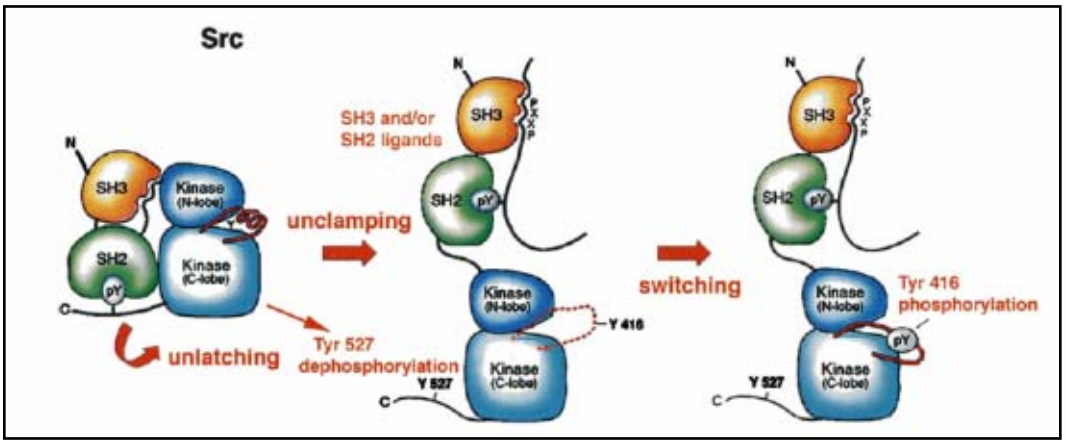
7. MOLECULAR DETERMINANTS FOR SRC-MEDIATED DGKα REGULATION

7.1. Src family of non-receptor tyrosine kinases [35]

Src and Src-family protein kinases (SFKs) are proto-oncogenes that play key roles in cell morphology, motility, proliferation, and survival. v-Src (a viral protein) is encoded by the avian cancer-causing oncogene of Rous sarcoma virus, while Src is encoded by a physiological gene, the first of the proto-oncogenes to be discovered. There are 11 members of the Src-family kinases in humans, ubiquitously expressed. These include Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm, and Yes, collectively identified as SFKs. In addition, the human genome contains a Yes pseudogene. Src, Fyn, and Yes are expressed in all cell types.

From the N- to C-terminus, Src contains a 14-carbon myristoyl group attached to an SH4 domain, a unique domain, an SH3 domain, an SH2 domain, an SH2-kinase linker, a protein tyrosine kinase domain (the SH1 domain), and a C-terminal regulatory segment. Myristoylation facilitates the attachment of Src to membranes. The seven terminal amino acids beginning with glycine are required for the myristoylation of Src and v-Src. One of the two most important regulatory phosphorylation sites in Src is tyrosine 527, six residues from the C-terminus. Under basal conditions *in vivo*, 90–95% of Src is phosphorylated at Tyr^{527}, and phosphotyrosine 527 binds intramolecularly with the Src SH2 domain. This intramolecular association stabilizes a restrained form of the enzyme. In the restrained enzyme, neither the SH2 nor the SH3 domain is readily accessible to external ligands. Tyr^{527} phosphorylation results from the action of other protein-tyrosine kinases including Csk and Chk. Src undergoes an intermolecular autophosphorylation at tyrosine 416; this residue is present in the activation loop, and its phosphorylation promotes kinase activity. The SH2 and SH3 domains have four important functions. First, they constrain the activity of the enzyme via intramolecular contacts. Second, proteins that contain SH2 or SH3 ligands can bind to the SH2 or SH3 domains of Src and attract them to specific cellular locations. Third, as a result of

displacing the intramolecular SH2 or SH3 domains, proteins can activate Src kinase activity. And fourth, proteins containing SH2 or SH3 ligands can enhance their ability to function as substrates for Src protein-tyrosine kinase. This structural design allows for Src regulation at multiple levels including competition between intramolecular and external ligands.



Mechanism of Src activation.
From Roskoski, BBRC 2004 (Ref. 35).

7.2. Interplay between Src and cell surface receptors [36,37]

Src family kinases are involved in signaling from many receptor tirosine kinases, including PDGF receptor (PDGF-R), epidermal growth factor receptor (EGF-R), fibroblast growth factor receptor (FGF-R), insulin-like growth factor-1 receptor (IGF-1R), hepatocyte growth factor receptor (HGF-R/Met), colony-stimulating factor-1 receptor (CSF-1R), stem cell factor receptor (SCF-R), muscle specific kinase (MuSK), and others. The requirement for SFKs in RTKs signal transduction pathways has been probed in different ways: by identification and mutation of SFKs-binding sites on RTKs, by introduction in cells of interfering mutants of SFKs, by pharmacological inhibition, and by cell lines derived from mice lacking the three ubiquitously expressed SFKs, Src, Fyn, and Yes (SYF cells).

The synergy between SFKs and RTKs is complex and bidirectional. SFKs have been shown to modulate the activity and signaling of RTKs, particularly EGF-R, PDGF-R, and IGF-R. In most cases, Src-dependent phosphorylation of EGF-R can positively regulate EGF-R signaling and cellular proliferation. SFKs also regulate PDGF-R by

phosphorylating critical tyrosines. Src-dependent tyrosine phosphorylation of IGF-1R increases the levels of phosphorylation of poly(Glu,Tyr) as well as IRS-1 *in vitro*, suggesting that Src-dependent regulation of IGF-1R enhances its catalytic activity. Additionally, SFKs regulate the turnover of RTKs, through regulation of RTKs endocytosis and ubiquitination. Cbl is a substrate of SFKs and, once phosphorylated, its conformation is changed and its interaction with the ubiquitin-conjugating enzyme UbcH7 is destabilized.

SFKs may participate in RTK-initiated cytoskeletal reorganization, migration, and survival. PDGF stimulation of fibroblasts results in translocation of Src to the cell periphery, in a process that requires small GTPases and an intact cytoskeleton. Acting through the tyrosine kinase Abl, SFKs are required for the generation of membrane ruffles in response to PDGF. However, PDGF-induced chemotaxis occurs normally in SYF cells. Following EGF stimulation of cells, the p190RhoGAP protein is phosphorylated on tyrosine in an SFK-dependent fashion. This phosphorylation acts to relieve an autoinhibitory conformation of p190, and is required, but not fully sufficient, for p190RhoGAP to exert its effects on actin stress fibres disassembly. Two recently characterized GTPase-binding proteins, the mouse diaphanous-related formins mDia1 and mDia2, associate with Src and play a role in cytoskeletal reorganization through Rho GTPases. Activated mDia1 and mDia2 induce signaling downstream of Rho GTPases along with Src. More recently, it was shown that the mDia-interacting protein DIP is phosphorylated by Src following EGF stimulation. Phosphorylated DIP in turn associates with p190RhoGAP and Vav2. As a result, DIP negatively regulates Rho and positively regulates Rac, in an SFKs-dependent manner.

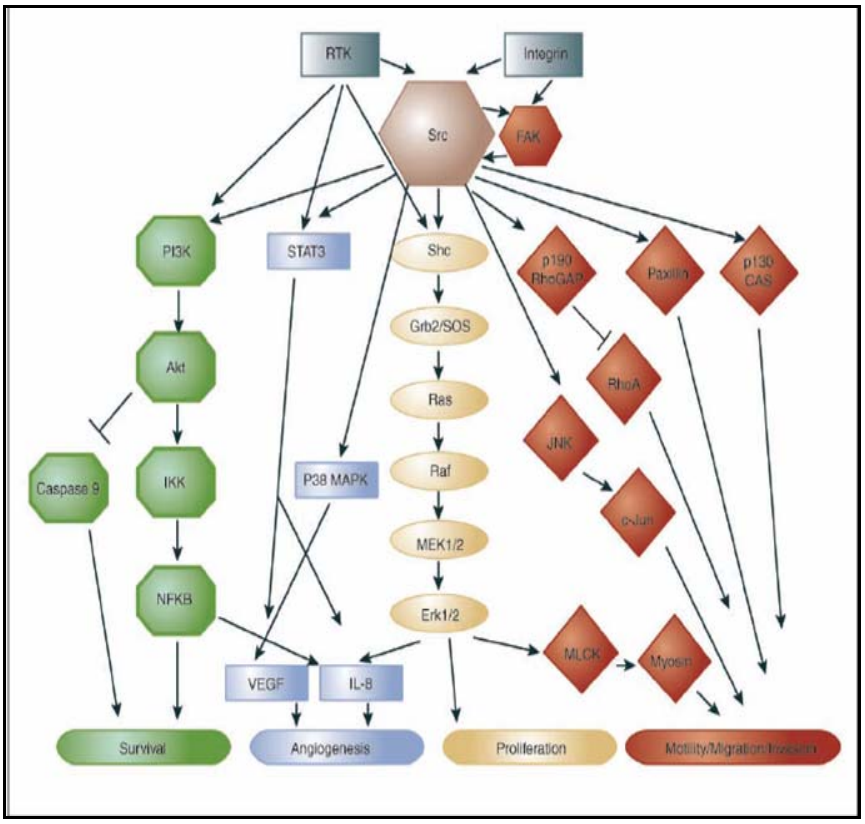
The effect of increased Src activity in cells appears to be pleiotropic. Cells with elevated Src kinase activity exhibit altered cell–cell adhesion, apoptosis, angiogenesis and tumor cell growth and invasion. Src-transformed cells are particularly characterized by a loss of actin organization and reduced cell–ECM adhesion, leading to cell rounding and occasional cell detachment. In addition, Src-mediated transformation of epithelial cells alters the known cuboidal cell shape to one resembling a fibroblast, in that process known as epithelial-to-mesenchymal transition (EMT).

Among the first clues indicating that Src was involved also in integrin signaling was the observation that v-Src localized to podosomes and to focal adhesions. In contrast to the localization of activated Src to focal adhesions, localization of non-activated Src is primarily perinuclear and in endosomes. The predominant localization of Src to these other compartments suggests that a small fraction of the total Src is localized to focal adhesions or that Src is transiently localized to these structures upon cell adhesion. A second line of evidence suggesting that Src functions in integrin signaling came from the identification of tyrosine-phosphorylated substrates in Src-transformed cells. These included FAK focal adhesion kinase, p130Cas and paxillin. In addition, each of these proteins was shown to associate with Src *in vivo*. Integrin signaling via Src and FAK leads to the activation of Rac and Cdc42 through p130Cas and Crk. It is plausible that the paxillin-Crk interaction is an alternative to p130Cas–Crk signaling pathway leading to Rac and/or Cdc42 activation.

Rho inhibition transiently reduces contractility, thereby allowing Rac- and Cdc42-mediated membrane spreading and ruffling in the early stages of cell migration. Two different mechanisms may function in inhibition of Rho activity upon cell adhesion. Cell adhesion promotes SFK-dependent 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. As previously mentioned, growth factor signaling leading to the inactivation of Rho via p190RhoGAP phosphorylation has also been reported and is mediated via a complex of Src, mDia and DIP, where Src phosphorylates DIP. Hence it is plausible that the phosphorylation of DIP by Src may play a critical role in the integrin-mediated suppression of Rho. It should be noted that DIP also links Src signaling to Rac activation, via recruitment of the Vav2 exchange factor. Therefore, DIP may be a central component in the coordination of integrin- and growth factor-mediated Rac and Rho signaling.

A second proposed mechanism for integrin-mediated regulation of Rho activity is via paxillin. Co-immunoprecipitation of p120RasGAP with paxillin is mediated by the two SH2 domains of p120RasGAP. The docking sites on paxillin are the phosphorylated tyrosine residues 31 and 118. Thus, tyrosine-phosphorylated paxillin competes with

p190RhoGAP for binding to p120RasGAP. Hence, cell adhesion-dependent tyrosine phosphorylation of paxillin may titrate p120RasGAP from p190RhoGAP. This results in an elevation in the activity of p190RhoGAP, and consequently a reduction in the level of Rho activity.



Integrins and RTKs signaling converge on Src. From Summy and Gullik, Clin. Cancer Res. 2006.

7.3. First clues to a Src-dependent Dgkα regulation

Src-dependent regulation of Dgkα downstream of HGF was firstly investigated by Cutrupi *et al.* [19]. HGF was shown to activate Dgkα, but no Dgk activity was found to associate with HGF receptor following HGF stimulation. On the other hand, Sugimoto *et al.* reported an enhanced synthesis of PA in v-Src-transformed fibroblasts, while a Dgk activity was found to co-purify with v-Src [38]. Since HGF activates Src in several cell lines, Cutrupi *et al.* hypothesized that Src might mediate HGF-induced activation of Dgkα. Indeed, a R59949-sensitive Dgk activity is found to co-precipitate with Src from HGF-stimulated PAE and GTL16 cells lysates, and in COS cells both endogenous and transfected Dgkα are found to co-precipitate with Src. Moreover, HGF-induced activation

of Dgkα is inhibited by either PP1 treatment or by expression of a kinase-inactive dominant-negative mutant of Src. In addition, Src is shown to be able to activate Dgkα *in vitro*. Finally, co-expression of Dgkα and Src in COS cells induces tyrosine phosphorylation of Dgkα, suggesting that it may be tyrosine phosphorylated, albeit this phosphorylation does not appear to correlate with enzymatic activation.

The second clue for a role of Src in Dgkα regulation came from the work of Fukunaka-Takenaka *et al.* [39]. Their work moves from the study of the molecular target of D-α-tocopherol treatment, which prevents early changes of diabetic renal dysfunctions. In diabetes, hyperglycemia is responsible for renal disfunction, neuropathy and retinopathy through, among other mechanisms, the increase of diacylglycerol, resulting in the activation of PKCβ. D-α-tocopherol acts normalizing both DAG levels and PKC activity by promoting Dgk ativity. D-α-tocopherol treatment induces specifically the translocation of Dgk isoform α from the cytosol to the plasma membrane in DDT1-MF2 cells and concomitantly it induces Dgkα activation, tough indirectly. In fact, D-α-tocopherol fails in inducing activation of the purified Dgkα *in vitro*. Cell treatment with the generic tyrosine kinase inhibitor genistein and the Src-family specific inhibitors PP2 and herbymicin prevents both Dgkα activation and translocation induced by D-α-tocopherol, suggesting that in this system too activation of Dgkα is mediated by Src kinase activity. The $Y^{334}F$ mutant of Dgkα does not translocates upon D-α-tocopherol treatment and can not be phosphorylated upon Src overexpression, differently from the wild-type Dgkα. Moreover, D-α-tocopherol-induced tyrosine phosphorylation of Dgkα increases upon over-expression of Src, while expression of kinase-dead Src abolishes Dgkα phosphorylation. Overall, this findings confirm that Src is able to phosphorylate Dgkα and mediates its activation in different contexts, and identify the tyrosine 334 of Dgkα as the phosphorylation site substrate for Src kinase activity.