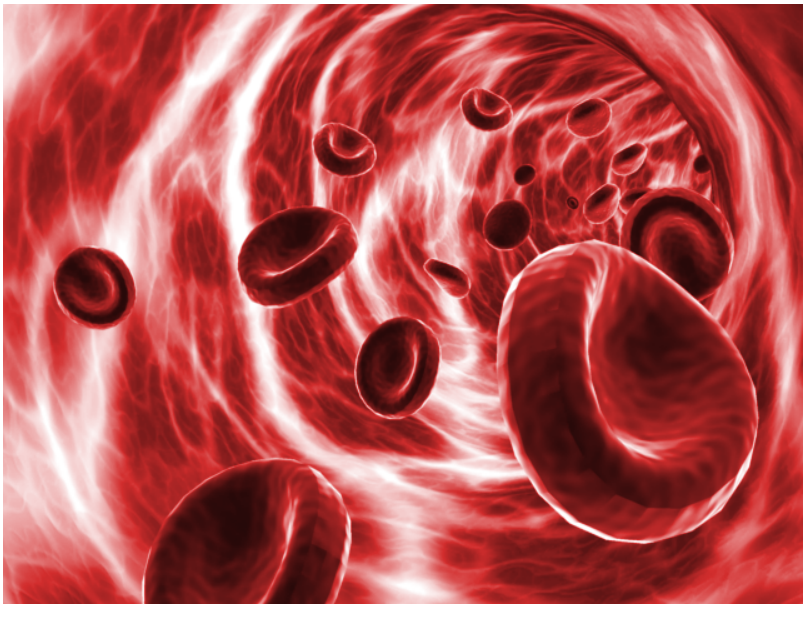
Reversible acetylation regulates Vascular Endothelial Growth Factor Receptor-2 activity

Annalisa Zecchin



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Ph.D. Thesis in Molecular Genetics and Biotechnologies

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Alla mia famiglia

La sapienza è figliola della sperienzia.

Wisdom is the daughter of experience.

-Leonardo Da Vinci-

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SYNOPSIS

The tyrosine kinase receptor Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) is a key regulator of angiogenesis. Activity of the receptor in endothelial cells follows interaction with its cognate ligands, primarily the members of the VEGF family, and involves phosphorylation of various tyrosine residues in the intracytoplasmic portion of the receptor. By combining biochemical and proteomics studies, here we provide the first evidence that membrane-associated VEGFR2 is acetylated in endothelial cells at four lysine residues forming a dense cluster in the kinase insert domain, and in a single lysine located in the receptor activation loop. These modifications are under the dynamic control of the acetyltransferase p300 and the two deacetylases HDAC5 and HDAC6. We demonstrate that VEGFR2 acetylation essentially regulates receptor phosphorylation. In particular, VEGFR2 acetylation counteracts the process of receptor desensitization following VEGF stimulation, still allowing receptor phosphorylation and intracellular signaling upon prolonged ligand treatment. Cells expressing VEGFR2 mutants that cannot be acetylated display reduced levels of receptor phosphorylation and impaired migratory capacity. Consistent with these findings, molecular dynamics simulations indicated that acetylation of the lysine in the activation loop contributes to the transition to an open active state, in which tyrosine phosphorylation is favored by better exposure of the kinase target residues. Taken together, these findings indicate that post-translational modification by acetylation is a critical mechanism that directly affects VEGFR2 function.

Chapter 1

INTRODUCTION

INTRODUCTION

Protein post-translational modifications

The sequencing of the human chromosomes, achieved in the past decade, has revealed that the human genome comprises between 20,000 and 25,000 genes, a number surprisingly lower in respect to all the previous predictions (HumanGenomeSequencingConsortium, 2004). Being a number unexpectedly close to that of the simple roundworm *C. elegans*, which has about 20,000 genes, the real complexity of organisms seems to reside within proteome rather than in the coding DNA. Accordingly, the human proteome has been estimated to encompass over 1 million proteins, supporting the notion that single genes encodes for multiple proteins (Jensen, 2004). The diversification can be initially built at the mRNA level, since several mRNA transcripts may be generated through different mechanisms, including transcription initiation at alternative promoters, differential transcription termination and alternative splicing (Ayoubi & Van De Ven, 1996). In addition, proteins might be processed post-translationally in various ways, both chemically and structurally, thus resulting in a remarkable increase in the diversity and heterogeneity of gene products. More than 300 types of protein post translational modifications (PTMs) have been identified so far, the vast majority of which are known to be mediated by enzymatic activity (Beltrao et al, 2012; Choudhary & Mann, 2010; Walsh, 2006). Shortly after translation newly synthetized proteins are subject to stable modifications, such as disulphite formation, lipidation, biotynilation and

lipoylation, which are necessary for the maturation and proper protein folding. Conversely, the functional complexity of the proteome is dictated by reversible PTMs, which consist in the transient and covalent addition of functional groups to the side chain of protein residues, typically tyrosine, serine, threonine, proline, and lysine.

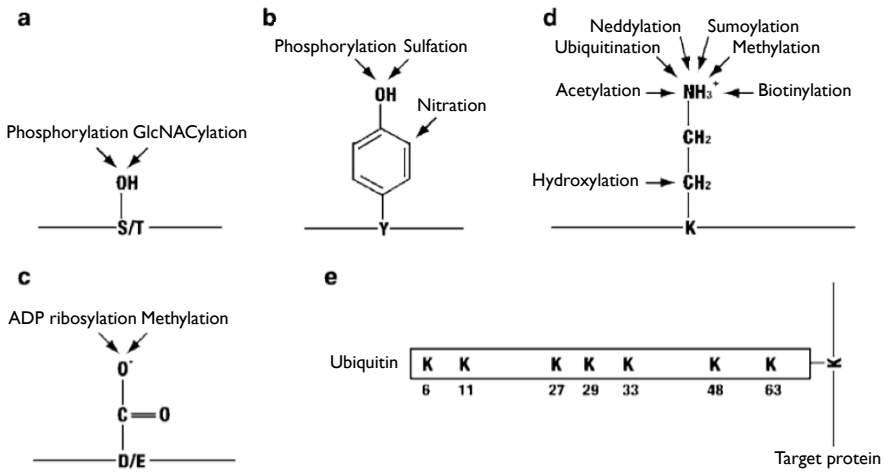

Figure 1.1. Multisite protein PTMs. The side chain of aminoacidic residue can be modified by several covalent modifications. Some prominent PTMs are reported, occurring on **a)** serine (S) and threonine (T), **b)** tyrosine (Y), **c)** aspartate (D) and glutamate (E) and **d-e)** lysine (Lys). *Adapted from Yang, Oncogene 2005*

Prominent PTMs are phosphorylation on tyrosine, serine, and threonine; proline isomerization; lysine ubiquitination, sumoylation, acetylation and neddylation; methylation on lysine and arginine residues. One single PTM can exert its effect by regulating protein activity as well as turnover, structure, intracellular localization and interaction with other molecules, through both "loss-of-function" and "gain-of-function"

mechanisms. More importantly, since many proteins can be modified at multiple sites, increasing evidence indicates that a crosstalk among PTMs exists, which can be considered as a "protein modification code", according to which PTMs act combinatorially, resulting in a significant increase in information content (Hunter, 2007; Yang & Seto, 2008a). Inevitably, to make the cellular interaction networks more intelligible, there is an urgent need to unravel PTM biological functions and decipher how distinct PTMs coordinates each other.

Protein acetylation

Protein acetylation is a common term used to identify two deeply different processes: protein N-terminal acetylation, N^{α}-terminal acetylation or Nt-acetylation, and protein lysine acetylation, N^{ε}-acetylation or Lys-acetylation.

The former refers to one of the most widespread chemical modification of eukaryotic proteins, consisting in the transfer of an acetyl group (CH3CO) from acetyl coenzyme A (AcetylCoA) to the α-aminogroup of the first aminoacid of nascent polipeptides. The reaction is irreversible and occurs co-translationally, catalyzed by N-terminal acetyltransferases (NATs) associated with ribosome (Gautschi et al, 2003; Polevoda et al, 2008). Despite it occurs on approximately 80-90% of human proteins and 50-70% of yeast proteins (Arnesen et al, 2009), little is known about its general biological implications and some hypothesis might only be derived from data of a handful of cases, highlighting a possible role in protein stability and membrane targeting (Arnesen, 2011; Behnia et al, 2004; Scott et al, 2011).

On the contrary, protein lysine acetylation is a post-translational and extremely dynamic process, which consists in the addition of the acetyl moiety to the ε-amino group of lysine residues, using acetylCoA as donor. By masking the positive charge of the lysine side chain and impairing its ability to form hydrogen bonds, the modification has a significant influence on the electrostatic properties of the protein and therefore on the regulation of protein function itself (Hodawadekar & Marmorstein, 2007). As a reversible process, a water molecule might be used to regenerate the lysine amino group by removing the acetyl group and releasing an acetate molecule. Moreover, although the vast majority of acetylated proteins have been studied in eukaryotic cells, considerable evidence of lysine acetylation has been found also in archea and bacteria (Bell et al, 2002; Soufi et al, 2012).

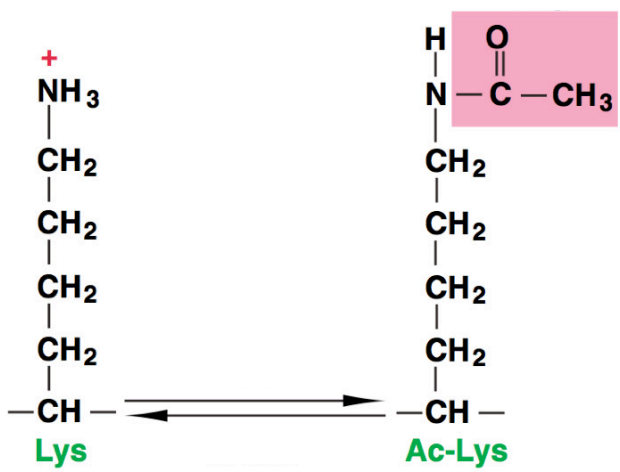

Figure 1.2. Protein lysine acetylation. Reversible acetylation of a lysine (Lys) residue, consisting in the transfer of an acetyl group from acetyl CoA to the e-amino group of the lysine residue. Adapted from Yang, Oncogene 2007

The first proteins that have been reported as acetylated were histones in the early 1960's (Allfrey et al, 1964; Gershey et al, 1968; Phillips, 1963; Vidali et al, 1968). Since the beginning, lysine acetylation was proposed as a mechanism of gene expression regulation; in fact, by neutralizing histone charge, it allows chromatin structure relaxation and therefore makes easier access to nuclear factors, such as the transcription complex (Turner, 2000). Fifteen years later, two non-histone proteins, HMG1 (high-mobility group protein 1) and α–tubulin, were found acetylated (L'Hernault & Rosenbaum, 1985; Piperno & Fuller, 1985; Sterner et al, 1979); afterwards, p53 and others transcription factors were reported to be controlled by HATs activity, thus confirming the notion that acetylation is not exclusive for nuclear histones (Gu & Roeder, 1997). Since then, the field has experienced an exponential growth and a plethora of proteins have been shown to be acetylated by biochemical techniques in one or more residues, referring to eukaryotic as well as viral and bacterial proteins (Glozak et al, 2005; Sadoul et al, 2010). In particular, among prominent PTMs, lysine acetylation has emerged in the last decade as a crucial modification which could rival phosphorylation in regulating several cytoplasmic processes by targeting non-histone proteins and giving rise to complex regulatory programs in synergy with other PTMs (Matthias et al, 2008; Spange et al, 2009; Xiong & Guan, 2012).

The acetylome

Acetylation is nowadays considered one of the major post-translational modifications, which can modulate protein-protein interactions, as well

as protein stability, activity and localization. Importantly, in the last few years, the remarkable advances in mass spectrometry have established the ubiquitous nature of protein acetylation by identifying thousands of putative acetylated proteins. The first study aimed at determining the extent of acetylation at the whole-proteome level in 2006 uncovered the acetylation of 195 proteins, of which more that 20% were resident in the mitochondria. Moreover, besides the well-defined modification of histones and transcription factors, many other acetylation sites were found in cytoskeletal proteins as well as in splicing factors, chaperones and signaling proteins (Kim et al, 2006). Three years later, an even more in-depth view of the in vivo acetylome were accomplished through a more sophisticated high-resolution mass spectrometry screening (Choudhary et al, 2009). This elegant study reported about 3600 acetylation sites on 1750 proteins, involved in all major cellular processes. Hence, it now appears that the acetylome size is highly comparable to that of the phosphoproteome, with a large array of proteins displaying exclusive cytoplasmic localization. Intringuingly, there was a preference for components of large macromolecular complexes, suggesting protein acetylation as an important event in the regulation of the multiprotein machinery. Moreover, an account was given of the differences in acetylation events following treatment with two deacetylase inhibitors (KDACi), SAHA and MS-275. More recently, a mass spectrometry analysis from 16 different rat tissues contributed to a 2-fold expansion of the available acetylome, and a 4-fold extension of the acetylation sites. Even more striking was the finding that the patterns of protein acetylation are unexpectedly different across tissues; in particular, the subcellular distribution of acetylated proteins appears to

be tissue specific (Lundby et al, 2012).

A forthcoming interesting frontier in this field of research would be the investigation of potential changes in tissue acetylation patterns during disease progression, such as cancer, and the uncovering of the underlying molecular mechanism of KDACi function, possibly towards the development of therapies for various disorders (Haberland et al, 2009; Kazantsev & Thompson, 2008).

KATs and KDACs

Protein acetylation is dynamically controlled by two different types of enzymes, commonly known as histone acetyltransferases (HATs), which add the acetyl moiety on the ε-aminogroup of lysines, and histone deacetylases (HDACs), which, conversely, remove the acetyl group (Haigis & Guarente, 2006; Lee & Workman, 2007; Yang & Seto, 2008b). Since both enzymes display broad activity towards non-histone substrate, more general terms have recently been adopted to refer to these enzymes; protein lysine acetyltransferases (KATs) and protein lysine deacetylases (KDACs) respectively (Allis et al, 2007). It is noteworthy that single protein can be substrates for multiple KATs, as well as KDACs, which mainly act as catalytic components of distinct multiprotein complexes, typically more active than the catalytic subunits alone (Lee & Workman, 2007). Thus, most non-catalytic interacting partners can bridge the interaction between enzyme and substrate and regulate acetyltransferase activity in several ways (Berndsen & Denu, 2008). Many KATs contain specific protein modules, termed bromodomains, which are able to recognize and bind the

docking sites created by lysine acetylation (Mujtaba et al, 2007). Besides being a pivotal feature in the regulation of chromatin structure and transcription by modulating KATs and histones interactions, bromodomains can trigger the physical association also between acetyltransferases and non-histone substrates, as it happens in the case of p53, Tat and MyoD (Bres et al, 2002; Dorr et al, 2002; Mujtaba et al, 2002; Mujtaba et al, 2004; Polesskaya et al, 2001).

In contrast, there are no obvious consensus motifs in the target substrates recognized by KATs prior to acetylation. Computational analysis of the lysine acetylation sites discovered so far, however, has allowed the identification of some common features of the target sequences, by which there are some residue preferences at particular positions in the proximity of the lysines subject to acetylation. For instance, the position -1 relative to the acetylated residue is often occupied by glycine residue while in position -2 and +1 reside more frequently aromatic residues, such as phenylalanine, tyrosine and proline. For the most part, acetylation appears to occur in regions with an ordered secondary structure; there is a broad preference for lysine-rich regions containing phosphorylatable residues (serine and threonine), with a particular predilection for negative charge residues in the immediate surroundings of the modified site (Basu et al, 2009; Gnad et al, 2010; Schwartz et al, 2009). The consensus acetylation sites so far identified differ depending on the subcellular compartment in which the acetylated protein resides, thus supporting the hypothesis of the existence of distinct KATs among subcellular organelles (Choudhary et al, 2009; Kim et al, 2006; Lundby et al, 2012). In this regard, cluster analysis of the acetylation sites identified in a proteomic survey of mitochondrial

acetyl-proteome has distinguished three distinct classes differentially regulated under calorie restriction, of which only one responds dynamically to the deacetylase activity of SIRT3. Consistent with the previous notion, such a difference could be due to the peculiar characteristic in the amino acid sequence and secondary structure of the surrounding sequence displayed by each class (Hebert et al, 2013).

KATs

Since the first histone acetyltransferase was discovered in *Saccharomyces cerevisiae* (HAT1) (Kleff et al, 1995), over 30 other proteins, mainly transcriptional co-activators, were shown to possess HAT activity. Although KATs are evolutionary conserved from yeast to humans, they display a large diversity both in structure and function (Yang, 2004) and in substrates recognition, the last largely depending on the subunit composition of the multiple subunits complex they belong (Kimura et al, 2005; Waterborg, 2002). The historical classification divides these enzymes into two categories, type A KATs, located in the nucleus, and type B KATs, located in the cytoplasm (Brownell & Allis, 1996). While type A KATs are linked to transcription by exerting their enzymatic activity on nucleosomal histones within chromatin (Grant & Berger, 1999), the cytoplasmic B-type KATs catalyze acetylation of newly synthetized histones, promoting their transport into the nucleus (Allis et al, 1985; Ruiz-Carrillo et al, 1975). Since many KATs have been shown to operate in different cellular compartments, a much more accurate classification could be made according to sequence similarity and conservation of structural motifs, identifying five different groups: the

general control non-derepressible 5-related N-acetylatransferases (GNAT superfamily), the p300/CBP family, the MYST proteins, the basal transcription factors and the nuclear receptor cofactors (Roth et al, 2001; Sterner & Berger, 2000). The HAT domain sequence is dissimilar between families, a divergence meaning that these proteins have evolved for more distinct functions rather than just histone acetylation (Marmorstein, 2001).

The GNAT superfamily comprises GCN5 (general control non derepressible-5), P/CAF (p300/CREB-binding protein-associated factor) and other related proteins, such as HAT1, Elp3 and Med5. Both human GCN5 and P/CAF possess a HAT domain, a bromodomain and a specific N-terminal domain, which is critical in the recognition of chromatin substrates (Xu et al, 1998). Their activity is highly conserved among species; besides being crucial for transcriptional activation (Georgakopoulos & Thireos, 1992; Wang et al, 1997), they have been shown to catalyze the acetylation of non-histone proteins as well (Gupta et al, 2008; Paolinelli et al, 2009; Sabo et al, 2008). Elp3 is the catalytic subunit of the Elongator, a multiprotein complex engaged in transcriptional elongation (Svejstrup, 2007). Apart from possessing obvious ability in acetylating the histone core, this KAT has been shown to be critical in the control of tubulin acetylation (Creppe et al, 2009). HAT1 has a pivotal role in chromatin assembly (Parthun, 2007), while Med5, which is a component of the Mediator complex, is involved in the control of telomeric silencing in *Saccharomyces cerevisiae* (Liu & Myers, 2012; Zhu et al, 2011).

CBP (CREB binding protein) and its cognate protein p300 possess several conserved domains, among which a HAT domain, a bromodomain and an

acetyl-CoA binding domain or Motif A, also shared with MYST proteins (Ogryzko et al, 1996). These are the most intensively studied and characterized nuclear HATs, able to acetylate both histone and non-histones protein and to interact with a variety of other factors, a large interactome counting more that 400 partners (Bedford et al, 2010; Cereseto et al, 2005; Gu & Roeder, 1997). Both CBP and p300 are found in mammals, whereas only CBP exists in *Drosophila* and in *Caenorhabditis Elegans* (Goodman & Smolik, 2000).

The acronym of the MYST family derives from the four founding members: human MOZ, yeast Ybf2, yeast Sas2 and the mammalian Tip60. In human the family comprises three additional members, namely MORF, HBO1 and MOF. Both the eukaryotic enzymes and the composition of the multisubunit protein complexes through which they function are extremely conserved in evolution. They are involved in a wide range of cellular regulatory functions, including transcription, DNA replication and DNA damage response and have been associated with several human diseases, among which cancer (Avvakumov & Cote, 2007; Yang, 2004).

KDACs

The first proof of histone deacetylase activity date back to 1960s (Inoue & Fujimoto, 1969), just a few years after the discovery of histone acetylation, introducing immediately the concept of high dynamicity in lysine residue modification.

According to cofactor requirement and mechanism of catalysis used, the KDAC superfamily is divided into two distinct families: the "classical family" or HDACs, the members of which are structurally related to the

yeast Hda1/Rpd3 proteins (de Ruijter et al, 2003), and the silent information regulator 2 (Sir2)-related proteins or sirtuins (Haigis & Guarente, 2006).

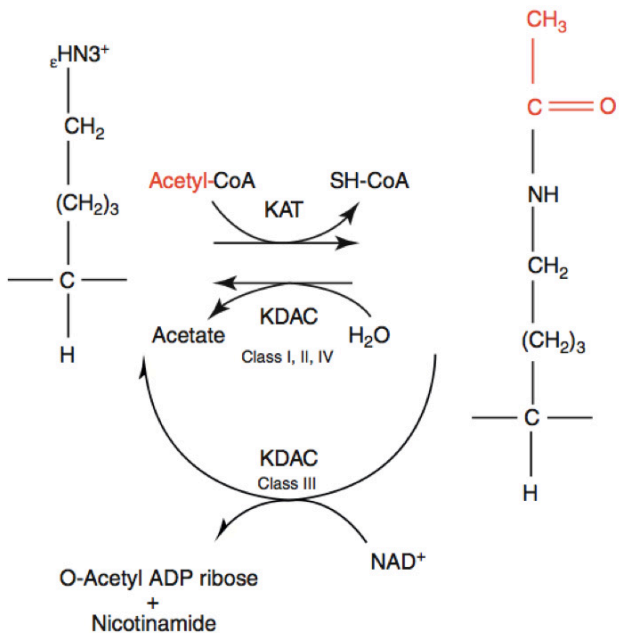

Figure 1.3. Mechanisms of lysine acetylation and deacetylation. KAT catalyzes the transfer of an acetyl moiety to the side amino group of lysine. Conversely, with the involvement of a water molecule, KDAC class I, II and IV remove the acetyl group from lysine and release acetate, whereas sirtuins utilizes NAD+ as a coenzyme and generate O-acetyl-ADP-ribose and nicotinamide. *Adapted from Xing, Trends in Plant Science 2005*

The members of the classical family possess a high degree of homology in the catalytic domain and deacetylate substrates by hydrolysing the amide bond with water, relying on an electrophillic divalent zinc cation (Hodawadekar & Marmorstein, 2007); on the other side, sirtuins need the oxidized form of nicotinamide adenine dinucleotide (NAD^+) as cofactor,

to which they transfer the acetyl group generating two products, nicotinamide and 2'-O-acetyl-ADP-ribose (Denu, 2005).

Moreover, depending on phylogenetic conservation, lysine deacetylases are further grouped into four classes (Gregoretti et al, 2004): class I, II and IV, which incorporate all the members of the classical family, and class III, or sirtuins, with a catalytic core highly conserved from bacteria to humans (Marmorstein, 2004).

Class I comprises protein orthologues to the yeast Rdp3, namely HDAC1, -2, -3 and -8, with a simple structure, predominantly consisting of the deacetylase domain and short N- and C-terminal extensions. They are ubiquitously expressed and mainly localized in the nucleus (Kurdistani & Grunstein, 2003; Taunton et al, 1996; Yang & Seto, 2008b).

Class II are related to yeast HdaI and, according to their structural organization, further subdivided into two classes: class IIa (HDAC4, -5, -7, -9) and class IIb (HDAC-6 and -10). These enzymes are considerably larger than class I members, about 1000 residues, and possess multiple regulatory region besides the catalytic domain. In particular, multiple binding sites for 14-3-3 chaperones are located in the N-terminal region, which render these enzymes responsive to nuclear-cytoplasmic shuttling (Martin et al, 2007; Vega et al, 2004a; Wang et al, 2008). Moreover, in the C-terminal part a nuclear export sequence (NES) is found involved in the control of class IIa HDACs nucleocytoplasmic distribution (McKinsey et al, 2001). The structure of HDAC6 is more specific, with two functional catalytic domains and a C-terminal zinc finger; it exerts its activity towards a large number of targets in the cytoplasm, the compartment where this enzyme is mainly localized (Kovacs et al, 2005; Tang et al, 2007; Zhang et al, 2007). Notably, class IIa

enzymes have the unique feature of being expressed in a tissue-specific manner; HDAC4 is enriched in the brain and in chondrocytes (Bolger & Yao, 2005; Vega et al, 2004b); HDAC7 is highly expressed in endothelial cells and in thymocytes (Chang et al, 2006; Parra et al, 2007); HDAC5 and HDAC9 are particularly abundant in brain, heart and skeletal muscle (Chang et al, 2004; Kim et al, 2008a; Mejat et al, 2005).

HDAC11 is the solitary member of class IV, which shares sequence similarity with both Rpd3 and Hda1 (Gao et al, 2002). Although its function is still not fully elucidated, there is much information about its expression, enriched in brain, heart, kidney, muscle and testis (Bradbury et al, 2005; Liu et al, 2008b; Villagra et al, 2009).

Finally, the yeast Sir2 is the founding member of the sirtuins family, which comprises seven paralogs in mammals, SIRT1 to 7 (Frye, 1999; Frye, 2000). Since their enzyme activity strongly depends on the ratio between NAD^+ and NADH, they are considered to serve as energy sensors, besides being transcription factors (Imai et al, 2000a; Imai et al, 2000b). Sirtuins show great variation in both subcellular localization and substrate specificity, playing key roles in a plethora of metabolic processes (Haigis & Sinclair, 2010; Michishita et al, 2005). SIRT1 is a nuclear protein, but it also carries out some cytoplasmic function (Jin et al, 2007; Tanno et al, 2007); SIRT2 is a cytoplasmic protein, but shows nuclear localization in the G2-M phase transition of the cell cycle (North & Verdin, 2007; Vaquero et al, 2006); SIRT6 and SIRT7 are found in the nucleus, with SIRT7 highly enriched in the nucleolus (Ford et al, 2006; Mostoslavsky et al, 2006). SIRT3, 4 and 5 are primarily located in the mitochondria (Huang et al, 2010).

KATs and KDACs regulation by acetylation

Reminiscent of kinases and phosphatases phosphorylation, several acetyltransferases and deacetylases exhibit ability in modulating their activity one another.

The best characterized example is the autoacetylation of p300, considered as a key event in the regulation of its enzymatic activity, a sort of autoregulatory loop that serves as an activation switch (Thompson et al, 2004). The rapid acetylation of p300 occurs by an intermolecular mechanism in a charge patch within the intact regulatory loop, containing a cluster of 12 acetylation sites (Karanam et al, 2006). Besides dramatically enhancing its activity, this sharp modification induces structural changes (Arif et al, 2007; Black et al, 2006) and thus may facilitate the interaction between p300 and the components of the multiprotein complexes in which the enzyme is active (Arif et al, 2007; Stiehl et al, 2007). Consistent with this notion, deacetylation of p300 by SIRT2 negatively affects its activity (Black et al, 2008). Moreover, both HDAC6 and SIRT1 repress p300 activity by mediating its sumoylation (Bouras et al, 2005; Girdwood et al, 2003). P/CAF acetylation targets lysine residues located within the nuclear localization signal (NLS); this modification, which can be catalyzed by both P/CAF itself and p300, increases the activity of the enzyme and is required for its nuclear localization (Herrera et al, 1997; Santos-Rosa et al, 2003). In support to this notion, it has been reported that P/CAF deacetylation by HDAC3 is responsible for the cytoplasmic retention of the acetyltransferase (Blanco-Garcia et al, 2009; Gregoire et al, 2007).

Within the MYST family as well, the mechanism of autoacetylation has been shown to regulate the catalytic activity of some members, namely Tip60 and MOF (Peng et al, 2012; Wang & Chen, 2010); in particular, the functioning of both enzymes is negatively regulated by SIRT1-dependent deacetylation (Yang et al, 2012a; Yang et al, 2012b).

In contrast, KDACs are negatively regulated by acetylation. As reported in several independent studies, p300 is responsible for the acetylation of HDAC1 as well as HDAC6 and SIRT2, thus leading to a strong attenuation of their deacetylase activity (Han et al, 2009; Han et al, 2008; Luo et al, 2009; Qiu et al, 2006).

Protein acetylation in the regulation of cellular metabolism

A growing body of evidence sustains an important link between protein acetylation and the control of cellular metabolism, according to which the concentration of metabolic fuels influence the rate of protein acetylation, especially metabolic enzymes, which in turn dictates the proper metabolic strategy in response to rapid environmental changes.

The activity of KATs strongly relies on the availability of Acetyl-CoA, a crucial intermediate of many metabolic reactions, which is also the source for the acetyl groups transferred to the epsilon aminogroups of proteins. In mammalian cells, the enzyme Acetyl-CoA synthetase 2 is responsible for the production of the mitochondrial pool of this metabolite, while the nuclear and cytoplasmic pools is catalyzed by two different enzymes, ATP-citrate lyase (ACL) and Acetyl-CoA synthetase 1 (AceCS1). ACL generates Acetyl-CoA from mitochondria-derived citrate, while AceCS1 from acetate (Hallows et al, 2006; Hatzivassiliou et al,

2005). The activity of ACL is predominant upon glucose abundance, the main carbon source for citrate production under glycolytic conditions. Strikingly, it has been recently shown that histone acetylation is nutrient-sensitive in both human glycolytic cancer cells and adipocytes, since it increases according to glucose uptake through an ACL-dependent pathway, thereafter affecting the expression of genes involved in glucose metabolism (Wellen et al, 2009). The yeast homolog to ACL, Acs2p, was correlated to the regulation of histone acetylation as well (Takahashi et al, 2006). Accordingly, in yeast KATs activity is favored in nutrient-rich environments, probably due to a higher amount of Acetyl-CoA in respect to coenzyme A (CoA) (Friis et al, 2009; Gao et al, 2007), the ratio of which has additionally been shown to fluctuate during metabolic cycle (Tu et al, 2007).

By contrast, Sirtuin-dependent deacetylation regulates the activity of both AceCS1 and AceCS2 (Hallows et al, 2006; Schwer et al, 2006), thus coordinating the production of Acetyl-CoA under low nutrient conditions. As a matter of fact, it is broadly established that the ratio of NAD^+/NADH influences the activity of Sirtuins, which are mainly functioning under nutrient deprivation, when the concentration of their essential cofactor NAD^+ is higher (Denu, 2005; Haigis & Sinclair, 2010; Imai et al, 2000a). Consistent with this notion, many studies have reported that SIRT1 activity is higher during energy limitation than under glucose excess (Boily et al, 2008; Chen et al, 2005a; Imai & Guarente, 2010). A pivotal substrate of SIRT1 is the peroxisome proliferator-activated receptor-γ co- activator 1α (PGC1α), the activity of which is turned off by GCN5-mediated acetylation; SIRT1 promotes the adaptation to fasting by deacetylating this transcriptional coactivator

and thus regulating the expression of metabolic genes involved in both gluconeogenesis and glycolysis (Lerin et al, 2006; Rodgers et al, 2005). Similarly, transcriptional profiling in yeast demonstrates a strong correlation between Gcn5 activity and several genes associated with different metabolic processes (Johnsson et al, 2006).

The regulation of cellular metabolism by acetylation occurs also at the protein level; of note, almost all enzymes involved in the catalysis of intermediate metabolism are post translationally modified, and, more importantly, their acetylation status could be reprogrammed in response to diet changes in a tissue specific manner (Kim et al, 2006; Schwer et al, 2009; Wang et al, 2010; Xiong & Guan, 2012; Zhao et al, 2010), besides being surprisingly connected also to the circadian clock (Masri et al, 2013). In particular, the mitochondrial protein acetylome is primarily controlled by SIRT3, which can sense calorie restriction and activate the adaptation response through direct deacetylation of metabolic enzymes involved in oxidative metabolism (Hirschey et al, 2010; Kendrick et al, 2011; Lombard et al, 2007; Someya et al, 2010).

How does lysine acetylation exert its effect?

The protonation occurring at physiological pH provides lysine side chain with a positive charge, frequently exploited by proteins to interact with negatively charged targets. As in the case of histones, lysine acetylation weakens their association with the negatively charged DNA molecule, resulting in nucleosome remodeling and higher accessibility of DNA to the transcription machinery (Brownell et al, 1996; Garcia-Ramirez et al, 1995; Shogren-Knaak et al, 2006; Wang et al, 2001). Similarly, acetylation

of transcription factors, such as FoxO (Matsuzaki et al, 2005a), Yin Yang 1 (YY1) (Yao et al, 2001), High mobility group protein A1 (HMG-A1) (Munshi et al, 1998), reduces their DNA-binding capability even if, in most cases, transcription factors acetylation enhances their ability to bind DNA (Chen et al, 2001). For instance, P/CAF dependent acetylation of E2F-1 results in an enhanced DNA-binding and transactivation ability (Martinez-Balbas et al, 2000; Marzio et al, 2000).

Resembling this mechanism, protein-protein interactions might also be affected by acetylation-induced conformational changes. This is the case of proteins containing the aforementioned bromodomains, able to recognize the docking sites generated by lysine acetylation and promote protein association (Mujtaba et al, 2007), and it is true also for proteins that do not contains such a particular module, such as transcription factor TFIIB, where acetylation stabilizes the interaction with transcription factor TFIIF (Choi et al, 2003), or Stat1, which is able to associate with NF-kB p65 only if acetylated (Kramer et al, 2006). On the contrary, protein-protein interactions could be disrupted following acetylation, as in the case of Nuclear Hormone Receptor Interacting Protein (RIP) 140 and transcriptional Co-repressor CtBP, the association of which is abolished following RIP140 acetylation (Vo et al, 2001); likewise, the acetylated form of the DNA-end joining protein Ku70 loose the ability to bind the proapoptotic factor Bax, as well as p53 acetylation causes its dissociation from Ku70 (Cohen et al, 2004; Yamaguchi et al, 2009).

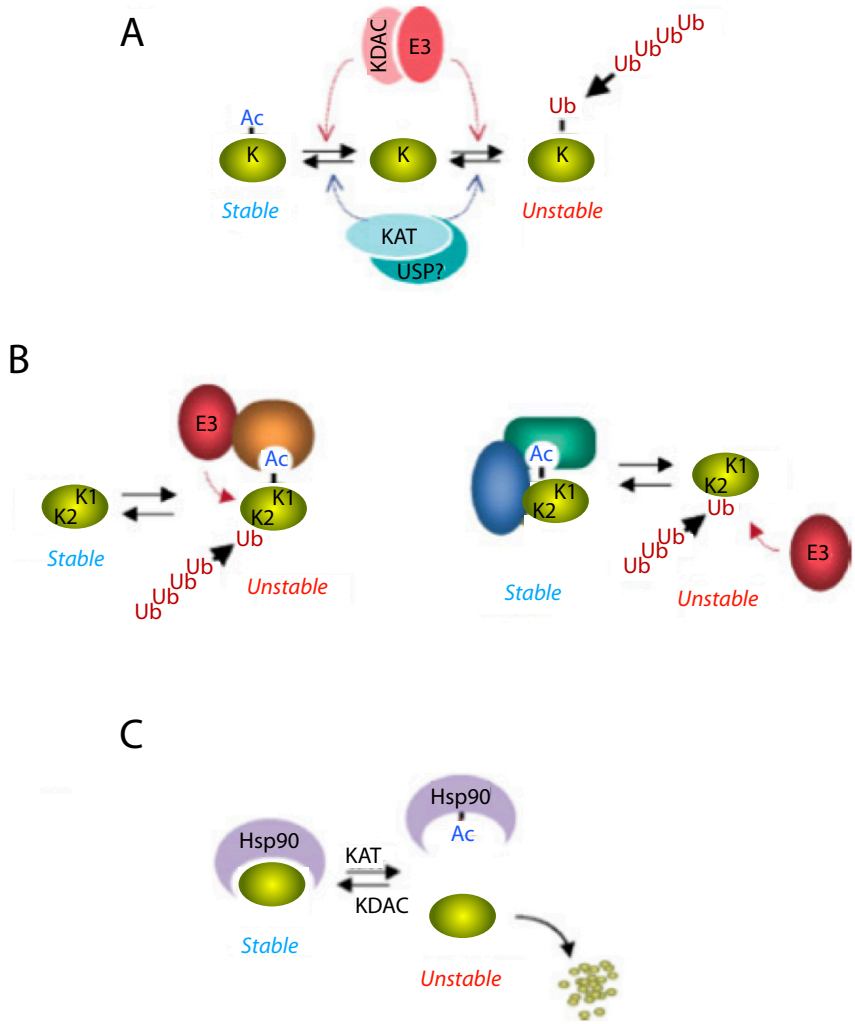

Figure 1.4. Control of protein stability following lysine acetylation. A) Same site-competition, the lysine is acceptor of both acetylation and ubiquitination. Acetylation stabilizes protein by preventing their ubiquitination. B-C) By regulating complex formation, acetylation might create binding site for E3-containing complexes, leading to protein ubiquitination (B) or, in contrast, causing complex dissociation and render components accessible for component of protein degradation machinery. *Adapted from Caron, BioEssays 2005*

In addition to acetylation, the lysine side chain can be target of several modifications which could happen simultaneously in a mutually exclusive manner, meaning that a specific modification can prevent further modifications by others and vice versa. One good example is the interplay between protein acetylation and ubiquitination in the control of target protein stability. The major mechanism through which this regulation occurs is the protection exerted by lysine acetylation that impedes protein degradation by means of direct competition with ubiquitination. Several crucial regulatory factors have been already shown to undergo stabilizing lysine acetylation, such as the oncoprotein c-Myc (Vervoorts et al, 2003), the nuclear protein Smad7 (Gronroos et al, 2002), the transcription factor Runx3 (Jin et al, 2004), the multi-functional protein WRN (Li et al, 2010), the sphingosine kinase SPHK1 (Yu et al, 2012). In contrast, it has been shown that, in some cases, protein acetylation is unexpectedly capable of increasing the rate of protein degradation by presumably modulating protein-protein interaction. As a matter of fact, the association between Hypoxia-inducible factor 1 (HIF1α) and von Hippel-Lindau tumor suppressor gene product (pVHL) is enhanced following HIF1α acetylation by ARD1, thus favoring HIF1α ubiquitination and subsequent proteasomal degradation (Jeong et al, 2002). In a similar fashion, acetylation stimulates the interaction between phosphoenolpyruvate carboxykinase (PEPCK1) and the E3 ubiquitin ligase URB5, again prompting ubiquitination and proteasomal degradation (Jiang et al, 2011; Zhao et al, 2010). Acetylation leads to protein destabilization also in the case of the retinoblastoma tumor suppressor protein RB, targeted for proteasomal degradation subsequent to Tip60-mediated acetylation (Leduc et al, 2006). Another

indirect way for lysine acetylation to stimulate protein degradation is the control of HSP90 chaperone activity (Bali et al, 2005; Scroggins et al, 2007), altering HSP90 ability to interface client proteins, such as HIF1α and glucocorticoid receptor (Kong et al, 2006a; Kovacs et al, 2005).

Alternatively, lysine acetylation might mingle with other PTMs directed on neighboring residues, either hindering or favoring secondary modifications. Recent studies reiterate the reciprocal regulation between phosphorylation and acetylation occurring on different residues, either adjacent or far away, which in turn affect each other in response to external signals (as depicted in cartoon 1.5).

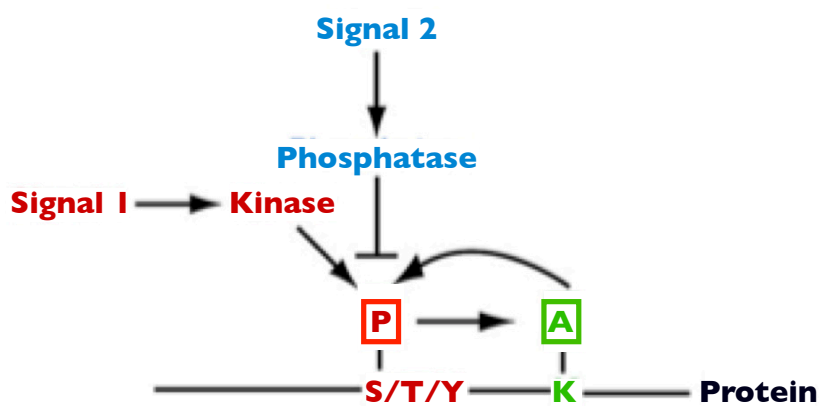

Figure 1.5. Crosstalk between phosphorylation and acetylation. Acetylation can regulate phosphorylation on serine (S), threonine (T), or tyrosine (Y), and vice versa. The lysine can be either N-terminal or C-terminal from the phosphorylation site. *Adapted from Yang, Molecular cell 2008*

Acetylation enhances the interaction between glycogen phosphorylase (GP), a key enzyme in the glucose metabolism, and protein phosphatase 1 (PP1), thereby inducing the enzyme dephosphorylation with a consequent negative regulation of its activity (Zhang et al, 2012). In the

same way, phosphorylation of Akt is prevented when the kinase is acetylated at two residues located in the pleckstrin homology domains, which mediate phosphatidylinositol 3,4,5- trisphosphate (PIP3) binding (Sundaresan et al, 2011). A counteraction was demonstrated between phosphorylation and acetylation also in the case of STAT1, since the T-cell protein tyrosine phosphatase (TCP45) binds specifically to the acetylated transcription factor, catalyzing its dephosphorylation and thereby blocking its activity (Kramer et al, 2009). RB acetylation also hampers its phosphorylation, thus leading to cell cycle arrest (Nguyen et al, 2004). Acetylation may have a broad role in the regulation of cyclin dependent kinase (CDK) function, as the modification of highly conserved residues in the catalytic core of both CDK2 and CDK9 inhibits enzyme kinase activity (Mateo et al, 2009; Sabo et al, 2008).

On the contrary, acetylation might favor phosphorylation, as it happens in the case of GCN5-mediated acetylation of the cell-division cycle (CDC)-6 protein, which is essential for the subsequent phosphorylation and activation of the enzyme (Paolinelli et al, 2009). Similarly, transcription factors FOXO1 is translocated from the nucleus to the cytoplasm in response to Akt-mediated phosphorylation, which is, in turn, induced by FOXO1 acetylation (Matsuzaki et al, 2005b).

Acetylation and the function of specific non-histone proteins

To illustrate the complexity of some of the molecular programs previously described, this section reports a few representative examples of the crosstalk between acetylation and others PTMs in non-histonic proteins.

p53

The best example of crosstalk among PTMs in the control of protein function is represented by the well-refined and tight post-translational modulation of the activity of the tumor suppressor p53. Considered as "the guardian of the genome", this transcription factor has an undisputed and pivotal role in gene expression regulation in response to a variety of stimuli (Beckerman & Prives, 2010; Vousden & Lane, 2007). More than 36 amino acids within p53 undergo different modifications, with phosphorylation, ubiquitination and acetylation having the major impact on such a complex regulatory network (Kruse & Gu, 2009). The levels of p53 acetylation are particularly elevated in response to a variety of stress-inducing stimuli. In particular, CBP/p300 is responsible for the *in vivo* acetylation of five lysines located in the p53 C-terminal region of p53 (Lys370, Lys372, Lys373, Lys381, Lys382) (Gu & Roeder, 1997; Pearson et al, 2000). Interestingly, the interaction with p300 is triggered by the phosphorylation of serine residues located in the N-terminal region of p53 (Sakaguchi et al, 1998), while acetylation of Lys373 induces hyperphosphorylation of p53 N-terminal residues (Knights et al, 2006). Furthermore, p53 hyperacetylation abrogates Mdm2-mediated ubiquitination and degradation, correlating with enhanced activity and stability of the transcription factor (Ito et al, 2001; Li et al, 2002). Mutually exclusive modifications of Lys320 in the nuclear localization signal are under the control of P/CAF and the ubiquitin ligase E4F1, the latter being responsible for the induction of a transcriptional program involved in cell cycle arrest (Le Cam et al, 2006; Liu et al, 1999). In contrast, the cytoplasmic E4 activity exerted by p300/CBP towards p53 targets it for ubiquitination, thus causing its destabilization (Shi et al,

2009). Nonetheless, it still remains elusive whether p53 carboxi-terminal acetylation has a primary role in the transcriptional activity and stability of the protein rather than only fine-tuning the p53-mediated response (Feng et al, 2005; Krummel et al, 2005; Yamaguchi et al, 2009). In addition to those mentioned above, there are two other important lysines lying in the DNA-binding core domain, Lys120 and Lys164, targets of hMOF or TIP60, and CBP/p300 respectively. Lys120 specific acetylation occurs after DNA damage and is required for p53-driven activation of the proapoptotic target genes bax and puma, and serves as a key switch between the p53 cell cycle arrest and apoptotic functions (Sykes et al, 2006; Tang et al, 2006). Unexpectedly, methylation on Lys372 by the protein methyltransferase Set7/9 serves as an anchor for TIP60 to exert its action on lysine 120 (Kurash et al, 2008). The simultaneous mutation at Lys120, Lys164 or the six C-terminal lysines greatly impairs p53 ability to activate p21 and suppresses cell cycle progression, while loss of acetylation at individual sites can be compensated by modification of the other sites, without any significant effect on p21 induction (Tang et al, 2008).

The complex regulation of p53 acetylation also comprises the action of almost four different KDACs, namely HDAC1, HDAC3, SIRT1 and SIRT7, which are fundamental in maintaining the delicate equilibrium between p53 acetylation and deacetylation, even though their site specificity is not clearly defined yet (Cheng et al, 2003; Luo et al, 2001; Luo et al, 2000; Vakhrusheva et al, 2008; Vaziri et al, 2001; Zeng et al, 2006).

α-tubulin

Dynamic and proper changes in eukaryotic cytoskeletal organization are

strictly required during cell division, as well as in carrying out a variety of essential functions in cell motility and transport of vesicles through the cells. A key component of the cytoskeleton is the microtubules network, the building blocks of which are subject to a variety of reversible PTMs that influence the interactions with other proteins (Westermann & Weber, 2003). Even though the acetylation of α - tubulin on Lys40, a residue that points toward the lumen of the microtubule, was discovered in mammalian cells almost three decades ago (L'Hernault & Rosenbaum, 1985), the responsible acetyltransferase, Elp3, has been identified only recently (Creppe et al, 2009). Tubulin acetylation was correlated to binding of cargo proteins and therefore to protein trafficking and vesicular transport (Dompierre et al, 2007; Reed et al, 2006). High levels of α-tubulin acetylation are present in stable microtubules that, in contrast, result hypo-acetylated during cytoskeleton dynamic processes, such as cell migration. In agreement, the overexpression of HDAC6 and SIRT2, the most active tubulin deacetylases, promotes chemotactic cell movement and counteracts oligodendrocyte differentiation respectively (Hubbert et al, 2002; Li et al, 2007; Matsuyama et al, 2002; North et al, 2003). HDAC6-deficient mice show hyperacetylated tubulin in most tissue, but are fertile and develop normally (Zhang et al, 2008).

Cortactin

As a filamentous actin-binding protein, cortactin has the ability to coordinate membrane dynamics and cytoskeleton remodeling (Wu & Parsons, 1993). Cortactin undergoes acetylation in 11 different lysines due to P/CAF action, eight of which (Lys87, Lys161, Lys189, Lys198,

Lys235, Lys272, Lys309, and Lys319) are located in a highly repeated region of the protein. The acetylation of one single lysine does not lead to significant effects, while the hyperacetylation in nine residues prevents the interaction with F-actin and impedes cortactin traslocation to the cell periphery, thus causing impairment in cell motility (Zhang et al, 2007). Likewise, cortactin has been recently pointed out as a novel substrate of both p300 and SIRT1, which cooperate in regulating cell motility, similar to P/CAF and HDAC6 (Kaluza et al, 2011; Zhang et al, 2007; Zhang et al, 2009b).

Plasma Membrane Receptors

Acetylation has been recently implicated in the control of transmembrane growth factor receptor activity and cytokine-mediated signal trasduction.

The Interferon α (INFα) antiviral response is mainly triggered by type I interferon receptor (INFαR) 1 and 2 oligomerization, which in turn mediate intracellular activation of STAT1 and -2 proteins (Schindler et al, 2007). Acetylation is involved in many steps of type I INF signalling pathway, as IFNαR2 as well as interferon regulatory factor 9 (IRF9) and the activator of transcription STAT2 have been found acetylated in response to IFNα. In particular, upon INFα treatment, CBP is exported in the cytoplasm, where it interacts with both INFαR2 and IRF9; IFNαR2 acetylation creates the docking site for IRF9, the acetylation of which, in turn, influences dimer formation and DNA binding. STAT1 and STAT2 interaction is regulated by acetylation as well. Interestingly, the crosstalk between lysine acetylation and serine phosphorylation in

IFNαR2 is required to initiate the IFNα-mediated intracellular signaling (Tang et al, 2007).

The acetylation of the Insulin-like Growth Factor 1 (IGF-1) receptor has been detected during a mass spectrometry survey in a conserved residue, Lys1088. Lying in a region of high similarity with the Insulin receptor, the modification of such residue suggests a more general mechanism for kinase activity regulation of transmembrane receptors (Choudhary et al, 2009). In support to this notion, the first evidence of the functional relevance of acetylation in the regulation of Receptor Tyrosine Kinase activity comes from the discovery of Epidermal Growth Factor Receptor (EGFR) acetylation by CBP. The receptor modification is linked to enhanced phosphorylation and oncogenic potential in tumor cell lines, besides being also pivotal for receptor internalization (Goh et al, 2010; Song et al, 2011). This recent finding unravels new unexplored possibilities to better comprehend the mechanisms controlling signaling transduction in response to growth factor stimulation.

Mechanisms of vessel development

The formation of an extensive and functional vascular network is essential for the proper delivery of oxygen and nutrients to every part of the body, besides allowing hematopoietic cells to patrol the organism for immune surveillance. The establishment of a highly branched vascular tree requires the perfect orchestration of several molecular and cellular players which direct both the processes of vasculogenesis and angiogenesis, the former occurring through *de novo* vessel formation and the latter through remodeling of preexisting vascular structure (Adams & Alitalo, 2007; Carmeliet & Jain, 2011).

During the early stages of embryogenesis, *de novo* blood vessels formation initiates with the recruitment of mesoderm-derived endothelial precursors cells (EPCs, hemangioblast), which differentiate and aggregate to form blood islands, as it was already reported at the beginning of the 20th century (Choi et al, 1998; Huber et al, 2004; Sabin, 1917). The subsequent assembly of these islands gives rise to a primitive vascular network, known as primary capillary plexus, a honeycomb shaped structure from which the dorsal aorta, the cardinal vein and the embryonic stems of yolk sac arteries and veins originate (Drake & Fleming, 2000; Ferguson et al, 2005). This immature vasculature then expands and undergoes remodeling via sprouting, branching, pruning and intussusceptive growth of preexisting capillaries, until a hierarchically complex vascular organization is eventually obtained, possessing all the different hemodynamic properties that enable transportation of blood throughout the organism (Adams & Alitalo, 2007; Jain, 2003). For the angiogenic sprouting to occur, some selected

endothelial cells (EC), called tip cells, take the lead and guide the new sprout towards a gradient of proangiogenic factors, such as that formed by the Vascular Endothelial Growth Factor A (VEGF-A). Behind the tip, the stalk cells are intended to support the sprout elongation by proliferating and forming the vascular lumen. The Notch pathway controls the specification of EC into tip and stalk cell (Eilken & Adams, 2010; Phng & Gerhardt, 2009; Potente et al, 2011).

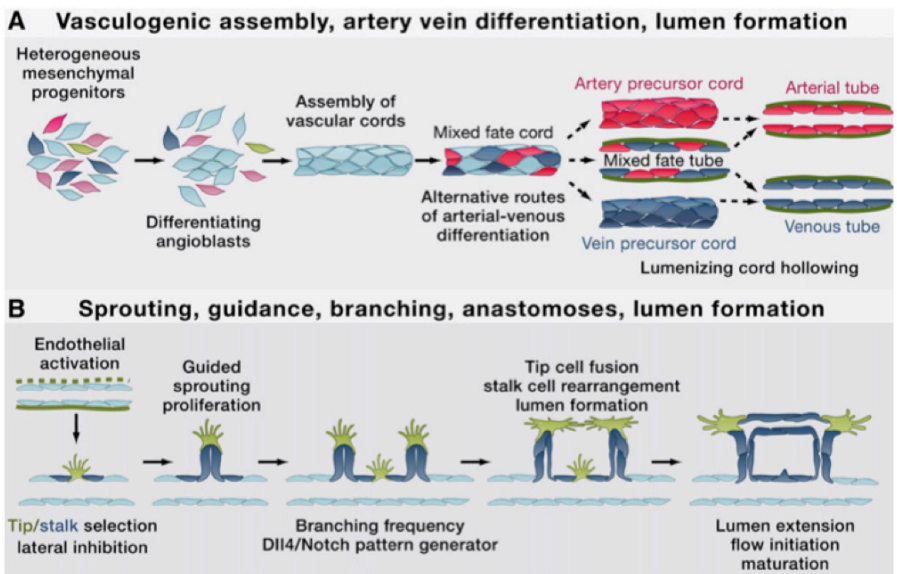

Figure 1.6. Blood vessel development. A) During the process of vasculogenesis, EC progenitors assemble a primitive vascular network, which is then remodeled until the stabilized and mature vessel network are formed. **B)** Steps of vessel sprouting. *Adapted from Potente, Cell 2011*

Arteries and veins are then formed through the process of artero-venous differentiation, during which they acquire their specific identities through the acquisition of specific anatomical and morphological composition (Gaengel et al, 2009; Swift & Weinstein, 2009). At the

molecular level, genetic specification determines the cell types, as Vascular Endothelial Growth Factor (VEGF) signaling promotes arterial specification upstream of the Notch pathway (Lawson et al, 2001; Lawson et al, 2002), whereas the COUP transcription factor II (COUP-TFII) regulates differentiation towards the venous phenotype, inhibiting both Neuropilin 1 (NP1) and Notch pathways (You et al, 2005). In turn, Notch regulates the expression of the best known arteriovenous markers, as it triggers the upregulation of the transmembrane ligand Ephrin-B2 in the arterial endothelium and suppresses the expression of the cognate receptor Ephrin B4, which is, conversely, confined to the venous endothelium (Kim et al, 2008b; Lawson et al, 2001). Additionally, blood vessel formation requires stabilization, which mainly occurs through recruitment of mural cells and generation of extracellular matrix, thus forming a basement membrane that keeps vessel structure in shape (Gaengel et al, 2009; Jain, 2003).

In the healthy adult organism, apart from cycling ovary and placenta during pregnancy, there is no evidence of active angiogenesis, until quiescent vessels are stimulated to respond to inflammatory or hypoxic stimuli, as it happens during wound healing. An imbalance of the angiogenic stimuli might contribute to several pathologies, both towards excessive angiogenesis, such as in tumor growth and inflammatory and ocular disorders, or insufficient angiogenesis, as in the case of ischemic and peripheral artery disease.

Vascular endothelial growth factors

Key actors in blood vessel formation are the members of the Vascular

Endothelial Growth Factor (VEGF) family, which comprises in mammals five distinct secreted dimeric glycoproteins of approximately 40 kDa, known as VEGF-A, -B, -C, -D and placental growth factor (PlGF). Their biological effects are mainly mediated by three tyrosine kinase receptors, Vascular Endothelial Growth Factors Receptors 1, -2, -3 (VEGFR1, VEGFR2 and VEGFR3). In addition, in parapoxvirus a protein structurally related to VEGFs, termed VEGF-E (Lyttle et al, 1994), exerts a VEGF-A-like activity by specifically binding to and activating VEGFR2 (Kiba et al, 2003; Meyer et al, 1999). VEGF homologues, known as svVEGF, have also been identified in snake venom (Junqueira de Azevedo et al, 2001; Suto et al, 2005), and are exploited by snakes to induce local vascular permeability in target animals, preferentially through VEGFR1 activation (Takahashi et al, 2004).

VEGFs share a high structural homology with the members of the platelet derived growth factor (PDGF) supergene family, generating dimers with intra- and intermolecular disulphide bonds between eight conserved cysteine residues. The crystal structure of human VEGF-A revealed two monomers organized in an antiparallel fashion and covalently linked by disulphide bridges between two conserved cysteines, thus defining two receptor-binding sites located at each pole of the dimer (Muller et al, 1997). Although VEGFs act predominantly by forming homodimers, VEGF-A and PlGF are able to form heterodimers, which have been detected in tumors and are able to potentiate VEGF-A driven angiogenesis under pathological conditions (Cao et al, 1996; Carmeliet et al, 2001; DiSalvo et al, 1995).

Since VEGFs exert their function regulating pivotal processes on a variety of cell types, their bioactivity is strictly regulated at many levels,

including gene expression, tissue specificity, alternative splicing and proteolytic processing.

VEGF-A

VEGF-A (also known as VEGF) is the main component of the family and key regulator of blood vessel formation. Abnormal blood vessel formation and circulatory disfunction are the causes of death at mid-gestation of mice lacking even only a single VEGF allele (Carmeliet et al, 1996; Ferrara et al, 1996). It is secreted by endothelial cells, as well as macrophages and activated T-cells (Freeman et al, 1995; Lee et al, 2007; Melter et al, 2000) and in adult it is robustly expressed in many tissues, such as brain, lung, liver, kidney and heart (Maharaj et al, 2006). Being pivotal in vascular maintenance, many stimuli including hypoxia, growth factors and nitric oxide (NO) controls VEGF at the transcriptional level (Dor et al, 2001; Kimura et al, 2002; Ladoux & Frelin, 1993). Moreover, VEGF mRNA undergoes alternative splicing that in humans gives rise to at least nine isoforms, of which $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ ($VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$ in the mouse) are the most abundant. Their distribution is determined by the presence of basic amino acidic stretches near the C-terminus, responsible for the interaction with extracellular matrix heparan sulphate proteoglicans (HSPG); thus, the shorter isoform $VEGF_{121}$ is completely diffusible, $VEGF_{189}$ is almost completely localized on the cell surface or in the extracellular matrix and $VEGF_{165}$ is found both as soluble and HSPG-bound factor (Giacca, 2010). The VEGF gradient that the differential expression of these isoforms create is a powerful tool for finely controlling the pattern of growing vessel networks, as it was exquisitely demonstrated in tip-stalk cell specification and vessel

branching (Phng & Gerhardt, 2009; Ruhrberg et al, 2002; Stalmans et al, 2002). Genetic studies in mice expressing single VEGF isoforms highlight the importance and the distinct roles of these molecules in arterial development and vascular morphogenesis. It is noteworthy that mice expressing only $VEGF_{164}$ mice are normal and healthy, whereas $VEGF_{120}$ mice die perinatally with severe defects in vascular remodeling and $VEGF_{188}$ alone results in abnormal arterial formation and vessel branching (Stalmans et al, 2002). In addition, mice lacking $VEGF_{164}$ and $VEGF_{188}$ show impaired myocardial angiogenesis (Carmeliet et al, 1999b).

VEGF exerts a direct action also in the prevention of motoneuron degeneration (Zacchigna et al, 2008a), in promoting survival and regeneration of skeletal muscle (Arsic et al, 2004; Germani et al, 2003), as well as in recruiting bone marrow derived cells at the sites of neoangiogenensis to promote vessel maturation (Carrer et al, 2012; Zacchigna et al, 2008b).

PlGF

Initially discovered in human placenta (Maglione et al, 1991), PlGF transcripts have been detected also in other tissue such as the heart, skeletal muscle and lung (Persico et al, 1999). Like VEGF, it undergoes differential splicing, giving rise to at least four different isoforms, PlGF-1, PlGF-2, PlGF-3 and PlGF-4 (Yang et al, 2003). Different from VEGF, PlGF-deficient mice are viable and healthy, suggesting that the factor is dispensable for embryonic and adult physiological angiogenesis; conversely, PlGF contribution is pivotal in various pathologies, such as tumor growth, inflammation and ischemia (Carmeliet et al, 2001; Fischer

et al, 2007; Fischer et al, 2008; Van de Veire et al, 2010). In agreement, overexpression of this growth factor was correlated to tumor development, including gastric and breast carcinoma (Chen et al, 2004; Parr et al, 2005), as well as hepatocellular and renal cancer (Ho et al, 2007; Matsumoto et al, 2003) and chronic myeloid leukemia (Schmidt et al, 2011). In other instances, the PlGF gene was shown epigenetically silenced, as it happens in colon and lung carcinoma (Xu & Jain, 2007). Consistent with its disease-specific activity, PlGF promotes tumor growth (Marcellini et al, 2006; Schmidt et al, 2011; Snuderl et al, 2013), and its specific blockage implies both antiangiogenic and antitumor effects (Lassen et al, 2012; Marcellini et al, 2006; Schmidt et al, 2011).

At the cellular level, PlGF is considered a pleiotropic factor, acting on various cell types and inducing a variety of biological responses. It is capable to stimulate angiogenesis acting as a mitogenic and chemoattractant factor for endothelial cells (Adini et al, 2002; Carmeliet et al, 2001; Roy et al, 2005; Ziche et al, 1997), besides being able to target mural cells as well (Bellik et al, 2005; Yonekura et al, 1999). Furthermore, PlGF recruits bone marrow-derived cells that consequently release proangiogenic factors, thus suggesting an indirect mechanism through which PlGF may induce angiogenic processes (Clauss et al, 1996; Hattori et al, 2002; Luttun et al, 2002; Pipp et al, 2003; Selvaraj et al, 2003). Consistently, the loss of PlGF was shown to skew the polarization of Tumor Associated Macrophages (TAMs), thus resulting in reduction of tumor growth and metastasis (Rolny et al, 2011).

PlGF-activity is mediated by binding to VEGFR1 and to the co-receptor NP1. An anti-PlGF blocking antibody, inhibiting binding to VEGFR1 and formation of VEGFR1-NP1 complexes, also inhibited the growth and

metastasis of various tumors (Fischer et al, 2007), an antitumoral effect strongly dependent on the expression of VEGFR1 in tumor cells (Yao et al, 2011). The same remarkable effect of PlGF/NP1 blockade in the inhibition of tumor progression was also demonstrated in medulloblastoma, the most common malignant pediatric brain tumor (Snuderl et al, 2013).

VEGF-B

Two protein isoforms are generated through alternative splicing of the VEGF-B gene, VEGF-B_{167} and VEGF-B_{186}, which give rise to 42 and 60 kDa homodimers, respectively. The two isoforms differ at the C-terminus, since the longer is diffusible, whereas the shorter exclusively contains a heparin-binding domain, which permits anchorage to cell surface and extracellular matrix HSPGs (Olofsson et al, 1996a; Olofsson et al, 1996b). In addition to VEGFR1 (Olofsson et al, 1998), both VEGF-B_{167} and VEGF-B_{186} also bind NP1 (Makinen et al, 1999), expressed on endothelial as well as on other cell types. Accordingly, VEGF-B has a wide tissue distribution, even if it is prominently expressed in the adult myocardium and skeletal muscle (Lagercrantz et al, 1998; Li et al, 2001; Olofsson et al, 1996a).

The VEGF-B knock out mouse develops normally. Instead, upon stress, VEGF-B deficiency results in altered heart size and atrial conduction abnormalities (Aase et al, 2001; Bellomo et al, 2000). Other studies have suggested that VEGF-B exerts proangiogenic activity under pathological conditions, most likely acting as a survival factor for vascular cells (Zhang et al, 2009a). Interestingly, unlike VEGF-A, this factor display a restricted role in angiogenesis (Li et al, 2008a), with particular high efficiency in

the ischemic heart, as its delivery in the infarcted myocardium significantly increases angiogenesis and arteriogenesis (Lahteenvuo et al, 2009; Li et al, 2008a). VEGF-B_{167} has a direct role in cardiomyocyte survival as well, either by exerting an antiapoptotic effect (Pepe et al, 2010; Zentilin et al, 2010) or by regulating lipid metabolism (Karpanen et al, 2008). In particular, its role in energy metabolism was also confirmed in endothelial cells, where it regulates fatty acid uptake, thus implying obvious important implications of VEFG-B functioning in organs with high metabolic stress, such as the heart (Hagberg et al, 2010). The potent antiapoptotic effect that VEGF-B_{167} exerts through activation of VEGFR1 was also demonstrated in neurons of both retina and brain (Li et al, 2008b). Similarly, VEGF-B_{186} was reported to be a neuroprotective factor, which drives cell survival in different types of neurons, such as motor, sensory and dopaminergic neurons (Dhondt et al, 2011; Falk et al, 2009; Poesen et al, 2008).

VEGF-C and VEGF-D

Dissimilarly from other VEGF members, both VEGF-C and VEGF–D are produced as a preprotein with unique N- and C- terminal extensions, which undergo proteolytic processing (Joukov et al, 1996; McColl et al, 2003; Yamada et al, 1997). Interestingly, the affinity for their cognate receptors is increased upon their proteolitic cleavage (Joukov et al, 1997; Leppanen et al, 2011; Stacker et al, 1999). They bind and activate both VEGFR3 and VEGFR2, and in addition, VEGF-C binds Neuropilin 2 (NP2) (Joukov et al, 1996; Xu et al, 2010). By activating VEGFR3, both growth factors are able to induce lymphatic endothelial cell growth and

migration (Makinen et al, 2001). VEGF-C is expressed in the heart, small intestine, placenta, ovary and the thyroid gland in adults, whereas VEGF-D is found particularly in lung, heart, skeletal muscle, and vascular endothelium (Roy et al, 2006). VEGF-C is essential for lymphangiogenesis, as its deficiency in mice leads to a complete absence of lymph vessels and embryonic lethality, whereas blood vasculature grows normally (Karkkainen et al, 2004). In agreement, transgenic expression of VEGF-C induces lymphangiogenesis (Jeltsch et al, 1997), and it occurs in an Akt-dependent manner (Zhou et al, 2010). Unexpectedly, the phenotype of the double KO mice for VEGF-C and VEGF-D does not resemble that of their cognate receptor KO mice, but mainly results in defects in lymphatic vessels without any involvement in embryonic angiogenesis (Haiko et al, 2008). Even if VEGF-D is not required for the development of the lymphatic system (Baldwin et al, 2005), it induces the formation of lymphatics within tumors (Stacker et al, 2001) and it seems to also affect tumor angiogenesis (Achen et al, 2001). Moreover, among the VEGF family members, VEGF-D is the most potent in inducing both angiogenesis and lymphangiogenesis upon gene transfer in skeletal muscle (Rissanen et al, 2003).

The biology of VEGF receptors

A large series of growth factors and their associated cell-surface receptor tyrosine kinase (RTKs) play pivotal roles in controlling the functionality of all the major cellular processes during the lifetime. As a matter of fact, deregulation of one or more RTKs causes severe pathophysiological consequences, including cardiovascular disorders, cancer, inflammation, diabetes (Lemmon & Schlessinger, 2010). There are 58 known human

RTKs, categorized into 20 subfamilies depending on their structure; despite the impressive advances that the field has experienced over the last 40 years, the sophisticated and complex signaling networks activated by RTKs are still not completely puzzled out.

According to the canonical view, once the ligand intercepts the cognate RTK in its inactive state, it induces receptor activation by primarily crosslinking two molecules into a dimeric complex. Afterwards, ligand binding drives conformational changes in the intracellular domain of the receptor, prompting the exposure of the ATP-binding site with consequent kinase activation and transfer of a phosphate group to tyrosine residues, either on the receptor dimer itself or on downstream effectors (Lemmon & Schlessinger, 2010). Despite this model has been widely confirmed, it has been recently evidenced that some RTKs, such as the epidermal growth factor receptor (EGFR) and the insulin receptor (IGF-1R), are able to associate even in the absence of ligand (Clayton et al, 2005; Gadella & Jovin, 1995; Ward et al, 2007), and that for some others (e.g. ephrins receptors and the Tie2 angiopoietin receptor) activation depends on the formation of oligomers rather than dimers (Barton et al, 2006; Himanen & Nikolov, 2003).

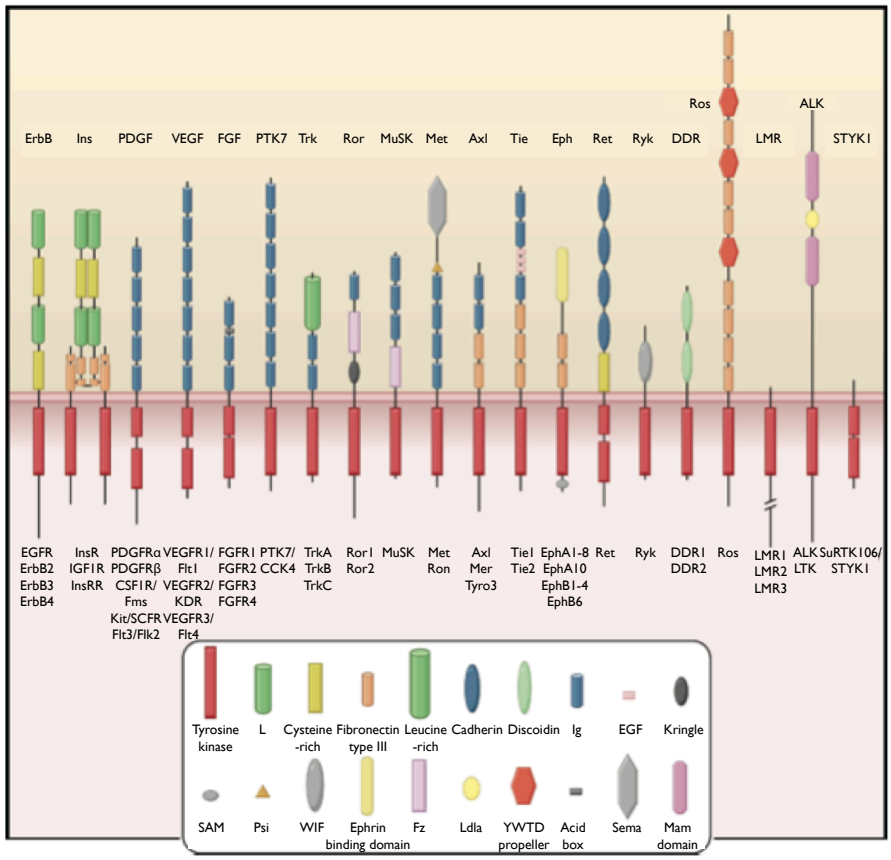

Figure1.7. Receptor Tyrosine Kinase family. Schematic representation of the 20 subfamilies of RTKs, with the family members listed beneath. *Adapted from Lemmon, Cell 2010*

As mentioned before, the members of the VEGF family remain the most critical regulators of blood vessels formation. The VEGF polypeptides bind in an overlapping manner to three RTKs, namely VEGFR1, also called fms-like tyrosine kinase 1 (Flt-1) in mouse (de Vries et al, 1992; Shibuya et al, 1990), VEGFR2, also known as kinase domain region (KDR) in human or fetal liver kinase-1 (Flk-1) in mouse (Matthews et al, 1991; Terman et al,

1991), and VEGFR3, also named Flt-4 (Pajusola et al, 1993). To date, VEGF-A, -B and PlGF bind to VEGFR1, VEGF-A and the proteolytically processed VEGF-C and -D bind to VEGFR2 and VEGF-C and -D bind to VEGFR3. Proteolytic processing of VEGF-C and -D allows binding to VEGFR2, even though with lower affinity compared to VEGFR3 (Koch et al, 2011). Thus, depending on which pair of ligand and receptor is involved, a great diversity of intracellular signaling cascades may be generated triggering various biological outcomes.

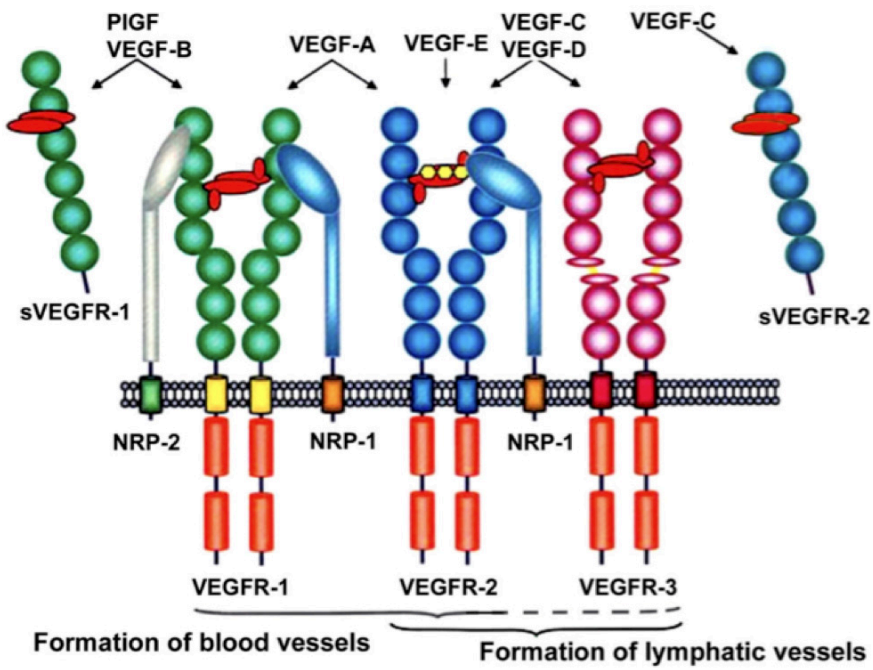

Figure 1.8. Mammalian VEGF-VEGFR interaction. Schematic representation of the interaction of the VEGF family members with their cognate receptors. *Adapted from Pavlacovic, Ann N Y Acad Sci. 2010*

All these three receptors display similar molecular architecture, both in

the extracellular and cytoplasmic regions. Because of the extracellular domain (ECD) organization, VEGFRs are classified as type V RTKs, with approximately 750 amino-acid residues organized in seven immunoglobulin (Ig)-like loops. The fifth Ig-like domain of VEGFR3 has a more peculiar characteristic, showing a disulphide bridge which serves as a connection between the proteolytically cleaved N-terminal part of the receptor and the rest of the protein (Pajusola et al, 1994). The ECD is linked to a single transmembrane portion, followed by the juxtamembrane domain, the tyrosine kinase domain and the C-terminal tail. Of note, the kinase domain is split into two parts by the insertion of a 70 amino acid sequence, defined as the kinase insert domain (KID), an unusual feature shared also with the other members of the RTK subclass to which they belong, e.g. PDGF receptors and fibroblast growth factor (FGF) receptor.

VEGFRs bind ligands both in *cis* and in *trans*. The former type of interaction implies that the receptor contacts either the free diffusible ligand or the VEGF polypeptide presented by co-receptor lying on the same cell; conversely, in the *trans* interaction the ligand is presented by a co-receptor expressed on neighboring cells, further increasing the signal amplitude and the communication among adjacent cells (Jakobsson et al, 2006; Miao et al, 2000). In addition to homo-dimers, VEGF polypeptides may induce the formation of hetero-dimers, depending on the availability and binding specificity of the ligands. VEGFR1/VEGFR2 interaction might be promoted by VEGF-A, as well as by VEGF-A/PlGF hetero-dimers (Autiero et al, 2003; Huang et al, 2001). This receptor hetero-dimerization has been predicted by computational modeling as unavoidable in cells expressing both receptors, with

minimal homo-dimerization of the less abundant receptor (Mac Gabhann & Popel, 2007). Moreover, the formation of VEGFR1/VEGFR2 hetero-dimers has been described to contribute to the regulation of EC homeostasis, by mainly mediating EC migration, *in vitro* tube formation and vasorelaxation of arterial tissue (Cudmore et al, 2012). VEGFR2 is also able to interface with VEGFR3 in response to VEGF-A, -C or –D stimulation, and in such cases the phosphorylation pattern of VEGFR3 in the homo-dimers substantially differs from the monomer in the hetero-dimers, which lack the phosphorylation of two C-terminal residues (Dixelius et al, 2003). Nevertheless, the association with VEGFR2 seems to be required for efficient VEGFR3 phosphorylation (Alam et al, 2004), with consequent implications in several cellular responses both in lymphatic and blood vessels, such as EC proliferation, angiogenic sprouting, lymphatic migration and proliferation (Goldman et al, 2007; Nilsson et al, 2010; Tvorogov et al, 2010). On the contrary, in EC VEGFR3 could decrease the level of phosphorylated VEGFR2, thus functioning as a negative modulator for its activity in vascular development (Zhang et al, 2010).

Among the VEGFRs, the binding of ligands to the cognate receptor is essentially determined by the differential way through which the ECD subdomains of each dimer component interact with one another. The crystal structure of VEGFR1, either alone or in complex with a ligand, exhibits hydrophobic interactions stabilizing the dimers, the formation of which mainly occurs through the second Ig-like domain with the important contribution of Ig-like domains 1 and 3 (Christinger et al, 2004; Davis-Smyth et al, 1996; Tanaka et al, 1997). Regarding VEGFR2, biochemical analysis demonstrate that subdomains 2 and 3 are required

for ligand binding (Fuh et al, 1998; Shinkai et al, 1998), while VEGFR3 binding of the ligand involves Ig-loops 1 and 2 (Jeltsch et al, 2006).

Vascular endothelial growth factor receptor 2 (VEGFR2)

Most VEGF-A-mediated downstream signaling requires VEGFR2, a 210-230 kDa glycoprotein, belonging to the type V receptor tyrosine kinase subclass. VEGFR2 is considered to be the most potent angiogenic mediator, both in physiological and pathological conditions. VEGFR2 deficient mice die in utero at E8.5, showing severe defects in the development of hematopoietic and endothelial cells (Shalaby et al, 1995), similarly to the homozygous VEGF-A-deficient embryo (Carmeliet et al, 1996; Ferrara et al, 1996). VEGFR2 displays stronger kinase activity than VEGFR1, even though it binds VEGF-A with a 10-fold lower affinity than VEGFR1 (Waltenberger et al, 1994). In this connection, it has been proposed that the naturally occurring variation of a single amino acid (conserved among type III RTKs) in the activation loop of VEGFR1 is responsible for its weaker autophosphorylation (Meyer et al, 2006). Remarkably, a pool of autocrine VEGF capable of activating VEGFR2 intracellularly exist in EC, and this mechanism seems to be required for adult vessels homeostasis (Lee et al, 2007).

VEGFR2 expression

The VEGFR2 gene is located on chromosome 4; its mRNA is predominantly detectable in vascular endothelial cells as well as in their embryonic precursors, with a particular abundance in proliferating cells (Oelrichs et al, 1993; Quinn et al, 1993; Shalaby et al, 1995). Of note, the

expression pattern of VEGFR2 and its high affinity ligand VEGF-A are correlated both temporarily and spatially (Millauer et al, 1993). Besides being highly expressed during embryonic vasculogenesis and angiogenesis, a major regulatory role of this protein has been observed in neovascularization, both in physiological conditions, such as in the utero in response to the estrous cycle (Tasaki et al, 2010), or in pathological processes, such as in tumors (Jinnin et al, 2008; Millauer et al, 1994; Plate et al, 1993; Shibuya & Claesson-Welsh, 2006).

The receptor is also present, even if at lower levels, in many other cell types, including megakaryocytes, hematopoietic stem cells, osteoblast, retinal progenitor cells, adult heart, brain and skeletal muscle (Asahara et al, 1999; Lyden et al, 2001; Matsumoto & Claesson-Welsh, 2001; Oelrichs et al, 1993; Youssoufian et al, 2007). Interestingly, VEGFR2 was found specifically expressed and constitutively phosphorylated *in vivo* in aortic endothelial cells, as well as in several adult mouse tissues, such as liver, lung, adipose tissue, and kidney (Maharaj et al, 2006).

A naturally occurring truncated, soluble form of human and murine VEGFR2 arises from alternative splicing, consisting of six Ig-like domains with a unique 13 or 16 amino acid C-terminal sequence. It was initially found in mouse and human plasma, but so far it has been described in many others mouse tissues, including skin, heart, spleen, kidney, ovary and in human cornea (Albuquerque et al, 2009; Ebos et al, 2004; Munaut et al, 2012). Soluble VEGFR2 (sVEGFR2) does not show any particular affinity for VEGF-A; instead, it contains the binding site for VEGF-C, with which it interacts, and consequently inhibits lymphangiogenesis, by probably trapping VEGF-C and preventing VEGFR3 activation (Albuquerque et al, 2009). Consistent with this possibility, sVEGFR2 has

been shown to play a role in the progression of human malignant tumors, being significantly down-regulated during the latest neuroblastoma metastatic stages (Becker et al, 2010). In addition, sVEGFR2 mediates the interaction between EC and mural cells, leading to mural cell migration and vessel coverage, thus contributing to vessel maturation (Lorquet et al, 2010).

VEGFR2 structure

The crystal structure of the phosphorylated kinase domain of VEGFR2 resembles closely that of FGFR-1 and the Insulin receptor, revealing a high similarity both in conformation and residue positions (McTigue et al, 1999).

The structural domains of which the receptor mainly consists are listed as follows:

- the extracellular domain (residues 20-764)
- the transmembrane segment (residues 765-785)
- the juxtamembrane segment (residues 790-833)
- the proximal kinase domain (residues 834-931).
- the kinase insert domain (KID) (residues 932-998);
- the distal kinase domain (residues 999-1158)

Although not directly involved in catalysis, the KID has been demonstrated to be relevant in maintaining competent kinase structure and downstream effectors (Heidaran et al, 1991; Severinsson et al, 1990; Taylor et al, 1989). The crystallization of the modified VEGFR2 kinase domain, containing 18 residues of the KID, yielded for the first time partial comprehension of the KID orientation in RTKs, according to which

the two strands at the end of the KID form a pseudo β–sheet structure that may have a key role in structural integrity (McTigue et al, 1999). The distal kinase domain contains both the catalytic and the activation loop (A loop). The former is referred to a stretch of residues (H1026-N1033) containing an invariant aspartic acid, essential for the phosphotransfer reaction; the latter (D1046-E1075) is a flexible region, mainly involved in directing protein kinase activity through the phosphorylation of two important tyrosine residues located herein.

As all protein kinases, the catalytic core of VEGFR2 has a typical bi-lobed architecture, presenting the proton acceptor, an active site in the cleft between the two lobes. The N-terminal lobe (residues 820-920) is the smaller one, folded into an antiparallel β-sheet and containing the glycine-rich ATP-phosphate binding loop; on the contrary, the C-terminal lobe (residues 921-1168) is predominantly formed by α-helices and includes the KID as well as the activation and catalytic loops (McTigue et al, 1999). The transition from inactive to active states of the receptor is governed by conformational changes at the level of these lobes. In particular, in the inactive state, a "closed" conformation of the activation loop prevents substrate binding, while upon activation tyrosine phosphorylation stabilizes it in an "opened" conformation (Roskoski, 2008) (see http://www.ebi.ac.uk/pdbsum/2oh4 for VEGFR2 crystal structure).

Ligand-induced dimer formation is necessary but not sufficient for receptor activation, since distinct orientation of the monomers is indispensable as well for driving receptor phosphorylation at specific tyrosine residues, with consequent induction of angiogenic signaling (Hyde et al, 2012; Kendrew et al, 2011; Tvorogov et al, 2010). Both the

transmembrane domain and homotypic contacts in membrane proximal Ig domains D4 and D7 are mandatory for the formation of active receptor dimers (Dosch & Ballmer-Hofer, 2010; Kisko et al, 2011; Ruch et al, 2007; Yang et al, 2010); in particular, the enthalpic penalty existing in the establishment of D4 and D7 interactions accounts for a possible role in preventing ligand-independent activation of VEGFR2 (Brozzo et al, 2012; Hyde et al, 2012).

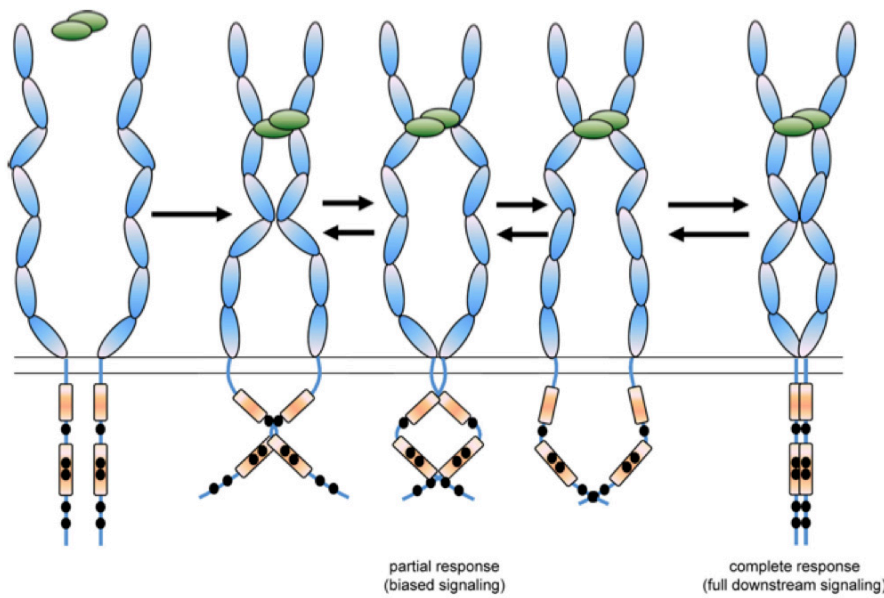

Figure 1.9. ECD involvement in VEGFR2 activation. Proper ligand mediate receptor dimerization, mainly guided by transmembrane domain and homotypic contacts in extracellular domains D4 and D7, are required for giving rise to complete transphosphorylation at selected tyrosine residues. *Adapted from Hyde, Molecular and Cellular Biology 2012*

Thus, the ligand-induced dimerization of VEGFR2 ECD, once stabilized by the interactions occurring among Ig-like loops, might be communicated across the membrane to induce the proper three-dimensional

organization of the intracellular kinase domain, essential for complete receptor transphosphorylation and activation.

VEGFR2 PTMs

As the role of VEGFR2 is essential in vascular development, its activity has to be finely tuned, and this occurs through gene expression control as well as through protein function regulation by post-translational modifications (PTMs).

Glycosylation. Soon after protein synthesis, the 150 kDa nascent VEGFR2 undergoes a first step of rapid N-glycosylation to an intermediate form of 200 kDa; then, the proper maturation and membrane presentation is achieved through a further glycosylation process at slower rate to reach the final size of 230 kDa, which corresponds to the only pool capable of being phosphorylated in response to ligand stimulation (Takahashi & Shibuya, 1997). Consistently, glucose deprivation induces an almost complete reduction in VEGFR2 protein levels, by most likely interfering with the process of receptor maturation (Adham & Coomber, 2009).

Phosphorylation. The activity of VEGFR2 is strictly dependent on receptor dimerization upon ligand binding and consequent autophosphorylation of tyrosine residues. The phosphotransfer reaction requires the formation of a ternary complex between VEGFR2, ATP and the peptide substrate; activation of the receptor seems to be dependent on the increased affinity for both substrates (Kendall et al, 1999). The phosphorylation of tyrosine residues creates binding sites, which modulate the interaction with downstream effectors, especially SH2-

domain-containing proteins, thus leading to the initiation of several intracellular cascades in EC. To date, at least six VEGFR2 tyrosine residues have been found to be modified in response to VEGF stimulation: Y951 and Y996, located in the kinase insert region, Y1054 and Y1059 within the distal kinase domain, and Y1175 and Y1214 which resides in the C-terminal tail (phosphorylated tyrosines, from now onward, are indicated as pY951, pY996, pY1054, pY1059, pY1175, pY1214) (Dougher-Vermazen et al, 1994; Matsumoto et al, 2005; Takahashi et al, 2001). In addition, the C-terminal tail shows phosphorylation at positions 1305, 1309, 1319, the exact functions of which have not been yet elucidated (Matsumoto et al, 2005), while the juxtamembrane domain is phosphorylated in Y801, at least when the intracellular domain of the receptor is selectively purified, and this event appears to precede the modification of the major autophosphorylation sites (Solowiej et al, 2009).

Tyrosines Y1054 and Y1059 are located in the activation loop of the receptor, and their VEGF-induced phosphorylation is critical for receptor maximal kinase activity (Dougher & Terman, 1999; Kendall et al, 1999). Interestingly, once phosphorylated, Y1059 is capable to recruit c-Rous Sarcoma (c-Src) kinase, which in turn promotes EC proliferation by facilitating the phosphorylation of Y1175 as well as downstream effectors, such as IQGAP1 (IQ-GTPase-activating protein) (Meyer et al, 2008). The phosphorylation of Y951, which occurs preferentially during active angiogenesis, creates a binding site for the T cell–specific adapter molecule (TSAd) (Matsumoto et al, 2005), alternatively referred as VRAP (VEGF receptor associated protein) (Wu et al, 2000). Tyrosine Y1175 (corresponding to Y1173 in mouse) is considered the essential residue for VEGFR2 function; as a matter of fact, knock-in homozygous mice

carrying a Y1173F mutation die between E8.5 and E9.5 because of severe defects in blood vessels formation as well as lack of mature endothelial and hematopoietic cells (Sakurai et al, 2005), a phenotype that mirrors that of VEGFR2 null mice (Shalaby et al, 1995). The signaling via Y1175 requires binding of several mediators crucial for vascular development, described in detail in the next section. The substitution of Y1214 (corresponding to 1212 in the mouse) with phenylalanine in knock-in mice results in viable and fertile animals, without any obvious phenotype, thus indicating that this residue is not essential during mouse embryogenesis (Sakurai et al, 2005).

The rate of phosphorylation of VEGFR2 is tightly controlled through the intervention of tyrosine phosphatases, responsible for the maintenance of the delicate equilibrium between kinase activation/deactivation, necessary to avoid VEGFR2 aberrant over-activation. Among the enzymes that limit VEGFR2 signaling, it is important to mention the vascular endothelial protein tyrosine phosphatase (VE-PTP) (Mellberg et al, 2009), the density-enhanced phosphatase 1 (DEP-1) (Chabot et al, 2009), the protein tyrosine phosphatase 1B (PTP1B) (Nakamura et al, 2008) the Src homology-2 domain-containing protein tyrosine phosphatase (SHP2) (Mitola et al, 2006) and the T-cell protein tyrosine phosphatase (TCPTP) (Mattila et al, 2008).

Ubiquitination. Besides dephosphorylation, another important mechanism used to counterbalance continuous receptor activation is ubiquitination, which routes the receptor for degradation. VEGFR2 is ubiquitinated in response to VEGF stimulation (Murdaca et al, 2004) and this event is mainly achieved through the involvement of the multi-

adaptor protein Cbl (Duval et al, 2003). The E3 ligase β-transducin repeats-containing protein (TRCP) has also been shown to mediate the poly-ubiquitination and subsequent degradation of VEGFR2. It is recruited to two phosphorylated serines, S1188 and S1191 (Singh et al, 2005), located in the PEST consensus sequence (rich in proline [P], glutamic acid [E], serine [S], and threonine [T]), present in the C-terminal tail of VEGFR2 (Meyer et al, 2011). Importantly, the epsin family of ubiquitin-binding endocytic clathrin-adaptor protein have recently been found to mediate VEGFR2 degradation by binding to the ubiquitinated receptor, and their absence leads to aberrant tumor angiogenesis due to excessive VEGFR2 signaling, which finally results in retarded tumor growth (Pasula et al, 2012).

Oxidation. Compelling evidences has established that the concentration of antioxidant present in the environment controls VEGF-induced cell signaling (Ushio-Fukai et al, 2002). More precisely, the VEGF-dependent oxidation of the reactive cystein thiol (cys-SH) renders VEGFR2 capable of sensing the redox state of the endothelium. The thiol oxidation in both VEGFR2 and c-Src promotes their interaction and thus c-Src activation (Lee et al, 2011). On the contrary, VEGFR2 oxidation in a critical C-terminal cystein residue is responsible for receptor inactivation and defective angiogenesis; peroxiredoxin II is the antioxidant enzyme that protects VEGFR2 against oxidative inactivation (Kang et al, 2011). The apparent discrepancy arising from these reports highlights the urgent need to better elucidate the mechanism connecting VEGFR2 activation to the local redox architecture of the endothelium, in both physiological and pathological conditions.

VEGFR2 signaling

As anticipated in the previous section, the capability of VEGFR2 to transmit signals inside the cells strongly relies on the phosphorylation of critical tyrosine residues, leading to downstream effector recruitment and activation of a multiplicity of cellular functions, such as EC proliferation, migration, survival and vascular permeability.

Proliferation. The main way through which VEGF exerts its mitogenic effect on EC requires the involvement of phospholipase C-γ (PLCγ), recruited by pY1175 on VEGFR2; the subsequent intracellular calcium increase and generation of diacylglycerol stimulates protein kinase C (PKC) function, which finally leads to the activation of mitogen-activated protein kinases (MAPK), among which extracellular-signal regulated kinase (ERK) 1 and 2 (Cunningham et al, 1997; Takahashi et al, 2001). Notably, the phenotype of PLCγ deficient mice resembles that of Y1173F VEGFR2 knock in mice, with embryonic death at E9.0 due to lack of both erythrogenesis and vasculogenesis, highlighting the key role of PLCγ in transmitting VEGF stimulation (Ji et al, 1997; Liao et al, 2002). Three PKC isoforms have been shown to modulate VEGF-induced proliferation: PKCα, PKCβ and PKCζ (Wellner et al, 1999; Xia et al, 1996). Although the PKC-dependent activation of MAPK pathway was initially shown to occur without the participation of Ras-specific guanine nucleotide exchange factors (GEFs) (Takahashi et al, 1999), other reports actually demonstrate its involvement, predominantly mediated by sphingosine kinase (SPK) activity (Meadows et al, 2001; Shu et al, 2002). These conflicting results

may indicate that different cell types and environment could guide the activation of different regulatory components. The PLCgamma/PKC pathway controls endothelial cells proliferation also through the phosphorylation and consequent activation of protein kinase D (PKD) (Wong & Jin, 2005). Finally, the induction of proliferation could alternatively be achieved through recruitment to pY1175 of growth factor receptor bound protein 2 (Grb2) and Shc [Src homology and collagen homology]-related adaptor protein (Sck) (Kroll & Waltenberger, 1997; Warner et al, 2000).

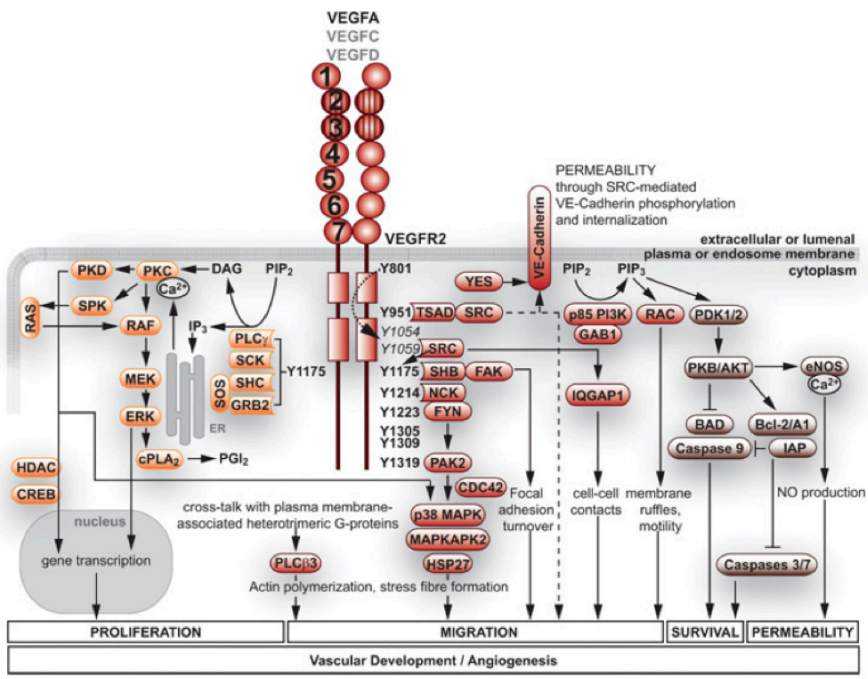

Figure 1.10. VEGFR2 signal transduction. Once the receptor is activated, upon VEGFs-induced dimerization, signaling molecules ('rocket' shapes) bind to respective tyrosine phosphorylation sites and initiate complex network of intracellular signal transduction pathways. *Adapted from Koch, The Biochemical Journal 2012*

Migration. Besides proliferation, EC migration is a critical step in blood vessels formation. Cell motility is directionally regulated by a concentration gradient of chemotactic stimuli and requires degradation of the extracellular matrix to allow cell movement. Several signal transducers implicated in EC migration have been found to bind different phosphorylated tyrosines in VEGFR2, including pY951-mediated recruitment of TSAd which drives VEGF-A dependent actin reorganization and cell migration (Matsumoto et al, 2005). In response to VEGF stimulation, Src phophorylates the adaptor protein SH2-domain-containing adaptor protein b (Shb), which is able to interact with pY1175 and to subsequently stimulate the phosphorylation of the nonreceptor tyrosine kinase focal adhesion kinase (FAK), ending with enhanced endothelial cell spreading and migration (Abu-Ghazaleh et al, 2001; Holmqvist et al, 2003; Holmqvist et al, 2004). pY1214 mediates complex formation between two members of the Src family, namely NCK and Fyn, and p21-activated protein kinase-2 (PAK-2) which, in turn, triggers subsequent activation of Cdc42 and stress activated protein kinase-2 (p38) (Lamalice et al, 2006; Lamalice et al, 2004); afterwards, p38 induces rearrangements of the actin cytoskeleton by phosphorylating the F-actin polymerization modulator, heat shock protein 27 (HSP27) (McMullen et al, 2005; Rousseau et al, 1997). Independently of p38, Hsp27 may be phosphorylated by PKD, thus inducing VEGF mediated cell migration and tubulogenesis (Evans et al, 2008). The phosphorylated PKD also controls the transcription of genes involved in endothelial cells migration by promoting nuclear export of HDAC5 and HDAC7 (Ha et al, 2008; Wang et al, 2008). VEGFR2 phosphorylation induces its interaction with the

docking proteins Grb-2 associated binder 1 and 2 (Gab1 and Gab2), most likely through Y801 and Y1214 since mutation of both abrogates Gab1 phosphorylation (Caron et al, 2009). Gab1-depleted cells show reduced ability to migrate (Dance et al, 2006; Laramee et al, 2007), and, more importantly, Gab1 knock out mice display impaired VEGF-induced angiogenesis (Lu et al, 2011). IQGAP1 has also been linked to VEGFR2-induced cell migration (Yamaoka-Tojo et al, 2006), as well as PLCβ3 (Bhattacharya et al, 2009) and Rho-dependent kinase (ROCK) (Le Boeuf et al, 2006).

Survival. Another event considerably relevant for vessel development and maintenance is the control of EC apoptosis. The survival function of VEGF has already been explained by the induction it exerts on the expression of antiapoptotic proteins, such as Bcl2 (B cell CLL/lymphoma 2), the X-chromosome linked inhibitor of apoptosis protein (XIAP) and survivin (Nor et al, 1999; Tran et al, 1999). VEGFR2 activity tightly governs this process by mainly signaling through the PI3K-Akt pathway, which mediates the survival cue (Gerber et al, 1998). Akt (also known as PKB) is capable of inhibiting the apoptotic activity of downstream targets, such as Bcl2-associated death promoter (BAD) and caspase 9 (Jiang & Liu, 2009). Akt1 is the predominant form in EC, and it was shown to be crucial for VEGF-mediated angiogenesis (Ackah et al, 2005; Chen et al, 2005b). Interestingly, the VEGFR2-dependent activation of Akt is strictly related to EC anchorage (Fujio & Walsh, 1999) and, in particular, to the proper function of the adhesion molecule Vascular Endothelial Cadherin (VE-cadherin) (Carmeliet et al, 1999a). On the other side, the impairment in p38 MAPK phosphorylation and activity has also been associated with

reduced EC apoptosis (Kumar et al, 2004; Yilmaz et al, 2003).

Permeability. The original designation of VEGF-A was "vascular permeability factor" (VPF), because of its critical action on microvascular permeability (Senger et al, 1983). Vascular permeability to solute and proteins as well as white blood cells is pivotal in both physiological and pathological conditions (Bates & Harper, 2002; Dvorak et al, 1999; Mehta & Malik, 2006), and it has been proposed to occur either through transendothelial pores formation or through transient opening of EC junctions (Vandenbroucke et al, 2008).

Mice lacking c-Src or Yes display reduced VEGF-mediated vascular permeability, despite their apparently normal vascular development (Eliceiri et al, 1999); in particular, c-Src was shown to elicit the disruption of intercellular junctions by phosphorylating VE-cadherin (Wallez et al, 2007; Weis et al, 2004), and thus promoting its rapid endocytosis and internalization into clathrin-coated vesicles (Gavard & Gutkind, 2006). Nevertheless, other signaling pathways may concur to cause loss of endothelial barrier function besides src-induced VE-cadherin phosphorylation (Adam et al, 2010). Importantly, the transmembrane binding partner VE-PTP decelerates the junctions opening by exerting its tyrosine phosphatase activity on VE-cadherin (Nawroth et al, 2002; Nottebaum et al, 2008). The maintenance of such a delicate equilibrium may also involve angiopoietin receptor Tie-2, with which VE-PTP interacts (Winderlich et al, 2009). Moreover, EC junction rearrangement in response to VEGF requires the TSAd-dependent Src activation, both of which are found in complex with VE-cadherin and VEGFR2 (Sun et al, 2012). Alternatively, VEGF may prompt vascular permeability through

the endothelial nitric oxide synthase (eNOS) (Fukumura et al, 2001), the activation of which is, in turn, dependent on PI3K/Akt pathway (Dimmeler et al, 1999; Fulton et al, 1999). However, as demonstrated in mice lacking the Akt1 isoform, its connection with permeability is bridged by the vascular regulator thrombospondins 1 (TSP-1) and 2 (TSP-2) (Chen et al, 2005b).

VEGFR2 internalization

Dynamic and quick VEGFR2 response to growth factors present in the tissue environment requires high rates of receptor turnover, either in terms of rapid clearing of activated receptor from plasma membrane as well as eventual sustained intracellular signaling.

Since in the absence of maximal receptor autophosphorylation the levels of internalization are significantly lowered (Dougher & Terman, 1999), a model has been proposed by which ligand binding and consequent receptor activation triggers clathrin-dependent endocytosis, which depends on the receptor interaction with both VE cadherin and ephrin-B2 receptor (Lampugnani et al, 2006; Salikhova et al, 2008; Sawamiphak et al, 2010). In particular, two ephrin-B2 partners, namely clathrin-associated sorting protein Disabled 2 (Dab2) and cell polarity regulator PAR-3, are pivotal in the regulation of VEGFR2 internalization (Nakayama et al, 2013). Nevertheless, extremely low rate of endocytosis persistence, even in the absence of VEGF-A stimulation, accounts for a plausible constitutive recycling in quiescent endothelial cells (Ewan et al, 2006; Jopling et al, 2011). As a matter of fact, in unstimulated cells, a substantial fraction of VEGFR2 is found localized in

early endosomes and in two distinct vesicular compartments characterized by the presence of Rab5 or Rab4 GTPase, the former compartment responsible for driving the transfer of protein from plasma membrane to early endosomes, the latter for targeting proteins back to the plasma membrane (Ballmer-Hofer et al, 2011; Jopling et al, 2009).

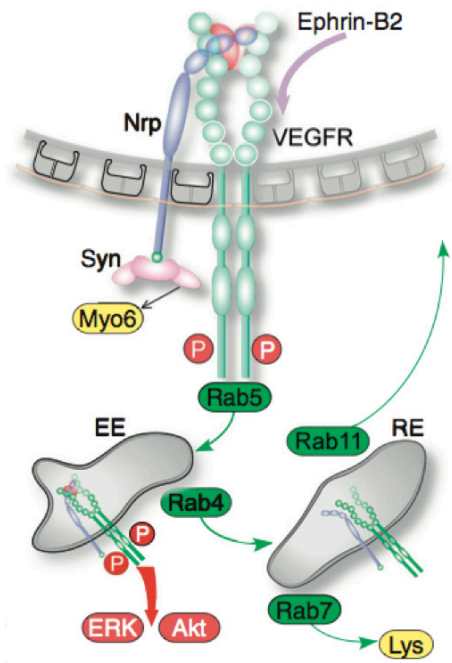

Figure 1.11. VEGFR2 trafficking. After receptor activation upon ligand binding, several factors, among which Ephrin B2, Neuropilin 1, Synectin, are engaged to induce VEGFR2 mobilization from plasma membrane to endocytic vesicles. Afterwards, the receptor might either be recycled back to the plasma membrane or targeted for degradation. See text for details. *Adapted from Horowitz, Cellular Signalling, 2012*

Upon VEGF stimulation, things appear more complex. First, VEGF-A elicits the mobilization of VEGFR2 from the trans-Golgi apparatus to cytoplasmic vesicles; Syntaxin 6, a Golgi-localized soluble N-

ethylmaleimide attachment protein receptor (t-SNARE), is responsible for the depletion of VEGFR2 from this Golgi pool, and its inhibition results in increased receptor lysosomal degration (Manickam et al, 2011). For VEGFR2 recycling back to the plasma membrane an additional passage from Rab4 to Rab11 associated vesicles is required (Ballmer-Hofer et al, 2011). Another unique feature is the dependence of VEGFR2 trafficking on its co-receptor, NP1, which most likely acts in concert with Sinectin (also referred as GAIP-interacting protein, C-terminus, GIPC) to allow the receptor to reach the external membrane. The dissociation between VEGFR2 and NP1 causes progression to Rab7-associated late endosomes and lysosomes, thus routing the receptor for degradation (Ballmer-Hofer et al, 2011; Jopling et al, 2009; Lanahan et al, 2010). Ubiquitination as well routes the receptor for degradation; proteasome inhibition prolongs the duration of VEGFR2 signaling, thus confirming the involvement of the proteasome proteolytic pathway in the negative regulation of VEGFR2 activity (Bruns et al, 2010; Ewan et al, 2006; Pasula et al, 2012).

Interestingly, even after internalization, the receptor retains the ability to transduce the signals, either from early or from recycling Rab4/Rab5-associated endosomes (Ballmer-Hofer et al, 2011; Bruns et al, 2010; Jopling et al, 2009; Lampugnani et al, 2006; Lanahan et al, 2010; Nakayama et al, 2013; Sawamiphak et al, 2010); in particular, the ERK and Akt pathways are upregulated upon VEGFR2 endocytosis, while, on the contrary, p38 activation is responsive to matrix-bound rather than soluble VEGF-A (Chen et al, 2010). On the other hand, PTP1b may counterbalance this sustained signaling by dephosphorylating the receptor near the plasma membrane (Anderie et al, 2007; Lanahan et al,

2010).

Caveolin-1, dynamin-2 and ADP-ribosylation factor 6 (ARF6) are other important interacting partners able to regulate the rate of VEGFR2 endocytosis and thus influence VEGF-induced angiogenesis (Bauer et al, 2005; Bhattacharya et al, 2005; Ikeda et al, 2005; Labrecque et al, 2003). The adaptor/scaffoding protein Cerebral cavernous malformation 3 (Ccm3), the mutation of which causes vascular malformation in the brain, is also linked to VEGFR2 internalization, by probably stabilizing it at the cell surface (He et al, 2010).

Other VEGFRs and coreceptors

VEGFR1

VEGFR1 is a 180 kDa glycoprotein expressed in vascular endothelial cells and in a range of non-endothelial-cells, among which macrophages and monocytes, vascular smooth muscle cells, dendritic cells, hematopoietic stem cells and cardiomyocytes (Barleon et al, 1996; Clauss et al, 1996; Dikov et al, 2005; Dikov et al, 2001; Grosskreutz et al, 1999; Kaipainen et al, 1993; Sawano et al, 2001). It is activated by three different components of the VEGF family, namely VEGF-A (de Vries et al, 1992), VEGF-B (Olofsson et al, 1998) and PlGF (Park et al, 1994).

Gene targeting studies demonstrate its essential role during embryogenesis, since Flt-1$^{-/-}$ mice die in utero between day 8.5 to 9.0 because of an overgrowth of endothelial-like abnormal cells and a failure in vascular channel organization (Fong et al, 1995; Fong et al, 1999). Unexpectedly, the importance of the receptor could not be attributed

to its signal transduction activity; on the contrary, this seems dispensable, since mice expressing the receptor lacking the tyrosine kinase domain (VEGFR1 TK(-) mice), but still able to bind VEGF, develop without any overt effect and with an essentially normal vasculature (Hiratsuka et al, 1998). As a matter of fact, VEGFR1 displays a strong affinity for VEGF-A, but, in contrast, an extremely weak kinase activity and tyrosine phosphorylation (de Vries et al, 1992; Waltenberger et al, 1994), explained in part by a natural occurring inactivating amino acidic replacement in the activation loop (Meyer et al, 2006) and in part by the presence of a repressor sequence in the juxtamembrane domain (Gille et al, 2000). Given these compelling evidences, VEGFR1 has been proposed as a "decoy" receptor, able to regulate vascular formation most likely by trapping VEGF-A and thereby preventing activation of VEGFR2 (Shibuya, 2001). Not only the full-length membrane-bound form of VEGFR1 but also a soluble, secreted form of the receptor, called sVEGFR1/sFlt, may be able to inhibit VEGF activity either by sequestering the growth factor or heterodimerizing with VEGFR2 (Kendall & Thomas, 1993; Kendall et al, 1996). Arising from alternative splicing, sVEGFR1 lacks the intracellular part of the protein and the seventh Ig-like extracellular domain but possesses a unique 31-aa hydrophilic C-terminal tail (Kendall & Thomas, 1993; Shibuya et al, 1990). Physiologically, this endogenous VEGF-A trap in adult life is essential for corneal avascularity (Ambati et al, 2006; Ambati et al, 2007), while, pathologically, its abnormal upregulation has been found in the hypertrophic myocardium at the onset of failure (Kaza et al, 2011) as well as in serum and placenta of patient with preeclampsia (Maynard et al, 2003). Surprisingly, about half of the mice expressing only sVEGFR1 die *in*

utero because of poor vessels development, thereby underlying the importance of the membrane-anchored form in VEGF-A signaling regulation (Hiratsuka et al, 2005).

Irrespective of this negative regulatory role assigned, the phosphorylation of several VEGFR1 tyrosine residues was found to drive the induction of different interacting partners (among which p38/PI3K, PLCγ, SHP2, GRB2 and Nkc) (Koch et al, 2011; Olsson et al, 2006). Additionally, different VEGFR1 ligands, such as VEGF-A and PlGF, may induce different phosphorylation patterns (Autiero et al, 2003).

Thus, VEGFR1 has been shown to transmit VEGF signals and stimulate the migration of both endothelial cells (Cai et al, 2003; Kanno et al, 2000; Wang et al, 2011) and non endothelial cells, such as monocytes/macrophages (Barleon et al, 1996; Clauss et al, 1996; Lyden et al, 2001; Zacchigna et al, 2008b) and tumor cell lines (Lesslie et al, 2006; Taylor et al, 2010). In addition, during pathological conditions the PlGF-induced activation of VEGFR1 might favor angiogenesis, either by inducing distinct intracellular signals or by displacing VEGF-A and making it available for VEGFR2 (Autiero et al, 2003). In cardiomyocytes, VEGFR1 is specifically upregulated in cells exposed to hypoxic conditions and oxidative stress. More importantly, activation of the receptor upon VEGF-B_{167} stimulation eventually improves cardiac function after myocardial infarction (Zentilin et al, 2010). Moreover, the increased expression and activation of VEGFR1 upon copper addition to primary cultures of neonatal cardiomyocytes was found to mediate the copper-induced regression of cardiomyocyte hypertrophy (Zhou et al, 2009). Finally, VEGFR1 was also involved in the initiation of the pre-metastatic niche by inducing the migration of haematopoietic bone marrow progenitors

(Kaplan et al, 2005), although this finding was later debated (Dawson et al, 2009a; Dawson et al, 2009b).

VEGFR3

VEGFR3 is the cognate receptor of VEGF-C and VEGF-D, that generates from the proteolytic cleavage of a 195 kDa precursor, resulting in an N-terminal peptide bound to the rest of the protein through disulphide bonds. Two conserved tyrosine residues in the kinase domain of VEGFR3 are responsible for its kinase activity, and five additional tyrosine phosphorylation sites through which it interacts with downstream effectors have been identified in the C-terminal tail (Dixelius et al, 2003; Salameh et al, 2005). VEGFR3 exists in two isoforms generated by alternative splicing, the shorter of which results in a protein lacking the C-terminal 65 amino acids (Hughes, 2001; Pajusola et al, 1993), and consequently the two autophosphorylation sites therein located (Dixelius et al, 2003). Signal transduction downstream to VEGFR3 involves SH2 domain protein C2 (Shc2) and Grb2, ERK 1/2, PI3K-Akt, and the adaptor protein C10 regulator of kinase (CRK) I/II, and contribute to proliferation, migration, survival of lymphendothelial cells (Koch et al, 2011).

In the adult its activity has been mainly linked to the maintenance of lymphatic endothelium (Alitalo et al, 2005), where it is predominantly expressed since the later stage of development (Kaipainen et al, 1995). Notably, mutations in VEGFR3 have been identified in patients with primary lymphoedema (Ghalamkarpour et al, 2006; Irrthum et al, 2000; Karkkainen et al, 2000). Nevertheless, during embryogenesis VEGFR3 has

an important function in blood vessel development, since VEGFR3 gene targeted mice exhibit defects in the organization of the vasculature and die at E9.5 because of cardiovascular failure (Dumont et al, 1998). As it happens for VEGFR1, mice expressing a kinase deficient VEGFR3, with the ligand-binding region still functional, present normal blood vessel development (Zhang et al, 2010). Thus, VEGFR3 might regulate angiogenesis either by trapping VEGF-C or by modulating VEGFR2 activity forming heterodimers. Consistent with this notion, antibodies neutralizing VEGFR3, by blocking both VEGFR3 homodimer and VEGFR3/VEGFR2 heterodimer formation, suppress vascular and lymphatic EC migration and vessel sprouting (Tvorogov et al, 2010). Recent data have extended the role of VEGFR3 in angiogenesis also during the adult life, according to which the blockage of VEGFR3 signaling results in decreased sprouting, vascular density, vessel branching and EC proliferation in the mouse (Tammela et al, 2008).

VEGFR3 is also found to be upregulated in the tumor vasculature, thus opening the possibility to exploit VEGFR3 targeting agents to inhibit tumor growth (Kubo et al, 2000; Laakkonen et al, 2007; Smith et al, 2010).

Finally, VEGFR3 has also been found in neuronal progenitor cells (Le Bras et al, 2006), osteoblasts (Orlandini et al, 2006) and macrophages (Schmeisser et al, 2006).

Neuropilins

VEGFs display different ability in interacting with co-receptors, such as Neuropilins and heparan sulphate proteoglicans (HSPGs) (Ruhrberg et al, 2002), which are broadly defined as cell-surface expressed molecules

that are devoid of any known intrinsic catalytic activity.

Neuropilins (NP1 and NP2) are transmembrane glycoproteins of about 130 kDa, initially identified as receptors for class 3 semaphorins, soluble axon guidance molecules implicated in the regulation of neural and vascular development (Chen et al, 1997; Fujisawa, 2004; He & Tessier-Lavigne, 1997; Kolodkin et al, 1997; Larrivee et al, 2009). They were later shown to also interact with VEGF family members, such as VEGF-A_{165}, to which they both bind with different affinity (Geretti et al, 2007; Soker et al, 1998) and $VEGF_{121}$ (Pan et al, 2007; Shraga-Heled et al, 2007). In addition, NP1 binds VEGF-B and PlGF, whereas VEGF-C and VEGF-A_{145} are ligands for NP2 (Neufeld et al, 2002; Xu et al, 2010). Even if NP1 and NP2 share only 44% homology, they have similar structural features with a short intracellular domain of 40 amino acids lacking enzymatic activity (Fujisawa et al, 1997). Nonetheless, despite structural homologies, NP1 and NP2 differ for signal properties, 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). Notably, the double NP1/NP2 knockout mouse has a more severe abnormal vascular phenotype than either NP1 or NP2 single knockouts, resembling the phenotypes of VEGF and VEGFR2 knockouts (Takashima et al, 2002). Importantly, Neuropilins are able to directly interact with VEGFRs and thus potentiate the ligand–induced activation by enhancing the association between growth factors and the main receptor. NP2 has been shown to be predominantly present in veins and lymphatics during development (Herzog et al, 2001; Yuan et al, 2002) and, in agreement, it interacts with VEGFR3 in response to VEGF-C stimulation (Xu et al, 2010).

On the contrary, NP1 is mainly expressed in arteries (Gu et al, 2003; Herzog et al, 2001), and forms complexes with VEGFR2 and VEGF-A (Prahst et al, 2008; Soker et al, 2002; Whitaker et al, 2001), thus enhancing VEGFR2-mediated signaling (Shintani et al, 2006), as it was demonstrated for sprouting angiogenesis (Kawamura et al, 2008), EC migration (Herzog et al, 2011) and permeability (Becker et al, 2005).

Moreover, it cannot be excluded that these molecules could transduce the signal independently of VEGFRs; as a matter of fact, compelling evidences suggest that NP1 may stimulate cell migration and adhesion also in the absence of VEGFR2 involvement (Murga et al, 2005; Wang et al, 2006; Wang et al, 2003).

Chapter 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Cell culture and treatment

PAE and PAE/KDR cells, kindly provided by L. Claesson-Welsh (Uppsala University, Sweden), were grown in HAM's F12 medium (Gibco, Life Technologies) supplemented with 10% FBS. Pooled human umbilical vein endothelial (HUVE) cells were cultured in EBM supplemented medium (both from Clonetics, Lonza) until the third passage. Cells were plated and grown in gelatin 0.1% coated dishes for at least 24 hours. HEK293T cells were maintained in Dulbecco's modified Eagle Medium with 10% FBS.

Recombinant human VEGF-A_{165} (rhVEGF) was purchased from R&D and was used after serum starvation at a final concentration of either 50 ng/ml in PAE and PAE/KDR cells or 20 ng/ml in HUVE cells for the indicated times. Cells were treated with trychostatin A (TSA; SIGMA) for 6 hrs, 500 nM for PAE/KDR cells, 1 μM for HEK293T cells and 10 μM for HUVE cells.

MS275 and MC1575 were provided by A. Mai and used at a final concentration of 10 μM. Nicotinamide (NAM) was purchased from (SIGMA) and used at a final concentration of 20mM.

The cellular treatment with Lys-CoA (synthesized at the ICGEB Peptide Synthesis Core Facility) was performed as described (Bandyopadhyay et al, 2002) with minor modifications. Briefly, $16x10^6$ exponentially growing HEK293T cells, transfected or not with pCDNA3-VEGFR2-Flag, were incubated with the ICB solution (10 mM HEPES, pH 7.0, 0.14 M KCl,

0.01 M NaCl, and 2.4 mM $MgCl_2$) 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 then lysed as described.

Transfection and plasmids

Transfections were performed either by the standard calcium phosphate co-precipitation procedure in HEK293T cells or using Lipofectamine (Invitrogen, CA) in PAE and PAE/KDR cells, according to the manufacturer's protocol.

pCDNA3-mouse VEGFR2 was kindly provided by F. Bussolino (University of Torino and IRCC, Torino, Italy). Starting from this plasmid, the construct pCDNA3-VEGFR2-Flag was obtained by inserting a Flag tag fused to the C-terminal part of VEGFR2. Site directed mutagenesis was carried out to construct the VEGFR2 mutants using pCDNA3-mouse VEGFR2 as a PCR template, with primers specific for the mutated version: K1053R and K929R-K937R-K939R-K947R. Starting from the flagged constructs of both wt and mutants VEGFR2, the catalytic domain (aa 806-1356) was amplified by PCR and cloned into pET-15b vector, carrying an N-terminal His Tag sequence (His-VEGFR2 constructs). pCDNA3-p300 was previously described (Marzio et al, 1998). The pCMV-p300-DY-myc mutant was kindly provided by T.P. Yao (Durham). pCDNA3-HA-GCN5 was generated by A. Sabo as previously reported (Sabo et al, 2008). Human Flag-tagged HDAC5 and HDAC6 were purchased from Addgene.

Antibodies

Anti-VEGFR2 antibody, either free or conjugated with agarose beads,

anti-HA, anti HDAC5 and HDAC6, and anti p300 antibodies were purchased from Santa Cruz. Anti-Flag, either free or conjugated with agarose beads, and anti-tubulin, were both from SIGMA. Anti-total acetyl-lysine, anti phospho-VEGFR2 1173, anti-phospho-ERK 1/2 and anti-ERK antibodies are from Cell Signaling Technology (MA); anti phospho-VEGFR2 1054, clone D1W, was purchased from Millipore; anti-total phospho-tyrosine, clone 4G10 was from Upstate. Horseradish peroxidase-conjugated secondary antibodies for western blotting detection were purchased from DAKO.

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 biotinylation lysis buffer (Tris HCl 10 mM, pH 7.5, NaCl 50 mM, 0.5% NP-40, 10% Glycerol, 5mM EDTA), supplemented with protease inhibitors tablets (Roche), Sodium Fluoride 1mM, Sodium Butirrate 10mM and Sodium Orthovanadate 1mM, TSA 10 µM and NAM 10mM (all from SIGMA). Protein lysate concentration was determined by Bradford Assay (BIORAD). Immunoprecipitation was carried out either with anti-Flag or anti VEGFR2 beads. Cells extracts where incubated for 3 hours, in co-immunoprecipitation assay, or overnight with conjugated beads, and then washed in lysis buffer. Immunoprecipitates were loaded onto 6% SDS page and electroblotted to PVDF transfer membrane (GE Healthcare); proteins were subsequently detected after incubation with specific antibodies. After detection of either phosphorylation or acetylation of proteins, membranes were incubated in stripping buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) at 60°C for

30 min and the amounts of the total protein were detected. Western blot are representative of at least three independent experiments. Band intensity was calculated using the ImageJ software.

In vitro acetylation assay

HAT assays were performed as previously reported, with minor modifications (Cereseto et al, 2005). Briefly, 500 ng His-VEGFR2 fusion proteins were incubated with the GST-HAT domain of p300 and ^{14}C-acetyl-CoA (Perkin Elmer,60mCi/mmol) in HAT buffer (50 mM Tris, pH 7.5, 1% glycerol, 50mM sodium butyrate) for 1 hour at 30°C. Acetylated proteins were then visualized by phospho-imaging (Cyclone) after separation by SDS–PAGE. To check protein loading, the 8% acrilamide gels were stain with Roti-Blue (Carl Roth GmbH) and dried 2hrs at 80°C. The extent of in vitro acetylated recombinant VEGFR2 was calculated as Density Light Unit (DLU) according to the Optiquant software analysis.

In vitro kinase assay

In vitro VEGFR2 kinase activity was assayed using an enzyme-linked immunosorbent assay kit. 500 ng of His-VEGFR2 recombinant protein were incubated with 20 µM ATP, 1.25 mM DTT and 6 µM Poly (Glu-Tyr) Biotinylated Peptide (Cell Signaling) in Tyrosine Kinase Buffer (60 mM HEPES pH 7.5, 5 mM Mg Cl_2, 5 mM Mn Cl_2, 3 µM Na_3VO_4) at room temperature for 30 minutes; the reaction was stopped with 50 mM EDTA pH 8 and the samples were subsequently transferred to a 96-well streptavidin-coated plate (Thermo Scientific) and incubated at room temperature for 1 hour to allow the binding of the biotinylated peptide

to streptavidine. Substrate phosphorylation was then monitored using a phopshotyrosine-specific monoclonal antibody, followed by recognition with an appropriate horseradish peroxidase-conjugated secondary antibody and colorimetric ELISA detection assay.

Immunofluorescent staining

HUVE cells were starved for 6 hours the day after plating, and then treated with rhVEGF for fixed times. After fixation with 4% buffered paraformaldehyde (PFA) and permeabilization with PBS-T-T buffer (Tween 0.2% and Triton 0.1%), cells were incubated with the primary antibody O/N at 4°C, followed by appropriate secondary antibodies. Nuclei were detected with Toto-3 stain, final concentration 100 nM (Life technologies). Coverslips were mounted using Vectashield mounting medium (Vector laboratories) and the samples were examined using an LSM 510 META confocal microscope (Carl Zeiss Microimaging) with an X63 NA 1.4 Plan-Apochromat oil objective. The pinhole of the microscope was adjusted to obtain an optical slice of <1.0 µm for any wavelength acquired.

Proteomic analysis

Immunoprecipitated proteins were separated by SDS-PAGE and the VEGFR2 bands, after Zinc staining (Pierce), were processed for analysis by mass spectrometry. In order to maximize the detection of acetylated peptides, the protein bands were treated with deuterated acetic anhydride (Sigma Aldrich), as this treatment normalizes the suppression of ionization caused by acetylation (Smith, 2005). This treatment results

in the addition of deuterated acetyl groups on the lysine residues that were not acetylated in vivo and results in a mass shift of +42 for the in vivo acetylated lysine residues and +45 for the in vitro acetylated residues. Following treatment with deuterated acetic anhydride, the samples were digested with trypsin, extracted from the gel, the N-termini were labeled with ICPL light reagents (Bruker Daltonics) and analyzed by LC-MALDI mass spectrometry essentially as previously described (Bish et al, 2008). The resulting spectra were analyzed using the X!tandem search engine using the ENSEMBL mouse protein database (release GRCm37).

Cell surface biotinylation assay

The assay was performed as previously described (Meyer et al, 2004), with minor modifications. Briefly, PAE cells were transfected with the indicated plasmids and serum starved over night. After 5 min of stimulation with rhVEGF, cells were washed three times with ice-cold PBS containing Ca^{2+} and Mg^{2+}(Ca2+/Mg2+ PBS) and then surface proteins were selectively labeled by treatment with 0.5 mg/ml of thiol-cleavable Sulfo-NHS-S-S-Biotin (Pierce, Thermo Scientific) for 30 min on ice. Unreacted biotin was quenched and removed by three washes with ice-cold H/S buffer (25 mM HEPES, pH 7.4 and 150 mM NaCl). Cells were then washed on ice twice with Ca2+/Mg2+ PBS and lysed with ice-cold lysis buffer. Equal amounts of proteins from each sample were incubated with Streptavidin agarose resins (Pierce, Thermo Scientific) on a rotating wheel overnight at 4°C. Beads were then washed five times with lysis buffer and boiled at 95°C in SDS sample buffer before proceeding with western blot analysis.

Computational modeling

Molecular Dynamics simulations were performed using the generalized Born model approach for implicit solvation as implemented in the AMBER package (Tsui & Case, 2000). A time step of 2 femtoseconds and a cut off of 1.8 nm was adopted. All the systems were stabilized by 0.2 nanoseconds, during which the temperature was linearly raised from 0 to 300 K coupling the systems to a Langevin thermostat with a collision frequency of 2/picosecond (Loncharich et al, 1992). Subsequently, 25 nanoseconds of production runs were performed for all the simulated systems. The solvent accessible surface (SAS), reported in Figure 5G was calculated on residues Asp1050 to Ala1063.

Wound healing assay

PAE cell transiently expressing either wt VEGFR2 or the M5 mutant were grown to 100% confluence on 12-well plates. After 24 h of serum starvation, the confluent monolayer was mechanically scratched with a sterile p200 pipette tip to create a cell-free zone. Cells were then washed to remove the debris and kept in the absence of serum or stimulated with rhVEGF; to block cell proliferation, thymidine 0.25 μM was added to the medium during the 24 hours of stimulation. Phase contrast images were taken immediately after the scratch (time 0) and 24 h after, and the scratched areas were imaged at both time points using a DM IL LED microscope (Leica). The area of the wound (area between the edges of the scratched cell monolayer) was determined at both time points using Volocity 5.5 software (Perkin Elmer). Endothelial cell migration was calculated by subtracting the wound area at time 24 h

with that at time 0.

Statistical analysis

Pair-wise comparison between groups was performed using the two-tailed Student's t test. *P* values of 0.05 or less were considered as statistically significant. Data analysis was performed using the PRISM 5.0d software.

Chapter 3

RESULTS

RESULTS

VEGFR2 is acetylated in endothelial cells

Given the essential role that VEGFR2 exerts in vascular development, it is not surprising that many mechanisms exist to finely tune its activity. Among these, post-translational modifications of the mature protein have a preeminent role. Besides phosphorylation (Dougher & Terman, 1999; Matsumoto et al, 2005; Takahashi et al, 2001), receptor glycosylation is required for proper maturation and membrane presentation (Takahashi & Shibuya, 1997), while Cbl-mediated ubiquitination routes the receptor for degradation (Duval et al, 2003; Murdaca et al, 2004). As extensively discussed in the previous section, among the most prominent PTMs, lysine acetylation has emerged in the last decade as a crucial modification that regulates several cytoplasmic processes by targeting non-histone proteins and giving rise to complex regulatory programs in synergy with other PTMs (Choudhary et al, 2009; Spange et al, 2009; Xiong & Guan, 2012; Yang & Seto, 2008a; Zhao et al, 2010).

Therefore, we wondered whether VEGFR2 might also be modified by acetylation in EC. To address this question, we first immunoprecipitated the endogenous receptor from whole cell lysates (WCL) of primary human umbilical vein endothelial cells (HUVE cells), and then probed the immunoprecipitated proteins with an antibody specific for acetyl-modified lysines. The endogenous VEGFR2 is ordinarily detected as a doublet of bands in the cell lysates, of which the lower one corresponds to the immature, partially glycosylated form of the protein, and the

upper one to the mature, highly glycosylated receptor. Both bands were detected as acetylated (Figure 3.1, panel A).

Blockage of the deacetylase activity by cell treatment with the class I/II histone deacetylase inhibitor trichostatin A (TSA) (Yoshida et al, 1995) caused hyperacetylation of VEGFR2 (Figure 3.1 panel A, compare lane 1 and lane 2), without affecting protein level. The same result was confirmed in another cellular model, Porcine Aorthic Endothelial cells stably expressing human VEGFR2 (PAE/KDR) (Waltenberger et al, 1994) (Figure 3.1, panel B).

These indications clearly indicate that endogenous VEGFR2 is acetylated in EC.

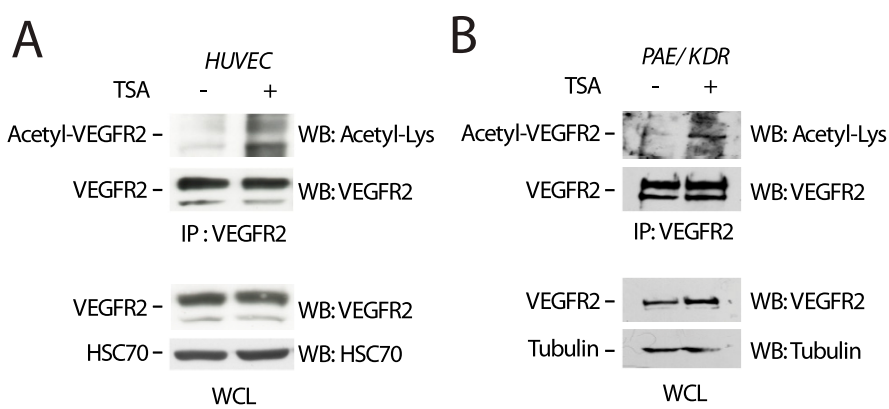

Figure 3.1. VEGFR2 acetylation in endothelial cells. WCL was immunoprecipitated using an anti-VEGFR2 antibody, and then immunoblotted with an anti-acetyl lysines antibody. Upon stripping, the same membrane was immunoblotted with an anti-VEGFR2 antibody. HUVE cells **(A)** or PAE/KDR cells **(B)** were treated either with TSA or DMSO as a control, as indicated on top of each lane.

p300 acetylates VEGFR2 in endothelial cells

In order to identify the lysine acetyltransferase (HAT) responsible for VEGFR2 acetylation, we tested the members of two main classes of KATs, namely p300, for p300/CBP family, and GCN5 and P/CAF, for the GNAT family. The *in vivo* acetylation assay were performed by overexpressing these enzymes in PAE KDR cells followed by receptor immunoprecipitation from WCL and blotting with anti-acetyl lysine antibody. Among all the acetyltransferases tested, p300 was the only enzyme effective at catalyzing VEGFR2 acetylation (Figure 3.2, panel A). The activity of p300 was neither cell type nor species specific, as these findings were reproduced in HEK293T cells cotrasfected with mouse VEGFR2 in combination with p300, GCN5-HA and P/CAF-Flag; moreover, in this condition, only p300 showed the ability to co-immunoprecipitate with VEGFR2 (Figure 3.2, panel B).

So far, p300 activity has been extensively characterized in the nucleus, where it regulates acetylation of both histones and transcriptional activators (Bedford et al, 2010; Goodman & Smolik, 2000), whereas VEGFR2 is a transmembrane receptor. Despite their apparently different compartment distribution, these proteins physically interact at the endogenous level inside EC, as demonstrated by the results obtained by immunoprecipitating the endogenous receptor and then probing the immunoprecipitates with an antibody specific for p300 (Figure 3.3). To check the specificity of this interaction, a WCL was incubated, in parallel, with beads conjugated with rabbit IgGs (lane corresponding to IgG only). TSA treatment did not exert any effect on the interaction between the two proteins.

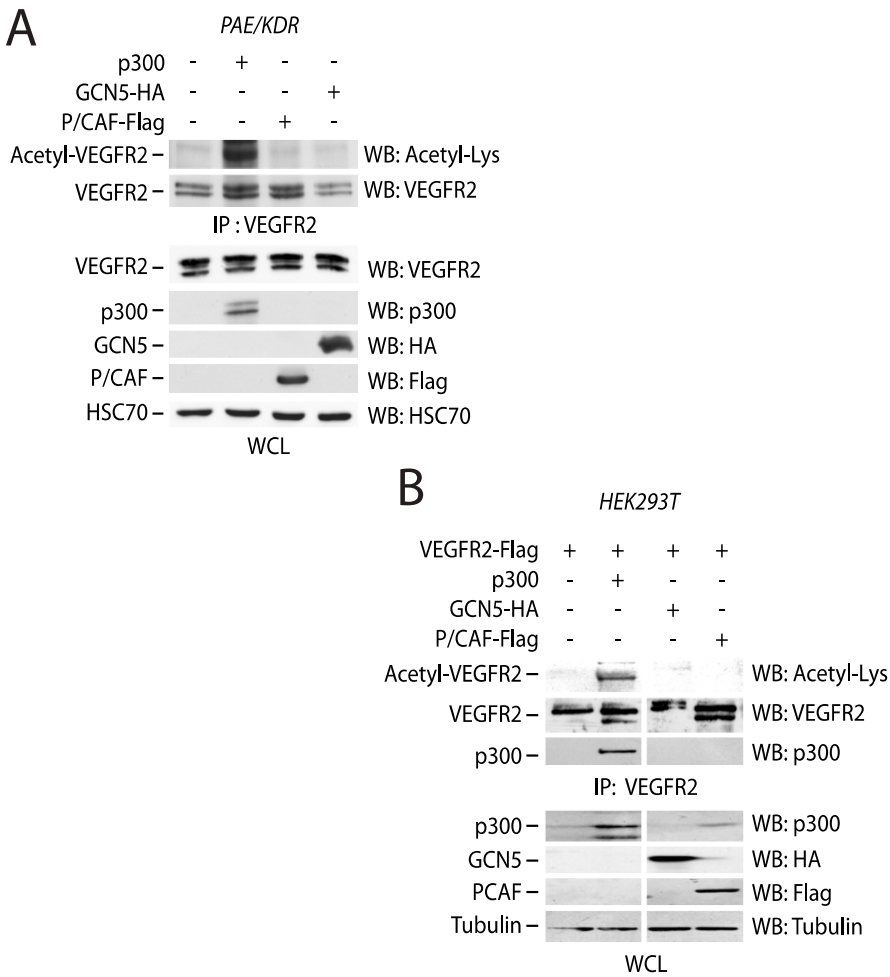

Figure 3.2. p300 induces VEGFR2 acetylation. (A) PAE/KDR cells were transiently transfected with p300, GCN5 or P/CAF and the acetylation levels of VEGFR2 were assessed by receptor immunoprecipitation and blotting with an anti-acetyl lysine antibody, followed by stripping and probing with an anti-VEGFR2 antibody. The WCL were probed with anti-p300, anti-HA, anti-Flag and anti-tubulin antibodies. **(B)** The same experiment was performed in HEK293T cells transiently transfected with mouse VEGFR2, p300, GCN5 or P/CAF. Additionally, the HEK293T immunoprecipitated proteins were probed with an anti-p300 antibody.

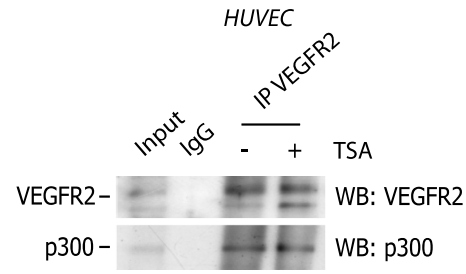

Figure 3.3. p300 co-immunoprecipitate with VEGFR2 in EC. HUVE cells were treated either with TSA or DMSO, followed by WCL immunoprecipitation using an anti-VEGFR2 antibody. The immunoprecipitated proteins were then immunoblotted either with anti-p300 or anti-VEGFR2 antibodies. Cell lysates (30 μg) were also immunoblotted as a control (Input). The WCL was also incubated with beads conjugated with rabbit IgGs (IgG only).

Even if in a handful of reports VEGFR2 was described to shuttle from the cytoplasm to the nucleus (Blazquez et al, 2006; Domingues et al, 2011; Feng et al, 1999; Fox et al, 2004; Santos et al, 2007), we were not able to detect the receptor in the nucleus by neither immunofluorescence nor western blotting of nuclear extracts. As shown in Figure 3.4 we found VEGFR2 exclusively localized in the cytoplasm, both in HUVE cells stained with an antibody that specifically recognizes the endogenous protein (Figure 3.4, panel A), and in HeLa cells overexpressing the YFP-tagged receptor (Figure 3.4, panel B).

The same observation was confirmed by separating nuclear and cytosolic fractions of EC with mild detergents, followed by immunoblotting analysis of protein expression. The purity of fractions was proven by analyzing α-tubulin and PARP as cytoplasmic and nuclear markers,

respectively. As anticipated, VEGFR2 was present only in the cytoplasmic fraction, which also includes cell membranes (Figure 3.4, panel C).

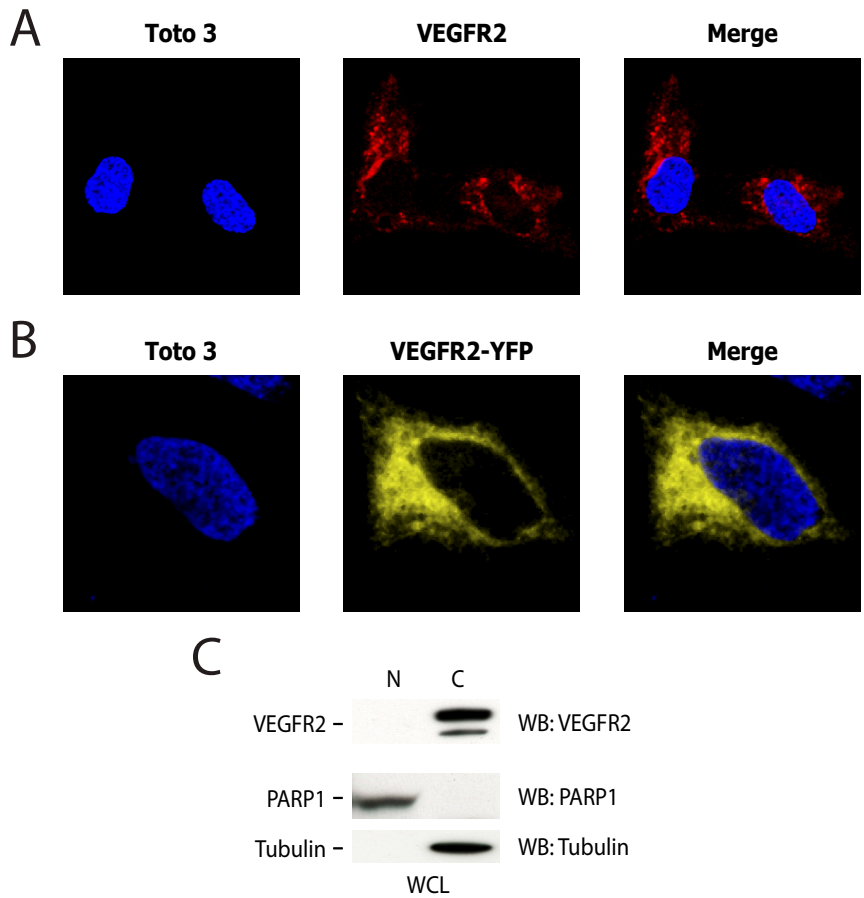

Figure 3.4. VEGFR2 subcellular localization in EC. (A) Confocal images of HUVE cells stained either with Toto 3 or anti VEGFR2 antibody as indicated above. **(B)** HeLa cells were transiently transfected with VEGFR2-YFP and stained with Toto3, followed by confocal image acquisition **(C)** HUVE cells were separated into cytoplasmic (C) and nuclear (N) fractions and analyzed for the distribution of the indicated proteins via immunoblotting. PARP-1 and Tubulin were used as nuclear and cytoplasmic markers, respectively.

Conversely, p300 was present in both the nucleus and the cytoplasm of

EC, as observed in immunofluorescence experiment. By using confocal microscopy, we found that endogenous p300 was preeminently detectable in the nucleus. At low levels, however, it was also detectable in the cytoplasm (Figure 3.5, panel A).

We corroborated this immunofluorescence results by performing cellular fractionation of HUVE cells and subsequent analysis of protein localization in different fractions, as previously discussed. The vast majority of p300 was present in the nuclear fraction, but a small part was also clearly detectable in the cytosolic and membrane fraction (Figure 3.5, panel B).

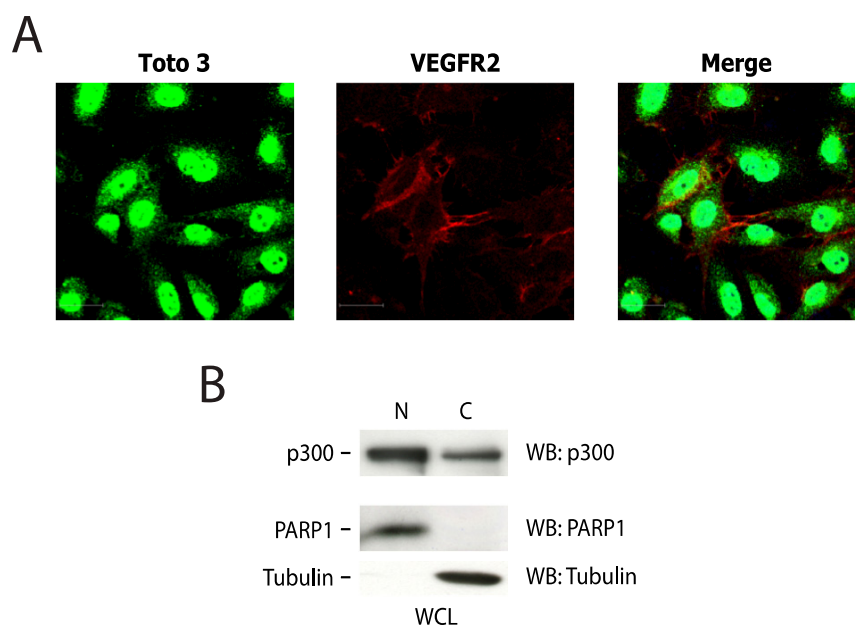

Figure 3.5. p300 subcellular localization in EC. (A) Confocal images of HUVE cells stained either with Toto 3, anti-p300 or anti-CD31 antibody as indicated. **(B)** Cytoplasmic (C) and nuclear extracts (N) from HUVE cells were analyzed by immunoblotting with antibodies against p300. PARP-1 and Tubulin were used as nuclear and cytoplasmic controls, respectively.

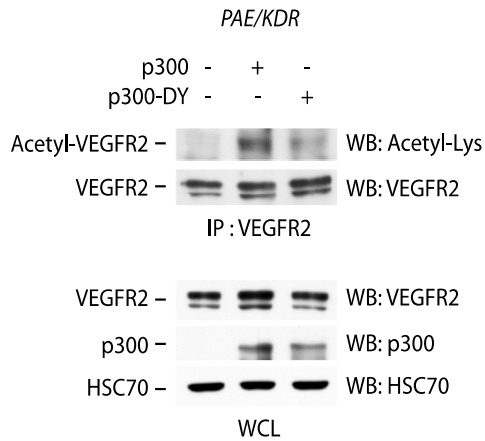

Figure 3.6. VEGFR2 acetylation relies on p300 activity. PAE/KDR cells were transfected with p300, the catalytically inactive mutant p300 DY or an empty vector. Anti-VEGFR2 immunoprecipitates were blotted with an anti-acetyl lysines antibody.

To further confirm that the enzymatic activity of p300 was directly required for VEGFR2 acetylation, we examined the level of receptor acetylation in PAE/KDR cells overexpressing the enzymatically inactive mutant p300 DY, carrying an inactivating, single amino-acid substitution (Ito et al, 2001; Sabo et al, 2008). As expected, this mutant was almost ineffective at inducing VEGFR2 acetylation in respect to the wt p300 protein (Figure 3.6).

Addionally, in HEK293T transfected with VEGFR2-Flag, the acetylation levels of the receptor was found to change in response to treatment with LysCoA, a synthetic and specific inhibitor of the p300 enzymatic activity (Cereseto et al, 2005; Lau et al, 2000). As it can be appreciated comparing lanes 1 and 2 of Figure 3.7, treatment with this inhibitor reduced the signal corresponding to the acetylated receptor in comparison to the vehicle control (Figure 3.7).

Taken together, these findings suggest that the cytosolic fraction of p300 physically interacts with VEGFR2 and is the main enzyme responsible for receptor acetylation.

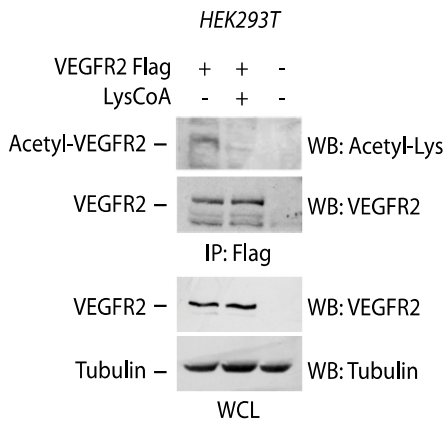

Figure 3.7. LysCoA treatment diminishes VEGFR2 acetylation. HEK293T cells overexpressing VEGFR2 were treated with LysCoA or vector alone. The acetylation levels were assayed by probing the immunoprecipitated VEGFR2 with an anti-acetyl lysine antibody.

VEGF increases VEGFR2 acetylation

As INFα treatment partially induces CBP relocalization from the nucleus to the cytoplasm, thus increasing INFR type 1 acetylation (Tang et al, 2007), we assessed the levels of acetylation of endogenous VEGFR2 in HUVE cells in response to VEGF stimulation. After serum starvation and addition of rhVEGF to the medium, VEGFR2 was immunoprecipitated from WCL and then probed with anti-acetyl lysine antibody. In parallel, the levels of receptor phosphorylation were checked using an antibody that specifically recognizes VEGFR2 phosphorylation on tyrosine 1173

(human Tyr1175), an essential residue in VEGF-induced signal transduction (Sakurai et al, 2005). As extensively reported, phosphorylation of the receptor was undetectable in serum-starved cells in the absence of stimulation, while it rapidly peaked at 5 min after addition of rhVEGF, to decrease afterwards (Figure 3.8, panel A). The kinetics of receptor acetylation was slightly delayed compared to that of phosphorylation, starting to be appreciable at 5 min and peaking at 15 min after rhVEGF addition (quantification in panel B of figure 3.8).

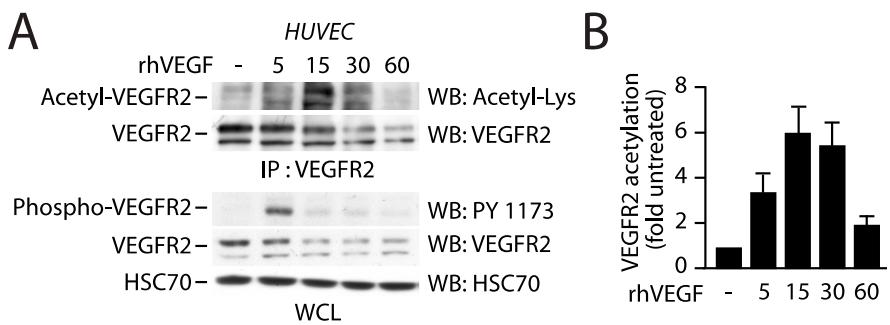

Figure 3.8. Induction of VEGFR2 acetylation in response to VEGF stimulation. (A) Immunoprecipitation of VEGFR2 in HUVE cells treated with or without rhVEGF for the indicated time. Receptor acetylation was detected with an anti-acetyl lysine antibody. WCL were probed with anti-VEGFR2, anti-phospho 1173 VEGFR2 and anti-HSC70 antibodies. **(B)** Relative quantification of VEGFR2 acetylation as fold induction over unstimulated receptor (mean±SEM of three different experiments).

In contrast of what happens to the subcellular distribution of CBP upon INFα or EGF treatment (Song et al, 2011; Tang et al, 2007), we did not observe any changes on p300 localization in endothelial cells consequent to VEGF stimulation. In fact, as shown in figure 3.9, cells treated with the recombinant factor for different time were immunostained with an anti-p300 antibody and confocal microscopy

images were acquired. In starved cells, p300 was predominantly present in the nucleus but also found in the cytoplasm; this distribution pattern remained unchanged even after prolonged treatment with VEGF.

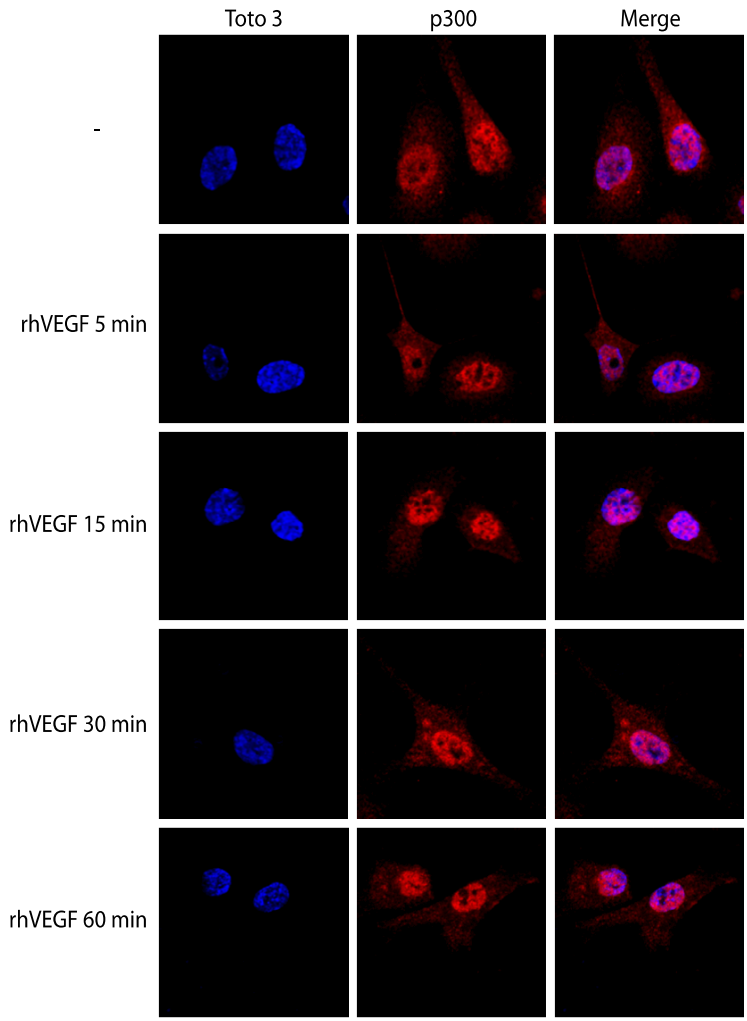

Figure 3.9. p300 subcellular localization upon VEGF treatment. Confocal images of HUVE cells treated with rhVEGF at different time points and stained with either Toto 3 or an anti-p300 antibody, as indicated above.

On the other hand, by immunoprecipitating the receptor from HUVE cell WCL and probing the immunoprecipitates with an anti-p300 antibody, we surprisingly observed that stimulation with recombinant VEGF induced the association of the KAT with the receptor as early as after 5 minutes of treatment, reaching a maximum at 15 minutes (Figure 3.10), thus mirroring the induction of VEGFR2 acetylation in response to VEGF stimulation.

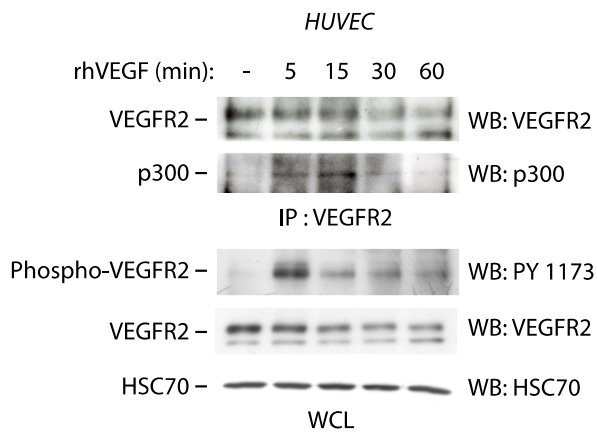

Figure 3.10. p300 and VEGFR2 association upon VEGF stimulation. HUVE cells were starved for 6 hours, and then treated with or without $VEGF_{165}$ for the indicated time. VEGFR2 were immunoprecipitated from WCL, and the immunoprecipitated proteins assayed with anti-p300 and anti-VEGFR2 antibodies. WCL were probed with anti-VEGFR2, anti-phospho 1173 VEGFR2 and anti-HSC70 antibodies.

Thereby, analysis of the kinetics of VEGFR2 acetylation indicates that the receptor undergoes acetylation in a ligand-dependent manner; recombinant VEGF stimulation does not have an effect on p300 subcellular distribution, whereas, in contrast, it positively affects the interaction between VEGFR2 and p300.

HDAC5 and HDAC6 interact with and deacetylate VEGFR2 in EC

Having shown that VEGFR2 is acetylated in EC and considering that acetylation is a reversible modification, we wanted to investigate which deacetylases recognized the receptor as a substrate.

A first experiment was performed with the purpose to understand whether pharmacological inhibition had an effect on VEGFR2 acetylation and, should this be case, which was the class of KDACs involved in the deacetylation process. We found that, in HUVE cells, inhibition of class III KDACs (sirtuins) by nicotinamide (NAM) did not significantly affect the acetylation levels of endogenous VEGFR2 (Figure 3.11, panel A).

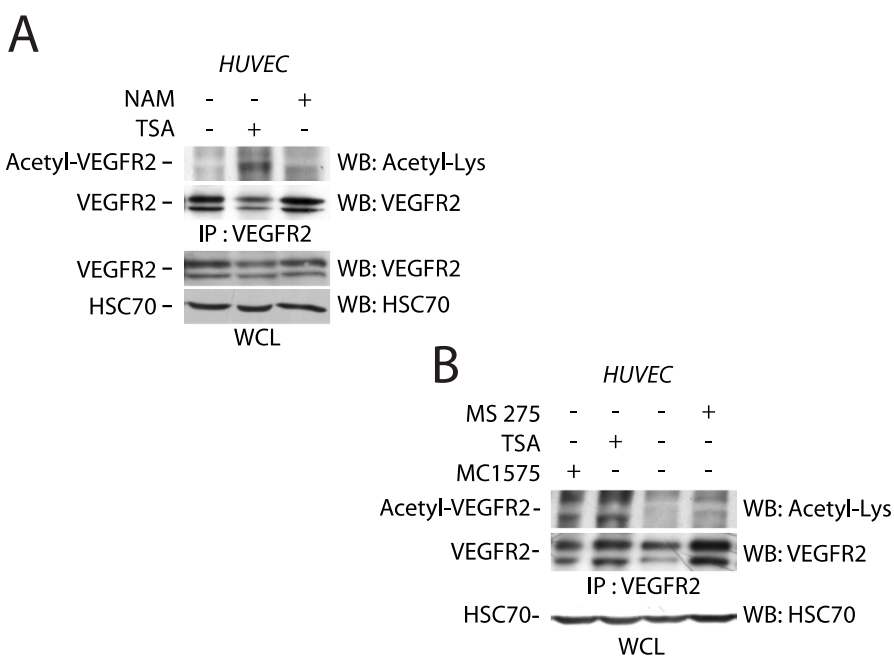

Figure 3.11. Effect of specific deacetylase inhibitors on VEGFR2 acetylation. HUVE cells WCL immunoprecipitation using an anti-VEGFR2 antibody, and then immunoblotting with anti-acetyl lysines and anti-VEGFR2 antibodies. In panel **(A)** cells were treated either with 10 μM TSA, 20 mM NAM or DMSO as a control, whereas in panel **(B)** either with 10 μM TSA, 20 μM MC1575, 10 μM MS 275 or DMSO.

In contrast, TSA, which inhibits both class I and class II KDACs, had a pronounced effect, as also already shown in figure 3.1. Next we tested two specific inhibitors for class I and class II KDACs, namely MS275 (entinostat) (Hu et al, 2003) and MC1575 (Mai et al, 2003; Mai et al, 2005), respectively.

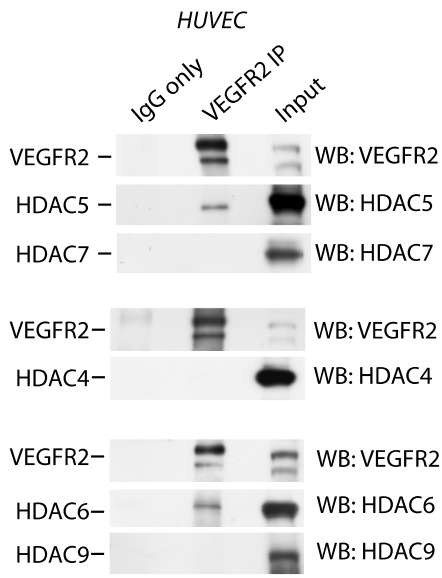

Figure 3.12. HDAC5 and HDAC6 interact with VEGFR2 in endothelial cells. VEGFR2 immunoprecipitates from HUVE cells contained both endogenous HDAC5 and HDAC6, whereas none of HDAC4, HDAC7 and HDAC9 co-immunprecipitated with the receptor. To discriminate among the different enzymes, specific antibodies were used for each member of class II KDACs. As controls, cell lysates (30 µg) were also immunoblotted in the absence of immunoprecipitation (Input), or incubated with beads conjugated-rabbit IgG (IgG only).

We found that the former compound did not show any detectable effect, while the latter (MC1575) significantly increased VEGFR2 acetylation at levels similar to those of TSA (Figure 3.11, panel B).

Thus we focused on the class II enzymes. Since KDACs often physically interacts with their substrates, we tested binding of VEGFR2 with the different class II KDACs by immunoprecipitating the receptor and then testing for the presence of KDACs in the immunoprecipitates. These experiments revealed that, in HUVE cells, HDAC5 and HDAC6 interacted with VEGFR2, while HDAC4, HDAC7 and HDAC9 did not (Figure 3.12). Consistent with these findings, we observed that the overexpression of both HDAC5 and HDAC6 in PAE/DKR cells significantly decreased the levels of endogenous VEGFR2 acetylation, with HDAC5 having a more modest effect compared to HDAC6.

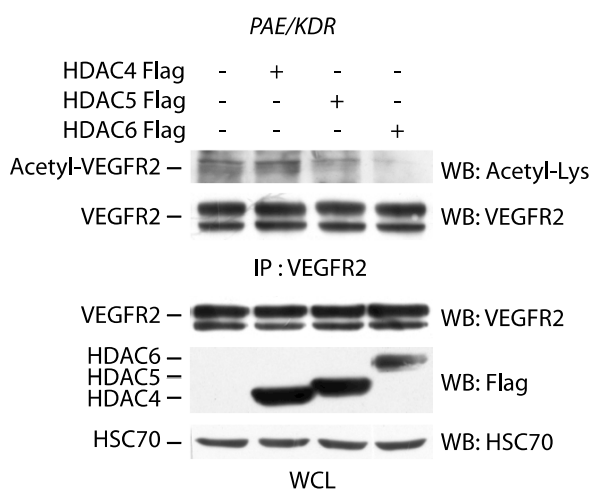

Figure 3.13. Deacetylation of VEGFR2. PAE/KDR cells were transiently transfected with HDAC4, HDAC5, HDAC6 or empty vector, as indicated. The receptor was immunoprecipitated, and then immunoblotted with anti-acetyl lysines and anti-VEGFR2 antibodies. The WCL were probed with anti-VEGFR2, anti-Flag and anti-HSC70 antibodies.

We determined the transfection efficiency by immunoblotting the WCL with an anti-Flag antibody. We noticed that HDAC6 was expressed at

lower levels than HDAC5, thus reinforcing the observation that this enzyme was the most powerful in exerting deacetylase activity on VEGFR2. HDAC4 was completely ineffective, albeit being expressed at the same levels as HDAC5 (Figure 3.13).

To determine the effect of class II KDACs downregulation, we established conditions to knock down HDAC5 and HDAC6, both individually and in combination; an anti-HDAC4 siRNA was used as a control for deacetylase specificity on VEGFR2 acetylation. siRNA-mediated knockdown of KDACs correlated with remarkable reduction of protein level, even below detectable levels via Western blot, as in the case of HDAC5 (Figure 3.14). HUVE cells were transiently transfected with the above indicated, specific siRNAs and allowed to incubate for 60 hrs.

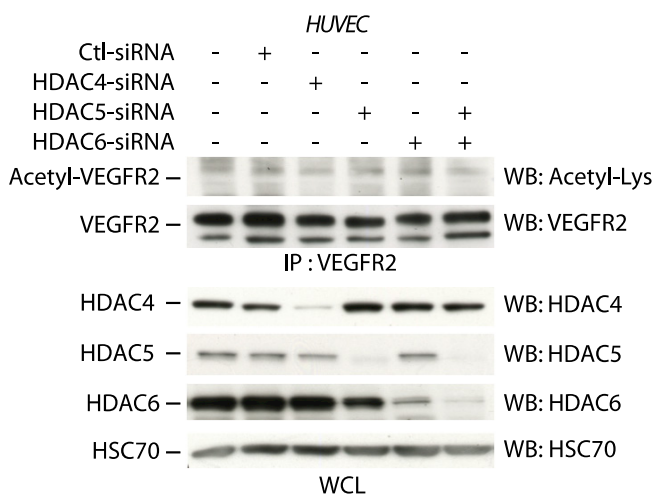

Figure 3.14. Class II KDAC redundancy. HUVE cells were transiently transfected with either HDAC4, HDAC5, HDAC6 or a non-targeting siRNA (Ctl-siRNA), as indicated. After 60 hours, cell lysates were analyzed by SDS-PAGE and immunoblotting for endogenous HDAC4, HDAC5 and HDAC6. VEGFR2 was immunoprecipitated with anti-VEGFR2 conjugated beads and then immunoblotted with anti-acetyl lysines and anti-VEGFR2 antibodies.

The cells were then harvested and immunoprecipitated with an anti-VEGR2 antibody, followed by anti-acetyl lysine blotting analysis. In contrast to our expectations, siRNA-mediated downregulation of both HDAC5 and HDAC6 in HUVE cells did not exert any effect on VEGFR2 acetylation, similar to the non-targeting or anti-HDAC4 siRNAs. Additionally, when HDAC5 and HDAC6 were simultaneously downregulated, there were no detectable differences in comparison with cells treated with a scrambled, control siRNA (Figure 3.14). Even if this finding seems to be apparently in conflict with the previous data obtained in KDACs-overexpressing cells, it simply suggests that these EC KDACs are redundant and thus the downregulation of one enzyme can be compensated by others activity. Similar findings have also been reported in other cell types, such as breast cancer cells, preadipocytes, myofibers and cardiac tissue (Chang et al, 2004; Clocchiatti et al, 2012; Potthoff et al, 2007; Weems & Olson, 2011).

Finally, since we observed a transitory association between p300 and VEGFR2 in response to rhVEGF stimulation, we wondered whether the receptor association with HDAC5 and HDAC6 also followed a similar kinetic. Thus, we looked at the co-immunoprecipitation of the deacetylases with the receptor in VEGF-treated HUVE cells. Both HDAC5 and HDAC6 were found associated with VEGFR2 in the absence of stimulation. Addition of the ligand, however, further promoted their binding, consistent with the recent findings that VEGF induces the phosphorylation and thus the nuclear export of several members of class II KDACs (Ha et al, 2008; Wang et al, 2008). In the case of HDAC5, the kinetics of association peaked at 15 min to rapidly decrease afterwards, while HDAC6 had a maximum at 5 min and then continued to remain

associated with the receptor at later time points (figure 3.15, representative blot in panel A and quantifications in panel B).

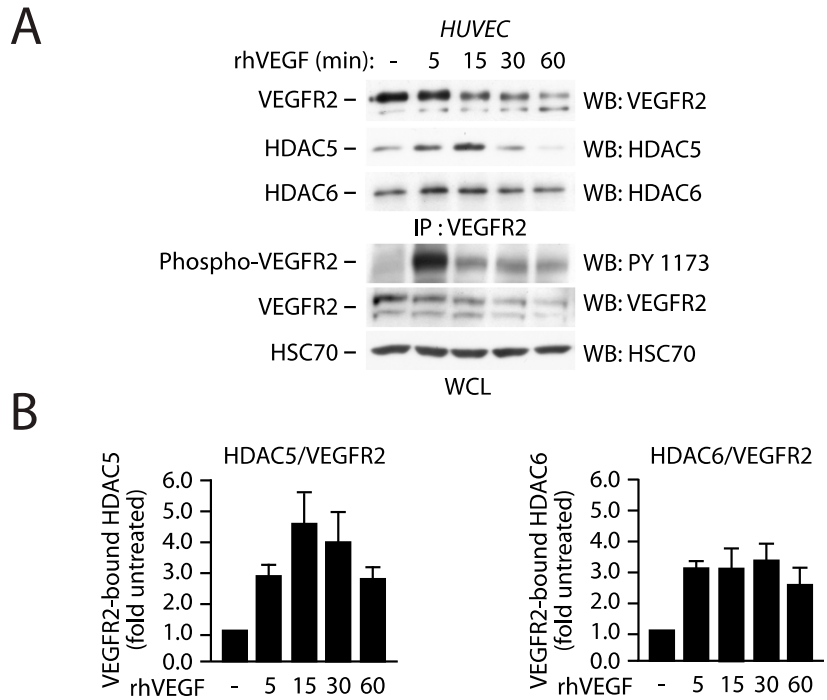

Figure 3.15. rhVEGF stimulates interaction of VEGFR2 with HDAC5 and HDAC6. (A) HUVE cells were starved for 6 hours, and then treated with or without $VEGF_{165}$ for the indicated time. VEGFR2 was immunoprecipitated from WCL, and the immunoprecipitated proteins then assayed with anti-HDAC5, anti-HDAC6 or anti-VEGFR2 antibodies. WCL were probed with anti-VEGFR2, anti-phospho 1173 VEGFR2 and anti-HSC70 antibodies. (**B**) Relative quantification of HDAC5 and HDAC6 co-immunoprecipitated with VEGFR2. Results are shown as fold induction over unstimulated receptor (mean±SEM of three different experiments).

Collectively, these results indicate that the enzymes responsible for VEGFR2 deacetylation are HDAC5 and HDAC6. These two factors associate with the receptor in a ligand-dependent manner, resembling the kinetics of interaction between VEGFR2 and p300.

Five lysines are acetylated in VEGFR2

The mouse VEGFR2 carries a number of lysines that are well conserved in different species; of these, 38 are located in its intracellular portion. No p300 acetylation consensus site has been identified so far, hence all the 79 lysines comprised in the receptor sequence could be possible sites of acetylation.

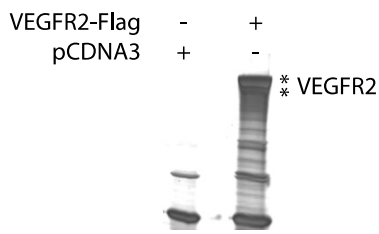

Figure 3.16. VEGFR2-Flag silver staining. Anti-Flag immunoprecipitates from WCL of HEK293T transiently transfected with VEGFR2-Flag or empty vector were loaded on a gel and silver stained. The asterisks indicate the bands corresponding to fully and partially glycosylated VEGFR2 that underwent protemic analysis.

To precisely determine which of these are the substrates for acetylation *in vivo*, we immunoprecipitated VEGFR2 from transfected HEK293T cells and recovered two bands from an SDS-PAGE gel, the higher of approximately 250 kDa and the lower of 220 kDa, corresponding to fully and partially glycosylated VEGFR2, respectively. Both bands were analyzed by MALDI-TOF mass spectrometry. One tenth of the total immunoprecipitate was loaded on a gel and silver stained as a control (Figure 3.16).

start	sequence	end	modifications
58	DLDWLWPNAQR	68	D [58] 105.021
58	DLDWLWPNAQR	68	D [58] 105.021
58	DLDWLWPNAQR	68	D [58] 105.021, N [65] 0.9848
58	DLDWLWPNAQR	68	D [58] 105.021, N [65] 0.9848
58	DLDWLWPNAQR	68	D [58] 105.021, W [61] 15.9949
58	DLDWLWPNAQR	68	D [58] 105.021, W [63] 31.9898
58	DLDWLWPNAQR	68	D [58] 105.021, W [61] 31.9898
58	DLDWLWPNAQR	68	D [58] 105.021, W [61] 15.9949, W [63] 15.9949, N [65] 0.9848
58	DLDWLWPNAQR	68	D [58] 105.021, W [61] 31.9898, W [63] 31.9898
58	DLDWLWPNAQR	68	D [58] 105.021, W [61] 31.9898, W [63] 31.9898
74	VLVTECGGGDSIFCKTLTIPR	94	V [74] 105.021, C [79] 57.0215, C [87] 57.0215, K [88] 45
74	VLVTECGGGDSIFCKTLTIPR	94	V [74] 105.021, C [79] 57.0215, C [87] 57.0215, K [88] 45
172	FVPDGNR	178	F [172] 105.021
540	VISFHVIR	547	V [540] 105.021
540	VISFHVIR	547	V [540] 105.021
651	HCLVKQLIILER	662	H [651] 105.021, C [652] 57.0215, K [655] 45
651	HCLVKQLIILER	662	H [651] 105.021, C [652] 57.0215, K [655] 45
651	HCLVKQLIILER	662	H [651] 105.021, C [652] 57.0215, K [655] 45
651	HCLVKQLIILER	662	H [651] 105.021, C [652] 57.0215, K [655] 45
727	KEDGGLYTCQACNVLGCAR	745	K [727] 45, K [727] 105.021, C [735] 57.0215, C [738] 57.0215, C [743] 57.0215
727	KEDGGLYTCQACNVLGCAR	745	K [727] 45, K [727] 105.021, C [735] 57.0215, C [738] 57.0215, C [743] 57.0215
727	KEDGGLYTCQACNVLGCAR	745	K [727] 45, K [727] 105.021, C [735] 57.0215, C [738] 57.0215, C [743] 57.0215
727	KEDGGLYTCQACNVLGCAR	745	K [727] 45, K [727] 105.021, C [735] 57.0215, C [738] 57.0215, C [743] 57.0215
725	VRKEDGGLYTCQACNVLGC/	745	V [725] 105.021, K [727] 45, C [735] 57.0215, C [738] 57.0215, C [743] 57.0215
790	ANEGELKTGYLSIVMDPDELI	814	A [790] 105.021, K [796] 42, M [804] 15.9949
790	ANEGELKTGYLSIVMDPDELI	814	A [790] 105.021, K [796] 45
790	ANEGELKTGYLSIVMDPDELI	814	A [790] 105.021, K [796] 42, M [804] 15.9949
790	ANEGELKTGYLSIVMDPDELI	814	A [790] 105.021, K [796] 42, M [804] 15.9949
818	LPYDASKWEFPR	829	L [818] 105.021, K [824] 45
818	LPYDASKWEFPR	829	L [818] 105.021, K [824] 45
818	LPYDASKWEFPR	829	L [818] 105.021, K [824] 45, W [825] 31.9898
818	LPYDASKWEFPR	829	L [818] 105.021, K [824] 45, W [825] 31.9898
818	LPYDASKWEFPR	829	L [818] 105.021, K [824] 45, W [825] 31.9898
818	LPYDASKWEFPRDR	829	L [818] 105.021, K [824] 42
818	LPYDASKWEFPRDR	829	L [818] 105.021, K [824] 42
818	LPYDASKWEFPRDR	829	L [818] 105.021, K [824] 45
815	CERLPYDASKWEFPR	829	C [815] 57.0215, C [815] 105.021, K [824] 45
815	CERLPYDASKWEFPR	829	C [815] 57.0215, C [815] 105.021, K [824] 45
818	LPYDASKWEFPRDR	831	L [818] 105.021, K [824] 42, W [825] 15.9949
832	LKLGKPLGR	840	L [832] 105.021, K [833] 42, K [836] 45
832	LKLGKPLGR	840	L [832] 105.021, K [833] 45, K [836] 45
832	LKLGKPLGR	840	L [832] 105.021, K [833] 45, K [836] 45
832	LKLGKPLGR	840	L [832] 105.021, K [833] 45, K [836] 45
830	DRLKLGKPLGR	840	D [830] 105.021, K [833] 45, K [836] 45
931	NEFVPYKSKGAR	942	N [931] 105.021, K [937] 42, K [939] 42
931	NEFVPYKSKGAR	942	N [931] 105.021, K [937] 42, K [939] 42
931	NEFVPYKSKGAR	942	N [931] 105.021, K [937] 45, K [939] 45
931	NEFVPYKSKGAR	942	N [931] 105.021, K [937] 45, K [939] 45
928	GKRNEFVPYKSKGAR	942	G [928] 105.021, K [929] 42, K [937] 45, K [939] 45
928	GKRNEFVPYKSKGAR	942	G [928] 105.021, K [929] 42, K [937] 45, K [939] 45
928	GKRNEFVPYKSKGAR	942	G [928] 105.021, K [929] 45, N [931] 0.9848, K [937] 45, K [939] 45
928	GKRNEFVPYKSKGAR	942	G [928] 105.021, K [929] 45, N [931] 0.9848, K [937] 45, K [939] 45
943	FRQGKDYVGELSVDLKR	951	F [943] 105.021, K [947] 45, K [958] 45
943	FRQGKDYVGELSVDLKR	951	F [943] 105.021, K [947] 45, K [958] 45
943	FRQGKDYVG	959	F [943] 105.021, K [947] 45
945	QGKDYVGELSVDLKR	959	Q [945] 105.021, K [947] 42, K [958] 45
945	QGKDYVGELSVDLKR	959	Q [945] 105.021, K [947] 45, K [958] 45
945	QGKDYVGELSVDLKR	959	Q [945] 105.021, K [947] 45, K [958] 45
943	FRQGKDYVGELSVDLKR	959	F [943] 105.021, K [947] 45, K [958] 45
943	FRQGKDYVGELSVDLKR	959	F [943] 105.021, K [947] 45, K [958] 45
943	FRQGKDYVGELSVDLKR	959	F [943] 105.021, K [947] 45, K [958] 45
1021	KCIHRDLAAR	1030	K [1021] 45, K [1021] 105.021, C [1022] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 42, K [1041] 42, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 42, N [1038] 0.9848, K [1041] 42, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 42 N [1038] 0.9848, K [1041] 42, C [1043] 57.0215
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1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 42, K [1041] 45, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 42, K [1041] 45, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 42, K [1041] 45, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 45, N [1038] 0.9848, K [1041] 42, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 42, N [1038] 0.9848, K [1041] 45, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 42, N [1038] 0.9848, K [1041] 45, C [1043] 57.0215

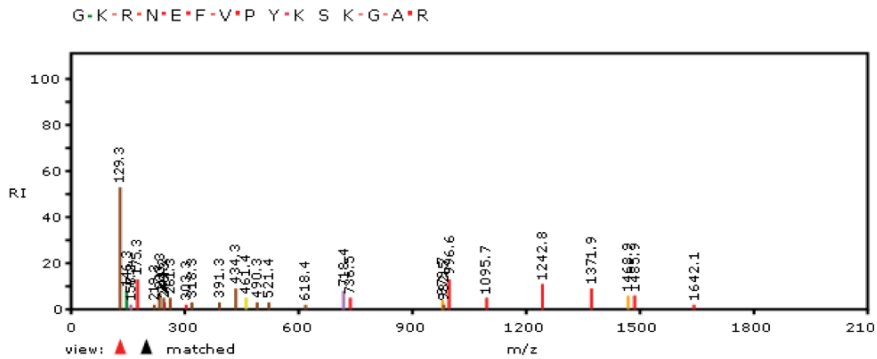
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 45, N [1038] 0.9848, K [1041] 42, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 45, K [1041] 45, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 45, K [1041] 45, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 45, K [1041] 45, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 45, K [1041] 45, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 45, K [1041] 45, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 45, N [1038] 0.9848, K [1041] 45, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 45, N [1038] 0.9848, K [1041] 45, C [1043] 57.0215
1031	NILLSEKNVVKICDFGLAR	1049	N [1031] 105.021, K [1037] 45, N [1038] 0.9848, K [1041] 45, C [1043] 57.0215
1050	DIYKDPDYVR	1059	D [1050] 105.021, K [1053] 42
1050	DIYKDPDYVR	1059	D [1050] 105.021, K [1053] 45
1050	DIYKDPDYVR	1059	D [1050] 105.021, K [1053] 45
1065	LPLKWMAPETIFDR	1078	L [1065] 105.021, K [1068] 42
1065	LPLKWMAPETIFDR	1078	L [1065] 105.021, K [1068] 45
1065	LPLKWMAPETIFDR	1078	L [1065] 105.021, K [1068] 45
1065	LPLKWMAPETIFDR	1078	L [1065] 105.021, K [1068] 45
1065	LPLKWMAPETIFDR	1078	L [1065] 105.021, K [1068] 45, M [1070] 15.9949
1117	LKEGTR	1122	L [1117] 105.021, K [1118] 45
1116	RLKEGTR	1122	R [1116] 105.021, K [1118] 45
1274	NKLSPSFGGMMPSKSR	1289	N [1274] 105.021, N [1274] 0.9848, K [1275] 42, K [1287] 45
1274	NKLSPSFGGMMPSKSR	1289	N [1274] 105.021, N [1274] 0.9848, K [1275] 42, K [1287] 45
1274	NKLSPSFGGMMPSKSR	1289	N [1274] 105.021, K [1275] 45, K [1287] 45
1274	NKLSPSFGGMMPSKSR	1289	N [1274] 105.021, K [1275] 45, M [1284] 15.9949, K [1287] 45
1274	NKLSPSFGGMMPSKSR	1289	N [1274] 105.021, K [1275] 45, M [1283] 15.9949, K [1287] 45
1274	NKLSPSFGGMMPSKSR	1289	N [1274] 105.021, K [1275] 45, M [1283] 15.9949, M [1284] 15.9949, K [1287] 45
1274	NKLSPSFGGMMPSKSR	1289	N [1274] 105.021, K [1275] 45, M [1283] 15.9949, M [1284] 15.9949, K [1287] 45

Figure 3.17. Summary of the peptide matches for VEGFR2 in the MS/MS experiments.

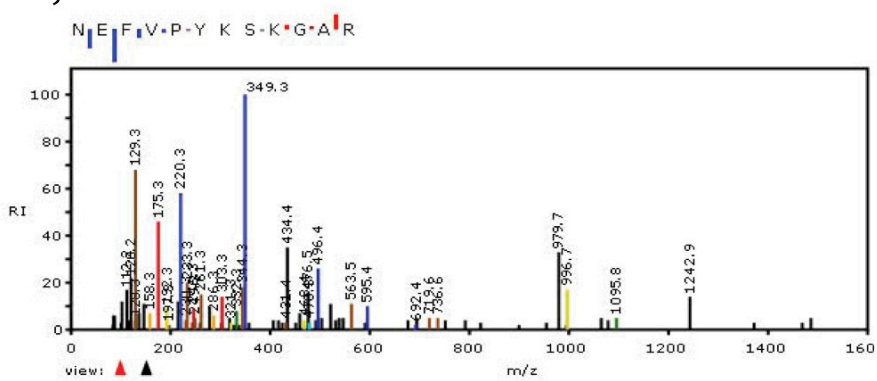
Five VEGFR2 lysines were detected as acetylated in three independent experiments (for a complete list of peptide matches see table in Figure 3.17). These residues were Lys929, Lys937, Lys939, Lys947 and Lys1053 (corresponding to Lys931, 939, 941, 949 and 1055 of human VEGFR2 respectively). The relative spectra are reported in Figure 3.18.

Notably, all these residues are distributed in the cytoplasmic tail of the receptor, as shown in the schematic representation in Figure 3.19. The intracellular portion of VEGFR2 mainly consists in the kinase domain, which is split into two parts by the insertion of the kinase insert domain (KID). Four of the residues that we identified as acetylated, namely Lys929, Lys937, Lys939 and Lys947, form a dense cluster in the KID. They are located near a well-characterized tyrosine, Tyr949 (human Tyr951), which was shown to be phosphorylated and to mediate TSAd-dependent cell signaling (Matsumoto et al, 2005).

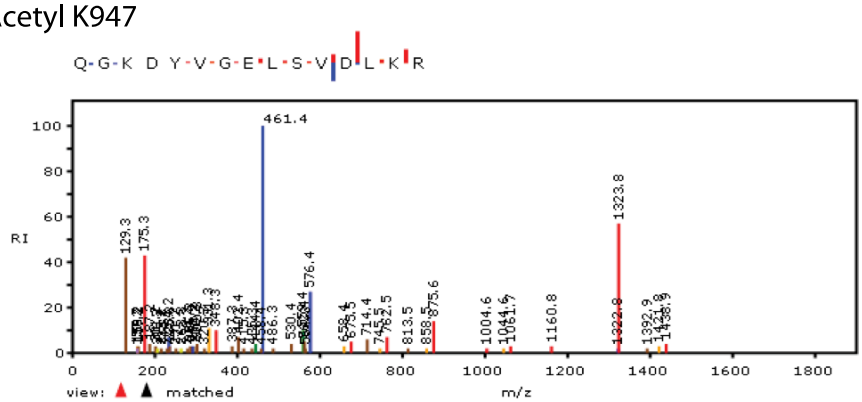
Acetyl K929



Acetyl K937/K939



Acetyl K947



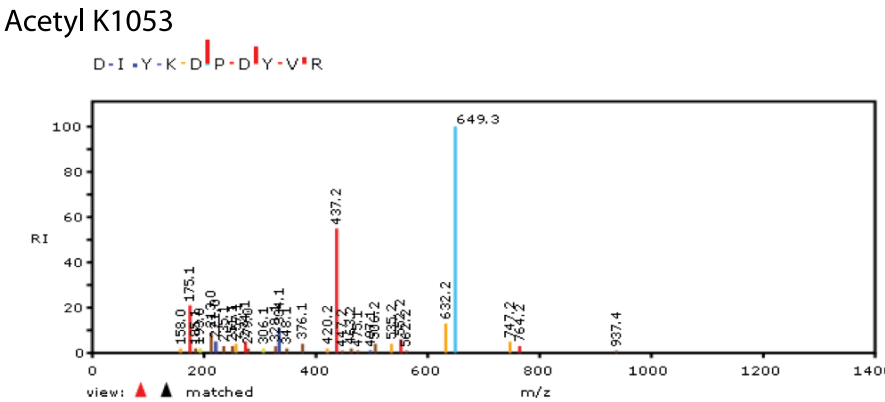


Figure 3.18. LC-MALDI tandem mass spectrometry (MS/MS) spectra showing acetylation of specific VEGFR2 lysine residues. Annotated spectra for acetylated lysine residues were produced using the GPM software. The acetylated residue is indicated for each spectrum and the b- and y- series ions are shown in blue and red, respectively. Other colors denote other ions such as neutral losses of water, ammonia, or internal fragment ions. The best scoring spectrum, based on log(e) value, is shown.

The fifth acetylated site, Lys1053, is positioned in the activation loop of the receptor, within the second kinase domain.

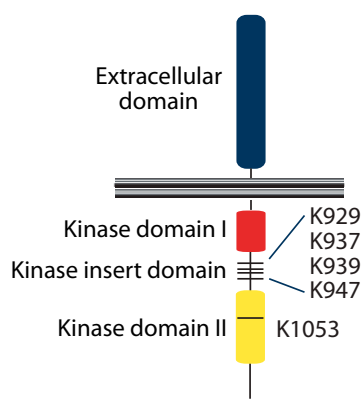


Figure 3.19. Mass spectrometry identified at least five acetylated lysines in VEGFR2. Schematic representation of the localization of the acetylated lysines in mouse VEGFR2, as detected by MS/MS.

Intriguingly, this acetylated lysine is flanked by two critical autophosphorylation sites (Tyr1052 and Tyr1057 in the mouse sequence), which are required for VEGFR2 maximal kinase activity (Dougher & Terman, 1999).

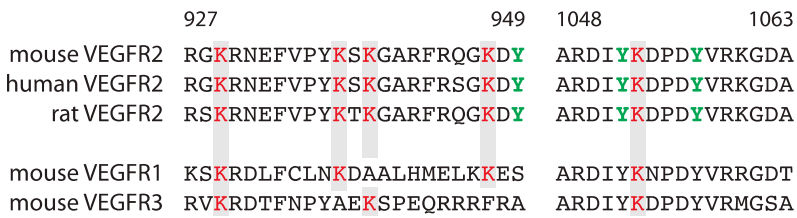


Figure 3.20. Comparison of VEGFRs sequences surrounding the acetylated lysines. Acetylated lysines are shown in red, reported phosphorylated tyrosines in green; conserved lysines are highlighted by a grey box.

All these acetylated lysines are highly conserved between human, mouse and rat, as we discovered by protein alignment; moreover both Lys929 and Lys1053 are also maintained in mouse VEGFR1 and VEGFR3 (Figure 3.20).

We constructed mutants of the full-length mouse VEGFR2 carrying substitution of lysines with arginines, which conserve the positive charge, but cannot be acetylated.

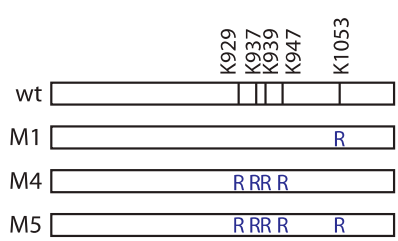


Figure 3.21. Construction of VEGFR2 mutants carrying Lys to Arg substitutions. (A) Schematic representation of Lys to Arg VEGFR2 mutants (M1, M4, M5). Lysines mutated to arginines are indicated as "R".

Three mutants were generated: M1, which is mutated in the lysine located in the activation loop only (Lys1053Arg), M4, mutated in the four lysines clustered in the kinase insert domain (Lys929,937,939,947Arg), and M5, mutated in all the five lysines detected as acetylated (Figure 3.21). After transfection in HEK293T cells and TSA treatment, acetylation of all three mutants was found significantly reduced, in particular that of M4 and M5 (representative experiments in Figure 3.22 panel A, and quantification in panel B).

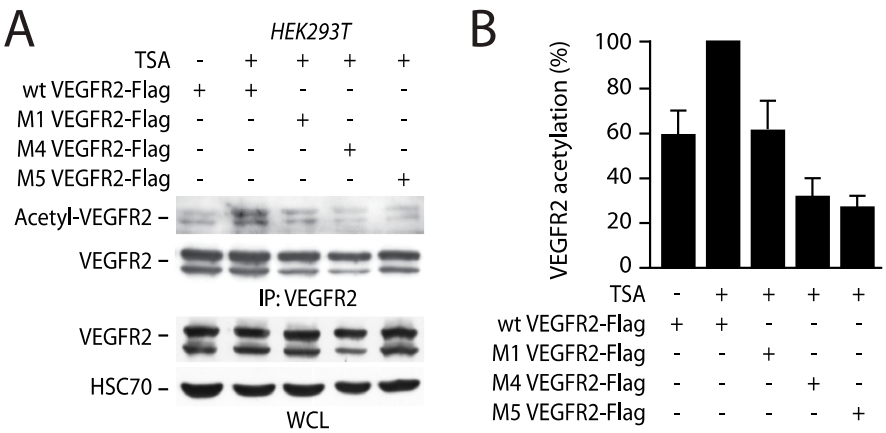


Figure 3.22. VEGFR2 mutant are less acetylated in response to TSA treatment (A) HEK293T cells were transfected with wt, M1, M4 or M5 flagged VEGFR2 and treated with TSA or DMSO as a control. Flagged proteins were immunoprecipitated from WCL and the acetylation levels assayed with an anti-acetyl lysine antibody. (**B**) Relative quantification of VEGFR2 acetylation as percentage variation over wt VEGFR2 in TSA-stimulated cells, after standardization over total VEGFR2 in each sample (mean±SEM).

To further confirm this observation, we also tested VEGFR2 acetylation *in vitro*. We produced His-tagged recombinant proteins corresponding to the wild type and mutated (M1, M4 and M5) intracellular domain of

mouse VEGFR2 (aa 805-1356). These were incubated with the recombinant HAT domain of p300 fused to GST, in the presence or absence of ^{14}C acetyl-CoA.

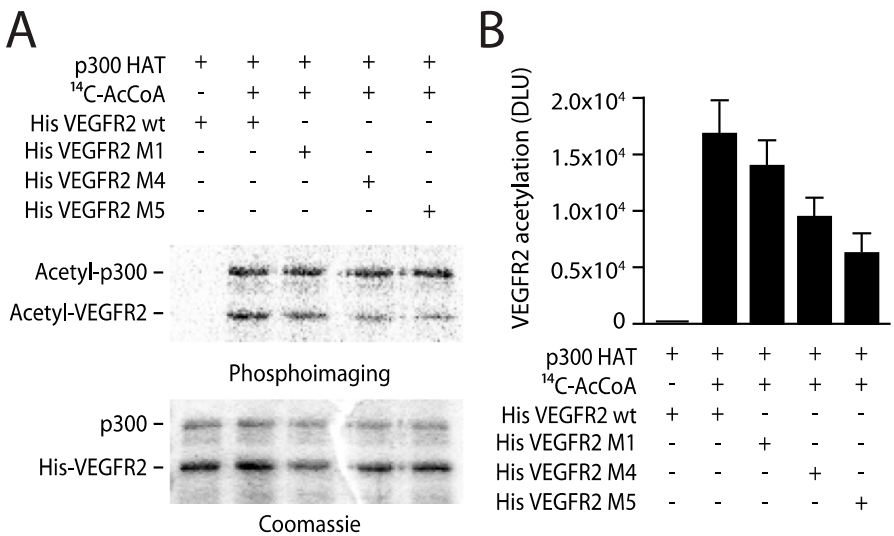


Figure 3.23. Impaired acetylation of VEGFR2 mutant *in vitro*. (**A**) His-tagged wt VEGFR2 and its mutants M1, M4 and M5 were assayed for acetylation by incubation with p300-HAT in the presence or absence of ^{14}C acetyl-CoA; after incubation, the reaction mixture was resolved by SDS–PAGE and the gel exposed to a phosphoimager. Upper panels: gels exposed to phosphoimager. Lower panels: Coomassie-stained gels. (**B**) Relative quantification of His-VEGFR2 acetylation as density light unit (DLU) variation; mean±sem of three experiments.

Consistent with the *in vivo* results, wt VEGFR2 scored clearly positive for acetylation, whereas the acetylation of mutants M4 and M5 appeared significantly impaired (representative experiment in Figure 3.23 panel A, and quantification in panel B). In addition to VEGFR2, the HAT domain of p300 was also acetylated due to the autocatalytic activity of the enzyme.

These results indicate that at least five lysine residues are acetylated in VEGFR2. All these residues are located in the intracellular portion of the receptor, four in the KID and one in the activation loop, and are absolutely conserved among species.

VEGFR2 acetylation has no effect on protein stability

We then wanted to understand the effect of VEGFR2 acetylation on the receptor biology. As extensively discussed in the Chapter 1, lysine acetylation might have an impact on protein stability by interplaying with lysine ubiquitination, either hampering or favoring protein degradation. In any of the previous experiments, we ever observed a variation in VEGFR2 total protein level upon hyperacetylating conditions. Nevertheless, since VEGFR2 was shown to undergo ubiquitination (Duval et al, 2003; Murdaca et al, 2004), we precisely investigate the eventual role of VEGFR2 acetylation in controlling the receptor stability. To address this question, we compared the half-life of VEGFR2 in HUVE cells treated with TSA or DMSO upon *de novo* protein synthesis inhibition with cycloheximide (CHX). The protein amount was evaluated by immunoblotting WCL with an anti-VEGFR2 antibody, followed by normalization to HSC70; the quantification of protein decay was expressed as the percentage of reduction over cells not treated with CHX. As soon as after one hour of treatment with CHX, we observed an approximately 50% decrease of VEGFR2 protein levels, with an additional halving in the subsequent hour. As initially hypothesized, we did not notice any differences in the kinetics of degradation of VEGFR2 in cells treated with TSA compared to control treated cells (Figure 3.24,

representative blot in panel A and quantification in panel B).

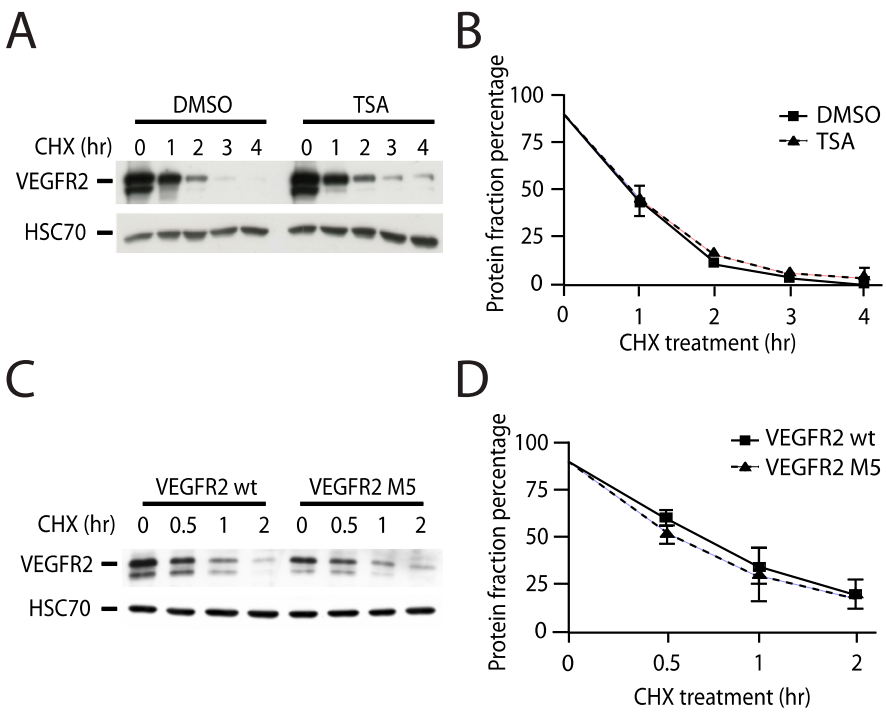


Figure 3.24 VEGFR2 acetylation does not affect protein stability. (A) HUVE cells were treated with either TSA or DMSO, and CHX was added to the medium for the indicated times to allow protein synthesis inhibition. WCL were loaded onto an SDS-PAGE gel and the membrane probed with anti-VEGFR2 and anti-HSC70 antibodies. (**B**) VEGFR2 protein levels are quantified and plotted on the graph over time as a percentage of reduction over cells not treated with CHX. Squares correspond to DMSO treatment, whereas triangles indicate TSA treatment. **(C)** Same as in (A) in PAE cells transiently expressing either wt or M5 VEGFR2. **(D)** Same as in (B), squares correspond to wt VEGFR2, triangles indicate VEGFR2 M5.

A similar experiment was also performed in PAE cells, which lack endogenous VEGFR2 and the Neuropilin-1 co-receptor, transfected to express either VEGFR2 wt or mutant M5. Also in this case, no difference

between the two proteins was observed (Figure 3.24, representative blot in panel C and quantification in panel D).

We conclude that VEGFR2 acetylation does not regulate receptor stability.

Acetylation regulates VEGFR2 phosphorylation

VEGFR2 transduces signals for endothelial cells most likely via ligand-induced tyrosine phosphorylation followed by coupling to intracellular signal transducers (for a pan-phopshorylation site map see (Olsson et al, 2006)). Since acetylation is known to modulate phosphorylation of other proteins (Sundaresan et al, 2011; Tang et al, 2007; Yang & Seto, 2008a) and given the proximity of the acetylated lysines to main VEGFR2 autophosphorylation sites, we wondered whether acetylation of VEGFR2 might influence its tyrosine kinase activity. To address this issue, we transfected PAE/KDR cells with wt p300, p300 DY or an empty plasmid, serum starved the cells and then stimulated them with rhVEGF. VEGFR2 was immunoprecipitated from the WCL and, finally, probed with an anti-total phospho-tyrosine antibody. In cells overexpressing p300, the magnitude of VEGF-induced receptor phosphorylation was markedly increased in respect to the control transfected cells. In contrast, in cells transfected with p300-DY, rhVEGF stimulation was almost ineffective, consistent with the hypo-acetylation status of the receptor and mirroring a potential transdominant negative effect of the p300 inactive mutant. In keeping with these observations, both p300 and p300-DY were found to co-immunoprecipitate with VEGFR2, irrespective of VEGF stimulation (Figure 3.25, blots in panel A and quantification of the ratios

between phosphorylated and total receptor in panel B).

These results strongly support the existence a crosstalk between acetylation and phosphorylation of VEGFR2, as the hyper-acetylation of the receptor upon p300 overexpression markedly enhances its autophosphorylation in response to ligand stimulation.

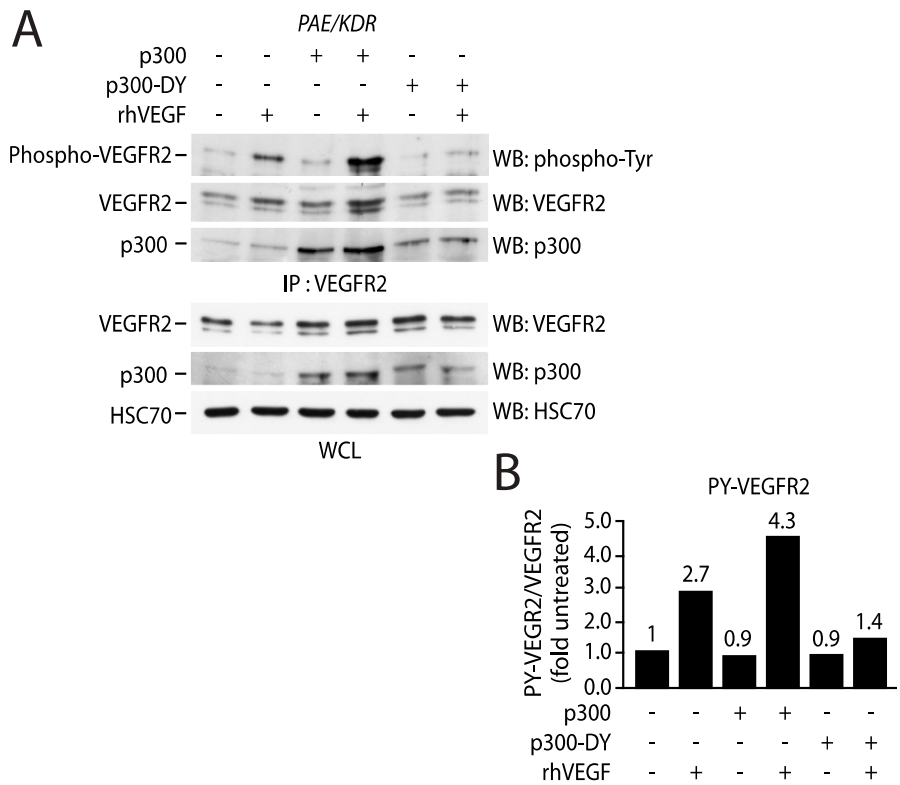


Figure 3.25. Acetylation regulates VEGFR2 phosphorylation. (A) VEGFR2 was immunoprecipitated from WCL of PAE/KDR cells transfected with p300, p300 DY or an empty vector, with or without rhVEGF treatment, and then immunoblotted with an anti-phospho tyrosine antibody. **(B)** Quantification of the ratio between phosphorylated and total VEGFR2, shown as fold induction over untreated receptor.

To gain insight into the role of the different lysine residues in tyrosine phosphorylation, we also assessed the levels of VEGF-induced phosphorylation of the VEGFR2 mutants in PAE cells. VEGFR2 phosphorylation was analyzed in WCL using antibodies specific for phospho-Tyr1173. The mutant, non-acetylated variants of the receptor, especially the M4 and M5 mutants, were less phosphorylated compared to the wt protein (Figure 3.26, blots in panel A and quantification of the ratios between phosphorylated and total receptor in panel B).

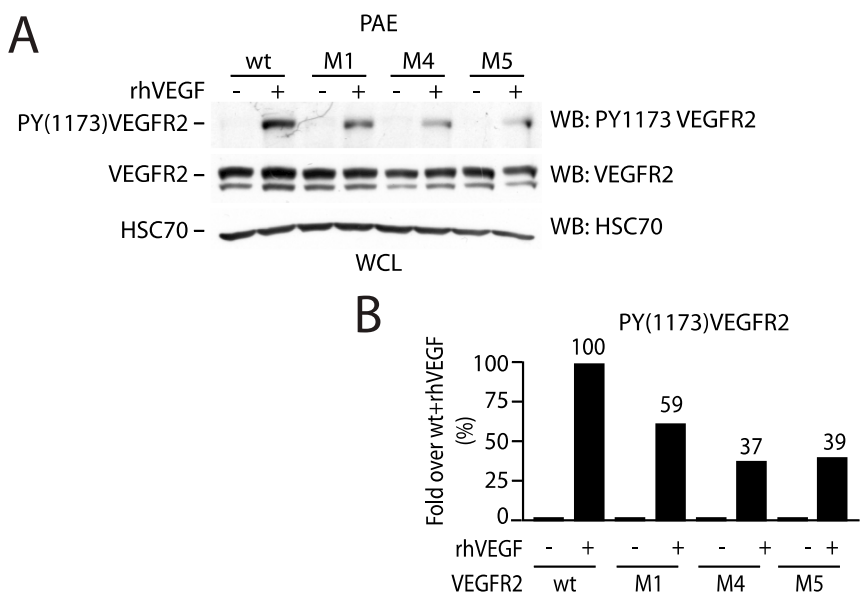


Figure 3.26. The non-acetylable VEGFR2 mutants show impaired autophosphorylation. (**A**) PAE cells were transfected with wt, M1, M4 or M5 VEGFR2 and treated with or without rhVEGF for 5 minutes. Phosphorylation levels of Tyr 1173 were measured and reported in (**B**) as percentage variation over wt VEGFR2 after standardization over total receptor.

Since it is the fully glycosylated form of the receptor, expressed on the cell surface, that undergoes phosphorylation in response to external

stimuli (Takahashi & Shibuya, 1997), we also performed the same experiment by selectively labeling the cell surface molecules with activated biotin, a membrane impermeant compound, followed by recovery of biotinylated proteins by streptavidine beads. VEGFR2 phosphorylation was analyzed using the antibody specific for phospho-Tyr1173 and also an antibody recognizing phospho-Tyr1052 (human Tyr1054), a critical autophosphorylation site located in the activation loop (Dougher & Terman, 1999; Dougher-Vermazen et al, 1994). We found that the M4 and M5 mutants showed a significant decrease in VEGF-induced phosphorylation on Tyr1173 (Figure 3.27, representative blot in panel A and quantification of the results of three independent experiments in panel B). As far as phosphorylation of Tyr1052 was concerned, instead, this was significantly reduced in mutants M1 and M5, while it was similar to wt in mutant M4 (Figure 3.27, blots and quantification in panel C and D, respectively). Of note, both M1 and M5 carry the Lys to Arg substitution at residue 1053, which is adjacent to the analyzed phosphorylation site.

Finally, we wanted to verify that the observed reduction in the autophosphorylation levels of the VEGFR2 mutants was not due to an impairment in their catalytic activity. For this purpose, the recombinant VEGFR2 proteins were incubated *in vitro* with the Poly (Glu-Tyr) peptide as a substrate, followed by an enzyme-linked immunosorbent assay kit to quantify peptide phosphorylation. The receptor kinase activity was comparable between wild type and M1 VEGFR2, whereas only an approximately 25% decrease was observed in the case of the M4 and M5 mutants (Figure 3.28).

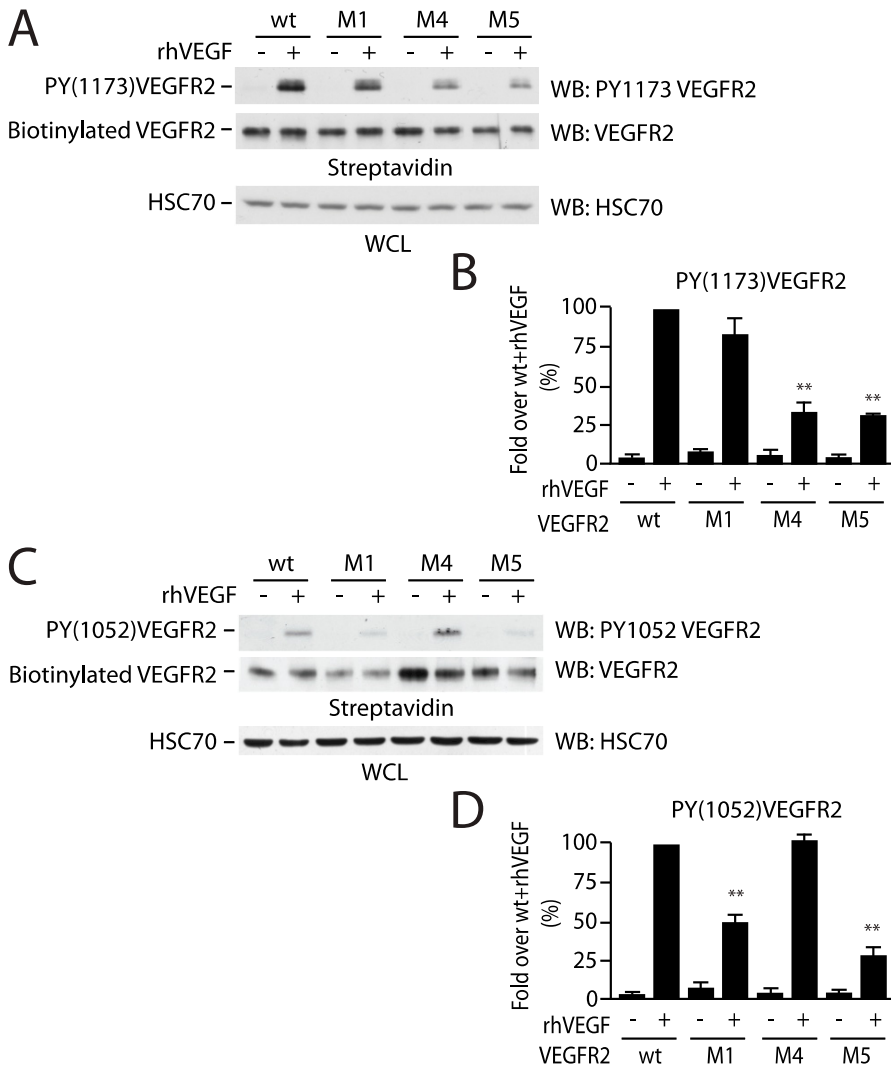


Figure 3.27. The membrane-exposed fraction of VEGFR2 mutants is less phosphorylated in response to VEGF treatment. PAE cells were transfected with wt, M1, M4 or M5 VEGFR2, starved and then treated with or without $VEGF_{165}$ for 5 minutes. Phosphorylation levels of Tyr 1173 **(A)** and Tyr 1052 **(C)** were assessed after cell surface biotinylation with thiol-cleavable Sulfo-NHS-SS-Biotin. Relative quantification of ratio percentage of VEGFR2 phosphorylation in Tyrosine 1173 **(B)** and 1052 **(D)** upon total VEGFR2 are shown on the right of the panels.

This finding rules out that the major impairment observed for the mutant receptor phosphorylation might be consequent to an intrinsically decreased kinase activity. This observations, in combination with our previous findings, indicate that acetylation of the receptor by p300 markedly enhances its autophosphorylation in response to ligand stimulation. Moreover, mutation of the acetylated residues significantly impairs ligand-induced VEGFR2 autophosphorylation at Tyr1052 and Tyr1173, without significantly affecting its tyrosine kinase activity.

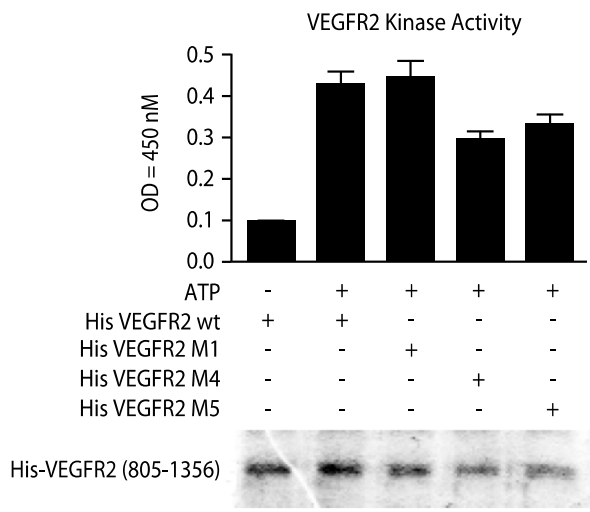


Figure 3.28. Mutation of the acetylated lysines in VEGFR2 does not impair receptor kinase activity. His-tagged VEGFR2, either wt or mutants, was incubated with Poly(Glu-Tyr) peptide in the presence or absence of ATP, and substrate phosphorylation was monitored by enzyme-linked immunosorbent assay. Data are represented as absorbance values at 450 nM (mean±SEM of three independent experiments). Lower panel: Coomassie-stained gel used to verify the quantity of protein loaded for each reaction.

Structural modeling of the VEGFR2 activation loop

Next we wanted to get structural insights into the role of Lys1053 acetylation in the conformation of the activation loop. An exhaustive search in the Protein Data Bank (www.pdb.org) revealed that, among the 38 structures reported for VEGFR2 in complex with different inhibitors, only 7 contain the complete structure of the activation loop in its unphosphorylated form.

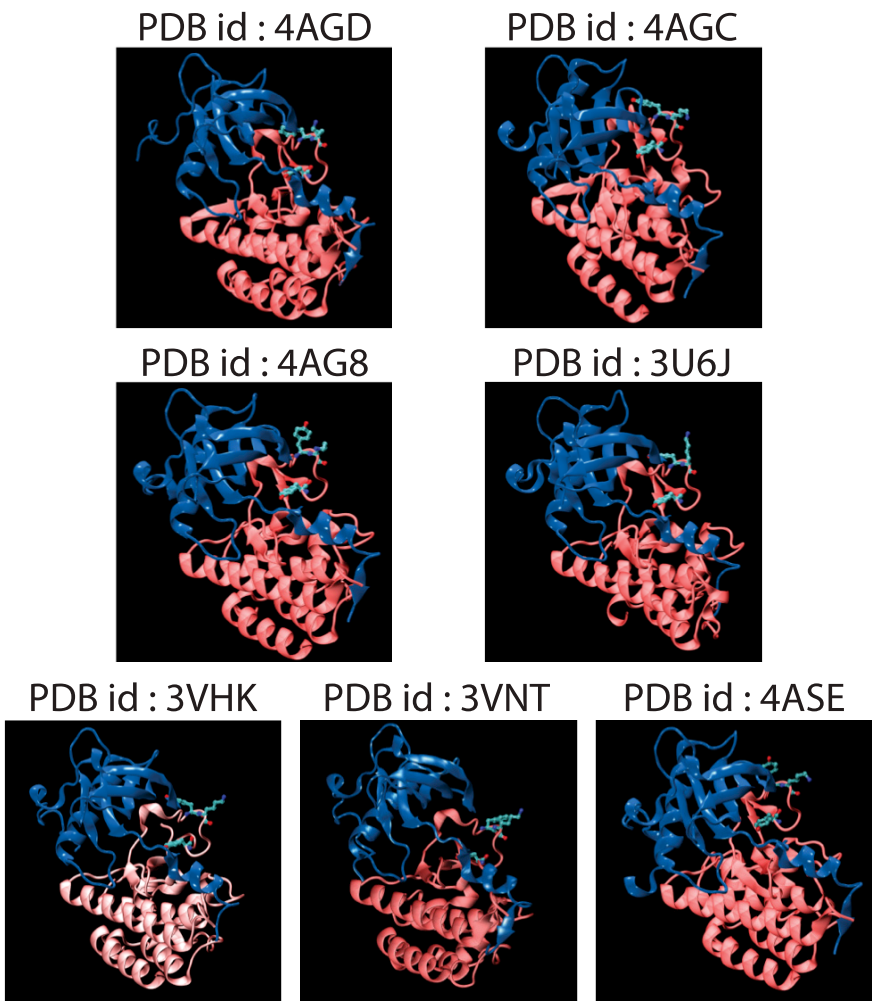


Figure 3.29. X-ray structures of VEGFR2 in complex with different inhibitors. The structures contain the complete receptor activation loop in its unphosphorylated form (PDB ids: 4AGD, 4AGC, 4AG8, 3U6J, 3VHK, 4ASE and 3VNT). The protein segments, before and after the kinase activation loop, are represented as pale blue and orange cartoons, respectively, whereas residues pY1052, Lys1053 and pY1057 are shown in sticks.

Notably, in all these X-ray structures (PDB ids: 4AGD, 4AGC, 4AG8, 3U6J, 3VHK, 4ASE and 3VNT), Lys1053 is completely exposed to the solvent (Figure 3.29) and both, or at least one, of the tyrosines present in the activation loop (Tyr1052 and Tyr1057) are completely buried.

Therefore, we sought to perform molecular dynamics simulations starting from the structure of VEGFR2 phosphorylated at Tyr1052 and Tyr1057 and in complex with a benzimidazole inhibitor (PDB id: 2OH4, Figure 3.30, panel A) (Hasegawa et al, 2007). Simulations were performed removing the inhibitor from the following systems: wt VEGFR2 kinase domain phosphorylated at Tyr1052 and 1057 (Figure 3.30, panel B); wt VEGFR2 kinase domain unphosphorylated (Figure 3.30, panel C); wt VEGFR2 kinase domain phosphorylated at Tyr1052, 1057 and acetylated at Lys1053 (Figure 3.30, panel D); wt VEGFR2 kinase domain unphosphorylated and acetylated at Lys1053 (Figure 3.30, panel E); and VEGFR2 kinase domain Lys1053Arg mutant (Figure 3.30, panel F).

Analysis of these computational models revealed that the removal of the phosphoryl groups from Tyr1052 and Tyr1057 resulted in a reduction of the Solvent Accessible Surface (SAS) of the phosphorylation motif, most likely due to a reduction of the electrostatic repulsion (Figure 3.31).

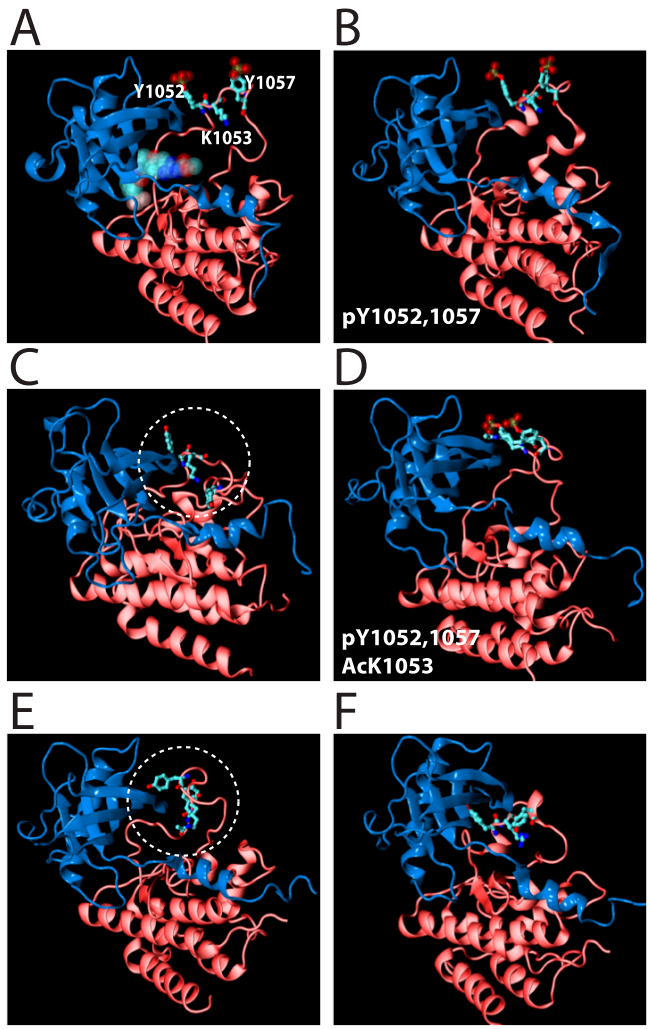


Figure 3.30. Structural modeling of acetylated VEGFR2. (A) X-ray structure of VEGFR2 (PDB code 2OH4). The color scheme is the same as in figure 3.25. The benzimidazole inhibitor is shown in semitransparent space filling representation. (**B to F**) Final structures of the simulations of different VEGFR2 systems are shown. **(B)** VEGFR2 phosphorylated at Y1052 and Y1057. (**C**) Unphosphorylated receptor. The circular section marks the activation loop. (**D**) VEGFR2 phosphorylated at Y1052, 1057 and acetylated at Lys1053. (**E**) VEGFR2 unphosphorylated and acetylated at Lys1053. The circular section marks the activation loop. (**F**) VEGFR2 mutant Lys1053R.

This result is in agreement with the more compact conformation obtained for the activation loop in its unphosphorylated form (cf., for instance, ref. (Oguro et al, 2010)) in which lysine 1053 is partially buried inside the middle of the activation loop, flanked by Tyr1052 and Tyr1057 (Figure 3.30, panel A and C). Lysine acetylation of the unphosphorylated protein resulted in a more exposed conformation of the activation loop, in which tyrosine residues were uncovered and thus accessible for phosphorylation (Figure 3.30, panel E).

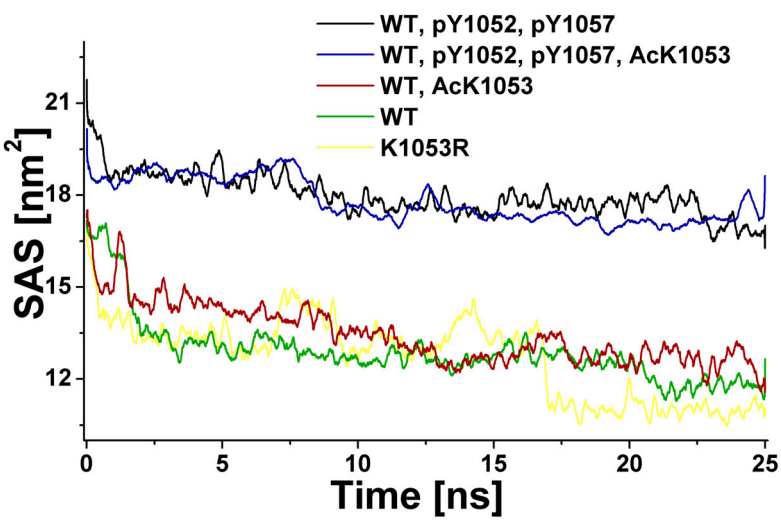


Figure 3.31. Activation loop solvent accessible surface. Time evolution of the solvent accessible surface area (SAS) calculated on the activation loop for the different systems that were simulated.

On the contrary, acetylation at Lys1053 of the phosphorylated protein did not affect significantly the structure (Figure 3.30, panel B and D and Figure 3.31). Furthermore, mutation of Lys1053 into arginine generated a more compact conformation, comparable to that of the unmodified protein (Figure 3.30, panel F and A); this simulation resulted in the least

flexible system, in which the entire activation loop was stabilized by the establishment of a network of hydrogen bonds (Figure 3.30, panel F and magnification in Figure 3.32). This conformation showed a clear impairment in the exposure of the tyrosines, which could thus be phosphorylated less favorably.

Taken together, these results provide a structural model to explain the increased levels of VEGF-induced VEGFR2 phosphorylation imparted by acetylation by enzymatically active p300 and, more specifically, the remarkable reduced levels of Tyr1052 phosphorylation in the Lys1053Arg mutant.

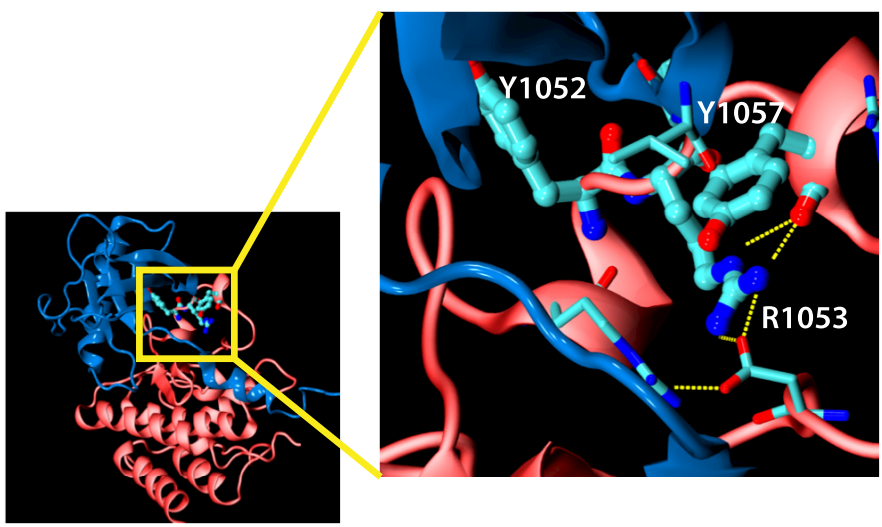


Figure 3.32. Magnification of the activation loop in Lys1053R VEGFR2. The activation loop is shown from a different orientation to highlight the network of hydrogen bonds established by R1053, shown as dashed yellow lines.

Acetylation prevents receptor desensitization

Different Receptor Tyrosine Kinases (RTKs), including VEGFR2, can undergo receptor desensitization as a means of rapid dephosphorylation soon after activation (Ewan et al, 2006; Lanahan et al, 2010; Lemmon & Schlessinger, 2010; Nakamura et al, 2008).

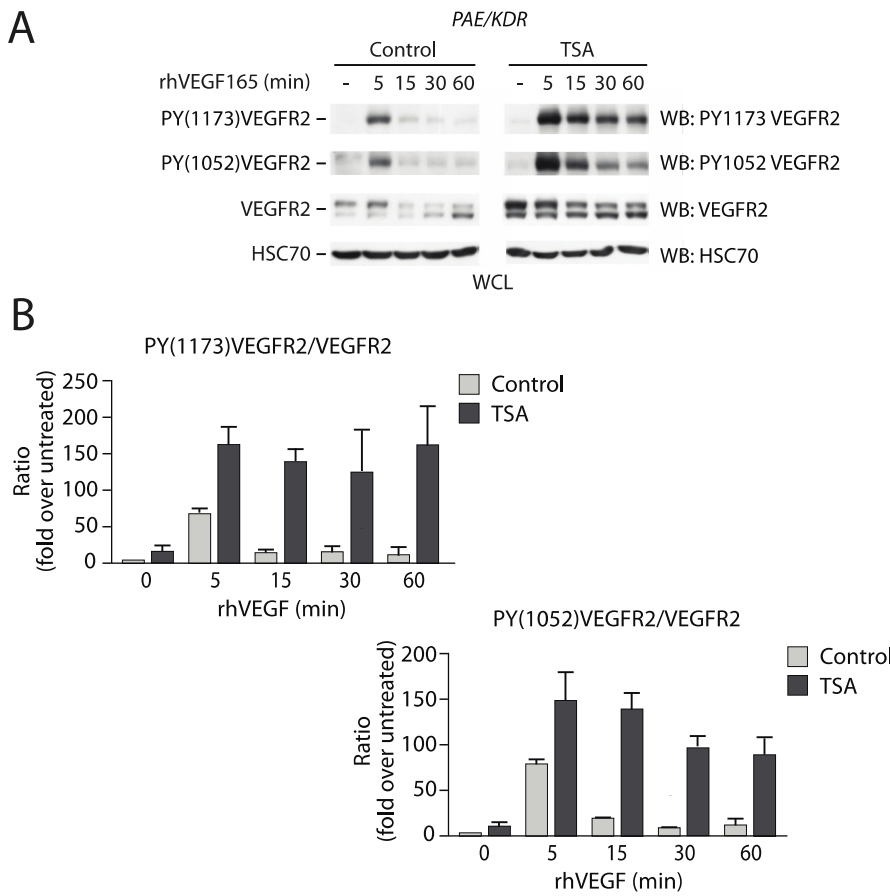


Figure 3.33. Acetylation regulates VEGFR2 desensitization. (A) PAE/KDR cells were treated or not with TSA and stimulated for different time points with rhVEGF. WCL were probed with antibodies against phospho-Tyr1052, phospho-Tyr1173, VEGFR2 and HSC70. **(B)** Relative quantifications of VEGFR2 phosphorylation at Tyrosine 1173 and 1052 are shown after standardization over total VEGFR2 and expressed as fold over untreated receptor (mean±SEM of three indepedent experiments).

Given the striking role of acetylation in enhancing VEGFR2 phosphorylation, we wanted to explore the possible role of acetylation in modifying the kinetics of VEGFR2 phosphorylation after VEGF-induced activation.

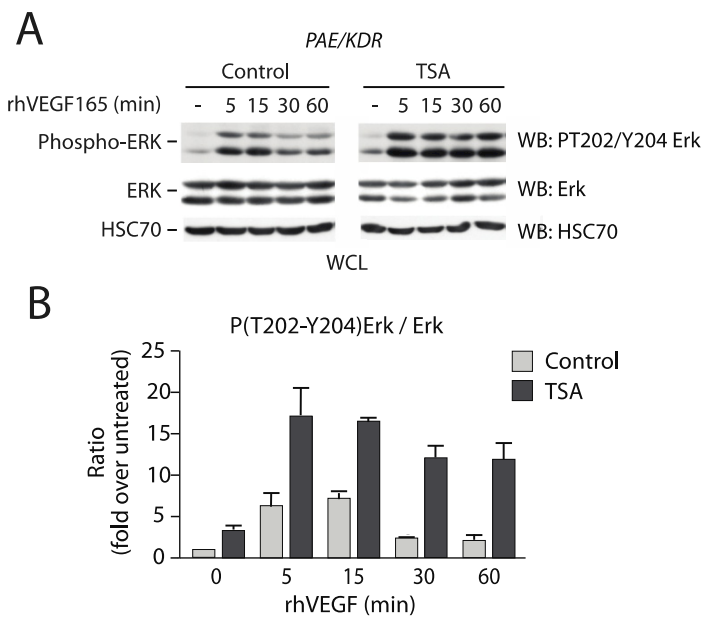


Figure 3.34. VEGF-induced signal transduction upon VEGFR2 hyperacetylation. (A) PAE/KDR cells were treated or not with TSA and stimulated for different time points with rhVEGF to induce receptor desensitization. WCL were immunoblotted with anti-phospho ERK 1/2, anti-ERK 1/2 and anti-HSC70. **(B)** Relative quantification of the ratio between ERK 1/2 phosphorylated at threonine 202 and tyrosine 204 and total ERK 1/2. The results are shown as fold induction over unstimulated cells (mean±SEM of three independent experiments).

Serum starved PAE/KDR cells were treated with rhVEGF and collected at different times after stimulation, as indicated; the levels of VEGR2 phosphorylation on Tyr1173 and Tyr1052 were analyzed by WCL western blotting comparing DMSO control conditions and TSA treatment, which

preserves VEGFR2 acetylation. In control conditions, the levels of phosphorylated VEGFR2 at both residues peaked at 5 min to sharply decrease at 15 min and remain very low in the subsequent hour. In contrast, in the TSA-treated cells, phosphorylation was remarkably higher after 5 min of stimulation and, most notably, was maintained over time (Figure 3.33, representative blots in panel A).

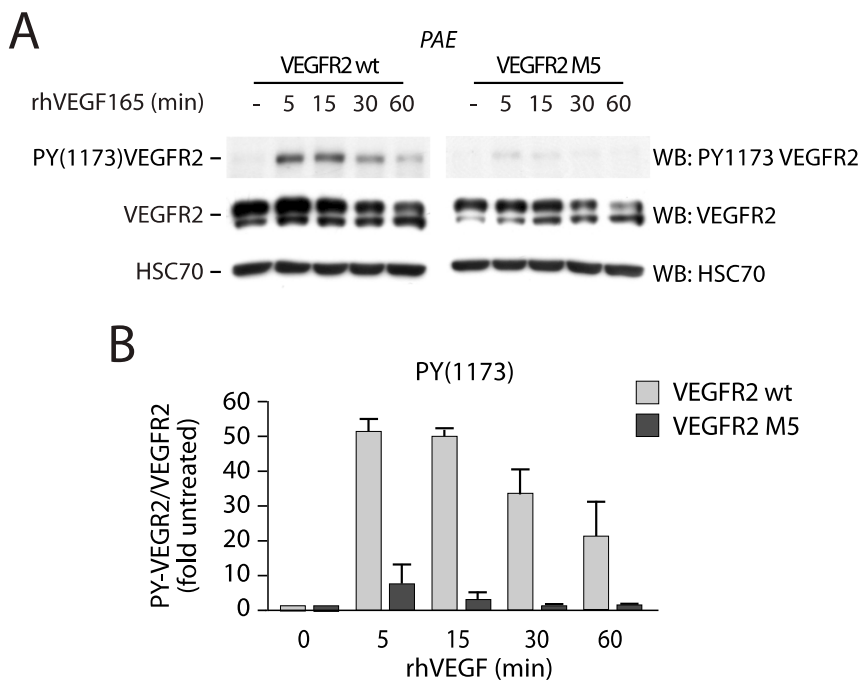


Figure 3.36. The non-acetylable M5 VEGFR2 mutant does not respond to prolonged VEGF treatment. (**A**) PAE cells were transfected with wt or M5 VEGFR2 and treated with rhVEGF for different time points. VEGFR2 phosphorylation levels of Tyr 1173 were quantified and reported in (**B**) as fold over untreated receptor (mean±SEM of three different experiments), after standardization over total VEGFR2.

The levels of phosphorylated VEGFR2 over total VEGFR2 was quantified and plotted on the graph as fold over untreated receptor (Figure 3.33,

panel B). The increased levels of phosphorylated VEGFR2 in TSA-treated cells were paralleled by increased phosphorylation of Erk1/2, a pivotal downstream effector of the receptor (Figure 3.34, representative blots in panel A and quantification in panel B).

The same experiment was performed comparing the kinetics of activation of wt VEGFR2 and the non-acetylable mutant M5. Consistent with the previous observations, this mutant was remarkably impaired in VEGF-induced phosphorylation on Tyr1173 (Figure 3.36, panel A for representative blots and panel B for quantification).

Together these data support the conclusion that VEGFR2 acetylation contrasts receptor desensitization, counteracting its dephosphorylation, without any detectable effect in the process of receptor downregulation.

Acetylation enhances VEGFR2 function

To further elucidate the functional significance of acetylation in VEGFR2 activity, we monitored the efficiency of wound healing of PAE cells transiently transfected with VEGFR2 wt or mutant M5.

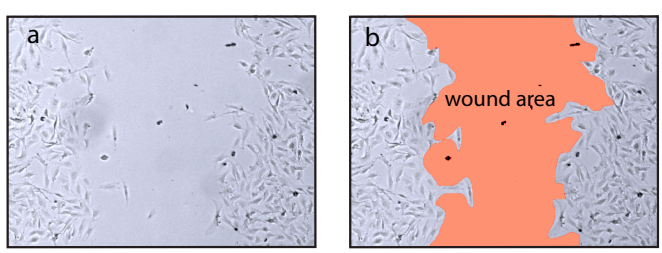


Figure 3.37. Wound size analysis. PAE cells transiently expressing VEGFR2 wt were imaged by phase contrast microscopy (a); the calculated wound area was pseudo-colored in orange (b).

In this assay, cell motility depends on VEGF stimulation. The cells were grown to 100% confluence, serum starved and treated with thymidine to block proliferation. We then mechanically created a cell free zone in the monolayer and kept the cells in the absence of serum or in the presence of rhVEGF for 24 hrs.

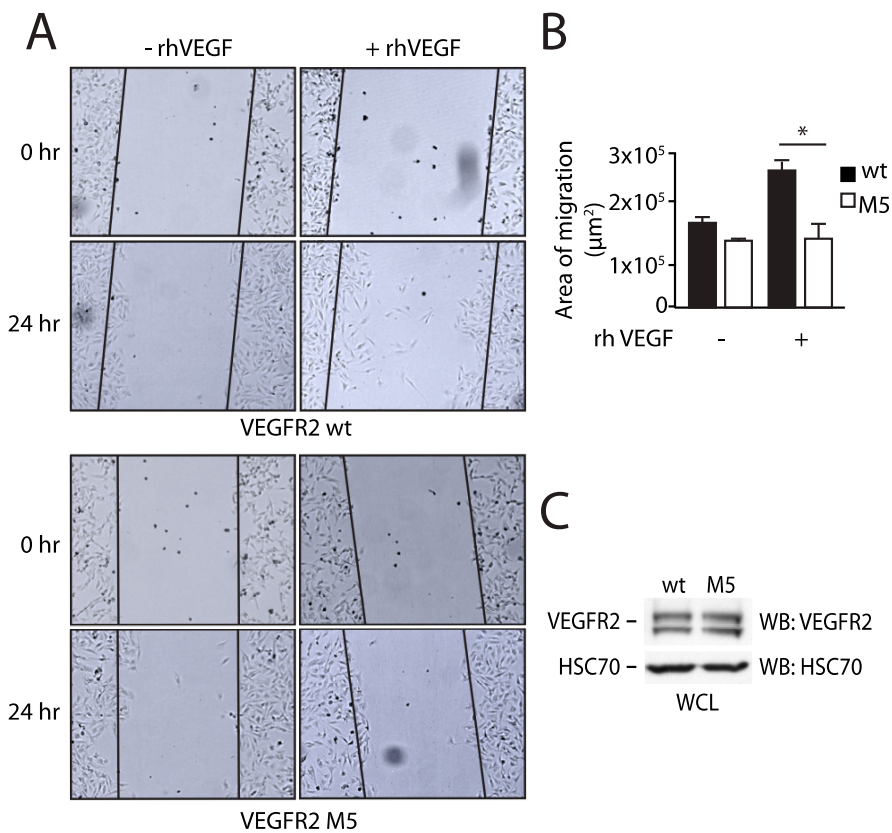


Figure 3.38. Expression of the M5 VEGFR2 mutant in PAE cells affects their response to migratory stimuli. (**A**) The migratory capability of PAE cells transiently expressing either wt or M5 VEGFR2 was measured by a wound healing assay, in the presence or absence of rhVEGF. (**B**) Quantification of cell migration area into the wound area. The extent of wound closure quantified by measuring the wound area at 24 hours compared to time 0. * indicates $P<0.05$. (**C**) After 24 h of stimulation with rhVEGF, PAE cells subjected to wound healing assay were collected and the levels of expressing proteins were analyzed by western blotting.

Phase-contrast images were taken immediately after scratch (t=0) and 24 hours after rhVEGF stimulation (t=24) and the migration rate of the endothelial cells was determined after quantification of the wound size at fixed time points (Figure 3.37).

We found that, in wt VEGFR2-expressing cells, VEGF treatment for 24 hours clearly induced cell motility to fill the wounded monolayer; on the contrary, this effect was markedly impaired in cells expressing mutant M5, which failed to respond to VEGF stimulation (Figure 3.38, representative images in panel A and quantification in panel B). Wild type VEGFR2 and mutant M5 were equally expressed in the PAE cells (Figure 3.38, panel C).

Thus, VEGFR2 acetylation enhances the receptor activity in inducing endothelial cell migration.

Chapter 4

DISCUSSION

DISCUSSION

VEGFR2 is modified by acetylation

Over the last few years, a large body of evidence has shown that post-translational modification by acetylation has a major role in the regulation of protein function. Most notably, protein acetylation is not confined to the nuclear compartment but also occurs for cytoplasmic proteins, such as α-tubulin, cortactin and Hsp90 (Bali et al, 2005; Matsuyama et al, 2002; Zhang et al, 2007). Information is more scant as far as membrane-associated factors are concerned. The type I interferon receptor (IFNαR2), together with interferon regulatory factor 9 (IRF9) and the activators of transcription STAT1 and STAT2, were all found to be acetylated by CBP (Tang et al, 2007), implying a role for acetylation in the control of cytokine receptor signal transduction. Post-translational modification by acetylation was also suggested to regulate clathrin-mediated endocytosis (Goh et al, 2010) and activity (Song et al, 2011) of the epidermal growth factor receptor (EGFR). Moreover, the acetylation of the Insulin-like Growth Factor 1 (IGF-1) receptor has been detected during a mass spectrometry survey (Choudhary et al, 2009).

We provide evidence that VEGFR2 is acetylated *in vivo* and that receptor acetylation has an important role in regulating VEGF-induced receptor phosphorylation. Given the essential function of VEGFR2 in vascular development, it does not appear surprising that the functionality of this receptor is finely tuned at the level of gene expression regulation (Olszewska-Pazdrak et al, 2009; Plate et al, 1993), as well as through intracellular trafficking (Lampugnani et al, 2006; Lanahan et al, 2010;

Manickam et al, 2011; Mukherjee et al, 2006; Sawamiphak et al, 2010) and post-translational modifications of the mature protein (Duval et al, 2003; Koch et al, 2011; Murdaca et al, 2004; Takahashi & Shibuya, 1997). Thereby, the acetylation of VEGFR2 enlightens another crucial mechanism that dynamically modulates receptor activity. More generally, our findings, together with those regarding EGFR, as well as IFNαR2 and IGF-1, strengthen the concept that protein acetylation represents a novel mechanism of regulation of receptor function which might broadly applies to RTK family members.

Conserved acetylated residues in VEGFR2 intracellular domain

By exploiting proteomic analysis, we found several acetylation sites in murine VEGFR2, mainly located in the intracellular part of the receptor. In particular, the peptides encompassing Lys929, Lys937, Lys939, Lys947 and Lys1053 were identified several times in three independent experiments. Notably, all this residues are conserved in mouse, human and rat, thus suggesting their relevance in regulating receptor function. In addition, lysines 929 and 1053 are also conserved in VEGFR1 and VEGFR3 of mouse origin. In agreement, we found acetylated VEGFR2 of both mouse and human origin; moreover, the receptor acetylation is not cell type specific as well, since we were able to reproduce our findings in different cell lines.

Lysines at positions 929 to 947 form a dense cluster in the KID. Interestingly, acetylation of lysine residue groups has also been found to form positive charged patches in other proteins, such as p53, cortactin and EGFR (Goh et al, 2010; Kawaguchi et al, 2006; Zhang et al, 2007); of

notice, the enzymatic activity of p300 is tightly regulated by the overall acetylation of 13 lysines located in the acetyltransferase activation loop (Thompson et al, 2004). Importantly, in VEGFR2 this cluster of residues is adjacent to tyrosine 949, an important autophosphorylation site, known to mediate TSAd-dependent cell signaling (Matsumoto et al, 2005).

In contrast to the 929-947 lysine cluster, lysine 1053 is not surrounded by other acetylated lysines. Instead, this lysine is located in the middle of the VEGFR2 activation loop, near two critical autophosphorylation sites (Tyr 1052 and Tyr 1057) (Dougher & Terman, 1999). It is compelling to note that the regions surrounding VEGFR2 acetylated lysines appear to conform to the general residue preferences in the proximity of acetylated sites, as detected by various computational analysis, according to which acetylation preferentially occurs in regions with ordered secondary structure, with a widespread preference for lysine-rich regions containing a residue that can be phosphorylated and with a particular predilection for negative charge residues in the immediate surrounding of the modified site (Basu et al, 2009; Choudhary et al, 2009; Gnad et al, 2010; Schwartz et al, 2009).

Crosstalk between acetylation and phosphorylation

Protein acetylation has been frequently linked to other PTMs. A growing body of evidence supports the existence of a crosstalk among the various PTMs, a sort of "protein modification code" responsible for the final regulation of protein function (Hunter, 2007; Yang & Seto, 2008a).

Lysine acetylation has an important regulatory potential in the control of protein stability by mingling with protein ubiquitination (Caron et al,

2005; Sadoul et al, 2010). Nevertheless, even if VEGFR2 has been found subjected to ubiquitin-dependent degradation (Duval et al, 2003; Ewan et al, 2006), we were not able to observe any differences between the half-life of either mutant M5 VEGFR2 or TSA-induced hyperacetylated receptor compared to the wt, not-treated protein. Thereby, since we provide evidence that it does not exist any correlation between VEGFR2 acetylation and stability, the possible impact of acetylation on receptor function might reside elsewhere.

Interestingly, many examples have been already reported stating that acetylation act in concert with phosphorylation to regulate protein functions. Most notably, both in the case of EGFR and IFNαR2, lysine acetylation has already been shown to regulate receptor function in coordination with phosphorylation (Song et al, 2011; Tang et al, 2007). Our work indeed shows that acetylation of VEGFR2 was effective at inducing an increase in the overall phosphorylation level of the receptor in response to VEGF stimulation. In particular, computational modeling of the VEGFR2 activation loop indicates that acetylation of lysine 1053, by quenching the positive charge of the residue, aids in the transition of the activation loop towards a more open state, in which the two tyrosine residues 1052 and 1057 are better exposed on the surface of the protein and become accessible for phosphorylation. Mutation of lysine 1053 into arginine, instead, generated a less flexible system, in which the entire activation loop was partially buried and stabilized by a network of hydrogen bonds. Our structural modeling is corroborated by the observation that the non-acetylable VEGFR2 mutants showed impaired auto-phosphorylation capability. More specifically, the lack of acetylation at lysine 1053 correlated with a decrement in the phosphorylation at

tyrosine 1052, an effect that our *in vitro* phosphorylation studies showed to be not consequent to an intrinsic loss of receptor tyrosine kinase activity in the absence of ligand following residue substitution. Thus, VEGFR2 joins the growing list of proteins in which lysine acetylation regulates enzymatic activity, including Cdk2, Cdk9 and p38 (Mateo et al, 2009; Pillai et al, 2011; Sabo et al, 2008).

The hypothesis that residue charge influences receptor autophosphorylation in the activation loop has been already put forward by mutational data on both VEGFR1 and VEGFR2, which indicate that mutation of an invariable, negatively charged aspartic acid to non-polar asparagine at position 1054 lowers the autophosphorylation of activation loop tyrosines 1052 and 1057 (Meyer et al, 2006). The presence of this same asparagine in VEGFR1 might explain the poor autophosphorylation and signal transduction activity of this receptor in endothelial cells (Meyer et al, 2006). In this respect, it is worth mentioning that VEGFR1 was also found modified by acetylation in a recent mass spectrometry survey aimed at identifying new acetylated proteins (Chen et al, 2012). It will be of interest to understand to what extent VEGFR1 acetylation is also involved in the regulation of this receptor.

The acetylation occurring in lysine-rich region has been widely hypothesized to affect protein function most likely by leading to protein conformational shift, as in the case of p300 and p53 (Arif et al, 2007; Black et al, 2006; Laptenko & Prives, 2006). Unfortunately, we were not able to provide a molecular modeling of the region surrounding the 929-947 lysine cluster, since so far the high electrostatic charge and hydrophilicity of the KID sequence did not render the crystallization of

the VEGFR2 catalytic domain with the entire KID sequence possible (Hasegawa et al, 2007; McTigue et al, 1999). Nevertheless, we could not exclude that a mechanism of acetylation-induced conformational change, similar to that observed in the VEGFR2 activation loop, might affect several VEGFR2 catalytic residues, in particular tyrosine 1173.

Molecular players in VEGFR2 reversible acetylation

Our experiments reveal that, among the KAT enzymes analyzed, p300 is responsible for VEGFR2 acetylation in both endothelial and non-endothelial cells. Besides its well established function as a transcriptional co-activator in the nucleus, p300 is known to shuttle in and out of the nucleus and to act also in the cytoplasm (Shi et al, 2009; Tang et al, 2007). We found p300 partially localized in the cytoplasm in HUVE cells; most likely, it is the cytosolic fraction of the KAT the one responsible for the acetylation of VEGFR2. Previous work indicates that the depletion of p300 significantly reduces angiogenic tubule formation (Pillai et al, 2010). This report is fully consistent with our findings, in particular with the experiments showing that the receptor mutated in the lysines that undergo p300-mediated acetylation has impaired ability to induce endothelial cells migration in response to VEGF. Thus, in addition to enhancing VEGFR2 expression by acting at the level of the gene promoter (Pillai et al, 2010), p300 has the additional role of promoting receptor function by acetylating the mature protein.

Increasing evidence indicates the contribution of class IIa KDACs, such as HDAC5, HDAC6 and HDAC7, to the angiogenic function of endothelial cells. Both HDAC5 and HDAC7 are negative regulator of angiogenesis, by

having an impact on transcriptional regulation of gene expression in EC (Urbich et al, 2009; Wang et al, 2008). On the contrary, HDAC6 has been shown to promote angiogenesis *in vitro* and *in vivo* by deacetylating the cytoplasmic actin-remodeling protein cortactin in endothelial cells, thereby regulating endothelial cell migration and sprouting (Kaluza et al, 2011). We found that specific inhibition of class II KDACs markedly increased the level of acetylation of VEGFR2, with both HDAC5 and HDAC6 being able to co-immunoprecipate with and deacetylate VEGFR2. In addition, the two KDACs interacted with the receptor in a VEGF-dependent manner, in agreement with recent findings showing that treatment with VEGF promotes nuclear export of class II KDACs in endothelial cells (Ha et al, 2008; Wang et al, 2008).

VEGF induces VEGFR2 acetylation

Similar to acetylation of IFNαR2, which was demonstrated to be dependent on INFα stimulation (Tang et al, 2007), we found that in HUVE cells VEGFR2 acetylation was induced by VEGF treatment. In contrast to what happens to the CBP subcellular distribution upon INFα or EGF treatment (Song et al, 2011; Tang et al, 2007), we did not observe any variation on p300 localization in EC consequent to VEGF stimulation. Nevertheless, the formation of a complex between VEGFR2 and p300 was VEGF-dependent, and correlated with the increment of receptor acetylation, reaching the maximum after 15 minutes of growth factor stimulation. At this time point, the activity of the deacetylases associated with the receptor prevails, with the subsequent, progressive deacetylation of the receptor. An interplay between p300 and HDAC6 is

already known to exist in other settings. In particular, p300 has been involved in the attenuation of HDAC6 activity, by directly interacting with and acetylating the class II histone deacetylase (Han et al, 2009; Liu et al, 2012). Additionally, HDAC6 has been shown to hamper the interaction between p53 and p300, interfering with p300-mediated p53 acetylation (Ding et al, 2013). Thus, we could not rule out a further synergism between p300 and HDAC6 in regulating VEGFR2 acetylation, by indirectly affecting their activity one another. Indeed, VEGF might play a pivotal role in keeping the balance between the action of the abovementioned KAT and KDACs.

VEGFR2 acetylation prompts receptor activity

Different RTKs, including VEGFR2, undergo desensitization upon prolonged ligand stimulation, involving dephosphorylation, ligand-induced removal from the plasma membrane by endocytosis and subsequent lysosomal proteolysis of both ligand and receptor molecules (Duval et al, 2003; Ewan et al, 2006; Gampel et al, 2006; Singh et al, 2005; Singh et al, 2007). Consistent with the previous data regarding the kinetics of p300 and HDAC5 and -6 association with VEGFR2, the inhibition of KDAC activity preserves receptor phosphorylation over time after ligand stimulation and maintains its capacity to transduce intracellular signals for longer times in endothelial cells. In contrast, the mutant M5 failed to respond to VEGF stimuli, thus confirming all the previous observations. Therefore, it is likely that under VEGF-stimulating conditions, receptor acetylation contributes to the maintenance of VEGFR2 phosphorylation and activation. Consistently, the impaired

receptor activation of the non-acetylable M5 VEGFR2 was reflected by the mutant incapability to induce EC migration in response to VEGF stimuli.

Taken together, our findings support the conclusion that VEGFR2 acetylation favors and sustains receptor phosphorylation and activation in endothelial cells, and reinforces the concept that a strict crosstalk exists between post-translational modification by acetylation and other PTMs (Hunter, 2007; Yang & Seto, 2008a).

Protein acetylation in angiogenesis

Protein acetylation exerts a pleiotropic role in the control of EC function, having an impact on both gene expression and regulation of non-histone protein.

The effect of KDAC activity in the activation of VEGF-responsive genes in EC is undoubted. As already mentioned, VEGF directly controls the nuclear export of class II KDACs, therefore promoting angiogenesis through activation of several VEGF-responsive genes (Wang et al, 2008; Ha et al, 2008;Urbich et al, 2009). Consistently, the inhibition of KDACs activity by TSA has been found to decrease angiogenesis *in vitro* and *in vivo*, an effect that appears to be directly dependent on the negative modulation of VEGFR1, VEGFR2 and NP1 gene expression (Deroanne et al, 2002). In addition, the upregulation of HDAC1 by hypoxia leads to the induction of angiogenesis by increasing the expression of both VEGF and HIF-1α, a transcription factor pivotal in the angiogenic process (Kim et al, 2001). Of notice, under hypoxic condition, HDAC7 forms a complex in the

nucleus with p300 and HIF-1α, finally potentiating HIF-1α transcriptional activity (Kato et al, 2004).

The acetylation in EC of many non-histone proteins has also been reported to markedly influence the angiogenic process. Besides cytoplasmic cortactin (Kaluza et al, 2011; Zhang et al, 2007), the transmembrane protein Notch-1 is acetylated as well, a modification reversed by the deacetylase SIRT1. Notch-1 acetylation alters its intracellular domain turnover, thus increasing its stability and the duration of Notch signaling in EC (Guarani et al, 2011). HIF-1α itself has been found modified by acetylation, but the functional consequence of this modification is still under debate, since HDAC6 and HDAC4 inhibition has been demonstrated to target the transcription factor for proteasomal degradation (Geng et al, 2011; Jeong et al, 2002; Qian et al, 2006), whereas the acetylation at lysine 674, reversed by SIRT1, seems to be required for its transcriptional activation (Lim et al, 2011).

Concluding remarks

Being the KDAC expression frequently altered in solid tumor malignancies and given their global effect on histone modulation, it is not surprising that several KDAC inhibitors (KDACi) have recently emerged as potential therapeutic agents for the treatment of different human cancers (Ellis et al, 2009). The apparent contradiction between the use of KDACi for tumor angiogenesis inhibition and our novel finding linking VEGFR2 hyperacetylation with increased receptor activation might be partially explained by the fact that prolonged deacetylases

inhibition might have a major impact on genome expression, rather than fine tuning VEGFR2 activity.

Notably, a lack of therapeutic effects was however observed when a KDACi was used as a single therapeutic agent, while the combined treatment with other agents, such as Tyrosine Kinase Inhibitors (TKI), appeared to increase KDACi anti-tumor activity (Qiu et al, 2013; Thurn et al, 2011). In this respect, being also the phosphorylation of EGFR enhanced upon TSA treatment in cancer cells (Song et al, 2011; Zhou et al, 2006), a challenging issue would be to understand whether the acetylation-dependent misregulated activity of both VEGFR2 and EGFR might be responsible for the KDACi monotherapy failure in cancer therapy.

More generally, it remains a matter for interesting future investigation whether this novel mechanism of regulation of receptor function also applies to other VEGFRs and RTKs. In particular, the recent implication of KDACi in the control of cardiac hypertrophy (McKinsey, 2011) is worth of note. Preclinical results have reported a profound efficacy of these drugs in suppressing chronic cardiac hypertrophy and pathological cardiac fibrosis (Cao et al, 2011; Kee et al, 2006; Kong et al, 2006b; Liu et al, 2008a). The actual mechanisms by which KDACi exert their function in the cardiac tissue still need to be fully elucidated. Current interpretation of these findings implies a combination of deacetylase activity on both histone and non-histone targets, such as the sarcomere components (Gupta et al, 2008). Given the key role of VEGFR1 in cardiac function, it appears tempting to speculate that the activity of VEGFR1 might also be involved in the protective effects of KDACi.

Despite all the efforts that have been invested over the last years to unravel the complexity of the acetylome, we are still far from entirely clarifying the many roles of protein acetylation in the control of cellular function as well as its implication in the onset of disease. The identification of new acetylated protein targets offers indeed the possibility to focus on precise biochemical KDAC targets and to elucidate their mechanism of action. With this knowledge, more specific drugs could be developed, and, thereby, more suitable therapeutic treatments for chronic and non cancer-related pathologies might also be designed.

Chapter 5

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