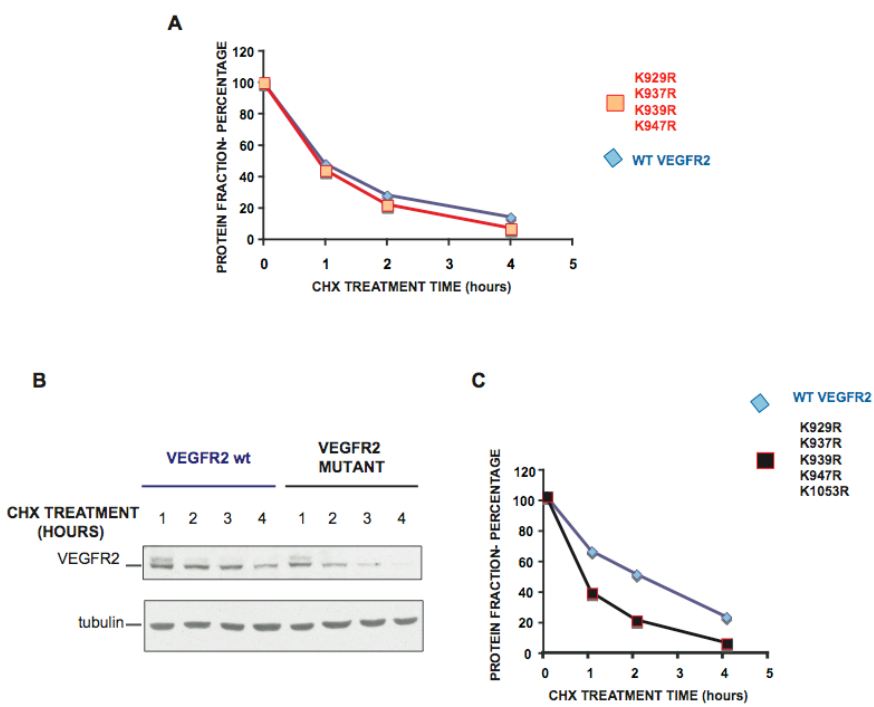

Figure 3.13. VEGFR2 with five mutated lysines is less stable than the wild type protein. Wt and mutant VEGFR2 stability curves are shown. HEK293T cells transfected with wt or mutant VEGFR2, were treated for the indicated hours with cycloheximide (CHX) to inhibit protein synthesis. Protein decay over time was quantified from blots using an anti-VEGFR2 antibody and tubulin to normalize protein levels. Protein decay is expressed

as a percentage of the protein at time 0 (without CHX treatment). A) Comparison between wild type and four-lysine mutant VEGFR2; B) Cell lysate from HEK293T cell trasfected with wt VEGFR2 or five-lysine mutant and treated wth CHX for indicated times were blotted with an anti-VEGFR2 and anti tubulin as a loading control; C) Protein decay is plotted over time.

Strikingly, the VEGFR2 mutant was undetectable at 4 hours of CHX treatment, while wt VEGFR2 was still detectable (compare lane 4 and 8 of panel B in Figure 3.13).

Effect of VEGFR2 acetylation on VEGF-dependent desensitization

The observation that VEGFR2 acetylation can affect both protein stability and receptor phosphorylation on tyrosine residues prompted us to investigate the role of acetylation in receptor desensitization, a process that involves both VEGFR2 down-regulation and inhibition of phosphorylation (Duval et al., 2003; Ewan et al., 2006; Gampel et al., 2006; Marmor and Yarden, 2004; Singh et al., 2005; Singh et al., 2007). For this purpose HEK293T cells transfected with wt VEGFR2 were treated with TSA in order to inhibit HDAC activity, in combination with rhVEGF for different time points as indicated. Surprisingly, TSA treatment inhibited VEGF-induced VEGF down-regulation, a shown in Figure 3.14. Additionally, acetylated VEGFR2 retained the ability to respond to VEGF stimulation by tyrosine phosphorylation (lower panels).
Therefore, VEGFR2 acetylation seems to be linked to inhibition of receptor desensitization.

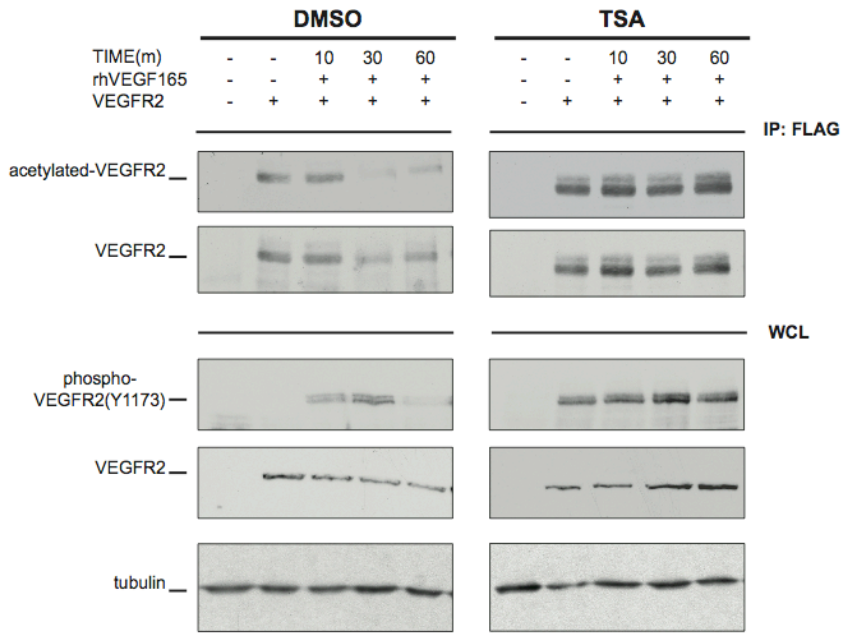

Figure 3.14. VEGFR2 acetylation is linked to receptor desensitization. HEK293T cells transfected with VEGFR2 were treated or not with TSA and stimulated for different time points with rhVEGF$_{165}$ to induce receptor desensitization. Anti-Flag immunoprecipitates were blotted with an anti-total acetyl lysine antibody, stripped and blotted with an anti Flag antibody. Lysates were blotted with an anti phopsho-VEGFR2 (Tyr1173), anti-flag and anti tubulin.

RESULTS PART II

Fluorescence Resonance Energy Transfer

On the basis of previous studies reporting VEGFR2-Neuropilin1 interaction in ECs, we further investigated by microscopy the formation of these membrane complexes. In particular we took advantage of a physical property, FRET, to analyze the protein-protein interaction occurring in the 1-10 nm range at the plasma membrane in endothelial cells. Briefly, FRET technique requires a pair of fluorophores with defined spectral properties. As a FRET pair suitable for our experiments, we selected ECFP and EYFP, due to the partial overlapping between ECFP (also defined as the donor fluorophore) emission spectra and EYFP (also defined as the acceptor fluorophore) absorption spectra. Among all the FRET detection methodologies available, we chose to apply acceptor photobleaching to study protein-protein interactions even in presence of low amounts of molecules. As shown in Figure 3.15, this technique exploits acceptor molecule bleaching to reduce energy transfer from the donor; only when donor and acceptor are in contact (less then 10 nm apart), an increase in donor emission can be detected by microscopy.

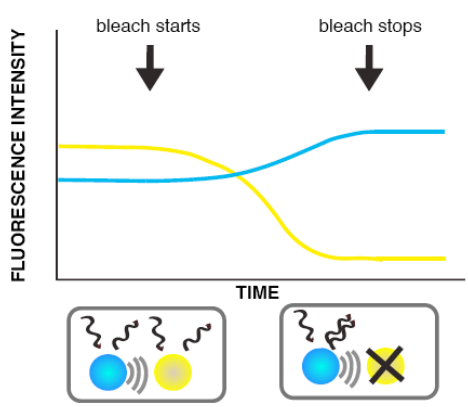

Figure 3.15. FRET detection using acceptor photobleaching technique.

As a first step we adapted this technique, formerly described by Karpova et al, to our experimental conditions (Karpova et al., 2003). In order to optimize image acquisition and, more importantly, to minimize possible cross talk between channels, we modified the acquisition setting for the LSM510 ZEISS microscope. Based on fluorescent proteins excitation spectra we used a 458 Argon laser line to excite ECFP and a 514 line to excite EYFP. A main beam splitter HFT 458/514 reflected laser lines. Since one of the major confounding factor in FRET detection is the bled-through between channels, we created a CFP filter spanning from 467 to 499 nm as highlighted in Figure 3.16; the EYFP filter was set to detect wavelength from 531 to 584 nm. In our experimental conditions channel cross talk was virtually absent using this optimized filter set. For brevity we named the combination of excitation and filters for ECFP or EYFP detection “ECFP channel” and “EYFP channel”, respectively.

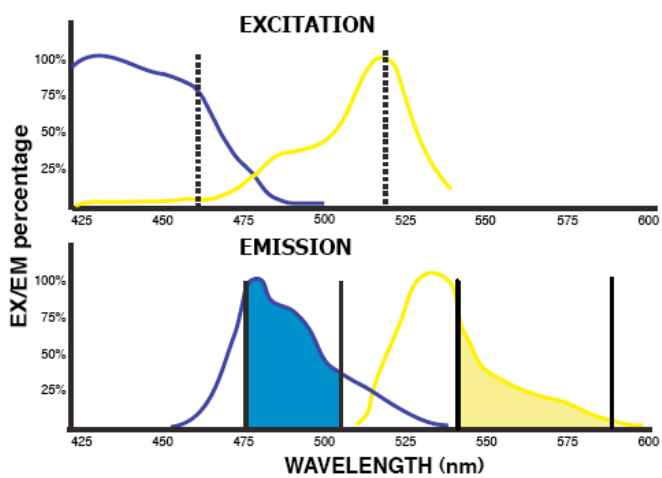

Figure 3.16. ECFP and EYFP spectra. ECFP (cyan) and EYFP (yellow) excitation spectra are shown in the upper panel, dotted bars represent laser lines. In the lower panel coloured area correspond to adopted filter set for fluorescence detection.

First, we tested these microscope settings for FRET detection using a fusion protein composed by ECFP and EYFP separated by a short, non-charged amminoacidic linker (Karpova et al., 2003). This construct should lead to a virtual 100% interaction between the two fluorophores.

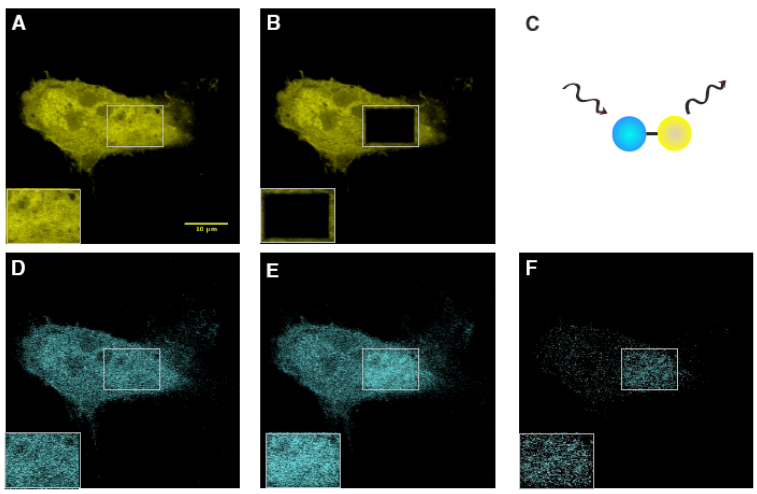

Figure 3.17. Acceptor photobleaching for FRET detection. Cell transfected with an ECFP-EYFP fusion protein (panel C) upon acceptor photobleaching in a ROI (B), displayed significant FRET signal (E), clearly visible upon digital subtraction (F).

We transfected PAE cells with this construct as a positive control and with equal amounts of ECFP and EYFP proteins as a negative control. Twenty-four hours after transfection PAE cells were fixed and analyzed at the confocal microscope using the above-mentioned FRET settings. A representative set of FRET images in a cell expressing the ECFP-EYFP fusion is shown in Figure 3.17. In panel A and D the EYFP and the ECFP channels images are shown separately before bleaching. We selected one region (ROI, Region Of

Interest), highlighted in the inset, and we applied only to the selected region a high intensity laser beam at 514 nm in order to bleach EYFP fluorescence. This bleaching resulted in a nearly complete loss of EYFP signal in the ROI (panel B), while FRET was detectable as a fluorescence increase in the corresponding ROI in the ECFP channel, as clear from the inset in panel E.

To better visualize FRET we used the image subtraction function of the ImageJ program: we digitally subtracted the ECFP image upon bleaching from the corresponding ECFP image acquired before bleaching. As shown in Figure 3.17 panel F this digital subtraction improved FRET visualization. Therefore, this digital subtraction method was applied to all other FRET images.

Remarkably, the LSM5 microscope not only allowed FRET visualization, but also FRET quantification using pixel-by-pixel fluorescence intensity measurement. As shown in Figure 3.18 fluorescence intensity in the EYFP and the ECFP channels variations during FRET acquisitions were plotted over time, and an increase in ECFP intensity (solid line) is visible upon acceptor photobleaching (dashed line).

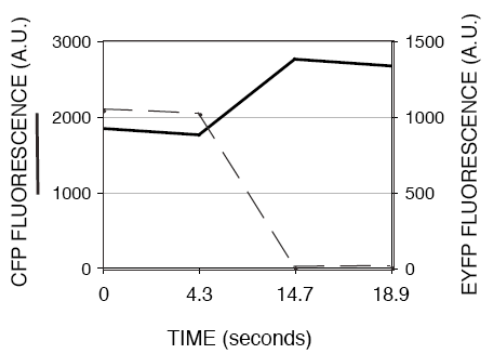

Figure 3.18. Fluorescence intensity variations in FRET. Pixel-by-pixel fluorescence variation is plotted over time in a FRET experiment with the ECFP-EYFP construct. FRET can be observed as an increase in ECFP fluorescence (-) upon EYFP (acceptor) photobleaching (_ _).

VEGFR2-EGFP characterization

To characterize the dynamics of VEGFR2 interactions with NP1 using FRET we created different fusion constructs containing the coding sequence of mouse VEGFR2. We fused the full length coding sequence in frame with different fluorescent protein tags: EGFP, EYFP and ECFP. In particular we cloned these tags fused to the VEGFR2 C-terminus by a linker of seven amino acids, to obtain an intracellular localization of the fluorescent protein.

In order to exploit these constructs for further FRET experiments, modified VEGFR2 should retain its original properties; in particular sub cellular localization and phosphorylation on tyrosine residues. To examine intracellular distribution, we transiently transfected ECs with the pCDNA-VEGFR2-EGFP plasmid. Among all the cell types tested, PAE were the most suitable for our work, in particular because they do not express nor VEGFR2 nor Neuropilin1, and we conducted all further FRET experiments in this cellular model. Cells were harvested 24 hours upon transfection and fixed to directly visualize VEGFR2-EGFP. WGA-TRITC staining for glycoproteins was then performed on samples to highlight membranes containing glycosylated protein, in order to provide an indirect indication of VEGFR2 distribution.

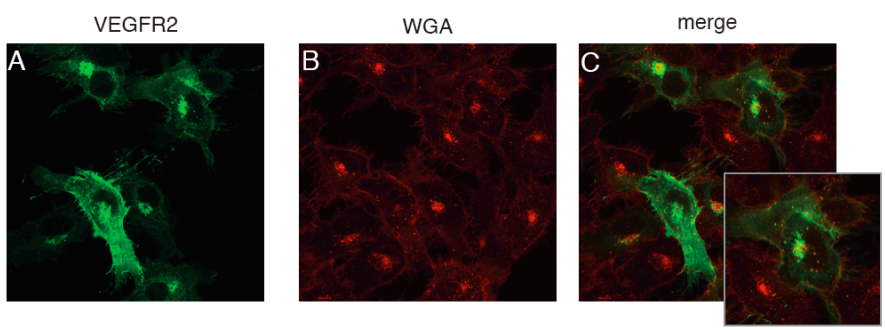

Figure 3.19. VEGFR2-GFP localization in EC. VEGFR2-GFP protein is detectable at the plasma membrane in PAE cells, in a perinuclear compartment with punctate staining, colocalizing with glycosylated proteins detected by WGA.

As expected VEGFR2-GFP showed a partial localization at the plasma membrane level and in filopodia, nonetheless protein accumulation was detectable in the perinuclear compartment and as punctate vesicular staining (Figure 3.19 panel A). Interestingly, HUVEC cells were previously reported to display a similar distribution for wt KDR (Bhattacharya et al., 2005; Gampel et al., 2006). Furthermore VEGFR2-EGFP colocalized with the WGA-TRITC staining characteristic of glycosylated protein, visible in Figure 3.19 panel B and C.

Taken together these observations suggested that VEGFR-EGFP expressed in PAE retained the normal sub-cellular localization of wt VEGFR2.

To assess the activity VEGFR2-EGFP we evaluated its levels of phosphorylation in cells.

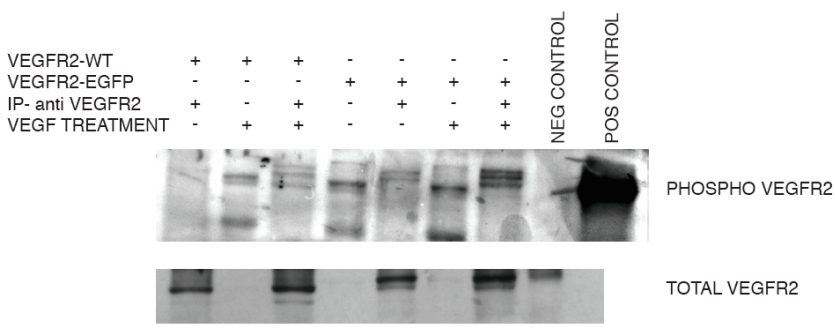

Figure 3.20. VEGFR2-EGFP phosphorylation. The upper panel show phospho-VEGFR2 as detected by anti-total phospho Tyrosine antibody upon IP form HEK293T cells. VEGFR2-EGFP (lane 7) is phosphorylated in response to VEGF165 stimulation.

In detail, HEK293T cells were transfected with pCDNA3-VEGFR2 or pCDNA-VEGF-EGFP, maintained for 16 hours in the absence of serum and then treated with 50 ng/ml of recombinant $VEGF_{165}$ for 5 min, as indicated in Figure 3.20; cells were lysed and whole cell lysates were subjected to immunoprecipitation using an anti-VEGFR2 antibody. As shown in Figure 3.20, VEGFR2-EGFP could undergo tyrosine phosphorylation upon VEGF

stimulation (compare lane 5 and 7) and this effect was comparable, if not higher, to that observed in the wt VEGFR2 (lane 1 and 3). Note that VEGFR2-EGFP displayed a higher molecular weight referred to its wt counterpart when detected in wb, due to the EGFP tag (Figure 3.20 lower panel, anti-VEGFR2).
Altogether these observation indicated that VEGFR2 with a c-terminal enhanced fluorescent protein tag (in these experimental settings EGFP) retained its characteristic localization in EC and, more important, can still undergo phosphorylation on tyrosine residues upon VEGF stimulation. This construct was therefore deemed suitable for FRET experiments.

Localization and activity of fluorescent Neuropilin1

In parallel to VEGFR2, in order to perform FRET experiments we created three different constructs coding for mouse Neuropilin1 with a C-terminal tail linked to EGFP, EYFP or ECFP.
Since Neuropilin1 activity is strictly coupled with its sub cellular localization and its ability to interact with other partners, we tested whether the main properties of the wt protein were maintained even in presence of a fluorescent tag.

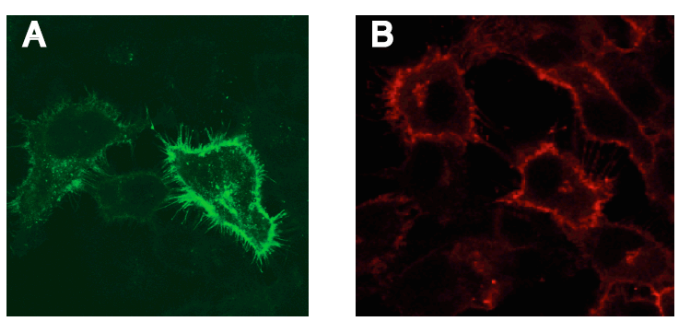

Figure 3.21 NP1-EGFP localization. NP-1-EGFP localization in PAE cells (A) and endogenous NP1 localization, detected in HUVEC cells by immunofluorescence with an anti-Neuropilin1 antibody (B).

To assess that the FPs tag had no effect on NP1 protein localization, PAE cells were transiently transfected with the pEGFP-N1-NP1 construct, coding for EGFP fused at the C-terminus of mouse Neuropilin1. As shown in Figure 3.21 panel A, twenty-four hours after transfection, the Neuropilin-1-GFP protein was mainly localized at the plasma membrane in PAE cells, displaying a characteristic distribution at cell filopodia, and a weaker accumulation in the ER. Immunofluorescence against the endogenous Neuropilin1 in HUVE cells revealed a nearly identical distribution (Figure 3.21 panel B), suggesting that the fluorescent tag was not influencing protein localization.

Next, we tried to elucidate NP1 activity. For this purpose, a cell contraction assay was used to measure the Neuropilin1 ability to transduce the signal triggered by its ligand SEMA3A. Cos-7 cells, which express neither Neuropilin1 nor PlexinA1, were transiently transfected with Neuropilin1-EYFP, alone or in combination with PlexinA1 (at the 1:1 ratio).

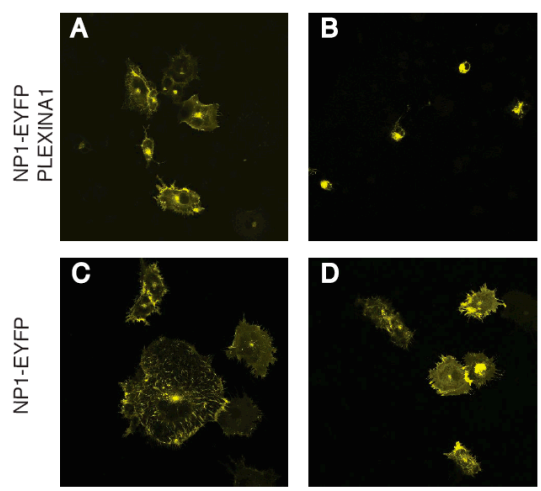

Figure 3.22. SEMA3A induced cell contraction. NP1-EYFP expression in COS7, combined with PlexinA1, triggers cell contraction in response to SEMA3A (B). In absence of PlexinA1, no response to SEMA3A is detectable (D).

Expression of Neuropilin1-EYFP in combination with PlexinA1 led to massive Cos-7 cells collapse only upon stimulation with SEMA3A (Figure 3.22 panel A and B). This effect was not observed when cells were subjected to SEMA3A treatment in absence of PlexinA1 expression (Figure 3.22 panel C D).

Since we were also interested in the preservation of the putative intracellular pathways immediately downstream of NP1, we decided to further investigate whether the interaction between fluorescently modified NP1 and its intracellular mediators was conserved. We therefore tested the formation of complexes formed by NP1 and its intracellular binding protein, GIPC1 (Wang et al., 2006). This issue was critical since our NP1 fusion construct consisted of the fluorescent protein tags separated by only a short linker from the NP1 C terminal amino acids; this region codes for the SEA motif that constitutes the recognition site for GIPC1 and has been reported to be required for NP-1-VEGFR2 interaction (Prahst et al., 2008).

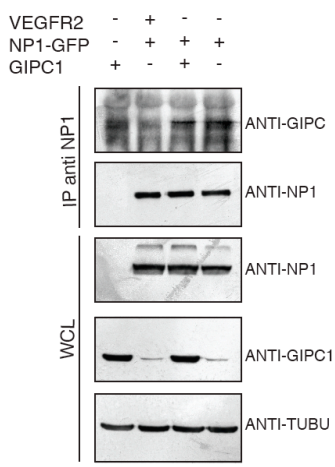

Figure 3.23. NP1-GFP interaction with GIPC. NP1-GFP was immunoprecipitated from HEK293T cells, transfected as indicated at the top. Anti-GIPC antibody was used to detect GIPC-NP1 co-immunoprecipitation.

HEK293T cells were transfected with pEGFP-NP1, pEGFP-NP1 in combination with pCDNA3-GIPC1 or pCDNA3-VEGFR2; 36 hours after

transfection whole cell lysates were subjected to immunoprecipitation against Neuropilin1 using an anti-GFP antibody. NP1-EGFP was able to specifically co-immunoprecipitate both over expressed and endogenous GIPC1, as visible in Figure 3.23, upper panel, lane 3 and 4.

Taken together, immunoflourescence, cell contraction and IP experiments indicated that modified NP1 had properties comparable to its wild type counteroart and could be used in FRET experiments.

Visualization of VEGFR2-NP1 interaction in EC using FRET

We applied FRET in order to better clarify the dynamics of VEGFR2-Neuropilin1 interaction in response to different soluble ligands.

In particular, we investigated the effects of two VEGF-A isoforms in the formation of angiogenic receptors complexes. The VEGF 165 aa long isoform ($VEGF_{165}$) is able to bind both Neuropilin1 and VEGFR2, while the properties of the 121 aa long isoform 121 ($VEGF_{121}$) are still controversial (Guttmann-Raviv et al., 2007; Pan et al., 2007b; Shraga-Heled et al., 2007). SEMA3A was also tested for its selective Neuropilin-1, but not VEGFR2, binding.

VEGFR2-EYFP and Neuropilin1-ECFP were transiently cotransfected in PAE cells. Twenty-four hours after transfection cells were grown in the absence of serum for additional 16 hours. Cells were then treated with either 50 ng/ml $VEGF_{165}$, 50 ng/ml $VEGF_{121}$, 100 ng/ml SEMA3A or maintained in the absence of serum. After 5 minutes stimulation, cells were washed, fixed and mounted for microscopy analysis. For FRET evaluation, we applied the microscope settings previously validated using the ECFP-EYFP control construct. The FRET experiment is outlined in Figure 3.24 using three images: two showing the EYFP channel before and after bleaching (corresponding, in this case, to

VEGFR-EYFP)(panels A, B, D and E) and one with the resulting FRET channel (obtained as discussed before)(C and F).

As shown in Figure 3.24, after $VEGF_{121f}$ (full length) stimulation and acceptor photobleaching in the ROI, in basal conditions FRET between VEGFR-EYFP and NP1-ECFP was barely detectable (panel C). Conversely, $VEGF_{165}$ treatment results in a clear FRET signal in correspondence to the bleached region (panel F).

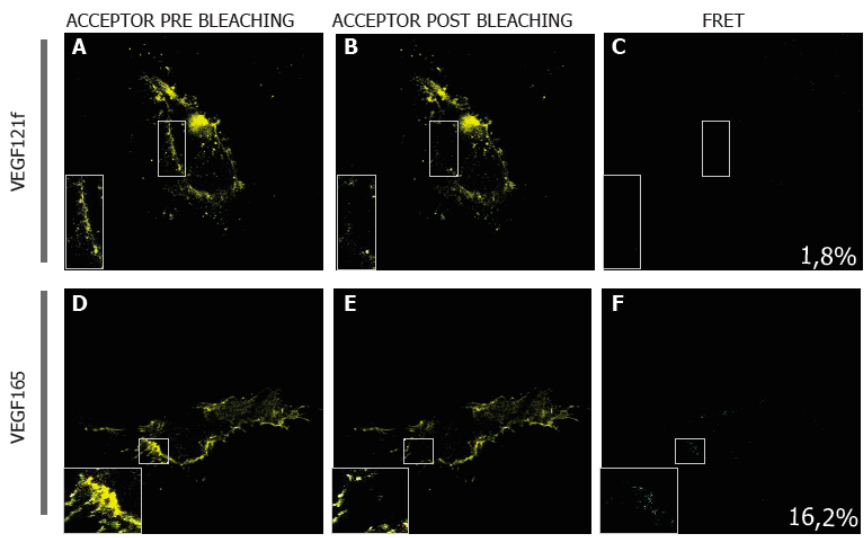

Figure 3.24. Interaction between VEGFR2 and NP1 by FRET. PAE cell transfected with NP1-ECFP and VEGFR2-EYFP showed no positive FRET signal in the bleached ROI upon treatment with 50 ng/ml $VEGF_{121f}$ (A, B, C). When the cells were treated with 50 ng/ml $VEGF_{165}$, a clear FRET signal was detected in correspondence of the bleached ROI (D, E and F).

Next we quantified FRET efficiency; the results, obtained form at least 10 cells for each treatment group, are plotted in Figure 3.25. We detected a positive FRET signal between VEGFR2 and Neuropilin1 only upon $rhVEGF_{165}$

treatment; conversely stimulation with two different batches of rhVEGF$_{121}$ (VEGF$_{121f}$ and VEGF$_{121m}$, lacking 9 C-terminal aminoacids) was not able to induce a significant increase in the FRET signal, referred to as a negative control (untreated cells). Conversely, cells treated with SEMA3A displayed no increment in the FRET signal corresponding to VEGFR2 Neuropilin1 interaction over control cells.

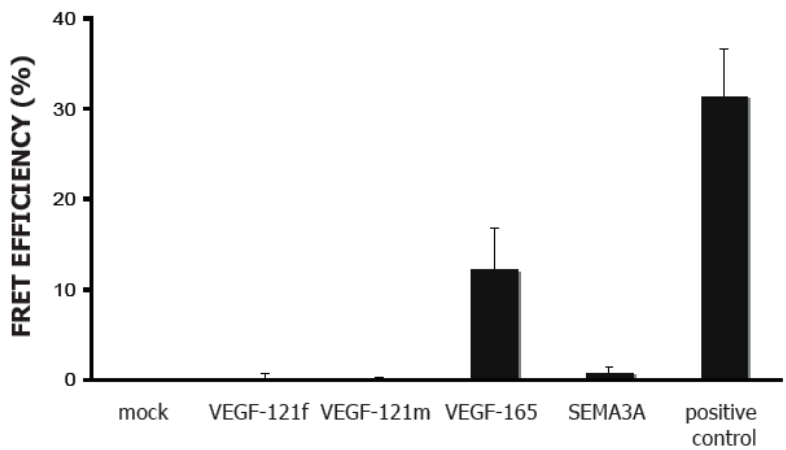

Figure 3.25. Quantification of FRET occurring between VEGFR2 and NP1. Consistently with microscopy analysis, VEGF165 induced the bridging between VEGFR2 and NP1 in endothelial cells detected by FRET analysis. VEGF121 and SEMA3A treatments did not induce a positive FRET signal over mock (transfected but untreated cells). Data are presented as FRET efficiency, obtained from almost 10 cells for each point. The positive control is FRET occurring in the ECFP-EYFP fusion protein.

Collectively, the results obtained indicated that the FRET technique was suitable for studying VEGFR2-NP1 interaction in EC. Interestingly, only VEGF165 was able to modify the interactions between Neuropilin1 and VEGFR2 at the plasma membrane level in EC, in contrast to VEGF121 and SEMA3A.

FRET for the visualization of Neuropilin1 complexes

Neuropilin1 has been recognized as a coreceptor, acting in combination with diverse transmembrane receptors. Nonetheless NP1 has been recently shown to induce intracellular response even in the absence of its canonical receptors, suggesting an independent signal transduction pathway for this protein. Since receptor dimerization constitutes a common response to ligand binding for transmembrane receptor signal transduction, we took advantage of FRET imaging to visualize NP1 homocomplexes.

PAE cells were transfected with plasmids coding for NP-1-ECFP and NP-1-EYFP (ratio 1:1), maintained in the absence of serum for 16 hours and then treated either with 50 ng/ml of VEGFs ($VEGF_{165}$ or $VEGF_{121f}$), or 100 ng/ml of SEMA3A.

Remarkably, using acceptor photobleaching to evaluate FRET, despite complete co localization between NP-1-ECFP and NP-1-EYFP, treatment with $VEGF_{121f}$ did not result in FRET signalling between NP-1 molecules (Figure 3.26 A, B and C). Conversely, treatment with both $VEGF_{165}$ and SEMA3A (panels E-I) led to a positive FRET signal detected in the ROI.

Next, we quantified FRET efficiency in this experiment from at least 10 cells for each treatment. As shown in Figure 3.27, a 2-fold and 3-fold increase over control in FRET efficiency was observed upon $VEGF_{165}$ and SEMA3A treatment respectively; $VEGF_{121}$-induced FRET was indistinguishable from untreated cells.

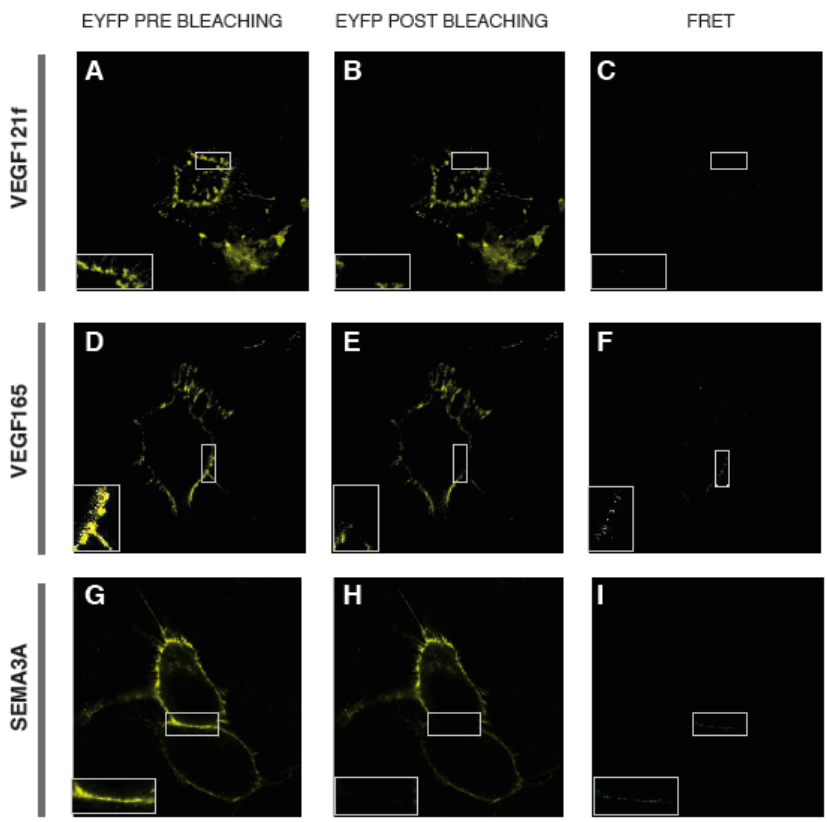

Figure 3.26. NP1 homocomplexes detection by FRET. Representative images of FRET in cell transfected with NP1-ECFP and NP1-EYFP are shown. (A-C) $VEGF_{121}$ treatment did not resulted in detectable FRET signal in the ROI corresponding to the bleached region. (D-I) Conversely both $VEGFG_{165}$ and SEMA3A stimulation resulted in a positive FRET signal shown in the insets panel F and I.

These results suggest that NP1 is able to form homocomplexes detectable by FRET only in response to the $VEGF_{165}$ isoform and to SEMA3A.

We further investigated the effect of different doses of $VEGF_{165}$ on the formation of NP1 homocomplexes by FRET. PAE cells were transfected with NP1 ECFP and NP1 EYFP and stimulated with increasing concentrations of

$VEGF_{165}$, ranging from 25 ng/ml to 200 ng/ml. As shown in Figure 3.28, FRET efficiency increased in parallel to$VEGF_{165}$ concentration.

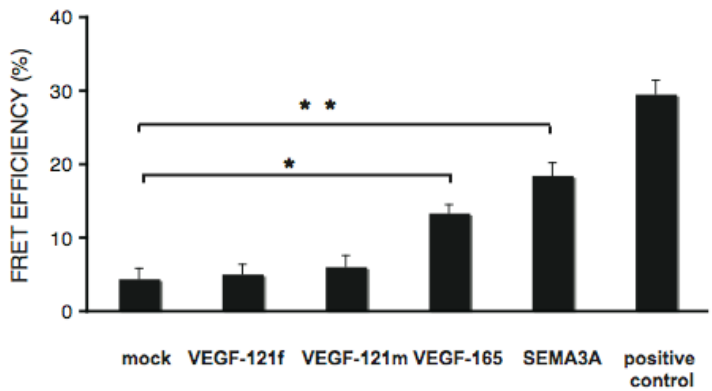

Figure 3.27. Quantification of NP1 homocomplexes formation. Upon acceptor photobleaching FRET efficiency % was quantified from more than 10 cells for each treatment. SEMA3A treatment resulted in a three-fold increase in FRET signals compared to untreated control cells. $VEGF_{165}$ induced a two-fold increase; $VEGF_{121}$ treated cells were similar to control.

Next we performed a similar experiment to test the effect of different SEMA3A doses on NP1 complex formation. Surprisingly we observed a peak in FRET detection (measured as FRET efficiency) corresponding to 100 ng/ml of SEMA3A, while higher concentrations determined a decrease in the FRET signal (Figure 3.29).

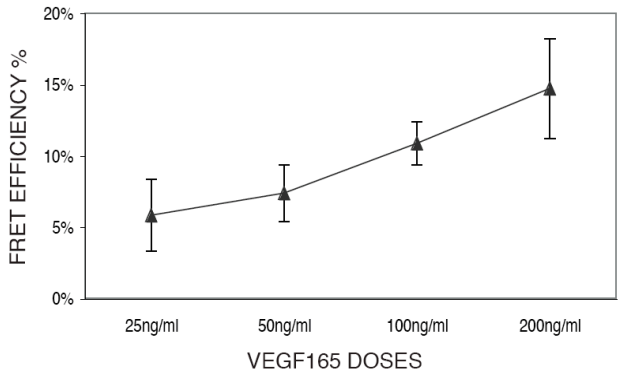

Figure 3.28. FRET efficiency correlates with VEGF165 doses. Stimulation with increasing doses of $VEGF_{165}$ was paralleled by an increase in FRET efficiency due to the formation of Np1 homocomplexes. Quantification was obtained from 15 cells for each point.

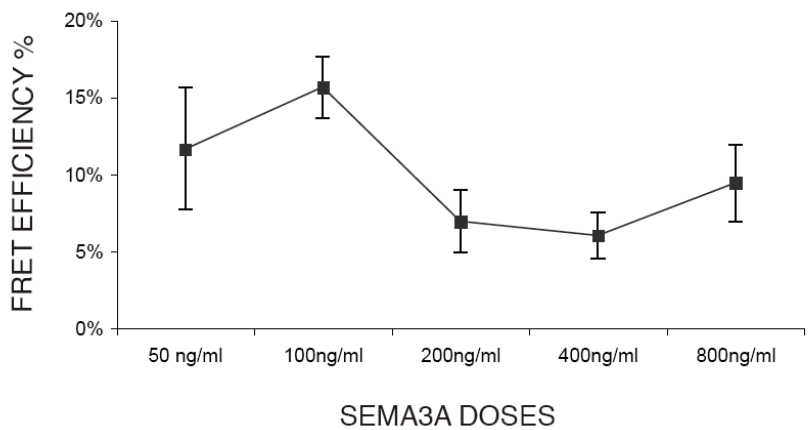

Figure 3.29. FRET efficiency variation with SEMA3A doses. At 100 ng/ml SEMA3A had a maximal activity in Np1 complexes formation measured with FRET, conversely higher doses resulted in a decrease FRET signal.

Finally, to validate the formation of NP1 homodimers we decided to exploit an in vitro angiogenic model such as tube formation. PAE cells were transfected with an equal amount of NP1-ECFP and NP1-EYFP and plated onto Matrigel-covered microscope slides the day after transfection. In order to stimulate

endothelial tube formation 50 ng/ml of $VEGF_{165}$ were added to cells. Since we observed tube formation starting from 6 h after treatment, cells were fixed and FRET analysis was performed to detect NP1 dimerization (Figure 3.30).

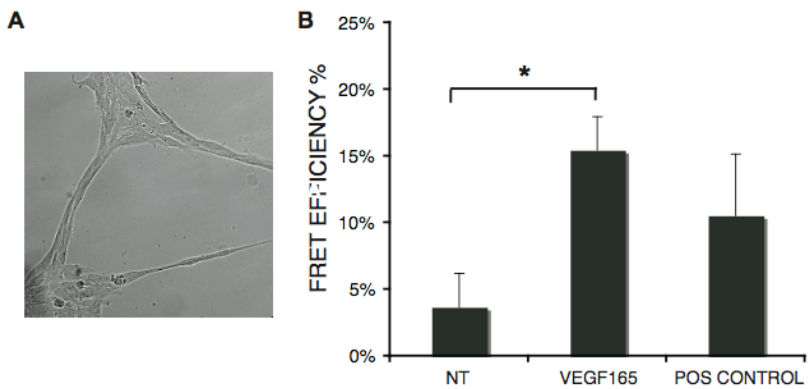

Figure 3.30. NP1 complexes detected in tube formation. In accordance with data obtained in PAE cells, positive FRET signal was observed upon VEGF165 treatment in cells contributing to tube formation on matrigel. FRET quantification in obtained form >10 cell for each point.

A 4-fold increase was observed in FRET efficiency, corresponding to the formation of NP1 dimers upon VEGF165 treatment, while control cells expressing NP1 and in the absence of any stimuli were not responsive (Figure 3.30).

Thus, FRET could not detect NP1 complexes formation in response to $VEGF_{165}$ stimulation at the plasma membrane of endothelial cells, in the tube formation assay.

Effect of anti-NP1 blocking antibodies on FRET-detected NP1 complexes

We hypothesized that if the formation of NP1 homocomplexes had an important role in Neuropilin1 biology, inhibition of NP1 activation at the plasma

membrane level should be paralleled by the disruption of NP1 complexes as revealed by FRET.

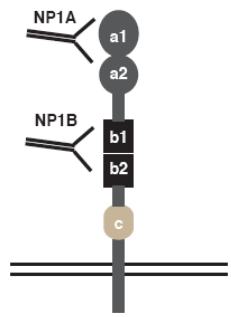

Figure 3.31. Anti-NP1 blocking antibodies.

To test this hypothesis we exploited two anti-NP1 blocking antibodies developed by Antibody Engineering, Tumor Biology and Angiogenesis laboratories at Genentech, South San Francisco (USA). These antibodies were generated using a VH/VL synthetic phage antibody library in order to cross react with human and mouse Neuropilin1. One antibody, termed NP1A, mapping to the a1a2 domain (CUB domain) of NP1 showed a strong inhibition of SEMA3A bioactivity, in particular in DRG cone collapse. In contrast, the NP1B antibody mapping to the b1b2 domain of NP1 prevented VEGF binding to NP1 and HUVEC migration in response to VEGF (see Figure 3.31)(Liang et al., 2007). NP1B was able to reduce tumor growth *in vivo*, while both antibodies displayed a potent inhibition of vascular remodelling and vessels pericyte association (Pan et al., 2007a).

To elucidate the activity of the NP1B antibody, we transfected PAE cells with NP1-ECFP and NP1-EYFP. Cells were pre-treated for 30 min with either a control antibody (IgG) or scalar concentration of the NP1B antibody (10, 25 or 50 μg/ml), and then with 50 ng/ml rhVEGF$_{165}$. FRET evaluation for NP1-

complexes formation was carried out by analyzing at least 50 cells for each experimental point, and quantified as FRET percentage of FRET efficiency as discussed above.

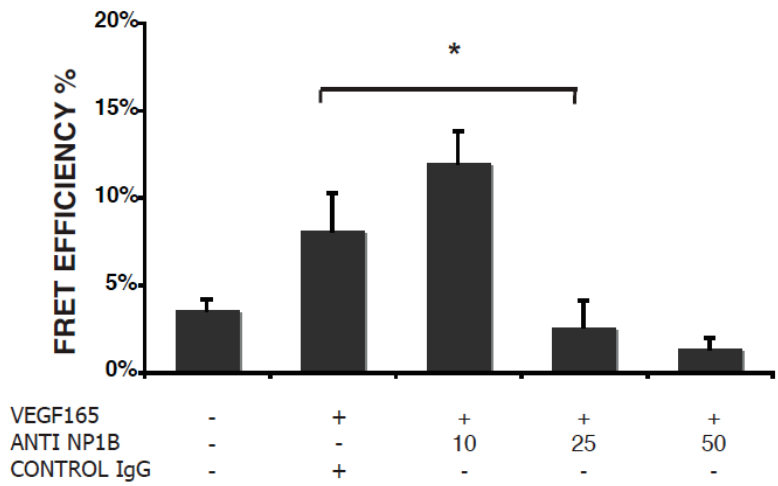

Figure 3.32. Quantification of NP1B antibody effect. An increasing inhibitory effect of NP1B antibody on $VEGF_{165}$-induced NP1 complexes could be observed with antibody scalar doses. Quantification of FRET efficiency in >50 cells for group is showed.

The addition of 25 and 50 μg/ml of NP1B antibody dramatically reduced the formation of NP1 complexes at the plasma membrane induced by $VEGF_{165}$, while 10 μg/ml NP1B had no effect (Figure 3.32). Analysis of SEMA3A-dependent NP1 complex formation revealed a marked inhibition even at the lower NP1A antibody dose (50 μg/ml), and an almost complete disruption of complexes at higher doses (Figure 3.33).

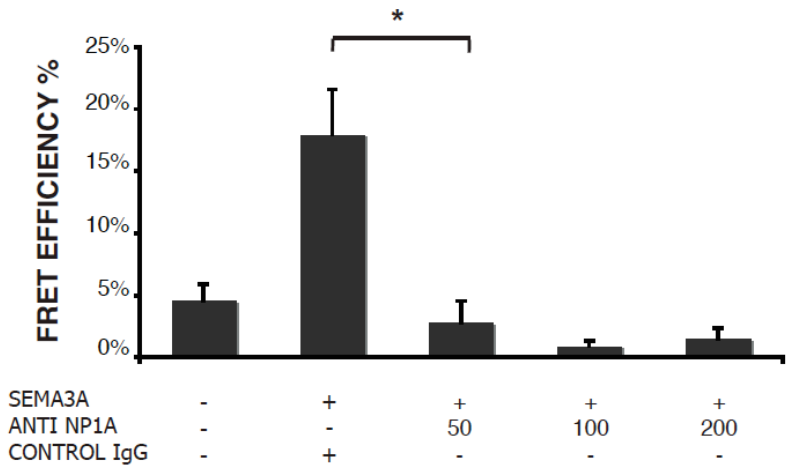

Figure 3.33. Effect of NP1A antibody on NP1 complexes. Complete inhibition of NP1A complexes formation detectable with FRET could be observed even at 50 μg/ml of NP1A and in presence of SEMA3A. FRET efficiency quantification is obtained from at least 50 cells per point.

To elucidate the activity of the Abs in cross inhibition of Neuropilin1 complexes we combined NP1B (which recognizes the NP1 VEGF binding site) with SEMA3A or NP1A (which recognizes the NP1 SEMA3A binding site) with $VEGF_{165}$. The NP1B antibody treatment markedly reduced SEMA3A triggered Np1 dimerization, while the NP1A antibody did not lead to a significant decrease in $VEGF_{165}$-induced dimerization (Figure 3.34).

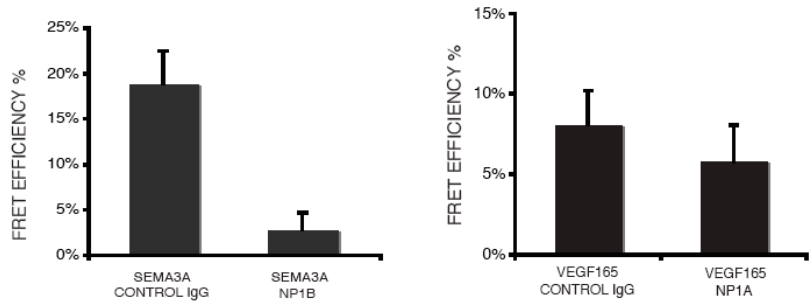

Figure 3.34. Cross-inhibition of NP1A and NP1B antibodies. NP1B treatment inhibited NP1 homocomplexes triggered by SEMA3A, while NP1A showed only a modest effect in the inhibition of NP1 complexes upon $VEGF_{165}$ treatment. FRET efficiency quantification are shown.

The marked inhibition of NP1 dimerization observed in FRET experiments upon anti-NP1A and anti-NP1B antibody treatment suggest that their anti-NP1 activity observed in vivo is in part mediated by receptor complexes disruption.

Anti-PlGF inhibits NP1 complexes formation

At this point we wanted to establish whether NP1 complex formation detected by FRET analysis was a mechanism shared by different NP1 ligands, apart from VEGF and SEMA3A. For this purpose we exploited our FRET system to study the effect of another NP1 ligand, PlGF2, in the induction of NP1 complexes.

As shown in Figure 3.35 PlGF treatment (100 ng/ml) induced a strong formation of NP1 complexes at the level of the plasma membrane in PAE cells.

To gain further insight in the role of NP1 dimers in response to PlGF, in collaboration with the Department for Transgene Technology and Gene Therapy, VIB, Leuven, Belgium (Professor Peter Carmeliet), we used a neutralizing monoclonal anti-PlGF antibody. This antibody had the ability to

inhibit PlGF binding to both VEGFR1 and NP1 and more importantly inhibited in vivo tumor growth and metastatization (Fischer et al., 2007). Pre-treatment of PAE cell expressing fluorescently tagged NP1 with the anti PlGF antibody dramatically reduced FRET signal as shown in Figure 3.35, lower panel.

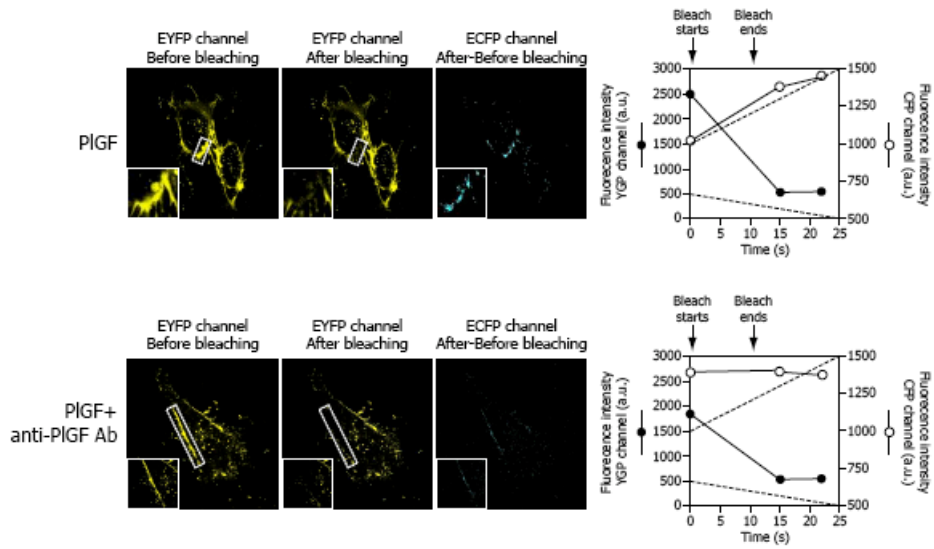

Figure 3.35 PlGF-induced FRET. Positive FRET signal was observed between NP1 molecules upon PlGF treatment (upper images), and fluorescence was quantified over time (graph). Combination of PlGF and anti-PlGF blocking antibody led a clear inhibition of the FRET signal (lower panle ad graph). Two representative experiments are shown.

Strikingly, the anti-PlGF antibody displayed a dose dependent effect, reducing the formation of PlGF induced NP1 complexes to control levels, as it is clear in the FRET quantification shown in Figure 3.36.

Taken together, the results of FRET with PlGF and anti-PlGF on NP1 suggested that NP1 dimerization is a common mechanism for NP1 activation. More interestingly, the FRET system here developed was able to detect the disruption of NP1 complexes upon antibody treatment.

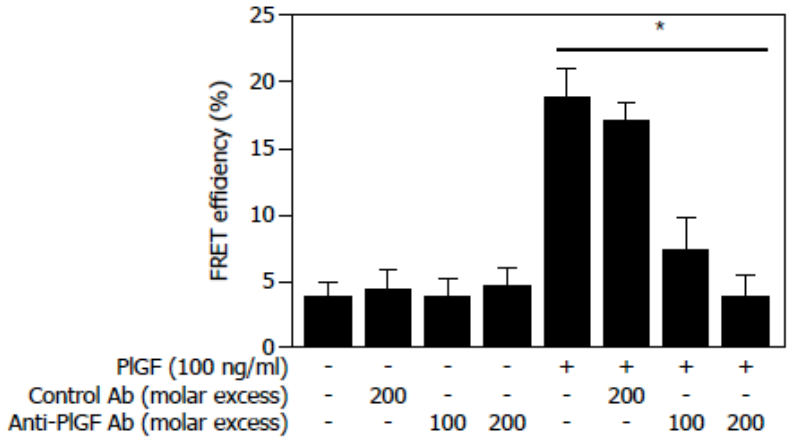

Figure 3.36. Anti-PlGF quantification. FRET efficiency measurement provided a quantitative analysis of anti-PlGF activity. A positive correlation was observed between anti-PlGF doses and Np1 complexes inhibition, while non effect was observed in presence of a control AB. Quantification were obtained from >15 cell for each group.

Chapter 4

DISCUSSION

DISCUSSION

DISCUSSION PART I

VEGFR2 is modified by acetylation

Due to its major role in VEGF-signalling in both pathological and physiological angiogenesis, it is not surprising that VEGFR2 tyrosine kinase receptor activity is tightly controlled at several levels, including protein expression and intracellular localization. This regulation is in part achieved by different post-translational modifications in addition to tyrosine residue phosphorylation, such as glycosylation and ubiquitination; nonetheless, other PTMs, which might regulate receptor activity, have not been investigated until now.

Here, we provide evidence that VEGFR2 is acetylated *in vivo* and that receptor acetylation has a role in regulating its function, in particular tyrosine phosphorylation and protein stability. This is, to our knowledge, the first indication that a tyrosine kinase transmembrane receptor can be modified by acetylation. Until now only one transmembrane receptor, INFαR2, has been reported as acetylated; in this case acetylation on Lysine 399 triggers a signalling cascade that finally leads to gene regulation (Tang et al., 2007). Even if acetylation was discovered as an histone modification, the number of identified acetylated non-histone proteins is rapidly growing, suggesting that even more proteins are acetylated and that acetylation might possibly rival phosphorylation in controlling protein functions (Kouzarides, 2000). Our findings, together with those of Tang and co-workers, strengthen the concept that acetylation may have an important function in controlling cytoplasmic and membrane protein function, as it has already been shown for α-tubulin, cortactin and Hsp90 (Haberland et al.,

2009; Kim et al., 2006; Kovacs et al., 2005; L'Hernault and Rosenbaum, 1985; Tang et al., 2007; Zhang et al., 2007).

From our biochemical and proteomic studies we deduce that only a fraction of VEGFR2 protein is acetylated *in vivo.* In particular, the protein fraction that is modified by acetylation corresponds to the fully glycosylated protein, which includes the receptor mature pool presented at the level of plasma membrane (Takahashi and Shibuya, 1997). Acetylation of a lysine cluster in p53 tail has been shown to regulate its nuclear export, thus suggesting that acetylation can influence protein activity by controlling intracellular localization (Kawaguchi et al., 2006). VEGFR2 compartmentalisation and membrane presentation have a major role in controlling protein activity (Gampel et al., 2006; Lampugnani et al., 2006; Mukherjee et al., 2006); accordingly, it will be interesting to investigate whether a correlation might exist between receptor acetylation and its presence at the plasma membrane.

By exploiting proteomic analysis, we were able to identify the VEGFR2 lysines that were modified, which are listed in Figure 3.7. The VEGFR2 sequence codes for 79 lysines, the vast majority of which are conserved between species. We discovered that at least five lysine residues are modified in murine VEGFR2: lysines 929, 937, 939, 947 and 1053. All these residues are conserved among mouse, human and rat VEGFR2; it is compelling to note that lysines 929 and 1053 are conserved also in VEGFR1 and VEGFR3 of mouse origin.

In some proteins, acetylation occurs at lysine residue clusters forming charged patches. p300, for example, is acetylated on 13 lysines in the activation loop, and overall acetylation of these residues, rather than individual lysine modification, regulates its enzymatic activity (Thompson et al., 2004); p53 and cortactin acetylation also occurs at the level of lysine patches (Kawaguchi et al., 2006; Zhang et al., 2007).

In the VEGFR2 sequence, lysines 929 to 947 are located in the kinase insert domain and form a cluster that gives rise to a charged patch; therefore in this case we speculate that acetylation at all the lysine residues might be important for receptor function.

Additionally, VEGFR2 is phosphorylated at at least six tyrosine residues and, remarkably, lysine 947 is adjacent to phosphorylated tyrosine 949, while lysine 1053 is flanking tyrosine 1052 and is adjacent to another phosphorylated residues, tyrosine 1057 (Olsson et al, 2006).

Different from lysines 929, 937, 939 and 947, lysine 1053 is located in the middle of VEGFR2 activation loop and is not surrounded by other acetylated residues. Since activation loop tyrosines are required for efficient receptor autophosphorylation in response to VEGF stimulation (Dougher and Terman, 1999), one possibility is that modification oys 1053 might affect VEGFR2 enzymatic activity, similarly to Cdk9 (Sabo et al., 2008).

Therefore, VEGFR2 acetylation at different residues might possibly have a different impact on protein function. A similar concept has already been put forward for p53 (Tang et al., 2008).

Molecular players in VEGFR2 acetylation and deacetylation

Acetylation and deacetylation are catalyzed by several KATs and KDACs, respectively; therefore these proteins constitute an additional level in the control of target protein activity.

From the results obtained by co-immunoprecipitation, co-expression of wt and mutated p300 and p300-inhibition by LysCoA, we can conclude that p300 is able to modify VEGFR2 by acetylation *in vivo*. It is interesting to notice that the yolk sac of p300 mutant embryos is frequently poorly vascularized, and that VEGFR2 null mice blood vessels never organize in the yolk sac (Shalaby et al., 1995; Yao et al., 1998). Nonetheless, we cannot currently exclude that additional acetyltransferases can modify VEGFR2. With respect to this, it is worth mentioning that p53 is modified on

multiple residues by CBP/p300 (Appella and Anderson, 2000; Brooks and Gu, 2003), by PCAF on lysine 320 (Sakaguchi et al., 1998), while the MYST family KATs acetylate lysine 120 (Sykes et al., 2006; Tang et al., 2006). Interestingly, we were able to co-immunoprecipitate the acetyltransferase Elp3 in combination with VEGFR2 in proteomic experiments (Sinigaglia M. and Pattarini L., unpublished results). This protein was recently identified as the KAT responsible for α-tubulin acetylation (Creppe et al., 2009), and its cytoplasmic localization strengthens the possibility that Elp3 might acetylate VEGFR2, in addition to p300.

Additionally, even if p300 has been extensively studied for its ability to modify nuclear proteins, we were able to detect p300 by IF in the cytoplasm of HUVE cells, thus suggesting that this KAT can also act in the cytoplasm. This is in accordance with the data by Tang and co-workers, who showed that INFRαR2 could recruit cytoplasmic CBP and p300 (Tang et al., 2007). Even if VEGFR2 has been reported by two independent groups to translocate into the nucleus (Feng et al., 1999; Shay-Salit et al., 2002), we were unable to detect VEGFR2 nuclear localization in our experimental conditions. However, it could be interesting to analyze VEGFR2 acetylation and function for example during shear stress, a stimulus reported to induce VEGFR2 nuclear localization (Shay-Salit et al., 2002).

Finally, since CBP and p300 can be exported from the nucleus following INFα stimulation (Tang et al., 2007), we are currently investigating whether VEGF treatment can affect p300 localization in ECs. In this respect, our experiments suggest that short recombinant VEGF treatment (5-7 minutes) does not change VEGFR2 acetylation and VEGFR2-p300 co-immunoprecipitation.

A still unresolved question is how VEGFR2 is deacetylated. Increase in VEGFR2 acetylation following TSA treatment suggests that class I, II and

IV HDACs might have a role in VEGFR2 deacetylation. One candidate for VEGFR2 deacetylation might be HDAC6, the enzyme responsible for α-tubulin deacetylation, which is characterized by a cytoplasmic localization (Zhang et al., 2008). Additionally, the Olson group has recently reported that VEGF induces nuclear export of HDAC7 in ECs through a phosphorylation cascade (Wang et al., 2008). These investigators showed that HDAC7 export controls VEGF-induced cell proliferation and migration by repressing the expression of MEF2-dependent and independent genes. Accordingly, we are interested in analysing whether HDAC7 might deacetylate VEGFR2.

In addition, given the emerging role of Sirtuins in angiogenesis (Finkel et al., 2009; Potente and Dimmeler, 2008; Potente et al., 2007), their possible role in VEGFR2 acetylation is an additional intriguing issue.

Eventually, it would not be surprising to find that VEGFR2 acetylation is indirectly controlled by VEGF through a balance between different KATs and KDACs.

Crosstalk between VEGFR2 phosphorylation and acetylation

One important feature of lysine acetylation is its ability to crosstalk with other post-translational modifications, such as ubiquitination and phosphorylation (Yang and Seto, 2008). VEGF induces VEGFR2 phosphorylation on different tyrosine residues, thus initiating intracellular signalling (Olsson et al., 2006).

Therefore, we wandered whether VEGFR2 acetylation and phosphorylation are interconnected. We observed that receptor hyper-acetylation increased its overall tyrosine phosphorylation in response to VEGF stimulation (Figure 3.10), and that a VEGFR2 mutant, in which lysine 1053 was converted to arginine, was less phosphorylated than the wild type protein (Figure 3.11). Consistent with these observations, our modelling of the VEGFR2 activation loop suggested that lysine 1053 acetylation, by quenching lysine

positive charge, switches its conformation towards a more open state, characteristic of receptor activation and autophosphorylation at tyrosines 1052 and 1057, as shown in Figure 3.12. The hypothesis that residue charge influences receptor autophosphorylation in the activation loop has been recently proposed to explain VEGFR1 impaired autophosphorylation; in particular, substitution of VEGFR2 negatively charged aspartic acid 1054 with unpolar asparagine (the residue present in VEGFR1 sequence) was reported to reduce VEGFR2 autophosphorylation (Meyer et al., 2006).

In light of these observations, we speculate that acetylation of lysine 1053 has a major role in increasing VEGFR2 response to VEGF by phosphorylation, probably modifying the conformation of the activation loop. Additional mechanisms might contribute to increase acetylated-VEGFR2 phosphorylation. It has been reported that acetylation influences STAT and IRF9 protein dimerization (Tang et al., 2007), and it is thus possible that acetylation affects VEGFR2 phosphorylation indirectly by enhancing receptor dimerization, thus increasing trans-phosphorylation. Moreover, protein acetylation might favour VEGFR2 membrane presentation, thus augmenting the proportion of VEGFR2 that can be bound by VEGF.

Finally, VEGFR2 phosphorylation sites have been described as docking residues for downstream signalling molecules; for example phosphorylated tyrosine 949 recruits TSAd protein (Matsumoto et al., 2005). Acetylation has been demonstrated to both stabilize and disrupt protein-protein interactions (reviewed in (Yang and Seto, 2008)), and a future perspective of this work is also to investigate the role of VEGFR2 acetylation in protein-protein interaction and in VEGF-induced downstream signalling.

VEGFR2 stability and acetylation

Ubiquitination and acetylation are mutually exclusive lysine modifications, and, accordingly, several findings indicate that a cross-talk exists between these two modifications (Hunter, 2007).

In our work, we found by proteomic analysis that VEGFR2 can be both ubiquitinated or acetylated at lysine 929. VEGFR2 is subjected to ubiquitin-dependent degradation in response to activation, even if ubiquitinated residues have never been identified (Duval et al., 2003; Ewan et al., 2006). Consistently, we provide evidence that a VEGFR2 mutant lacking all identified acetylation sites is less stable than the wt protein (Figure 3.13). More importantly, receptor desensitization, a process involving ligand-mediated VEGFR2 downregulation, is counteracted by receptor hyper-acetylation induced by TSA treatment. Interestingly, not only VEGFR2 levels are maintained over time, but also VEGFR2 phosphorylation is retained upon 1 h of VEGF stimulation (Figure 3.14). As a final comment, it is worth noticing that we are probably still far from clarifying the pleiotropic effect of acetylation in endothelial cell function, and that the identification of new acetylated protein targets is required to better understand the role of acetylation in angiogenesis.

VEGFR2 acetylation and endothelial cell function

In addition to the direct effect of acetylation on VEGFR2 stability and phosphorylation, which is the focus of our work, others have reported the role of acetylation in the control of endothelial cell function (Ellis et al., 2009; Potente and Dimmeler, 2008).

In fact, it has been shown that HDAC inhibition suppresses VEGF-induced angiogenesis *in vitro* and *in vivo,* also by counteracting VEGF-induced VEGFR1, VEGFR2 and NP1 over-expression (Deroanne et al., 2002). Additionally, another group demonstrated that hypoxia is able to upregulate HDAC1, 2 and 3 at the mRNA and protein level. As a consequence of HDAC activity on p53 and VHL, HIF1α and VEGF are upregulated, while TSA inhibits hypoxia-induced angiogenesis (Kim et al., 2001).

These studies illustrate the complex function of HDAC and HDAC inhibition in angiogenesis. Another clear example suggesting how intricate is protein regulation by acetylation is the interplay between HIF1α and HDACs (Ellis et al., 2009). In the case of HDAC7, hypoxia induces protein translocation from the cytoplasm to the nucleus, finally potentiating HIF1α transcriptional activity via formation of a ternary complex formed by HIF1α, HDAC7 and p300 (Kato et al., 2004).

In parallel, other reports suggested a role for Sirtuin1 in the control of vascular endothelial function. By silencing experiments, Potente and coworkers showed that SIRT1 regulates sprouting angiogenesis in endothelial cells; this effect was almost in part mediated by SIRT1 interaction with Foxo1, a negative transcriptional regulator of vessel formation (Potente et al., 2007).

Taken together, these studies suggest that KDAC inhibition has a negative role in angiogenesis, providing a rationale for the use of HDAC inhibitors in the inhibition of tumor angiogenesis.

These results are apparently in contrast with our findings linking VEGFR2 acetylation to increased receptor phosphorylation. However, HDAC inhibition for long times probably has a major effect on genome expression, and possibly transcriptional regulation of VEGF-related genes prevails over fine tuning of VEGFR2 activity, thus finally resulting in an anti-angiogenic effect. Moreover, p300 is also upregulated during hypoxia and in some tumors, thus counteracting the effects of KDAC deregulation (Ishihama et al., 2007; Tan et al., 2009).

To this respect, a challenging issue is represented by the analysis of VEGFR2 acetylation in different tissues, with particular attention to conditions in which KDACs and KATs expression and activity is misregulated, such as during tumor growth and in hypoxic tissues.

DISCUSSION PART II

FRET as a powerful technique to study protein-protein interaction

In this thesis we provide evidence for the broad potential of a FRET assay that we optimized in order to disclose the dynamics of VEGFR2 and Neuropilin1 complex formation at the plasma membrane of endothelial cells.

FRET is a promising technique in elucidating dynamic protein interactions, as these play a significant role in many cellular processes. In particular, combination of FRET with fluorescent protein detection allows high-resolution assays for protein-protein interactions in living cells. FRET has been successfully exploited to study EGFR2 interaction with Shc and protein tyrosine phosphatase-1B (Carter and Sorkin, 1998; Sorkin et al., 2000), and VEGFR2-SHP2 dynamics (Mitola et al., 2006). Nonetheless, analysis of protein-protein interaction at the level of the plasma membrane is severely limited by their low protein levels, often resulting in undetectable FRET signals even in presence of protein interaction (Wouters et al., 2001). An additional limitation of FRET assays with fluorescent proteins is that it requires expression of chimeric proteins, exogenously introduced in living cells. On the other hand, FRET assays are unique for their sensitivity, detecting inter- and intra-molecular interactions in the range of 1-10 nm (1-6 nm in the case of the use of GFP derivatives) (Jares-Erijman and Jovin, 2003; Piston and Kremers, 2007).

It is worth mentioning that, in our case, FRET pair selection had a major role in the design of successful FRET experiments. Among all possible fluorescent protein pairs suitable for FRET, we choose to adopt ECFP/EYFP, one of the best FRET pairs due to large spectral superimposition (Piston and Kremers, 2007; Tsien, 1998). The major drawback in the use of the ECFP-EYFP combination in FRET essays is the

partial superimposition of their spectral behavior. We could overcame this limitation by optimizing the masking filter sets, as shown in Figure 3.16, in particular by creating a narrow (32 nm) acquisition range for the ECFP channel. In order to obtain a robust and quantitative FRET signal, we combined the ECFP-EYFP pair with the acceptor photobleaching technique (Bastiaens and Jovin, 1996; Bastiaens et al., 1996). We and other groups successfully applied this measurement method to the analysis of nuclear and cytoplasmic proteins (Cereseto et al., 2005; Karpova et al., 2003). This method is simple, fast and allows quantitative FRET measurement; the main disadvantage is irreversible fluorescence destruction. An additional limitation is the recently reported possibility that, due to laser excitation, EYFP could be converted to ECFP, a phenomenon defined as photoconversion (Valentin et al., 2005). The false positive signal, a consequence of photoconversion, is reduced using 458 nm laser as the ECFP excitation line (Valentin et al., 2005). However, we did not detect photoconversion in our experimental settings.

Finally, an absolute requirement for reliable FRET analysis using fluorescently tagged protein is the verification that the tagged proteins retain their native function. In our experiments, we confirmed that both VEGFR2 and Neuropilin1, upon C terminal fusion with GFP derivatives, retain the activity and subcellular localization characteristic of the wild type protein, as shown in Figures 3.19, 20, 21, 22 and 23.

FRET allows analysis of VEGFR2-Neuropilin1 interactions in ECs

We optimized FRET analysis, as previously described, in an effort to image the interactions between angiogenic receptors. We investigated the formation of VEGFR2-Neuropilin1 complexes in PAE cells in response to cytokines, in particular $VEGF_{121}$, $VEGF_{165}$ and SEMA3A. As shown in Figure 3.24 and in the graph in Figure 3.25, we could not observe any increase in the formation of VEGFR2-Neuropilin1 complexes on the plasma

membrane in the presence of full length or truncated $VEGF_{121}$, in comparison to untreated control cells. SEMA3A was also unable to trigger the formation of angiogenic complexes on the plasma membrane, while $VEGF_{165}$ treatment induced a strong FRET signal denoting complex formation. A model of VEGFR2-NP1 oligomer formation detected by FRET is shown in Figure 4.1

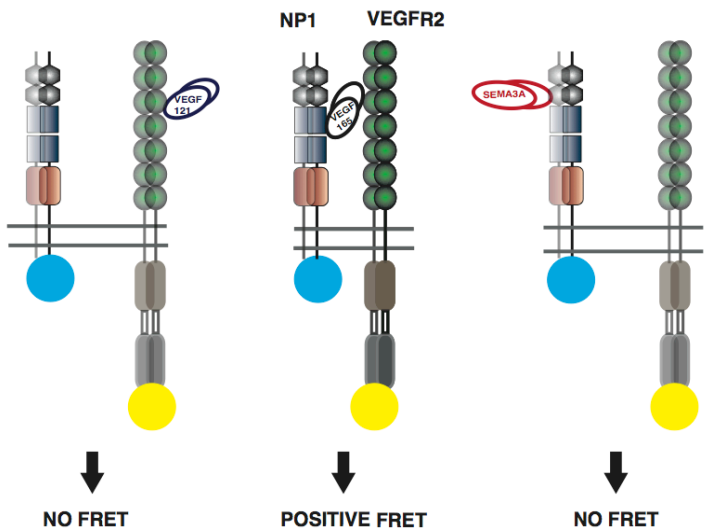

Figure 4.1. Modelling of VEGFR2-NP1 interactions by FRET. VEGFR2 and Neuropilin1 interactions on the plasma membrane are schematized in presence of $VEGF_{121}$ (blue ovals), $VEGF_{165}$ (black ovals) and SEMA3A (red ovals), respectively. ECFP and EYFP fusion proteins allowing FRET detection are depicted as cyan and yellow circles, respectively.

Our data confirm that $VEGF_{121}$ is not able to induce the formation of Neuropilin1-VEGFR2 complexes, while $VEGF_{165}$ is a powerful inducer (Soker et al., 2002; Whitaker et al., 2001). Our FRET assay, different from the vast majority of biochemical studies on VEGFR2, allowed us to image and measure the formation of angiogenic complexes directly at the level of the plasma membrane. Interestingly, SEMA3A, a NP1 but not VEGFR2

ligand, was not able to bridge together VEGFR2 and Neuropilin1 in a complex.

Recent data reported that, different from what has been thought for a long time (Soker et al., 1998), $VEGF_{121}$ might be able to bind to Neuropilin1 (Pan et al., 2007b). Nevertheless, in accordance with our observations and unlike $VEGF_{165}$, $VEGF_{121}$ anyhow does not induce the formation of a VEGFR2-VEGF-NP1 complexes (Kawamura et al., 2008a; Pan et al., 2007b; Shraga-Heled et al., 2007).

Finally, what is the biological function of VEGFR2-NP1 complexes?

Since its discovery as a VEGF co-receptor, it has been proposed that NP1-enhances VEGF-induced signalling through formation and stabilization of VEGFR2-VEGF-NP1 complexes (Soker et al., 2002; Soker et al., 1998). This model has mainly been based on the differential binding – and signalling potency - of the VEGF isoforms to NP1 and on the observation that NP1 has a short, catalytically inactive intracellular tail. This simplified model has been revised taking into account that NP1 can also transduce VEGF-mediated signalling in the absence of VEGFRs (Murga et al., 2005; Wang et al., 2007). One NP1-signal transducer candidate is NIP/GIPC, a molecule binding the NP1 tail (Cai and Reed, 1999; Chittenden et al., 2006; Wang et al., 2006). The observation that the GIPC binding site on NP1 might be required for NP1-VEGFR2 interaction suggests that NP1 can also act by bringing unique machinery, probabably comprising also GIPC, to the VEGFR2 complex (Prahst et al., 2008). Additionally, $VEGF_{165}$, but not $VEGF_{121}$ (which can not induce VEGFR2-NP1 complexes), activates p38 and NP1 is critical for VEGF-induced sprouting and branching of ECs (Kawamura et al., 2008a).

In conclusion, VEGFR2-VEGF-NP1 complex formation has an important function in vessel formation, stressed by the distinct phenotypes displayed by genetic models selectively expressing the different VEGF isoforms

(Grunstein et al., 2000; Ruhrberg et al., 2002; Stalmans et al., 2002).
The FRET assay we developed might be a useful tool to analyze in details the VEGFR2-NP1 interaction in different contexts.

NP1-NP1 complex formation

By FRET, we were able to investigate the formation of NP1-NP1 homocomplexes on the membrane of ECs and in response to different ligands. Based on the observation that NP1 can form homo-oligomers (Chen et al., 1998; Nakamura et al., 1998; Roth et al., 2008; Takahashi et al., 1998), we asked at what extent the formation of NP1 complexes might be influenced by different ligands.
First, we found that NP1 forms homo-oligomers at the plasma membrane even in absence of VEGFR2, as shown in Figure 3.27. Second, we found that $VEGF_{121}$ was not able to induce further NP1 oligomerization in comparison to mock treated samples. Third, we showed that SEMA3A is able to induce NP1 homo-oligomers on the plasma membrane, consistent with the data obtained using gradient ultracentrifugation by Roth and colleagues (Roth et al., 2008). Surprisingly, we also found that $VEGF_{165}$ was able to induce formation of NP-1 complexes, even if less efficiently than SEMA3A.
Accordingly, although we have no direct evidence that NP1 homo-complex formation is linked to VEGF- or SEMA3A-induced signalling, we speculate that the formation of these oligomers might represent the first step in NP1-triggered signalling. For this purpose, we attempted to set up a FRET assay to investigate the role of GIPC downstream to Neuropilin1 oligomers; however, these experiments were unsuccessful due to GIPC mislocalization upon fluorescent protein tagging (unpublished data). Interestingly, we previously showed that BM-derived $CD11b^+$ cells, expressing NP1, migrate in response to SEMA3A and $VEGF_{165}$, but not

$VEGF_{121}$, both *in vitro* and *in vivo* (Zacchigna et al., 2008c). Additionally, we observed that increasing concentrations of SEMA3A display a bell-shaped effect both on cellular migration (Zacchigna et al., 2008c) and on NP1 complex formation visualized by FRET (Figure 3.29).

In this context, our aim is to exploit FRET assays as a tool to unravel the role of NP1 oligomers in NP1-dependent signalling. Therefore, we first wonder whether NP1 acts as homo-complexes or homo-dimers when associated with VEGFRs. Second, do plexins have a role in the formation of such NP1 complexes in the absence of VEGFRs? In order to start address this issue, we first attempted to overcome the limitation of FRET analysis to the study of only two proteins each time. In fact, the use of a FRET pair restricts our FRET assay to a couple of protein each time; nonetheless the BIFC-based FRET allows the visualization of a ternary complex, such that one possibly formed by NP1 dimers and PlexinA1 (Shyu et al., 2008a; Takahashi et al., 1999).

We were able to detect NP1-NP1 dimer formation using protein complementation or BIFC (Kerppola, 2006), by creating two NP1 tagged protein, each one coding for half of an EYFP protein. As shown in Figure 4.2, only cotransfection of two differently tagged NP1s reconstituted EYFP fluorescence at the plasma membrane level, in correspondence to NP1 localization. This technique, in combination with a third ECFP tagged protein, such as PlexinA1, should allow triple FRET detection (Shyu et al., 2008a, b).

FRET for anti-NP1 antibody validation

Genetic studies have provided robust evidences that NP1 is required for vascular morphogenesis (Gu et al., 2003; Kawasaki et al., 1999; Takashima et al., 2002). Therefore, Neuropilins might represent an additional target in anti-angiogenesis therapies.

Here, we showed that two anti-NP1 monoclonal antibodies, generated to

selectively block SEMA3A or VEGF functions on NP1 (Liang et al., 2007), were able to disrupt the formation of NP1 oligomers in our FRET assay (see Figures 3.32, 3.33 and 3.34). Anti-NP1A, targeting SEMA3A binding site on NP1, completely disrupted SEMA3A-induced NP1 oligomers, and anti-NP1B, targeting the VEGF binding site, inhibited in a dose dependent manner VEGF-triggered NP1 complex formation. Interestingly, Pan and collaborators showed that anti-NP1A could reduce VEGF-driven cellular motility (Pan et al., 2007a), and we showed that anti-NP1A was able to slightly reduce VEGF-induced complexes. Likewise, anti-NP1B strongly inhibited SEMA3A-triggered Neuropilin1 oligomerization (Figure 3.34).

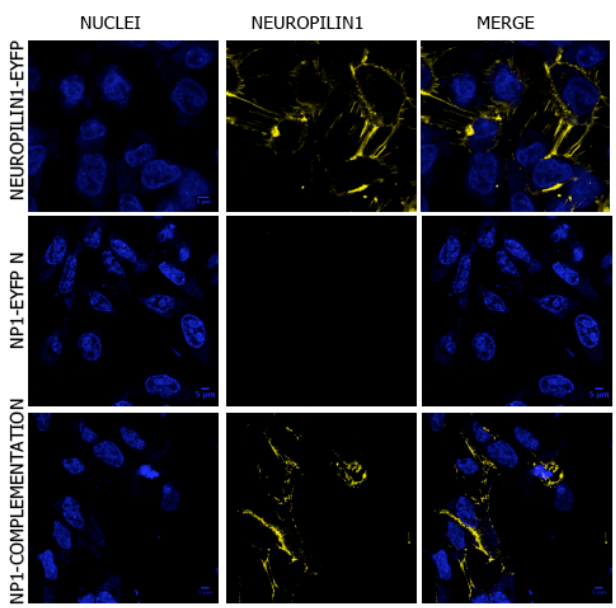

Figure 4.2 Neuropilin1 complementation. PAE cells were transfected with NP-1-EYFP (upper panel), NP1-EYFP N (which codes only for the N-terminal part of EYFP) or NP1-EYFP N plus NP1-EYFP C (which codes only for the C-terminal part of EYFP) in order to obtain protein complementation and fluorescent EYFP reconstruction, as shown in the lower panels. Nuclei are counterstained with Toto3 (Blue).

Therefore, combining evidences that NP1 can act in a VEGFR2-independent manner (Pan et al., 2007a) and our data on NP1 oligomer disruption by the antibodies generated by Pan and co-workers, we speculate that NP1 oligomer formation in response to ligand might be a key step in NP1-independent signalling.

To further validate this model, we tested an anti-PlGF blocking antibody usin our FRET system (Fischer et al., 2007). We found that PlGF, besides binding to NP1 (Mamluk et al., 2002; Migdal et al., 1998), was also able to induce NP1 oligomer formation, as shown in Figure 3.35. More importantly, the anti-PlGF blocking antibody inhibited NP1 oligomers formation on the plasma membrane of ECs, as quantified in Figure 3.36.

As a final remark, it is worth noticing that several strong evidences indicate that blocking NP1 function may represent a valuable approach to ameliorate anti-angiogenic therapy. Additionally, it is now becoming clear how combination of different angiogenic factors and their receptors has a major role in controlling pathological and physiological angiogenesis. In this context, imaging techniques, and in particular FRET, offer the unique possibility to image angiogenic receptor dynamics in cells.

Chapter 5

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APPENDIX

During my PhD course I have been personally involved in several research projects. These researches were mainly focused on *in vivo* work, with particular attention to different applications of AAV vectors in the cardiovascular field.

The first two papers describe the *in vivo* characterization of differential effects of VEGF isoforms, exploiting AAV vectors (Zacchigna et al., 2008a; Zentilin et al., 2006).

An additional paper describes the use of Positron Emission Tomography and Single-Photon Emission Computed Tomography techniques to characterize the effects of AVV-mediated therapeutical angiogenesis on blood vessel functionality (Zacchigna et al., 2007).

The fourth paper is focused on the application of AAV vectors to an *in vivo* model of nerve regeneration (Manasseri et al., 2007).

Two additional papers are focused on skeletal muscle regeneration and the description of a new myogenic cell line, with particular attention to the expression of VEGF-related genes in this cells line (Arsic et al., 2004; Zacchigna et al., 2008b).

Finally, the last paper is focused on the characterization of the anti-tumoral effect of an anti-PlGF blocking antibody (Fischer et al., 2007).

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