Review Article

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Archives in Cancer Research
ISSN 2254-6081

2024
Vol.12 No.1:001

# What are the Different Cross Talks between HIF1-α, TGFβ, Integrin and ECM in Mediating Breast Cancer?

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**Received:** December 14, 2023 Manuscript No. IPACR-23-14367; **Editor assigned:** December 19, 2023, PreQC No. IPACR-23-14367 (PQ); **Reviewed:** January 02, 2024, QC No. IPACR-23-14367; **Revised:** January 10, 2024, Manuscript No. IPACR-23-14367 (R); **Published:** January 18, 2024

**Citation:** Baccoche S, Fourati N, Siala W, Jlidi R, Daoud J (2023) What are the Different Cross Talks between HIF1-α, TGFβ, Integrin and ECM in Mediating Breast Cancer? Archives Can Res, Vol.12 No.01: 001

## Abstract

Extracellular Matrices (ECM) serve as the molecular scaffold for cell adhesion, migration, proliferation and differentiation and as a repository of cytokines and molecular cues that determine cell polarization and tissue organization. The EMC alteration conducts to Integrin β3 regroupement, after its TGFβ induced overexpression; important tumorogenesis step that mediates an amplified integrin-Fak-Src signaling leading to aggressive tumor phenotype.

The upstream of these events is the accumulation of stress sensor protein, HIF-1α, which in involved in a constitutive active TGFβ overexpression, epigenetic landscape remodeling and sustaining a Smad2/3 signaling by supressing VHL expression. Together, allowed TGFβ late target genes expression such as: SNAIL, MMP, galectin, paxillin and integrinβ3; and at the same time TGFβ early genes repression such as: p21 and E-cadherin.

This review highlight, subsequent to stress condition (as hypoxia), different feedbacks, whose HIF-1α, TGFβ, ECM alteration and integrin β3 interplay to promote breast cancerogenesis.

**Keywords:** TGFβ; Integrins β1/β3; HIF-1α; ECM; SMADs; EMT; Menstrual phases swich; Src and breast cancerogenesisa

**Abbreviations:** FAK: Focal Adhesion Kinase; Src: Non-receptor tyrosine kinases; Grb2: Growth factor receptor-bound protein 2, contains one SH2 domain and two SH3 domains; TET: A family of Ten-Eleven Translocation (TET) methylcytosine dioxygenases; NF-κB: Nuclear Factor-kappa B; SOS: Son of Sevenless (SOS) a guanine nucleotide exchange factors; Ras: Rat sarcoma protein, small GTPase or small G-protein; Erk: Extracellular Signal-regulated Kinases (ERKs) or classical MAP kinases; PI3K: PhosphoInosotide 3 Kinase; AKT: Protein kinase B (PKB); Raf: Rapidly Accelerated Fibrosarcoma: Serine/Threonine-Specific Protein Kinase; Rac1: Member of Rho family GTPase. MEK: Mitogen-activated protein kinase kinase also known as MAP2K, MEK, MAPKK; ATF-2: Activating Transcription Factor-2; CREB: CAMP-Responsive Element-Binding Protein; Ets: E-twenty-six, Erythroblast Transformation Specific; Elk1: ETS Like-1 protein? P130Cas: Breast Cancer Anti-oestrogen Resistance 1 (BCAR1) is a member of the Cas (Crk-associated substrate) family of adaptor proteins, DOCK: Dedicator of Cytokinesis: DOCK family members contain a RhoGEF domain to function as guanine nucleotide exchange factors to promote GDP release and GTP binding to specific small GTPases of the Rho family. YAP: Yes-Associated Protein, transcription regulator by activating the transcription of genes involved in cell proliferation and suppressing apoptotic genes. STAT: Signal Transducer and Activator of Transcription? PTP: Protein Tyrosine Phosphatases, group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. PELP-1: Proline-, glutamic acid- and leucine-rich protein 1 (PELP1) also known as modulator of non-genomic activity of estrogen receptor (MNAR) and transcription factor HMX3

## Introduction

It's evident that menstrual phases switch is dependent of ovary hormones, oestrogen and progesterone. But when we looked for their precise effect, we found that they impact firstly on Extracellular Matrix (ECM) structure.

### The impact of ECM on menstrual phases switch

Vogel and Coll, divided the morphologic variation of the mammary gland, related to the menstrual cycle, into two major phases: Proliferative phase (early phase) and secretory or differentiated phase (late phase) which caracterized specifically the behaviour of the normal epithial breast cell whitin the menstrual cycle [1].

Extracellular Matrix (ECM) is a non-cellular three-dimensional macromolecular network composed of collagens, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins and several other glycoproteins. Matrix components bind each other as well as cell adhesion receptors forming a complex network into which cells reside in all tissues and organs. Cell surface receptors transduce signals into cells from ECM, which regulate diverse cellular functions, such as survival, growth, migration and differentiation and are vital for maintaining normal homeostasis. ECM is a highly dynamic structural network that continuously

undergoes remodeling mediated by several matrix-degrading enzymes during normal and pathological conditions. Deregulation of ECM composition and structure is associated with the development and progression of several pathologic conditions [2].

**ECM of the mammary epithelium gland:** The epithelium of the mammary gland is composed of luminal cells, which line the ducts and alveoli and myoepithelial cells which form the basal cell layer that surrounds luminal cells and contacts the basement membrane, a specialized form of ECM rich in collagen IV, fibronectin, laminins and vitronectin [3].

Fibronectin (FN) serves as the molecular scaffold leading to ECM contractibility. FN matrix assembly is a cell-mediated process in which soluble dimeric. FN is converted into a fibrillar network. Binding of cell surface integrin receptors to FN converts it to an active form, which promotes fibril formation through interactions with other cell-associated FN dimers (Figure 1) [4].

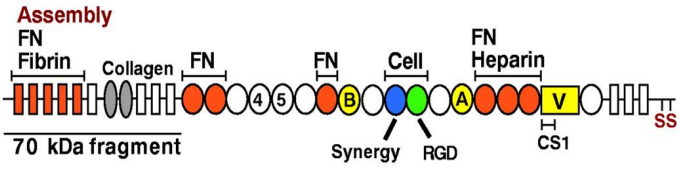


**Figure 1:** Domain structure of Fibronectin (FN).

FN consists of type I (rectangles), type II (ovals) and type III (circles) repeats. Sets of repeats constitute binding domains for fibrin, FN, collagen, cells and heparin, as indicated. The three alternatively spliced segments, EIIIA, EIIIB and V (or IIICS), are in yellow. The assembly domain and FN-binding sites are highlighted in orange. SS indicates the C-terminal cysteines that form the dimer. The RGD sequences (Arg-Gly-Asp) recognized by integrin.

FN fibrils are not static but are rearranged and recycled by cell movements, cell density and degradative processes [5]. This elasticity provides a dynamic and pliable ECM environment to accommodate cell activities within tissues and also provides the potential for regulation of fibril organization and availability of binding sites.

## Literature Review

### The impact of the cross talk between ERα, ECM and integrin β3 on early phase

**ERα promotes cell proliferation and inhibits the ECM effect:** ERα, specific molecule of the early phase, is a ligand-dependent transcription factor, across its transcriptional activating gene expression propriety, ERα targets a variety of mitotic genes. In fact, oestrogen, binding to ERα, induces its nuclear translocation. Once in the nucleus, ERα induces the expression of target genes, such as cyclin D1 and c-myc [6]. Also, among its target gene, the Matrix-Metalloproteinase (MMP) [7]. Matrix Metallopeptidases (MMPs), also known as matrix metalloproteinases or matrixins, are metalloproteinases that are calcium-dependent zinc-containing endopeptidases; other family members are adamalysins, serralysins and astacins [8]. Collectively, these enzymes are capable of degrading all kinds of extracellular matrix proteins, essentially the different type of collagen and fibronectin, but not vitronectin.

The deletion of fibronectin essentially in early phase, allows vibronectin, becoming the major component of ECM, to interact specifically to integrin β3 and initiating the integrin-FAK/Src signaling pathway [9,10].

**Integrin β3, a molecule of the early phase:** Integrin expression in the mammary epithelial cells is complicated as it is regulated spatially and temporally as the gland develops and through pregnancy, lactation and involution [11]. First, mammary epithelial cells are anchorage dependent and require cell-cell interactions or integrin-mediated attachment to the ECM; in the absence of such adhesion, a cell will not proliferate in response to growth factors and will succumb to a specialized form of apoptosis-anoikis-that occurs as a result of detachment from the E.C.M. Second, although integrin expression and activation can vary within the gland, a somewhat limited set of integrins are expressed-as assessed by immunohistochemistry-with certain integrins restricted to either the luminal or myoepithelial cells [12,13].

The β1 and β3 integrin subunits are expressed in epithelial cell of the gland, while the β4 subunit exhibits a more restricted expression pattern to myoepithelial cells [14]. Natural ligands of integrins are component of the ECM such as vitronectin, collagen or fibronectin. Thus, epithelial cells of the mammary gland are capable of assembling at least eight functional integrin receptors including two collagen receptors (α1β1 and α2β1), three laminin receptors (α3β1, α6β1 and α6β4) and three integrins (α5β1, αvβ1 and αvβ3) which recognize RGD sequences (Arg-Gly-Asp) present in certain ECM molecule: αvβ1/ fibronectin and αvβ3/vitronectin. The repertoire of integrins present at the membrane dictates therefore the extent to which a cell will behave on a specific matrix and respond to its environment. Once engaged with the ECM, integrins heterodimer and recruit various signaling and adaptor proteins to form focal adhesion complexes.

Integrin recognition of ECM proteins induces allosteric changes that allow the receptor to transduce this signal across the membrane, a process referred to as outside-in signaling. Once engaged with the ECM, integrins heterodimer recruits various signaling and adaptor proteins to form focal adhesion complexes.

**Integrin β3 involved in cell proliferation:** The integrin β3 activation by the heterodimerisation of αv, after interaction with specific EMC element, such as vibronectin, allows the binding of ctyplasmic integrin domain to paxillin which recruits the FAK protein (focal adhesion kinase) leading to its activation through releasing the catalytic domain from FERM domain (Four-point-one, Eszrin, Radixin, Moesi). Once the FERM domain inhibition was arised, the kinase domain is autophosphrylated at Y397 tyrosine residue which provinding to its hyprphosphrylation in certain tumoral circumstance (Figure 2). In fact, FAK is scaffolding kinase protein, once activated, it recruits different

types of proteins kinases whose their activation constitutes the integrin β3-FAK transdution signals (Figures 3 and 4).

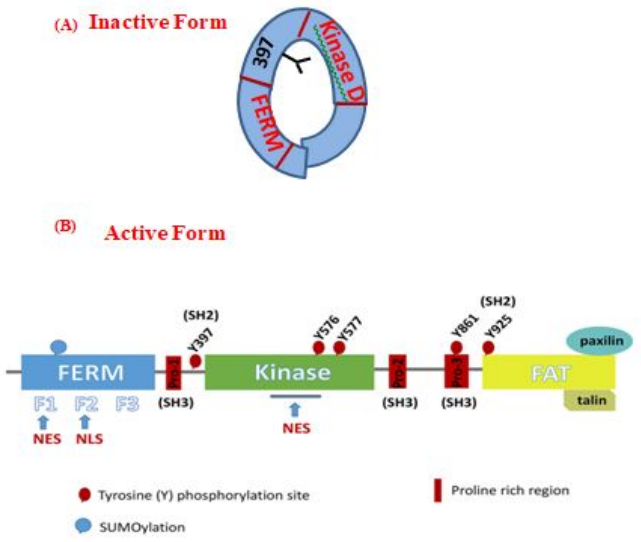


**Figure 2:** FAK is a scaffold kinase protein.

FERM is a kinase domain inhibiting. Binding with paxillin, the inhibition set is arised, providing kinase domain to autophosphrylation and potentially hyperphosphrylation at different sites of catalytic domain; of wich FAK transduces the integrin signaling pathway. Maximal FAK catalytic activation occurs after Src mediated phosphorylation of FAK within the kinase domain at Y576/577 and maximal Src activation occurs after FAK phosphorylation of Src within the kinase domain at Y419 [15].

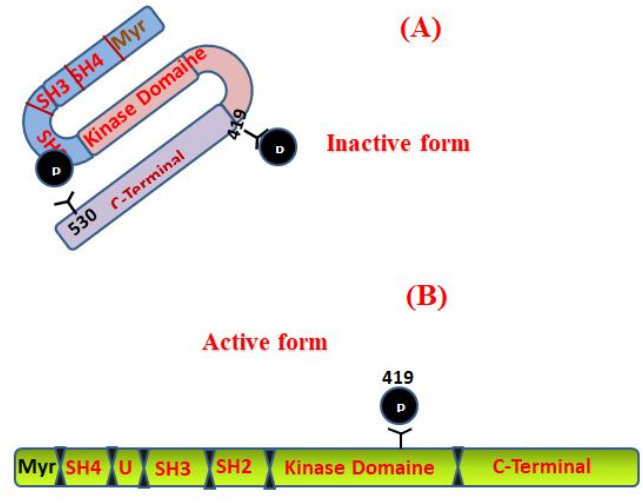


**Figure 3:** Schema of Src protein structure: In inactive form, where the C-terminal domain masks the kinase domain by binding the phosphorylated Tyrosine-530 with SH2 motif. In active form, after engagement the SH3 motif to proline-rich within activated form of FAK protein.

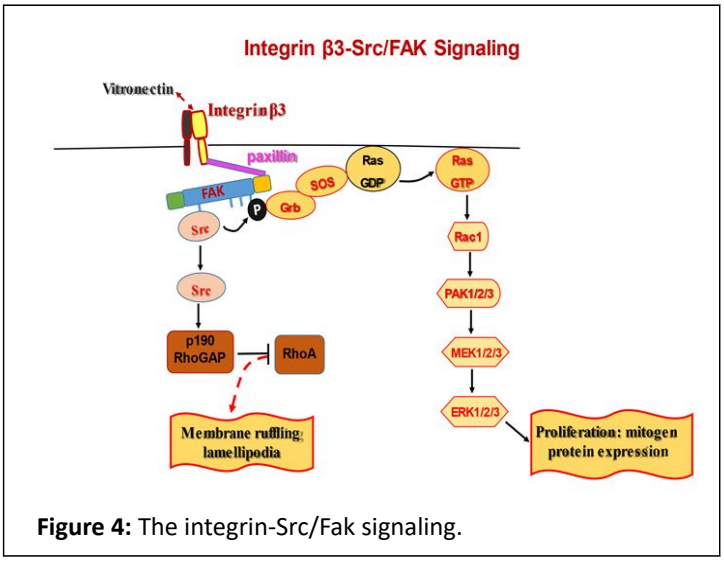


**Figure 4:** The integrin-Src/Fak signaling.

The activation of integrin β3 through its interaction to vitronectin allowed the paxillin to interact with the cytoplasmic tail domain of integrin β3. The binding FAT-domain of FAK to paxillin arises the catalytic domain from FERM domain. Once the FERM domain inhibition was arised, the kinase domain is autophosphrylated at Y39 which undertakes Src to initiate two downstream signals: Grb/Rac1 signaling leading to mitogen protein expression enhancing cell prolferation and Src/ p190RhoGAP signaling leading to membrane ruffling lamellipodia enhancing the morphogenesis.

**Integrin β3 involved in morphogenesis:** Cell motility is an essential cellular process involved in numerous physiological events including embryogenesis, wound healing, inflammation, tissue regeneration and mammalian gland morphogeneis.

Motility is largely dependent on localized actin polymerization at the leading edge of lamellipodia. The cell spreading and motility share a common requirement for dynamic remodeling of the actin cytoskeleton and focal adhesions through the activation of Rho-family GTPase. In fact, the integrin-triggered RhoA inhibition by p190RhoGAP enhances spreading and migration by regulating cell protrusion and polarity (Figure 3). The inhibition of RhoA activity that is induced transiently by adhesion was antagonized by expression of dominant negative p190RhoGAP [16].

## The impact of the cross talk between PR-TGFβ, ECM and integrin β1 on late phase

**TGFβ a clue element of the late phase:** The Transforming Growth Factor-β (TGF-β) superfamily is distinct from other cytokines owning to its more widespread and pleiotropic effects [17]. A plethora of cellular activities, including cell proliferation, differentiation, apoptosis, adhesion and migration, are controlled by TGF-β superfamily members in a context-dependent manner. Although cellular responses to TGF-β signaling are mainly induced *via* its transcriptional regulation of genes [18]. The TGF-β family consists of TGF-β1, 2 and 3 that have largely redundant fnctions. Each isoform contains nine highly conserved cysteine residues, mediating the formation of inter or intramolecular disulfide bonds that interlock two TGF-β polypeptides as a dimer. The dimeric TGF-β ligand associates

with the pro-region-derived Latency-Associated Peptide (LAP) and a Latent TGF-β Binding Protein (LTBP) and forms a Large Latent Complex (LLC), which is trapped in the Extracellular Matrix (ECM). Once activated, the dimeric TGF-β initiates signaling by promoting the assembly of two type I (TβRI) and two type II (TβRI) transmembrane receptors. Both of TβRI and TβRII possess Ser/Thr kinase activity in the cytoplasmic domain.

Ligand binding results in the tetramer receptor complex formation with two TβRI and two TβRII, in which TβRI is activated *via* phosphorylation of Thr and Ser residues. The phosphorylation-induced conformational change activates the TβRI kinase that relays the signal to the effector Smad proteins [19].

Upon activation of TβRI kinase activity, Smad2/3 is phosphorylated at two serine residues and subsequently is dissociated from the TβRI kinase domain, forming a trimeric Smad complex composed of two Smad2/3 and one Smad4. This Smad complex is then accumulated in the nucleus and acts as a transcription factor to regulate contextual expression of target genes through collaboration with diverse co-factors, as SP1.

In absence of stress conditions, the limit effect of TGFβ to differentiation phase provided by VHL protein which mediates Smad2/3 proteosome degradation; which attributed tumor suppressor propriety to either TGFβ and VHL (Figure 5) [20].

TGF-β receptor-I and II expression was higher in stromal cells than in epithelial cells during the secretory phase while no such variation was observed during the proliferative phase. Progesterone induces stromal decidualization indirectly, by enhancing the expression and secretion of TGF-β from epithelial cells [21]. The TGFβ involved in epithelial cell differentiation process through two ways:

**TGFβ remodels the ECM:** In fact, TGFβ increases the accumulation of the extracellular matrix proteins, fibronectin and type I collagen. The increase in fibronectin and type I collagen mRNA levels is an early response of cells to TGF-β [22].

**TGFβ induces p21 and E-cadherin expression:** TGFβ inhibits cell cycle progression, in part through up-regulation of gene expression of the p21WAF1/Cip1 (p21) cell cycle inhibitor. The intracellular effectors of TGFβ, smad3 and smad4, functionally cooperate with Sp1 to activate the human p21 promoter (Figure 5).

Smad proteins play important roles in regulation of the p21 gene by TGFβ and the functional cooperation of Smad proteins with Sp1 involves the physical interaction of these two types of transcription factors [23].

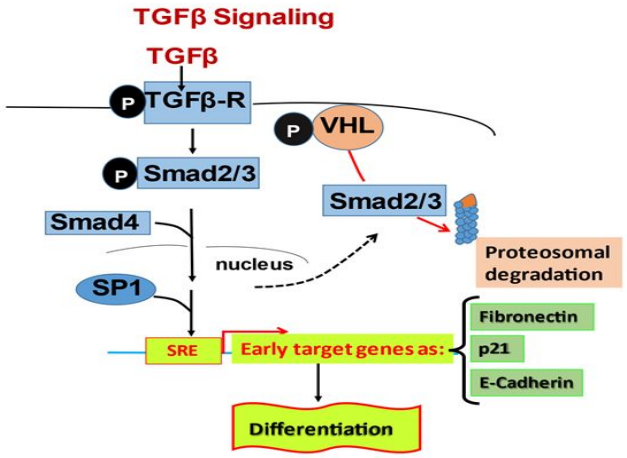


**Figure 5:** The TGFβ-Sma2/3 signaling.

Phosphorylation of TGF-receptor dimer is after TGFβ binding and initiates smad2/3 signaling, where smad2/3 are phosphorylated and recruits Smad4 to bind to SP1 transcription factor in nucleus. The complex: Smad2/3-Samd4-SP1, interacts with Smad-Reponsive-Element (SRE), promotor motif, to activate transcription of target early genes involved in late menstrual phase. The limit effect of this signling, on this differentiation phase, is provided by pVHL Sma2/3 proteosomal degradation.

In epithelial cells, E-cadherin-containing cell-to-cell junctions are often adjacent to actin-containing filaments of the cytoskeleton. The accumulation of E-cadherin-mediated adherens junctions, the membrane cytoskeleton and the Na/KATPase [24,25]. E-cadherin is involved in cell contact inhibition of epithelial mesenchymatous transition. Loss of function contributes to progression in cancer by increasing proliferation, invasion and/or metastasis. The establishment of spatial coordinates during the differentiation of polarized cells involves a positional cue from cadherins. E-cadherin is expressed mainly at the cell membrane after TGF-β stimulation [26].

## Integrinβ1/fibronectin interaction mediates stress fibers/actomyosinne contractibility, essential step to proliferation inhibition and cell differentiation induction

FN fibril elongation involves centripetal tensin-dependent translocation of α5β1-integrin from focal adhesions along actin stress fibers, forming fibrillar adhesions that promote conformational changes in soluble FN dimers and assembly of a fibrillar network [27]. During cell spreading, translocation of ligand-occupied α5β1 integrins away from focal contacts and along bundles of actin filaments generates ECM contacts. Which enhanced by TGFβ induction fibronectin secretion by fibfroblaste. Tensin is a primary cytoskeletal component of these ECM contacts and a novel dominant-negative inhibitor of tensin blocked ECM contact formation, integrin translocation and

fibronectin fibrillogenesis without affecting focal contacts. Whereas the vitronectin receptor αvβ3 remains within focal contacts, the fibronectin receptor α5β1 translocates from focal contacts into and along Extracellular Matrix (ECM) contacts [28].

As FN fibrils form on the outside of the cell, cytoplasmic domains of integrin receptors organize cytoplasmic proteins into functional complexes inside. Intracellular connections to the actin cytoskeletal network and stimulation of certain key intracellular signaling pathways are essential for FN-integrin interactions and propagation of FN fibril formation. Thus, assembly of native functional ECM depends on exquisite coordination between extracellular events and intracellular pathways (Figure 6).

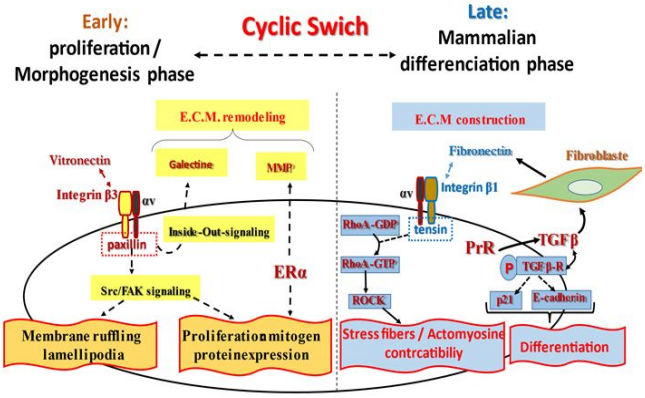


**Figure 6:** The different cross talks involved in the cycles swich of the epithelial breast cell during the menstrual cycle.

The entering into the early cycle is initiated by the estadiol-ERα binding which promotes the expression of ERα target genes launching eitheir the cell cycle and the alteration of the fibronectin-integrin β1 binding. This E.C.M. remodeling favors the interaction of vitronectin-integrin β3 launching ouside-in-integrin signaling leading to cell proliferation and membrane ruffling and lamllipoda which facilited by the E.C.M remodeling by the inside-out signaling. The late phase transition is promoted by the fibronectin synthesis by TGFβ; the subsequent inetgrinβ1-fibronectin interaction through the engagement of tensin specifically leads to activate the outside-in integrin β1 signaling guiding to stress fibers and actomyosine contractibility which in-turn participate in the E.C.M construction. TGFβ induces also other early target genes: p21 and E-cadherin allowing cell differentiation.

## The impact of the dysregulation of these different cross-talks on epithelial breast cell behaviour in stress conditions (hypoxia)

**Hif-1α, a stress sensor transcription factor:** Hypoxia-Inducible Factor-1α (HIF-1α) plays central roles in the hypoxia response. It is highly expressed in multiple cancers. In fact, HIF-1 is the term coined in 1993 by Gregg Semenza for a transcription complex bound to a Hypoxia-Responsive Enhancer (HRE) lying 3' to the erythropoietin gene. Since then, the key components of the HIF-1 system have been identified. HIF-1α overexpression has been associated with an unfavorable prognosis in most cancers, as it activates genes that play a role in promoting cancer metabolism, angiogenesis, invasion, maintenance of stem cell pools, cellular differentiation, genetic instability and metastasis.

The HIF family comprises 3 functional nonredundant a subunits, HIF-1α, -2α and -3α which form a heterodimer with the HIF-1β subunit. HIF-1α and HIF-2α are the most studied members of this family and have been thought to be largely overlapping in their proto-oncogenic function [29].

The β subunit is constitutively expressed and is also involved in xenobiotic responses. The α subunit protein is readily detectable in cells cultured under low oxygen conditions and is virtually undetectable in most cells under standard tissue culture conditions due to rapid proteasomal destruction. In hypoxia, the α subunit dimerises with a β subunit and translocates to the nucleus. After dimerization, HIF-1α/HIF-1β bind E-box motifs. As E-box is genome wide spread, make the HIF-1α a strong powerful gene regulatory networks involved in cell development, homeostasis and cellular behaviour. HIF-1α is target for posttranslational modifications (Figure 7). These modifications are related to metabolic stress, hypoxia, oxidative stress, pH and oncogenic signaling; making HIF-1α a principal sensor of stressful microenvironment.

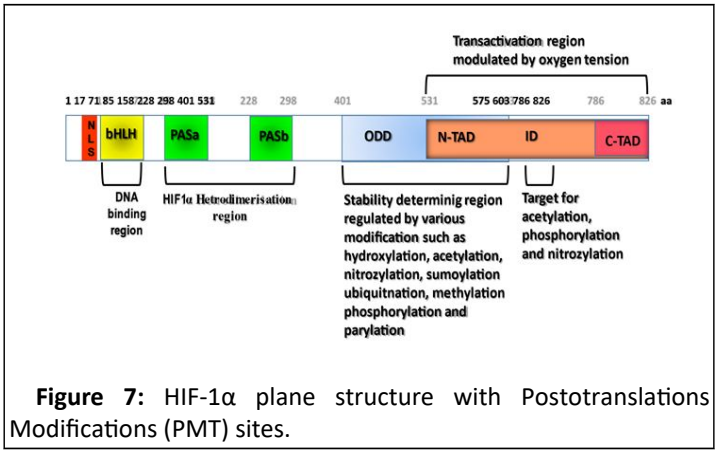


**Figure 7:** HIF-1α plane structure with Postotranslations Modifications (PMT) sites.

The HIF-1α subunit has two Transactivation Domains (TAD): $NH_2$-terminal (N-TAD) and COOH-terminal (C-TAD). These two domains are responsible for HIF-1α transcriptional activity. C-TAD interacts with co-activators such as CBP/p300 to modulate gene transcription of HIF-1α under hypoxia. N-TAD is involved in protein and DNA bindings. The Oxygen-Dependent Degradation Domain (ODDD) overlapping N-TAD in their structures. This ODDD domain is important in mediating $O_2$ regulation stability. Different types of PMT impacted on HIF-1α stability and on its protein and DNA binding activities. The HIF-1α belongs to bHLH-PAS protein family, because their structures are related to two nuclear proteins found in *Drosophila* (Per and Sim, PAS) which have basic-helix-loop-helix (bHLH) motif. In general, the PAS motifs are essential to allow heterodimer formation between HIF-1α and HIF-1β subunits and b-HLH is essential for binding to the HRE-DNA sequence on the target genes in the context of permissive chromatin.

## HIF-1α stabilized and accumulated in hypoxia

While hypoxia limits the proliferation of many cell types, some cancer cells, stem/progenitor cells and pulmonary vascular cells continue to grow and divide in low oxygen conditions. Hypoxia is an important environmental stimulus that causes genetic and metabolic reprogramming in cells to facilitate survival. This programmed response is mediated primarily through stabilization of Hypoxia-Inducible Factor 1α (HIF1α), a transcription factor that coordinates a shift in energy metabolism away from oxidative phosphorylation and toward glycolysis and lactate fermentation through the increased expression of Glucose Transporters (GLUT1), glycolytic enzymes, Lactate Dehydrogenase (LDHA) and pyruvate dehydrogenase kinase. In fact, in normaxi, HIF-1α is inactivated through hydroxylation by HIF-Prolyl Hydroxylases (HPHs) also referred to as Prolyl Hydroxylase Domain (PHD) proteins which form an evolutionarily conserved subfamily of dioxygenases that uses oxygen and 2-Oxoglutarate (2-OG) as co-substrates and iron and ascorbate as cofactors [30].

The hydroxylation of the HIFa protein causes interaction with the von Hippel Lindau (VHL) protein, a component of an E3 ubiquitin ligase complex. However, in the absence of oxygen the PHDs that use oxygen in the hydroxylation reaction are inactive and consequently HIF reaches a higher steady state level [31]. However, stability does not necessarily mean activity. In fact, once HIF-1α is stabilized, it undergoes acetylation at 709 lysine residue by p300 leading to its activation and enhacing its stabilization. After p300 was autoacetylated in the same context (Figure 8) [32].

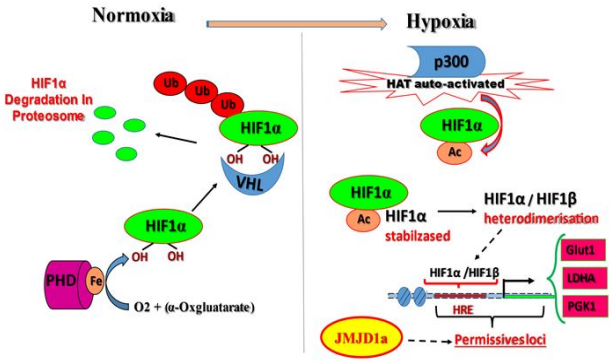


**Figure 8:** HIF-1α stability and activity regulation in normoxia and hypoxia conditions.

In normoxia, PHD hydroxylates HIF-1α with Fe and α-cetoglutarate as cofactors and leading to its degradation by proteasomes proteins. In hypoxia, HIF-1α escapes from its hydroxylation leading to its stabilisation but its transcriptional activaty is achieved by p300 whose HAT activity is autoactivated in hypoxia leading to the acetylation of HIF-1α. HIF-1α acetylated heterodimerisases with HIF-1β; the heterodimee can bind to its target consensus HRE sequences. But only permissive HREs were accecibles, which are in open chromatin that mediated by KDMs such as JMJD1a in this context.

## G9A could participate in HIF-1α stabilization and up regulation

G9A (KMT1C) methyltransferases is hypoxia-inducible at the post translational level, strikingly similar to the regulation of HIFa subunits (Figure 9) [33].

Given the hypoxia-inducibility of these KMTs, their extensive enzyme-substrate networks, as well as the involvement and regulation of their substrate proteins in hypoxia, the role of G9A in hypoxia may be larger than what is currently known. In the last decade, lysine methylation has been shown to participate in the complex combinatorial PTM code that regulates HIF1α function. HIF1α-K674 methylation was shown to occur in an oxygen-independent manner and is associated with impaired HIF-1α transcriptional activity.

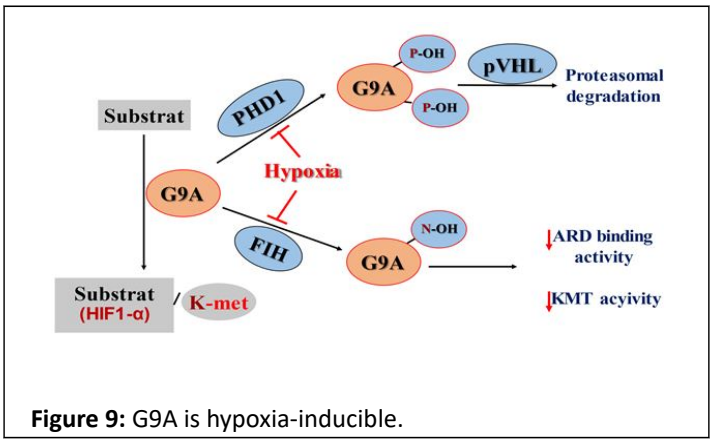


**Figure 9:** G9A is hypoxia-inducible.

G9A catalyzes lysine methylation of histone and non-histone substrates. Like HIFa regulation, PHD1 induces prolyl hydroxylation of G9A (P676 and P1207, occurring at higher stoichiometry at the former proline) and pVHL-mediated proteasomal degradation. FIH hydroxylated Ankyrin Repeat Domains (ARDs) of G9A-N779 become impaired in both the ability to bind mono- and dimethylated H3K9 products and the hydroxylation inhibits di- and trimethylation of H3K9.

**G9A could prepare an epigenetic landscape for HIF-1α gene expression:** A lot of data showed that under hypoxia stimulus the HIF-1α stabilization is associated with HIF-1α gene overexpression. In addition to the regulation of HIF-1α by protein stabilization, several *in vivo* studies showed increased levels of HIF-1α mRNA when mice, rats and ferrets were exposed to hypoxia. These suggest that G9A, stabilized under hypoxia, could be involved in displaying permissive chromatin in Hif-1α locus allowing transcription factor such as the NF-kB, activated during hypoxia, to trans activate HIF-1α gene leading to its over expression [34].

## HIF-1 accumulation is associated with glycolysis acceleration

Cancer cells undergo fundamental changes in their metabolism to support rapid growth, adapt to limited oxygen and nutrient resources and compete for these supplies with surrounding normal cells. The lack of energy, which can result from the absence of oxidative phosphorylation reactions, is compensated by the high rate of glycolysis overproducing

lactate, NAD+, polyolpathway and AGE. Glycolysis is the production of the building blocks required for cancer proliferation. In some cancer cells, a large proportion of glucose is used in the serine de novo synthesis pathway, wherein 3-phosphoglycerate is used by D-3-Phosphoglycerate Dehydrogenase (PHGDH). Serine is also converted to glycine and connected to the folic acid and methionine metabolism [35]. Thus, the serine biosynthesis pathway is also considered critical for sustaining the growth of cancer cells (Figure 10).

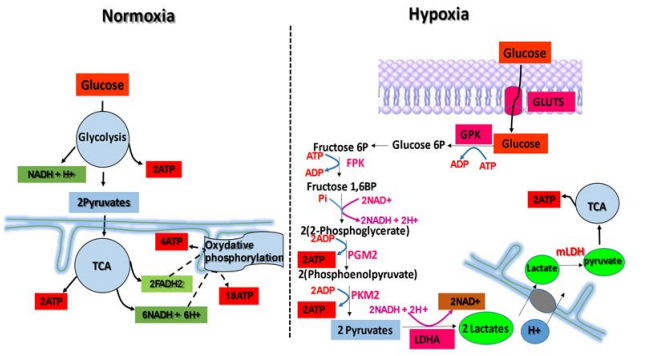


**Figure 10:** Metabolics sources of energy in normoxia and hypoxia conditions.

The absence of oxidative phosphorylation chain in hypoxia will be recompensed by glycolysis acceleration to approvision maximum of energy for cell adaptation in that stressfull condition (Figure 11).

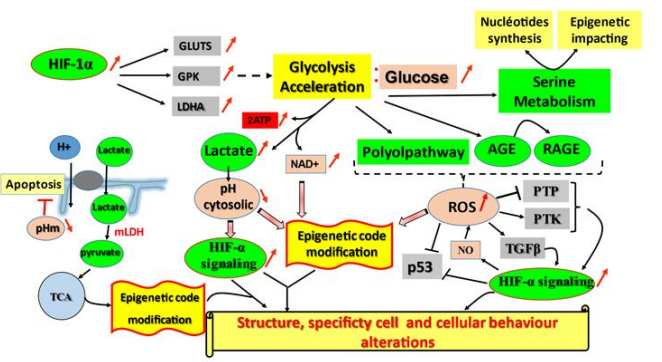


**Figure 11:** Cross talk between HIF1-α signaling and epigenetic landscape in mediating structure, specificity cell and cellular behaviour alterations.

HIF dependent glycolysis acceleration impacts on many different cell metabolisms and generates ROS, metabolic sub starts and cofactors with high availability; whose, each one can impact on the epigenetic landscape and enhance different types of HIF-1α signaling. Together affects the cell phenotype. These HIF-1α impacts are on non-specific type of cell. What be can the HIF1 impact on epithelial breast cell?

## HIF-1α, *via* its dioxygenase enzymes inactivation, inhibits VHL gene expression

**A conserved role of pVHL in the regulation of TGF-β/SMAD3 signaling:** Germline mutations of the VHL tumour suppressor gene cause von Hippel-Lindau (VHL) disease and are associated with a high risk of early onset and multicentric clear cell renal cell carcinoma. Somatic VHL gene mutations are found in 43%-57% of clear cell renal cell carcinoma cell lines and primary tumours. pVHL, as an E3 ligase for SMAD3 ubiquitination, directly interacts with conserved lysine and proline residues in the MH2 domain of SMAD3, triggering degradation. As a result, the level of pVHL expression negatively correlates with the expression and activity of SMAD3 in cells, Drosophila wing and patient tissues. In Drosophila, loss of pVHL leads to the up-regulation of TGF-β targets visible in a downward wing blade phenotype, which is rescued by inhibition of Smad activity. Drosophila pVHL expression exhibited ectopic veinlets and reduced wing growth in a similar manner as upon loss of TGF-β/SMAD signaling. Thus, our study demonstrates a conserved role of pVHL in the regulation of TGF-β/SMAD3 signaling in human cells and Drosophila wing development.

Many genes modified by promoter hypermethylation have classic tumor-suppressor function. Example is the VHL gene in renal cancer. 11%-19% of renal cell carcinoma cell lines and primary tumours demonstrate promoter hypermethylation and transcriptional silencing of the VHL gene.

**Epigenetic VHL gene inactivation:** The CpG islands of several tumor suppressor genes acquire cancer-specific methylation and many genes involved in familial forms of cancer undergo DNA methylation-associated silencing in sporadic cancers [36]. These changes are thought to contribute to uncontrolled proliferation and thus tumor development. The Ten Eleven Translocation protein 1 (TET1), a Fe/αcetoglutarate dioxygenase enzyme, involved in DNA demethylation. TET1 functions to regulate the lineage differentiation potential of embryonic stem cells [37]. TET1, as PHD, a Fe/αcetoglutarate dioxygenase enzyme, are vulnerables indirectly or directly to pH cytosolic variations.

In fact, the glycolytically overproduced lactate is associated with cytosolic acidification. A common feature of hypoxia, as well as the tumor and stem cell microenvironments, is metabolic acidosis [38]. One of the metabolic hallmarks of cancer is the activation of glycolysis and lactate production. Furthermore, lactate itself is used to further advantage by cancer cells. The conversion of pyruvate to lactate regenerates the NAD+ cofactor and contribute to cytosol acidification. The α-ketoglutarate (α-KG), an intermediate of the Tricarboxylic Acid (TCA) cycle, is an essential co-substrate for dioxygenases family due to its role in Fe (II) coordination in the catalytic center. In fact, cytosolic acidification moderately elevated 2-Hydroxyglutarate (2-HG) in cells and boosting endogenous substrate TCA cycle intermediate α-Ketoglutarate (α-KG) levels further stimulated this elevation. pH can independently drive elevated 2-HG levels, pH regulation of 2-HG may have important implications for 2-HG signaling in hypoxia. The downstream signaling roles of D-2-HG in cancer biology and of L-2-HG in hypoxia or stem cell biology are thought to be mediated by epigenetic effects (Figure 12) [39].

The acccumulation of 2-Hydroxyglutarate (2-HG) inhibited TET-dependent oxidation of 5 mC into 5 hmC in several cancers including gliomas and hematological malignancies [40].

Directly pH can interfere with the catalytic site of dioxgenase enzyme. In fact, the crystallographic studies on numerous members of the Fe(II)/2OG-dependent oxygenase superfamily have revealed two conserved structural features shared among its members. First, the Fe (II) is ligated by two his residues and (with the exception of the halogenases) a carboxylate from either a Glu or an Asp residue; this metal-binding motif is termed the 2-His-1- carboxylate facial triad. Second, the 2-His-1-carboxylate motif is located within a Double-Stranded-Helix (DSBH) fold, also known as the jelly-roll, cupin or jumonji C fold [41].

Thus, the cytosolic pH acid, in modifying the charges of the facial triad, according to pka of triad elements, alters the catalytic activity, inactivating directly the α-KG-dependent dioxygenase enzymes. Also, acidic pH is known to stabilize Hypoxia-Inducible Factor (HIF): Through neutralisation function of VHL by triggering its nucleolar sequestration and inhibition of HIF Prolyl Hydroxylases (PHD) by 2-HG. Acidosis is involved in HIF signaling feed back loop, that conducts to cell engagement to irreversible malignancy phenotype [42].

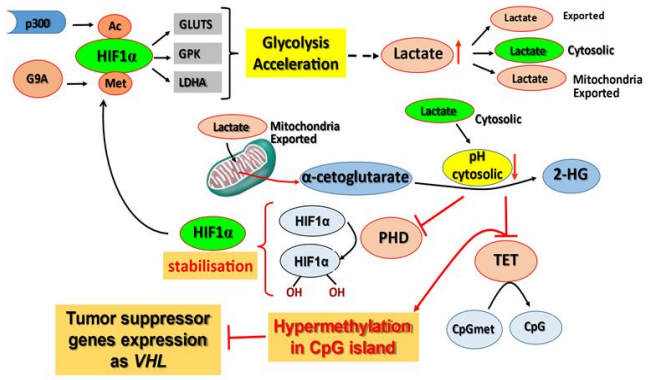


**Figure 12:** Epigenetic inactivation of VHL gene.

HIF-1α glycolysis acceleration induction conducts to pH cytosolic acid which promotes FeII/αcetoglutarate dioxygenase inactivation directly or through 2-HG production; causing an hypermethylation in CpG island, mainly in tumor suppressor gene promotors. Such as VHL.

## The impact of the feed-back between HIF-1α and ROS on TGFβ

ROS (Reactive Oxygen Species) are an intricate part of normal cellular physiology. In excess, however, ROS can damage all three major classes of macromolecules and compromise cell viability.

**Accelerated glycolysis and ROS production:** Through HIF-1α-glycolysis acceleration inducing, different derivative metabolisms can arise pathways releasing harmful reactive oxygen species (Figure 13).

The cell metabolism of glucose excess can product metabolic intermediates promoting unfavourable biochemical consequences. Such metabolic intermediates: (1) sorbitol/polyol and (2) hexosamine pathways; (3) augmented intracellular formation of AGEs and expression of the Receptor for AGE (RAGE). These products are usually sources of intracellular Reactive Oxygen Species (ROS) [43].

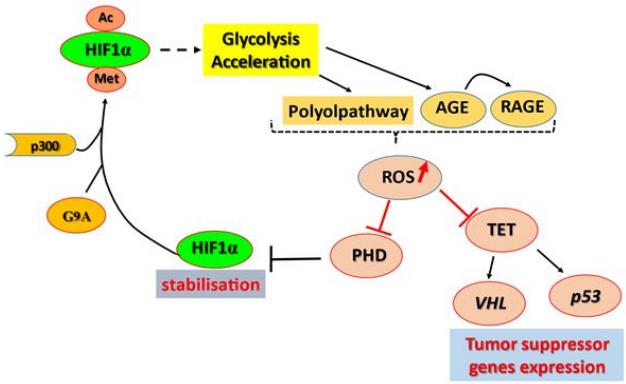


**Figure 13:** A constitutive feed back impact, in hypoxia, between HIF-1α and ROS on tumor suppressor gene.

## Feedback effect between NO synthesis and HIF-1α

NO radical is generated during the oxidation of L-arginine to L-citrulline by at least three different isoforms of the enzyme Nitric Oxide Synthase (NOS). However, NO generated by the inducible form of NO synthase (iNOS) has been implicated in many pathophysiological states. Hypoxia causes an increase in iNOS expression and that HIF-1 is essential for the hypoxic regulation of iNOS gene expression. HIF-1 or a closely related nuclear factor binds to the HIF-1 consensus sequence of the iNOS promoter [44]. In otherwise, accumulation of NO, by feedback, affect the stability and the level expression of HIF-1α.

In fact, NO transfer reactions between protein and peptide cysteines have been proposed to represent regulated signaling processes. Extensive biochemical and genetic data-including both mutational analyses of Cysteine (Cys) residues in over 30 proteins that are targets of NO and creation of plants and mice deficient in S-Nitrosothiol (SNO) metabolism-have led to the current understanding that most actions of NOSs are in fact conveyed by S-nitrosylation, the modification of protein Cys thiols by NO [45,46].

Importantly, endogenous formation of NO in RCC4 cells *via* inducible NO synthase elicited S-nitrosation of HIF-1 alpha leading to its stabilisation. All 15 free thiol groups found in human HIF-1 alpha are subjected to S-nitrosation, as the ractive Cys 800 [47]. NO can also inhibit PHD activity through nitrosylation of cysteine residues or by binding the catalytic iron. The ability of NO to bind the iron center of PHD appears to be affected by the concentration of 2-OG, because inhibition is only seen when 2-OG is unbound, indicating the metabolic status of the cell can alter the effects of NO on HIF-1α stability [48].

**NF-kB a knot of cross talk between ROS, HIF-1α and TGFβ expression:** NF-kB pathway signaling is a major regulator of many important biological processes, including cell proliferation, cell survival and elements of the immune response and is considered a drug target for a range of pathologies including inflammation and several kinds of cancer [49]. One of the NF-kB signalling pathway targets is HIF-1α. In addition to the regulation

of HIF-1α by protein stabilization, several *in vivo* studies showed increased levels of HIF-1α mRNA when mice, rats and ferrets were exposed to hypoxia [50].

In fact, HIF-1α encompassed in its promotor an NF-kB responsive element. Interestingly, hypoxia induced Nuclear Factor-kB (NFkB) nuclear translocation and activity. In line, expression of the NFκB subunits p50 and p65 enhanced HIF-1α mRNA levels, whereas blocking of NFkB by an inhibitor of nuclear factor-kB attenuated HIF-1α mRNA induction by hypoxia. In line, gel shift analysis and chromatin immunoprecipitation confirmed binding of p50 and p65 NFkB subunits to the HIF-1α promoter under hypoxia.

Reactive oxygen species directly link HIF-1α and NF-kB; ROS-mediated HIF-1α induction occurred on the transcriptional level and was dependent on NF-kB. (NEMO), also known as IkB Kinase γ (IKKγ), is a compelling but also a challenging target for drug discovery. In complex with the catalytic IKKα and IKKβ proteins, NEMO forms the active kinase IκB Kinase (IKK), which performs a key role in activating NF-kB pathway signaling. IKK phosphorylates IκB and triggers its proteolytic degradation, allowing transcription factor NF-kB to translocate to the nucleus where it modulates expression of biological effector genes.

Exposure to oxidizing conditions can induce NEMO homo-dimer formation through disulfide bond formation between Cys54-Cys54 and Cys347-Cys347. The disulfide-stabilized forms of NEMO are active and that covalent dimerization preorganizes the polypeptide in a conformation that confers relatively high affinity binding with IKKβ. Thus, ROS, which are product of HIF signalling, activate HIF-1 through PHD inhibition or S-nitrosation of HIF-1α and induce its expression through NF-kB DNA binding activation or NO radical accumulation. Marking an active loop promoting a harmful weight impact on cell destination.

## Feedback between ROS and TGF-β signaling

**Noxs, an important ROS producers, is target of TGFβ signaling:** NAPDH oxidases (Noxs) are a group of heme-containing transmembrane proteins and important ROS producers. Seven members have been identified in the Nox family: Nox1, Nox2, Nox3, Nox4, Nox5, Dual oxidase1 (Duox1) and Dual oxidase 2 (Duox2). TGF-β has been shown to induce the expression of several Nox enzymes including Nox1, Nox2 and Nox4 in different types of cells.

**TGFβ is up regulated by ROS products:** TGF-β is synthesized and secreted into the extracellular space as a large latent complex containing mature dimeric TGF-β bound to Latency-Associated Protein (LAP). Release of TGF-β from LAP, a process called latent TGF-β activation, is required for the binding of TGF-β to its receptors. An increase in ROS after radiation exposure leads to the activation of the TGF-β signaling pathway through the oxidation of cysteine residues of the Latency-Associated Peptide (LAP).

In fact, oxidation of LAP leads to a conformational change in LAP, which allows the release of TGF-β from the latent complex; it is known that ROS and TGF-β are interlinked by both feed forward and feedback mechanisms. Numerous studies have shown that ROS/RNS also up regulate TGF-β gene expression in various types of cells. TGF-β stimulation increases the basal level of ROS through several NADPH oxidases (NOXs), including NOX4, *via* the canonical smad2/3 signaling factors.

In cultured human alveolar epithelial cells, xanthine/xanthine oxidase derived ROS increased TGF-β1 production through a transcriptional mechanism whereas S-nitroso-N-acetyl-penicilamine generated RNS induced TGF-β1 through translational mechanisms. Nitrosylation of Cys162 in VHL prevents it from ubiquitinating of either HIF-1α and Smad2/3. Anette Teo et al. showed that, HIF-1 and Activator Protein 1 (AP-1) are involved in up regulation TGF-β expression, leading to activation of the transcription factor Smad3 through autocrine action.

We deduced that there are tree feedbacks: The one between HIF-1α and ROS, the second between TGFβ and ROS and the third is between HIF-1α/ROS and TGFβ; and these feedbacks are interconnected and continuously active (Figure 14).

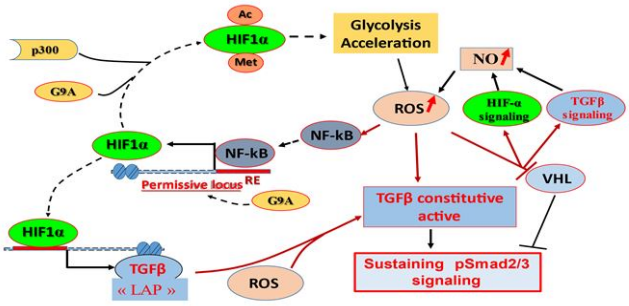


**Figure 14:** Different feed backs interconnected leading on the hand to constitutive HIF-1α signaling and on the other hand to sustaining smad2/3 signaling.

## The HIF-1α epigenetic impact can overlap TGFβ target genes (known as late genes) involved in proliferation, EMT and cell migration

Chromatin is not static but changes according to the regulatory cue including histone-modifying, histone modification-recognizing and histone modification-erasing proteins, so-called writer, reader and eraser proteins, respectively. The structure of chromatin determines the accessibility of DNA to transcriptional machinery; thus, it is closely related to gene activity.

The epigenetic as defined by Andrian bird, the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states. Subsequent to HIF-1α glycolysis acceleration induction, HIF-1α can remodels the epigenetic landscape overlapping Smad responsive element related to TGFβ target genes. These genes, called late genes, are involved in proliferation, Epithelia Mesenchymal Transition (EMT) and cell migration. Such as integrin β3, paxillin, snail, MMP galectin and EMT markers etc. HIF-1α can remodels the epigenetic landscape through many ways (Figure 15).

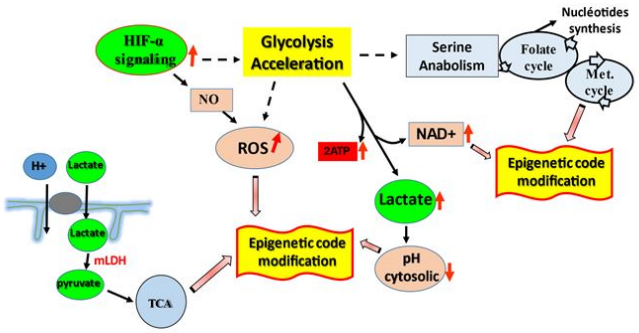


**Figure 15:** Different epigenetic impacts of HIF-1α signaling.

**Serine anabolism:** Consequently to high rate of glycolysis, glycolytic intermediate 3-phosphoglycerate could be converted to serine following three-step enzymatic reaction (Figure 16). The serine, glycine, one-carbon network generates carbon units that satisfy many metabolic demands including nucleotide precursors for anabolic metabolism, redox maintenance and substrates for methylation reactions that shape the epigenetic landscape. Serine anabolism is coupled by two cycles metabolism: Folate cycle associated with methionine cycle.

One metabolic intermediate of methionine cycle, S-adenosyl Methionine (SAM). The methionine cycle provides methyl units for a variety of reactions such as the methylation of proteins, DNA, RNA and lipids, allowing for the modulation of their biological functions (Figure 16).

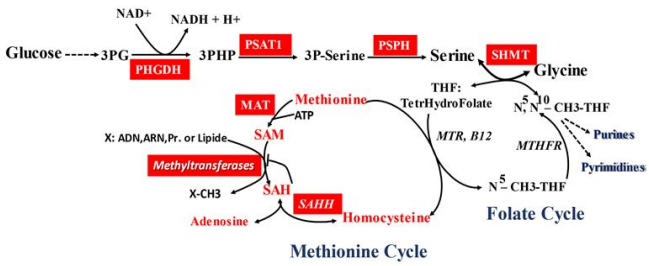


**Figure 16:** Serine anabolism associated with methionine cycle.

**NAD+:** NAD+ and its redox counterpart, NADH, are key metabolites influencing a large constellation of metabolic reactions. Nicotinamide Adenine Dinucleotide (NAD) is a co-enzyme that mediates redox reactions in various metabolic pathways, including glycolysis, Tricarboxylic Acid (TCA) cycle, oxidative phosphorylation and serine biosynthesis. There is an abundance of data from model systems and humans that age and conditions of metabolic stress challenge the NAD system in affected tissues.

Continuous replenishment of NAD promotes the proliferation and survival of fast-dividing cancer cells because elevated NAD levels enhance glycolysis *via* Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) and Lactate Dehydrogenase (LDH) that require NAD as a co-enzyme. PHGDH, a rate-limiting enzyme of the serine biosynthesis pathway, also uses NAD as a co-enzyme and the intracellular level of NAD is considered to be an important regulator for serine biosynthesis in cancer cells. Furthermore, NAD serves as a substrate for poly (ADP-ribose) polymerase (PARP) and sirtuins (NAD-dependent deacetylases) and mediates poly-ADP-ribosylation and deacetylation, respectively.

Therefore, the dynamic NAD+ and its metabolites levels, in response to diverse cellular stress and physiological stimuli, rewire biological processes *via* post-synthesis modification of fundamental biomolecules, including DNA, RNA and proteins.

**Acethyl-COA and epigenetic:** The lactate passively diffuses across the Mitochondrial Outer Membrane (MOM) into the Mitochondrial Intermembrane Space (MIS). An increase in lactate concentrations in the MIS facilitates conversion back into pyruvate catalysed by an isoform of Lactate Dehydrogenase (LDH) located in the mitochondria (mLDH). Pyruvate is then shuttled across the Mitochondrial Inner Membrane (MIM) into the matrix *via* a mitochondrial Monocarboxylate Transporter (mMCT), where it is oxidized. The two reactions were near of the equilibrium:

$$\text{Lactate} + \text{NAD+} \longleftrightarrow \text{Pyruvate} + \text{NADH+H+}$$

Thus, pyruvate is converted to Acetyl-coA, precursor of TCA, leading, in the absence of OXOPHOS, to accumulation of either acetyl-CoA, TCA intermediate metabolites and proton H+ intra-mitochondrial (Figure 17).

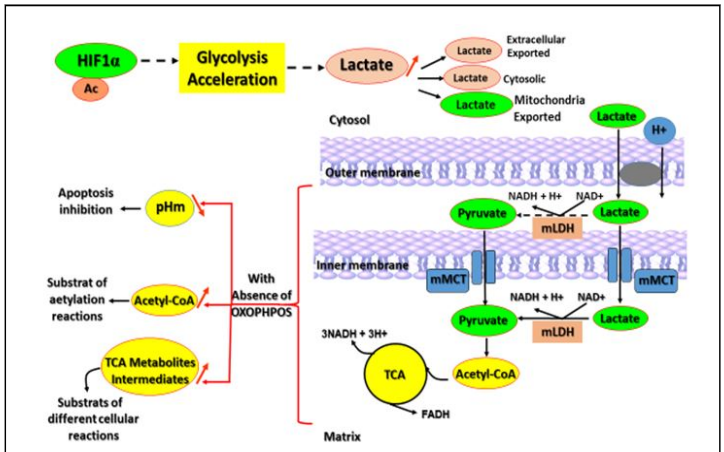


**Figure 17:** With concomitant of glycolysis acceleration and absence of oxophos, lactae is generated in high availability which will have three destnations: Extracellular export, cytosol or mitochondrial export.

This figure focuses on that lactate mitochondrial export leading to pH mitochondrial acid, affecting negatively the apoptosis and high level of either acetyl-CoA or TCA metabolites intermediates. There is a convention, that acetyl-CoA is not only a central intermediate in the oxidation of glucose to produce ATP, but also a precursor for the biosynthesis of numerous metabolites required to build a new cell, such as lipids and sterols.

It's now admitted that, metabolites downstream of acetyl-CoA could be signaling epigenetic modifications. In fact, Acetyl-CoA is the principal acetyl donor for acetylation reactions within the cell, essentially, which implicate Histone Acetyl Transferase

(HAT) relies on intracellular levels of acetyl-CoA, that stands as a prominent example of the interplay between metabolism and chromatin dynamics. The acetylation of such a protein might then enable it to perform some function required for growth or proliferation.

## pH cytosolic and epigenetic

**Through 2-Hydroxyglutarate (2-HG) production, pH impact on epigenetic landscape:** In fact, cytosolic acidification moderately elevated 2-Hydroxyglutarate (2-HG) in cells, and boosting endogenous substrate TCA cycle intermediate α-Ketoglutarate (α-KG) levels further stimulated this elevation. pH can independently drive elevated 2-HG levels, pH regulation of 2-HG may have important implications for 2-HG signaling in hypoxia.

The downstream signaling roles of D-2-HG in cancer biology and of L-2-HG in hypoxia or stem cell biology are thought to be mediated by epigenetic effects, because of competitive inhibition of the α-KG-dependent dioxygenase superfamily of enzymes. This includes the JmjC domain-containing histone demethylases, The TET 5-methylcytosine hydroxylases and the AlkB homolog family of DNA/RNA demethylases which can inhibit DNA and histone demethylating enzymes resulting in the glioma-CpG Island Phenotype (G-CIMP) and increased histone methylation marks (Figure 18).

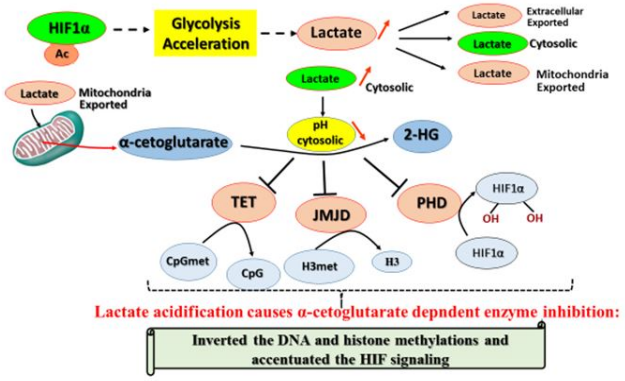


**Figure 18:** pH cytosolic acid modifies the epigenetic landscape by inactivation α-cetoglutarate dependent enzyme, directly or indirectly *via* 2-HG production.

Subsequent to lactate cytosolic availability, glycolysis acceleration dependent, pH cytosolic becames acide leading to 2-HG formation and inhibiting three enzymes dioxygenases: TET, JMJD and PHD, impacting thus on epigenetic landscape and accentuating HIF-1α signaling.

## ROS production and epigenetic

**ROS affect TET protein activity:** TET proteins contain a carboxyl-terminal core catalytic domain that comprises a conserved cysteine-rich domain and a Double Stranded β-Helix domain (DSBH). Within the DSBH domain, there are key catalytic residues that interact with Fe (II) and 2OG. Upon cofactor binding, molecular oxygen oxidizes Fe (II) in the catalytic pocket, thereby inducing the oxidative decarboxylation of 2OG and substrate oxidation. TET proteins also have an additional domain that potentially regulates their chromatin targeting. At the amino-terminal region, TET1 and TET3 have a DNA-binding domain called the CXXC domain, which is composed of two Cys4-type zinc finger motifs (Figure 19).

Redox regulation affects thiol posttranslational modification-altering molecule activity. In fact, in stressful condition, iterative of ROS production, the thiol group within these different domains of TET proteins could be undergone oxidative modifications These include sulfenic (SOH), Sulfinic ($SO_2H$) and Sulfonic ($SO_3H$) acids, disulfide bonds (PrSSPr) or nitrosothiols (SNO). Such modifications can alter automatically the TET protein activities such as TET-DNA-binding ability and α-KG-dependent dioxygenase activity.

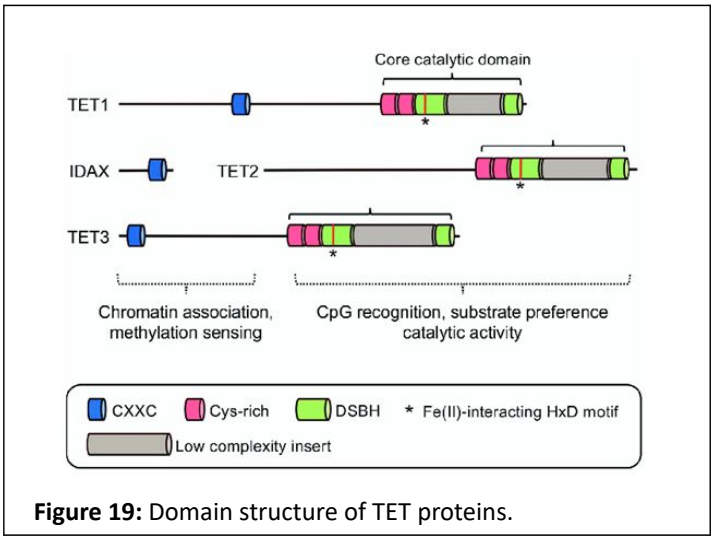


**Figure 19:** Domain structure of TET proteins.

The carboxyl-terminal core catalytic domain is highly conserved among all TET family members and consists of a DSBH domain and a Cysteine (Cys)-rich domain. The Cys-rich domain is comprised of two subdomains and modulates the chromatin targeting of TET proteins. The DSBH domain harbors key catalytic motifs, including the HxD motif, which interacts with Fe (II) and 2OG.

# Discussion

## The resultant of TGFβ overexpressed and constitutive active is: A large spectre of TGFβ permissive loci targets and sustaining smad2/3 signaling

The constitutive HIF-1α signaling, obtained through different feed-back signaling, leads to constitutive active TGFβ overexpression, enlarges the spectre of TGFβ target genes encompassed the genes involved in proliferation, EMT and cell migration. Such as: Integrin β3, MMPs, FAP, galectin, SNAILs (SNAI1, SNAI2, ZEB1 and TWIST1, genes central to EMT.

And at the same time, in this same context, the sustaining Smad2/3 signaling inhibits, in recruiting AP1 transcription factor, the early genes involved cell differentiation such as p21 and E-cadherin.

**Smad2/3 signaling recruits SP1 and AP1 to regulate TGFβ target genes:** Smads are the only substrate and signalling transducers of the activated TGF-β-receptors. Nevertheless, the positive and negative changes in the gene expression induced by TGF-β signalling cannot occur with the Smad proteins only. Thus Smad-dependent regulation of gene transcription is modulated by the interaction with transcriptional co-activators or co-repressors (Figure 20).

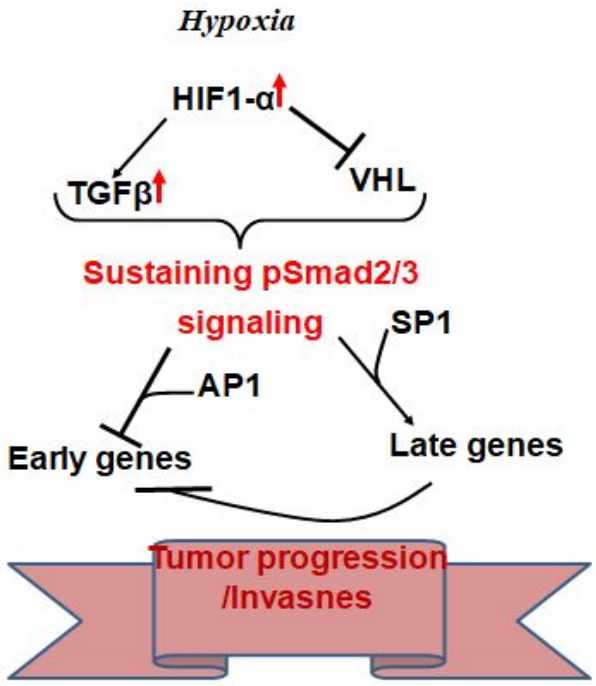


**Figure 20:** In hypoxia, the TGFβ overexpression and the VHL inhibition conduct *via* SMADs signaling to activate late gene expression in recruiting SP1 transcription factor and to repress early genes expression in recruiting AP1 transcription factor. Some lates genes which are under stress products participate in early genes repression.

**SP1 is involved in TGFβ late genes up regulation:** SP1 (Specificity Protein 1) is a well-known member of a family of transcription factors that also includes SP2, SP3 and SP4, which are implicated in an ample variety of essential biological processes and have been proven important in cell growth, differentiation, apoptosis and carcinogenesis.

SP1 acts as a co-activator of the smad-dependent transduction pathway. SP1 transcription factor is capable of potentiating TGF-β-induced promoter activity of induced target genes, through a physical interaction with smad3/smad4 complexes.

**AP1 is recruited to repress TGFβ early target genes:** A plethora of physiological and pathological stimuli induce and activate a group of DNA binding proteins that form AP-1 dimers. These proteins include the Jun, Fos and ATF subgroups of transcription factors. There is evidence that AP-1 proteins, mostly those that belong to the Jun group, control cell life and death through their ability to regulate the expression and function of cell cycle regulators such as Cyclin D1, p53, p21cip1/waf1, p19ARF and p16. Amongst the Jun proteins, cJun is unique in its ability to positively regulate cell proliferation through the repression of tumor suppressor gene expression and function, and induction of cyclin D1 transcription. These actions are antagonized by JunB, which up regulates tumor suppressor genes and represses cyclin D1. An especially important target for AP-1 effects on cell life and death is the tumor suppressor p53, whose expression as well as transcriptional activity, are modulated by AP-1 proteins.

The differential TGF-β regulation (transcription or repression) between its various target genes depends on the context. The remodelling of epigenetic landscape modulates the spectre of permissive loci, for TGFβ regulation. The epigenetic context, as well as recruitment of different cofactors of Smads, such as Sp1 or AP1, participate in this differentiation.

## Up regulated TGFβ target genes

**Integrin αVβ3:** Integrin αVβ3 was highly expressed in tumors than adjacent normal breast tissues. Over expression integrin αVβ3 in tumors than adjacent normal breast tissues was an indication of cancer progression with involvement of integrin signaling. Integrin β3 is induced by TGF-β in A549 cells (transformed cell line) to about 3.5 fold as compared to 1.8 fold in HPL1D (untransformed cell line).

**MMPS:** Matrix Metallopeptidases (MMPs), are capable of degrading the extracellular matrix proteins, essentially the different type of collagen and fibronectin, but not vitronectin. There is a considerable amount of evidence that Matrix Metalloproteinases (MMPs) play an important role at different steps of malignant tumor growth. The MMPs members family are overexpressed in breast cancer tissue compared to normal breast tissue. MMPs play an important role in the regulation of EMT. Early studies showed that MMP3 directly degraded the cell-cell adhesion receptor E-cadherin in mammary epithelial cells leading to Epithelial Mesenchymal Transition (EMT). EMT is a developmental process in which epithelial cells take on the characteristics of invasive mesenchymal cells and activation of EMT has been implicated in tumor progression. Recent findings have implicated MMPs as promoters and mediators of developmental and pathogenic EMT processes in the breast. Many of these MMPs have also been associated with EMT during cancer progression. However, a novel mechanism for MMP-induced EMT involving TGF-β has been reported.

TGF-β has been shown to upregulate a number of matrix Metalloproteinases (MMP) in epithelial cells, which may in turn play a role in developing metastatic potential in these cells.

**FAP:** Fibroblast Activation Protein (FAP), a member of the serine protease family, selectively expressed in the stromal fibroblasts associated with epithelial cancers, whereas with low or undetectable expression in the resting fibroblasts of normal adult tissues. FAP was abundantly expressed in the stroma across all breast cancer subtypes.

The Yixin Shi study further provided evidence that TGFβ mediates up regulation of FAP expression in U87 glioma cells through the canonical smad-dependent TGFβ signaling pathway, in which activated TGFβ receptor induces phosphorylation of

Smad (pSmad) and pSmad further directly activates transcription of the FAP gene by binding to its promoter.

**Galectin:** Galectins as matricellular molecules regulate integrin-mediated adhesion to the ECM. Galectins were discovered through their galactoside binding activity, in a quest to find proteins that decode complex cell-surface glycans. They were defined as a protein family based on conserved β-galactoside-binding sites found within their characteristic ~130 amino acid (aa) Carbohydrate Recognition Domains (CRDs).

Galectin-9 is one of the crucial proteins used by various types of cancer cells to suppress cytotoxic immune responses and thus, escape immune surveillance. Some cancer cells (Acute Myeloid Leukaemia (AML) and colorectal cancer) are capable of secreting this protein, while other cancer cells translocate galectin-9 onto the surface and use it to impair anti-cancer activities of cytotoxic lymphoid cells such as cytotoxic T lymphocytes and Natural Killer (NK) cells.

TGF-β1 expression, leading to activation of the transcription factor Smad3 through autocrine action, triggers upregulation of galectin-9 expression in both malignant (mainly in breast and colorectal cancer as well as Acute Myeloid Leukaemia (AML)) and embryonic cells. The effect, however, was not observed in mature non-transformed human cells.

**SNAIL:** Three SNAIL family proteins have been identified in vertebrates: Snail1 (Snail), Snail2 (Slug) and Snail3 (Smuc). All the family members encode transcriptional repressors and share a similar organization with a highly conserved C-terminal domain and bind to the E-box motif in target gene promoters.

The TGF-β pathway is a master regulator of EMT due to its ability to activate multiple transcriptional pathways that ultimately coordinate to drive a cell towards a mesenchymal phenotype. During TGF-β-induced EMT, SNAIL forms a transcriptional repressor complex with Smad3/4. This complex targets the adjacent E-boxes and Smad binding elements in genes encoding junction proteins such as E-cadherin.

Moreover, loss E-cadherin correlated with nuclear co-expression of snail1 and smad3/4 in a mouse model of breast carcinoma and at the invasive fronts of human breast cancer. In canonical TGF-β signaling, smad2/3 complexes with Smad4 to regulate target gene expression, including SNAI1, SNAI2, ZEB1 and TWIST1, genes central to EMT.

The TGF-β pathway is a master regulator of EMT due to its ability to activate multiple transcriptional pathways that ultimately coordinate to drive a cell towards a mesenchymal phenotype (Figure 21).

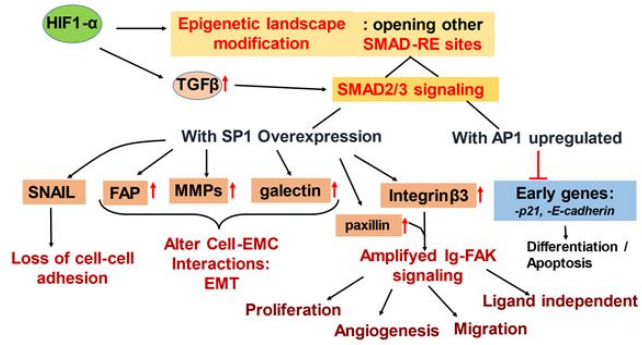


**Figure 21:** HIF-1α signaling conducts to TGFβ overexpression and in affecting epigenetic landscape, overlaps the SMAD-RE sites.

Sustaining smad2/3 signaling, thus, up regulates late genes in recruiting SP1 factor and inhibits at the same time early genes in recuiting AP1 factor. Subsequent to these signalings, the epithelial breast cell undergoes different hallmark of tumor behaviours leading to an aggressive tumor phenotype.

## *Via* integrin β3/FAK signaling, TGFβ promotes malignant tumor phenotype

Subsequent to proteases and galectin TGFβ induction, ECM undergone structural alterations; essential step of promoting outside-in signalings conducting to malignant cell tumor phenotype. In fact, ECM alteration allowed the integrins regroupement, the major element player of theses integrins is integrin β3. This integrins regroupement enhances different signaling pathways, arised from an amplified integrin-FAK-Src signaling. Where much number of FAK proteins were hyperphosphorylated downstream events of FAK/Src complex formation. FAK overexpression is widely observed in numerous tumor types and is used as a marker for invasion and metastasis. It is highly overexpressed and activated in basal-like breast cancer.

FAK is a scaffold protein; depending on this propriety, it activates, through its Kianse domain, a lot of factors that transduce cell proliferation, survival and motility. Thus, the FAK hyperphosphorylation, corresponding to the phosphorylation sites between YP397 and YP925, activates different signaling pathways (Figure 23). These phosphorylation sites serve as the binding site for the SH2 domains of other proteins such as Src, phosphatidylinositol-3-kinase (PI3K) and Grb2. The fully activated Src-FAK complex phosphorylates other proteins, including the adapter protein $p130^{cas}$ (Cas). Cas is phosphorylated on multiple tyrosine residues by Src, which forms binding sites for other signaling molecules bearing SH2 domains. Grb2 and $p130^{cas}$ (Cas) signalings are mediators of cell proliferation, survival, migration and angiogenesis.

**PI3K-AKT signaling:** FAK activates the PI3K/AKT-mTOR. Phosphorylation of PI3K activates AKT which regulates several downstream molecules, including mTOR. NF-kB, is main transcriptomic factor downstream of mTOR activation. As NF-kB

target genes: HIF-1α, iNOS, COX2, MMP2/9 and bcl2. This signaling pathway arises a constitutive active feed back between TGFβ and HIF1α (Figures 22 and 23).

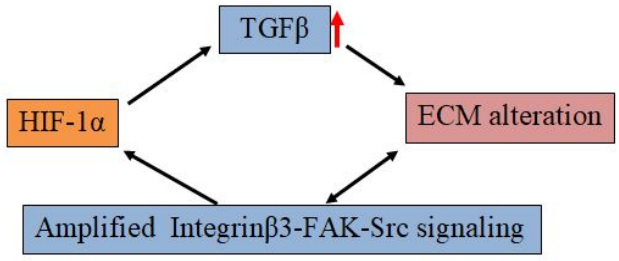


**Figure 22:** A constitutive feedback between the key elements of breast tumorogenesis process.

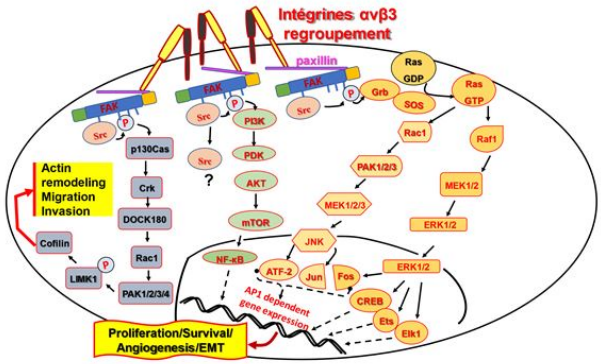


**Figure 23:** Different signaling pathways evoked downstream of amplified Integrin/FAK signaling.

**Src signaling:** The proto-oncogene c-Src (Src) is a nonreceptor tyrosine kinase whose expression and activity are correlated with advanced malignancy and poor prognosis in a variety of human cancers (Figure 24).

Breast cancer exhibits altered signal transduction pathways involving Src. Evidence of increased Src activity and protein expression levels has been found in human breast cancer tissue relative to normal tissue.

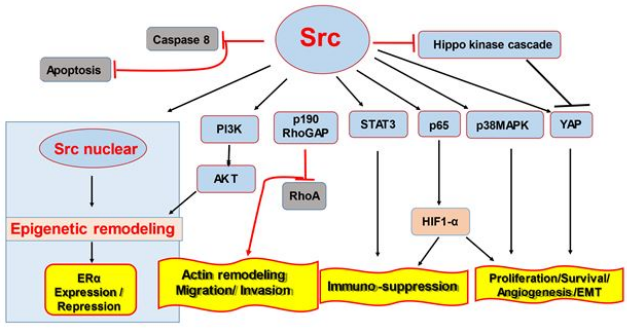


**Figure 24:** Different Src signaling pathways downstream of an amplified integrin-FAK-Src signaling.

**Src and motility:** An early event upon integrin engagement is the inactivation of the Rho GTPase in an Src-dependent manner.

The observation that Src can phosphorylate p190RhoGAP, resulting in a decrease in GTP-binding capacity of Rho, provided an early indication of a role for Src in the regulation of Rho. Src promotes tyrosine phosphorylation of p190RhoGAP and concomitantly, an activation of p190RhoGAP activity, which may be responsible for the observed reduction in RhoA activity upon cell adhesion.

**Src and apoptosis:** Caspase-8 is phosphorylated on amino acid Tyr380 residue in a Src dependent manner whose phosphorylation is very important for cell transformations and enhanced by hypoxic conditions. The phosphorylation of Tyr380 residue of Caspase-8 may present a molecule switch to turn its role from tumor suppressor to tumor activator. Caspase-8 represents the molecular switch that controls apoptosis, necroptosis and pyroptosis and prevents tissue damage during embryonic development and adulthood.

**Src *via* epigenetic regulates ER gene expression:** Beside the membrane cytoplasmic function, Src has been described in other subcellular compartments, as the nucleus; where it's involved in regulation of remodeling epigentic enzyme activity.

**Src *via* HDAC represses ER gene expression:** The tumor expression of Estrogen Receptors (ERs) is a very important marker for prognosis and a marker that is predictive of response to endocrine therapy. The loss of ER expression portends a poor prognosis. This repression can be a result of the epigenetic deacethylation mediated by histone deacethylase, HDAC or by hypermethylation of CpG islands within the ER-α promoter. Src was shown to phosphorylate and increase the activity of HDAC3. Src may activate a transcrptional repressor to associate with chromatin and/or alter its subcellular localisation.

DNMT1 has been found to interact physically with either HDAC1 or HDAC2 through its N-terminus, thereby forming a transcriptionally inactive chromatin structure that represses transcription. Thus, DNA methylation and histone deacetylation function through a common mechanistic pathway to repress transcription.

**Src *via* AKT regulates gene expression by targeting the DNMT, HDMT and HMT activities:** Histone phosphorylation that depends on amino acids in histone is a dynamic process. Histone phosphorylation occurs by altering many cellular processes, including the cell cycle, repair of DNA damage and cell apoptosis, so impaired regulation often leads to tumor formation. Hence, the kinases that regulate the phosphorylation of histones are always overexpressed in cancers.

In the same context, breast tumors over-express Src kinase and AKT, also known as protein kinase B, is downstream of Src effectors. AKT is linked to many of the cancer hallmarks and the metastatic cascade in breast cancer. Up regulation of AKT in cancer is associated with overall poor prognosis. AKT is responsible for the phosphorylation of various epigenetic regulators. Epigenetic regulators undergo extensive post-translational modifications, in particular, phosphorylation.

The Src/AKT pathway promotes transcriptional activation by reducing global genome DNA methylation. This signaling regulates DNMT1 through AKT-mediated phosphorylation at

S143. The Src/AKT pathway favours transcriptional activation through other means in addition to the reduction of H3K27me3. Promoter associated H3K4me3 is characteristic of transcriptionally active euchromatin and has been reported to be elevated in breast and colorectal cancers, which are commonly associated with Src-pathway activation. The Src/AKT pathway was recently shown to be essential in regulating H3K4me3 in *in vivo* models of AKT-activated breast cancer.

## ROS mediates constitutive active Src

The Src tyrosine kinase and some of the members of its family have been reported as redox regulated proteins. It has been reported that Src tyrosine kinase undergoes oxidation/activation in response to the formation of an S-S bond between Cys245 and Cys487, respectively located in the SH2 and in the kinase domain of the Src molecule (Figure 25).

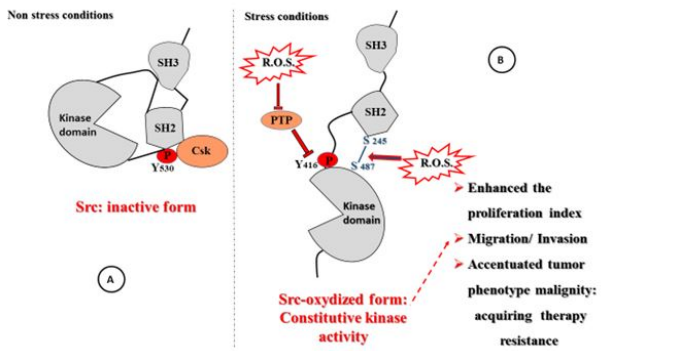


**Figure 25:** Src-oxydized form acquired a constitutive kinase activity.

ROS induce Cys-245-Cys487 disulfide bond promoting the release of Src tyrosine kinase (Csk) from the inhibitory tyrosine 530 residue of Src. This is followed by phosphorylation of the tyrosine 419 residue in the activation loop of the Src kinase domain.

Consequently, negative PTP oxidized/inhibited and activation loop Tyr hyperphosphorylated extend the Src-mediated cell proliferation to functional regulation of cytoskeletal rearrangement and the acquirement of a spread cell shape for anchorage dependent cells.

## Src mediates hormono-therapy resistance

**Src interferes with ERα and Her-2 signalings leading to ligand-independent phenotype:** ER, a ligand-dependent transcription factor, has been implicated in the progression of breast cancer; almost 70% of breast tumors are ER-positive at the time of early diagnosis. ERα stimulates the expression of a large number of protein involved in the regulation of the cell cycle across binding to the consensus binding site: ERE or ERS (Estrogen Responsive Element or Sites) in their promotors. Which suggests that the overexpression of ERα favours the overlapping binding sites, sharing some bases sequences homology with ERE, even if with less affinity for expression other oncogenes, whose involved in the proliferation/motility and antiapoptotic signals network. This context provides a further regulatory function of ERα.

Independent of its ligand E2, the ERα function is also regulated by phosphorylation through various kinase signaling pathways that will impact various ERα functions including chromatin interaction, coregulator recruitment and gene expression, as well impact on breast tumor growth and on breast cancer patient response to endocrine therapy.

AKT is recruited for ERα phosphorylation at specific sites, pathway named as the cytoplasmic ERα signaling pathway. The recruitment of PI3/AKT, from an amplified integrin-FAK-Src signaling and/or from Src signaling, overlaps to ERα phosphorylation, making ERα insensitive to its ligand described, as a ligand-independent phenotype which is responsible for the hormonal therapy resistance. By the same way, Src mediates the phosphorylation of Her-2, activating, thus, the signal outcome without its growth factor ligand, leading to ligand independent tumor phenotype, such as resistant to Trastuzumab therapy (Figure 26).

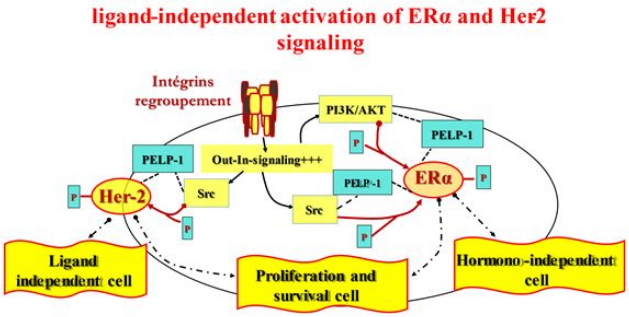


**Figure 26:** The PI3/AKT recruited through the amplifyed FAK trandusing integrin signal and the Src signaling play a major role in ligand independent phenotype. Gracefulness to the scafold protein PELP-1, the receptors Her-2 and ERα were phosphrylated independent of their ligand.

# Conclusion

Through this study some remarks were arised:

- TGFβ plays dual contradictory roles, in normal condition, TGFβ is involved in menstrual cyclic phases swich, in promoting epithelial cell differentiation; but in stress condition (as hypoxia) is involved in tumor promotion.
- In these two contexts, Smads are the only substrate and signalling transducers of the activated TGF-β-receptors. Nevertheless, the positive and negative changes in the gene expression induced by TGF-β signalling cannot occur with the Smad proteins only. Thus Smad-dependent regulation of gene transcription is modulated by the interaction with transcriptional co-activators (SP1) or co-repressors (Ap1).
- ECM remodeling constitutes essential step in menstrual cyclic phases swich; also its alteration constitutes an essential etiologic factors in tumorogeneis promotion.

- Also different feedbacks interconnected were highlighted, whose the efficient therapy must take in consideration each knot of this network.

## Author's Contribution

Sami Baccouche conceived of the manuscript, performed and wrote manuscript. The other authors read and approved the final manuscript.

## Ethics Approval and Consent to Participate

Not applicable here in this type of this study.

## Consent for Publication

Not applicable.

## Competing Interests

The authors declare no competing interests.

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