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Tumor Suppressor Role of Abl Kinase in CrkI-Transformed Fibroblasts and Its Implication in Human Cancer Cells

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University of Connecticut [2015]

Since the discovery of the first oncogenic Crk adaptor protein, v-Crk in 1988, various studies have been conducted to understand the signaling mechanism of Crk proteins. In our previous knockdown study to investigate the importance of several CrkI SH3-binding effectors in the tumorigenesis of CrkI, we were surprised to learn that Abl kinase negatively regulates CrkI transformation in NIH3T3 cells. Because constitutively active Abl kinase, in the form of BCR-ABL, is famously known to cause chronic myelogenous leukemia (CML) in humans, we initially expected a similar tumor promoting role for Abl in CrkI transformation. However, downregulated Abl kinase (through shRNA knockdown or inhibitory drug, Imatinib) enhances the anchorage-independent growth of CrkI-transformed NIH3T3 cells. This raises our concern over the popularity of Imatinib, a potent Abl inhibitor, in various combination therapies for cancer treatment. Here, we have identified Dok1 as the Abl-phosphorylated substrate that mediates a previously unknown negative regulatory pathway for CrkI tumorigenesis. In particular, we showed that the phosphorylation of two tyrosine residues in Dok1, Y295 and Y361, are essential for limiting the anchorage independent growth of CrkI-transformed cells. Through both *in vitro* and *in vivo* SH2-interaction assays and subsequent p120RasGAP shRNA knockdown, we then confirmed p120RasGAP as the next effector for the Abl/Dok1 pathway. Although we did not find any increased Ras or Erk activation in the whole cell lysates of control, CrkI-transformed and the subsequent Abl or Dok1 knockdown cell lines, we were able to detect the dysregulation of localized Ras activation during the spreading of CrkI-overexpressing cells via live-cell imaging and a Förster resonance energy transfer (FRET) biosensor for activated Ras. In addition, our preliminary data suggest that the Abl/Dok1 pathway is most likely regulating CrkI transformation

through small GTPases and its inhibition further induces Jnk activation. And finally, we demonstrated that Imatinib promotes the anchorage-independent growth of SF268, a human glioblastoma-derived cell line but more investigations are needed to understand the underlying mechanism(s).

Tumor Suppressor Role of Abl Kinase in CrkI-Transformed Fibroblasts and Its Implication in Human Cancer Cells

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Tumor Suppressor Role of Abl Kinase in CrkI-Transformed Fibroblasts and Its Implication in Human Cancer Cells

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Chapter 1

Introduction

Crk adaptor proteins

The first Crk protein was discovered from the chicken tumor virus isolate #10 (CT10), a type of avian sarcoma virus that induces tumor formation in chickens (Burk et al., 1941; Claude et al., 1947; Mayer et al., 1988). Besides the 521 bp Long Terminal Repeats (LTR) and other sequences essential for viral replication, the open reading frame (ORF) encodes a 440 amino acid long polypeptide. Of the 440 amino acids, 208 at the N-terminus encode the normal group specific antigen (gag) (which encodes major structural proteins of the virion) and the remaining 232 amino acids make up a protein derived from a cellular proto-oncogene. Interestingly, this oncoprotein lacks any catalytic domain and only consists of Src homology 2 (SH2) and Src homology 3 (SH3) domains (these domains are further described below). It was subsequently named v-Crk (CT10 regulator of kinase) because it significantly increases the tyrosine phosphorylations of endogenous proteins in infected chicken embryonic fibroblasts (CEF) (Mayer et al., 1988).

Following the discovery of Crk in the retrovirus, the first two cellular homologs were later identified and named CrkI and CrkII (Matsuda et al., 1992). In the human genome, the Crk gene is located at chromosome 17p13.3 and three alternative splice forms have been discovered so far (Figure 1.1). The identified cDNAs of CrkI and CrkII are detectable in various human cell lines at different levels and they encode proteins with 204 and 304 amino acids respectively (which correspond to 27 kDa and 34 kDa molecular weight). The study went on to overexpress CrkI and CrkII in 3Y1 rat fibroblasts, and found that CrkI but not CrkII was able to transform the rat fibroblasts. This observation marked the very first functional distinction between the two alternative spliced forms. Structurally, CrkII has an additional SH3 domain at the C-terminus and tyrosine residues that were later reported to be regulatory sites at position 221 and 251 (see below). Together with v-Crk, CrkI was later studied extensively for its tumorigenicity and the

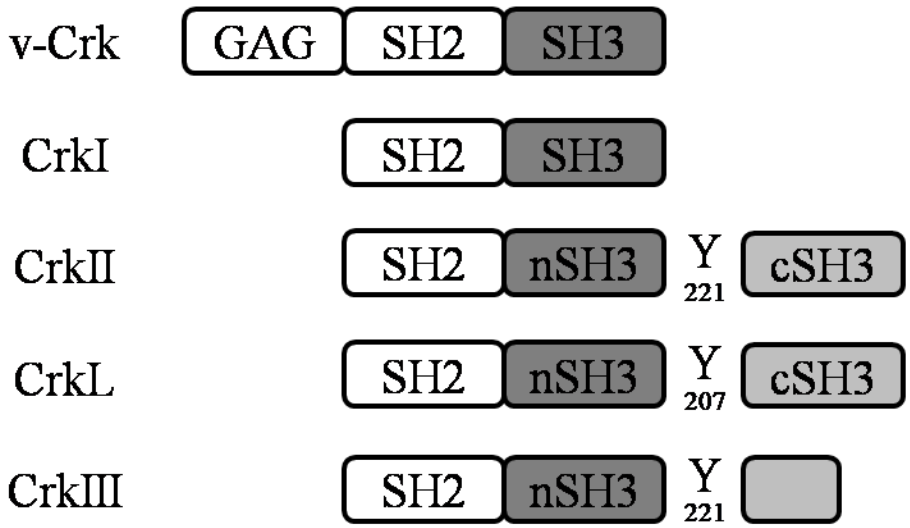

Figure 1.1: The basic domain structure of Crk family adaptor proteins.
All Crk family adaptor proteins are comprised of SH2 and SH3 domains.
GAG, retroviral group specific antigen; SH2, Src Homology 2; SH3, Src Homology; nSH3, N-terminal SH3; cSH3, C-terminal SH3; 3, Y, tyrosine (major site of regulatory phosphorylation).

majority of our current understanding on the transforming activity of Crk proteins is based on these two isoforms.

Then in 1993, ten Hoeve *et al.* reported the discovery of yet another Crk adaptor protein and named it Crk-L (Crk-like) for its resemblance to the previously described CrkII (~60% overall homology) (ten Hoeve et al., 1993). The Crk-L gene is located at 22q11 in the human genome and its ORF encodes a 303 amino acid polypeptide. As mentioned, its domain structure mirrors that of CrkII (Figure 1.1) and was later classified as a new member to the Crk family proteins. Expression-wise, unlike CrkI and CrkII, which are generally ubiquitous with higher expression in brain, lung, and kidney, CrkL is most abundant in adult hematopoietic tissues but relatively low in epithelial tissues (de Jong et al., 1995).

Apart from the better known CrkI and CrkII, a study later identified a third spliced form of the Crk gene, CrkIII, using the cloning of receptor targets (CORT) technique (Prosser et al., 2003). They described CrkIII as structurally similar to CrkII, but with a truncated cSH3 domain and total protein size of 283 amino acids. Unfortunately, we currently have limited understanding about this relatively new member of the Crk proteins.

Basic structure and regulation of Crk proteins

v-Crk and CrkI form the most basic structure of Crk proteins with one each of SH2 and SH3 domains. And being the ones with the simplest domain structures and also the more oncogenic Crk proteins, both v-Crk and CrkI have been studied intensively for their signaling in inducing cell transformation. In contrast, studies on CrkII and CrkL are generally focused on their cSH3 domain, their regulatory tyrosine residues as well as their roles in Abl-associated signaling pathways. Overall, the general interests in Crk proteins are i) how they differ from each other ii) how they interact with other signaling proteins and iii) how they are associated with human cancer. We will first focus on the basic structure of Crk proteins (with emphasis on the more

complex CrkII and CrkL self-regulatory tyrosines) and later address the other two aspects in the following sections.

All Crk family proteins consist of a single SH2 domain and at least one SH3 domain, which govern their functions. The Crk SH2 domain binds to phosphorylated tyrosine with the consensus sequence pYxxP (Songyang et al., 1993) whereas the SH3 (or nSH3 in the case of CrkII and CrkL) domain binds proline-rich motifs of the polyproline II (PPII) subtype with a consensus sequence of PxxPxK (Knudsen et al., 1994). However, the cSH3 domains found in CrkII and CrkL are atypical, and thus incapable to bind conventional PPII motifs (Jankowski et al., 2012; Muralidharan et al., 2006). To date, the cSH3 domain has no known effector proteins and is thought to play some roles in the self-regulatory mechanisms specific to CrkII and CrkL.

The presence of an inter-SH3 tyrosine residue in CrkII (Y221) has been long regarded to be the reason for its weaker transforming activity. When phosphorylated, it forms an intra-molecular interaction with its own SH2 domain (Figure 1.2) (Rosen et al., 1995), preventing the binding of other targets. Indeed, a structural study conducted by Kobashigawa *et al.* using NMR spectroscopy to analyze the solution structures of CrkI, CrkII and CrkII with phosphorylated tyrosine 221 (p221CrkII) found compelling evidence that phosphorylated Y221 in CrkII represses the binding ability of its SH3 domain in addition to the SH2. They first determined the radii of gyration (R_g) through small-angle X-ray scattering (SAXS) assay and concluded that even without phosphorylation, CrkII has a more compact and globular structure as oppose to the highly flexible and elongated CrkI. Then, by analyzing and modeling the hydrophobic peptide sequences in CrkII's inter-SH3 core (ISC) and its SH domains, they found that the nSH3 domain was partially occluded by its own inter-SH3 linker region. In addition, subsequent affinity studies confirmed that CrkII has a relatively lower binding affinity toward a Sos-derived peptide than CrkI, and that various mutations at hydrophobic residues in the ISC were able to alter its binding

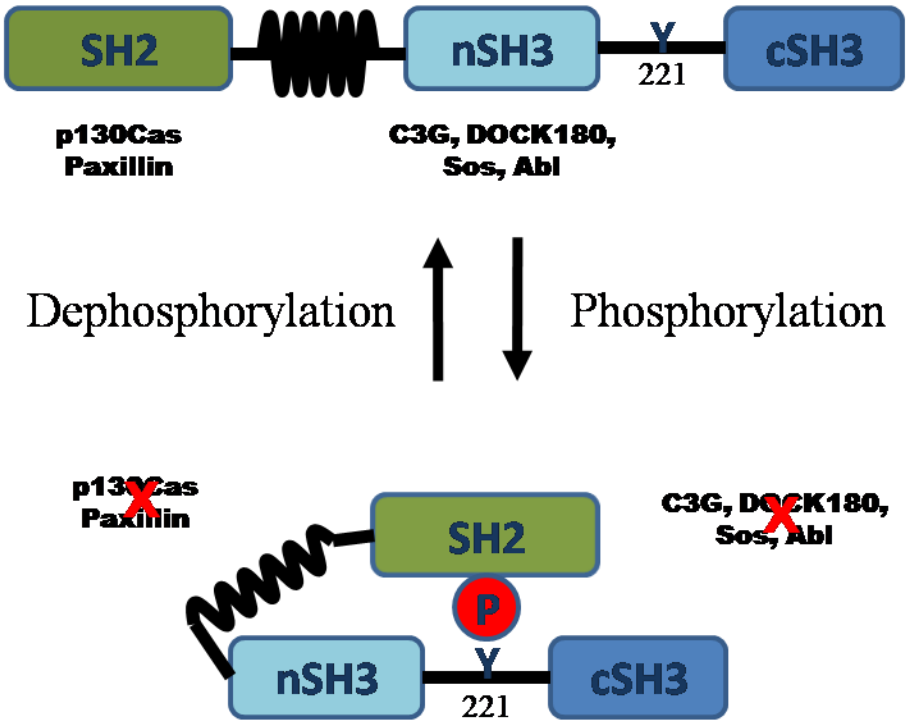

Figure 1.2: Self-inhibiting mechanism of tyrosine 221 in CrkII.
CrkII has been purported to regulate itself via the binding of its own SH2 domain to the phosphorylated tyrosine 221 and this prevents the binding of both its SH2 and SH3 binding targets.

affinity (Kobashigawa et al., 2007). Overall, the findings in this study correlated well with the conclusion of Matsuda *et al.* in 1992 that CrkII is less active than CrkI in ligand binding, and thus it is incapable of transforming 3Y1 rat fibroblasts.

A more recent study identified the regulatory significance of another tyrosine residue in CrkII, Y251 (Sriram et al., 2011). Their results suggested that the phosphorylation of tyrosine 251 promotes the trans-activation of Abl kinase via its binding to the Abl SH2 domain. They proposed that the phosphorylated Y251 now serves as a binding site for other unidentified SH2/PTB (phospho-tyrosine binding site) domains. And together with Y221, they regulate CrkII differently from the alternative spliced form, CrkI, which lacks these sites. Since Y221 and Y251 control the switch between SH2- and cSH3-dependent structural regulation, they are likely to affect proteins localization, complexes that they form, and ultimately the activation of different signaling pathways.

Despite being a close paralog of CrkII, several studies have so far indicated some differences for CrkL. For instance, BCR-ABL fusion kinase (see below) prefers CrkL over CrkII as its primary Crk family substrate (Sattler and Salgia, 1998; Senechal et al., 1996). Moreover, CrkL has been reported to be indispensible for the aberrant activity of BCR-ABL in Chronic Myelogenous Leukemia (CML) (Luo et al., 2008; Seo et al., 2010). A recent study carried out by Jankowski *et al.* suggested three detailed observations for the interaction of CrkL with BCR-ABL through their structural and affinity experiments (Jankowski et al., 2012). First, the lack of a proline-enriched DE flexible loop (which, in CrkII, binds the SH3 domain of Abl kinase (Donaldson et al., 2002)) within its SH2 domain implied that alternative binding modes, with unknown affinity exist in CrkL. In addition, unlike the occluded nSH3 domain in CrkII, CrkL has completely accessible nSH3 domain. And finally, phosphorylation of Y207 in CrkL only affects the affinity of its SH2 domain towards other target protein and has no effect on the nSH3 domain.

Interestingly, the study also identified a polar contact-mediated interaction between CrkL's SH2 and nSH3 domains that is highly unique to CrkL.

Once again, these findings highlighted the discrepancies among the various Crk proteins. Apparently, each Crk protein has both distinctive and overlapping regulations and functions in facilitating signal transduction.

SH2 and SH3 domains

The SH2 domain was first described in 1986 as an ~100 amino acid sequence in the vFps/Fes oncoprotein essential for cell transformation (Sadowski et al., 1986). It was named after its sequence homology to Src family proteins and since then, it has been found in more than 100 human proteins (Liu et al., 2006). A study in 1993 revealed that the binding affinity of SH2 domains to phosphorylated tyrosine (pY) relies on the amino acid sequence surrounding the pY (Songyang et al., 1993). The interaction of SH2 domains is highly specific and proteins within the same family will often share a preferred pY motif. For instance, the Src family kinases bind pY with pYEEI motif whereas the Phosphoinositide 3 kinases (PI3Ks) bind pY with pYΦxM motif (with Φ being a hydrophobic residue and x being any amino acid) (Songyang et al., 1993). Structural and biochemical studies showed that the positively charged binding pocket on the SH2 domain uses an Arginine (R) residue (within the conserved FLVR motif) (Hidaka et al., 1991; Koch et al., 1991) to bind the pY of target ligands and usually, the amino acid sequence of +1 to +6 residues from the pY dictates the specificity (Pawson, 1995; Pawson et al., 2001; Songyang et al., 1993). Several proteins like the PI3K, Shp2 and Zap-70 consist of tandem SH2 domains and the additional SH2 domain is thought to confer higher affinity and specificity (Ottinger et al., 1998). In general, the SH2 domain serves as molecular switch that modulates signal transduction. To date, SH2 domain has been reported to mediate recruitment of downstream effectors to activated receptor tyrosine kinases (RTKs) (eg. Grb2 (Li et al., 1993)), to maintain dormancy of

protein (eg. CrkII (Kobashigawa et al., 2007)) and to enhance and facilitate kinase activity (eg. Abl (Filippakopoulos et al., 2008)).

The SH3 domain was first noted as a ~60 amino acid sequence found in Crk and phospholipase Cγ (PLCγ) (Mayer et al., 1988; Stahl et al., 1988). It was later shown to bind a proline-rich motif, PxxP (Ren et al., 1993). The interactive surface of SH3domain is rather flat, hydrophobic and consist of three shallow pockets; it binds the proline-rich, polyproline-2 (PPII) helical motif, a region of sequence that has also been known to interact with WW domains and profilin (Kay et al., 2000; Zarrinpar and Lim, 2000). Unlike the SH2 domain, the SH3 domain has relatively weak affinity and low specificity (Mayer, 2001). The proline rich motif, ΦPxΦP (also known as PxxP) can be further categorized into two classes: class I with the general consensus +xΦPΦxΦP and class II with the general consensus ΦPxΦx+ (where Φ represents a hydrophobic residue, x any amino acid and, + a basic amino acid, usually Arg or Lys) (Feng et al., 1994; Lim et al., 1994; Mayer and Eck, 1995). Due to the low specificity of SH3 domains, it has been proposed that additional mechanisms like protein compartmentalization also help regulate the interaction of SH3 domain. Interestingly, the plasticity of SH3 interactions (due to the low specificity) provides the advantage of rapid interaction remodeling and therefore, more fluidity toward its signal transduction.

Crk binding partners, functionalities and oncogenic signaling

As adaptor proteins, Crk proteins nucleate protein complexes through their SH2 and SH3 domains. By connecting downstream effectors to upstream proteins with phosphorylated tyrosine, Crk proteins have been reported to regulate activities such as cell proliferation, cell adhesion, migration, apoptosis, and immune cell responses to pathogens (Birge et al., 2009; Feller, 2001).

The SH2 and SH3 domains of Crk proteins bind the consensus motif pYxxP and PxxPxK, respectively and to date, numerous binding proteins have been identified. The Crk SH2 domain

primarily binds paxillin (Birge et al., 1993), p130Cas (Cas, Crk-associated substrate) (Sakai et al., 1994) as well as other targets like Cbl (Sattler et al., 1996), Stat5 (Ota et al., 1998), Gab proteins (Furge et al., 2000) and Shp (Rohrschneider et al., 2000). On the other hand, the SH3/nSH3 domain of Crk proteins has been shown to bind the proline-rich region of several guanine exchange factors (GEFs) such as C3G (Tanaka et al., 1994), DOCK180 (Hasegawa et al., 1996), and Son of sevenless (Sos) (Matsuda et al., 1994). And apart from GEFs, the SH3 domain also binds non-receptor kinases, Abl1 and Abl2/Arg (Feller et al., 1994; Ren et al., 1994; Wang et al., 1996). The interactions of Crk proteins with Abl family kinases will be discussed later in the subsequent sections.

It has been suggested that the SH2 domain of a Crk protein binds and maintains tyrosine phosphorylation while the SH3 domain is required for cell transformation (Akagi et al., 2000). Indeed, overexpression of Crk's SH2 domain alone in the Cos7 cell line increases the phosphorylations of p130Cas and Paxillin (JA Jadwin and BJ Mayer, unpublished data) and in our previous study, the knockdown of either C3G or Sos were enough to significantly impair CrkI-transformation (Zheng et al., 2010). Over the years, both human cancer cell lines as well as cultured animal fibroblast models have been used to study the roles of Crk proteins in various signaling pathways, and they generally affect anchorage-independent growth and cell membrane regulations. For instance, the binding of CrkI (and/or CrkII) to p130Cas has been shown to regulate cell migration, invasion and adhesion downstream of ErbB2 (Spencer et al., 2000), uPAR (Smith et al., 2008), EphB (Nagashima et al., 2002), Integrins (Chodniewicz and Klemke, 2004; Gustavsson et al., 2004), PDGFR (Antoku and Mayer, 2009) and IGF-1R (Klemke et al., 1998). Due to the diverse signaling complexes that involve Crk proteins, please refer to a review article by Bell and Park (Bell and Park, 2012) for a more comprehensive description on other interacting proteins.

Crk and human cancer

Overexpression of CrkI/II and CrkL has been associated with various types of human cancers almost since their discovery. In particular, elevated levels of CrkI/II were reported in tumors originating from the breast (Fathers et al., 2012; Rodrigues et al., 2005), lung (Nishihara et al., 2002; Rodrigues et al., 2005), oral tissues (Yamada et al., 2011), ovary (Wang et al., 2011), colon (Nishihara et al., 2002), and brain (Takino et al., 2003; Wang et al., 2007). As for CrkL, elevated expression has been found in ovarian carcinoma (Wang et al., 2011), gastric cancer (Wang et al., 2013), chronic myeloid leukemia (Oda et al., 1994), and non-small cell lung cancer (Wang et al., 2013). Interestingly, mutation in the functional domains and regulatory tyrosines in Crk proteins has not been observed in human cancers so far.

Even though the transforming activity of Crk proto-oncoproteins is relatively "weak" and they were never considered as the main drivers to initiate human tumorigenesis, the increased expression of Crk proteins has been frequently shown in some advanced-stage tumors and it has also been implicated to have important roles in the metastatic potential of cancer cells (Fathers et al., 2012; Miller et al., 2003; Wang et al., 2011; Yamada et al., 2011). In lung cancer, the overexpression of Crk proteins correlates with the more aggressive type of lung tumors. In fact, this study is the first to suggest a correlation between elevated expression and high-risk stage I and III tumors (Miller et al., 2003). Later, a cDNA microarray-based genomic profiling on 128 adenocarcinomas and squamous cell carcinomas also found higher expression of CrkL in a majority of the tumor cells (Kim et al., 2010). In lung cancer-derived cell lines, the knockdown of CrkL and CrkI/II (Kim et al., 2010; Rodrigues et al., 2005) attenuates cell migration and invasion.

Apart from lung carcinomas, CrkI/II proteins are also found to be highly elevated in primary human breast tumors and once again, the knockdown of CrkI/II in breast tumor-derived cancel cell lines reduces their motility and invasiveness (Rodrigues et al., 2005). Moreover, by overexpressing CrkII in T47D, a human breast cancer cell line, the cells are found to lose their adherens junctions, a phenotype that mirrors the epithelial-mesenchymal transition (EMT) in

metastatic cancer cells (Fathers et al., 2010). Nevertheless, the role of CrkII in human breast tumors has been ambiguous, as the results of several mouse xenograft models seem to support a tumor suppressor role when coupled with Abl kinase (see below). The contrasting observations highlight the diverse roles of Crk proteins and the importance of protein expression profiling in treating human cancers.

Regardless, in most cases overexpression of Crk proteins is thought to be pro-oncogenic. For example, overexpression studies in glioblastoma (Takino et al., 2003), and knockdown studies using synovial sarcoma cells (Watanabe et al., 2009b) and human mucinous cystadenocarcinoma (MCAS; an ovarian-derived cell line (Linghu et al., 2006)) all concluded that Crk proteins support tumor progressions and metastasis.

CrkL is highly expressed in myeloid- and lymphoid-derived cells and has been closely associated with BCR-ABL, a fusion protein with over-reactive kinase activity that causes chronic myelogenous leukemia (CML; see below) (Nichols et al., 1994; Oda et al., 1994). In fact, BCR-ABL phosphorylates tyrosine 207 in CrkL in CML. This phosphorylation is undetectable in normal peripheral blood cells, and has been used as a prognostic indicator for a patient's responsiveness towards Imatinib, an Abl kinase inhibitor (Lucas et al., 2010). The exact function of CrkL in CML development remains elusive, but a 2009 mouse model showed that BCR-ABL lacking all Grb2, Cbl and CrkL binding sites failed to induce leukemia-like phenotypes (Johnson et al., 2009), strengthening the importance of CrkL in BCR-ABL oncogenesis.

More recent mechanistic studies related to altered Crk protein levels in cancer cells focus on micro-RNA (miR) regulation of gene expression, precisely miR-126, which targets CrkI/II mRNA for degradation. Forced expression of miR-126 in lung cancer cells attenuated the invasion and migration of these cells (Crawford et al., 2008). Furthermore, when Src-transformed cells were examined, a significant loss of miR-126 was found (Feng et al., 2010).

Abl kinases

Similar to Crk, the Abl kinase was first discovered as a viral oncoprotein, specifically in the Abelson murine leukemia virus (Abelson and Rabstein, 1970). More than a decade later, the human homolog was identified as part of a fusion oncoprotein, BCR-ABL (see below) (Ben-Neriah et al., 1986). Following this, ABL2, also known as Abl-related gene or ARG was found in the early 90's (Kruh et al., 1990; Perego et al., 1991). The two human paralogs shared a 90% identical SH3-SH2-TK (tyrosine kinase) domain cassette but differ in their actin- and DNA-binding domains, SH3 binding motifs and the presence of nuclear shuttling elements in ABL1 (Figure 1.3). Through knockout mouse models, ablation of Abl1 resulted in compromised hematopoiesis and impaired development of the immune system (Brightbill and Schlissel, 2009; Liberatore and Goff, 2009; Schwartzberg et al., 1991; Silberman et al., 2008), cardiac hyperplasia (Qiu et al., 2010) and osteoporosis (Li et al., 2000). On the other hand, disruption of Abl2/Arg showed subtle neuronal defects (Gourley et al., 2009; Koleske et al., 1998), and the double knockout of Abl1 and Abl2 is embryonic lethal (Koleske et al., 1998). Together, these studies confirmed the distinctive roles of each paralog as well as the overlapping functions they possess.

Clinically, Abl kinase is best known to be the causative agent in most of the cases of chronic myelogenous leukemia (CML). It usually occurs when a reciprocal chromosomal translocation, t(9;22)(q34;q11) (Westbrook et al., 1985) results in the creation of a fusion protein known as BCR-ABL (BCR: break point cluster region) with constitutive Abl kinase activity. The presence of homophilic BCR at the N-terminal end has been shown to promote oligomerization of BCR-ABL protein, a mechanism that facilitates trans-phosphorylation and thus, activation of Abl kinase (Tognon et al., 2004; Zhao et al., 2002). In addition, it was suggested that although the fusion proteins vary a little in size, the breakpoint position consistently includes the removal of the myristoylation site, the Cap sequence and the SH3 domain at the N-terminus of Abl. This has been widely perceived to disrupt the autoinhibitory mechanism and the stability of inactive ABL1,

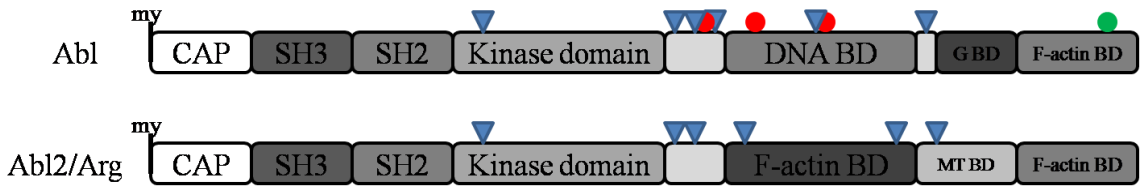

Figure 1.3: ABL domain structure and motif conservation.
Linear domain structure of Abl and Abl2/Abl related gene (Arg).
my, myristoylation site; G BD, G-actin binding domain; MT BD, microtubule binding domain. Green circle, nuclear exporting signal (NES); red circle, nuclear localizing signal (NLS); and blue triangle, proline-rich motif with capacity to bind SH3 or WW domains.

therefore leading to over-reactive ABL1 kinase. An ATP-competitive inhibitor, Imatinib mesylate (also known as STI571 or Gleevec) is known to stabilize the inactive ABL kinase conformation and has since become one of the first-line treatments for CML (Druker et al., 2001).

The myristoyl group (Hantschel et al., 2003), Cap sequence (Pluk et al., 2002), SH3 and SH2 domains (Nagar et al., 2006; Smith et al., 2003) in Abl1 are all involved in *cis*-regulating the kinase activity by stabilizing the inactive conformation. Other than these intrinsic regulating mechanisms, the kinase activity of Abl1 is also regulated by several kinases and phosphatases including i) tyrosine phosphorylation of Abl via Src (Brasher and Van Etten, 2000; Furstoss et al., 2002; Tanis et al., 2003), platelet-derived growth factor receptor (PDGFR; (Plattner et al., 2004)) or other ABL1/2 molecules in the form of *trans*-phosphorylation (Brasher and Van Etten, 2000; Tanis et al., 2003) ii) serine phosphorylation by p21-activated kinase 2 (PAK2 (Jung et al., 2008)) and iii) tyrosine dephosphorylation by protein tyrosine phosphatases (PTPs) like PTPN1 (LaMontagne et al., 1998)(LaMontagne et al., 1998)(LaMontagne et al., 1998)(LaMontagne et al., 1998)(LaMontagne et al., 1998)(LaMontagne et al., 1998)(LaMontagne et al., 1998), PTPRO (Motiwala et al., 2009), PTPN6 (also known as SHP-1 (Bruecher-Encke et al., 2001; Kharbanda et al., 1996)), PTPN11 (also known as SHP-2 (Mitra et al., 2008)), PTP-PEST (also known as PTPN12 (Cong et al., 2000)) and PTP-HSCF (also known as PTPN18 (Cong et al., 2000)).

The preferred Abl kinase phosphorylation site has been identified as (L/I/V)pYxxP through *in vitro* kinase assay (Cujec et al., 2002; Songyang et al., 1995). The SH2 domain in Abl kinase also plays an important role in the successive phosphorylation of multiple tyrosines on the same substrate, a mechanism known as processive phosphorylation (Mayer and Eck, 1995; Pellicena and Miller, 2001). The SH2 domain facilitates tyrosine targeting and a few examples of the substrates that undergo this phosphorylation process include Cbl (Andoniou et al., 1996), Dok1 (Woodring et al., 2004), Gab2 (Dorsey et al., 2002), p130Cas (Mayer et al., 1995) and others. Besides the intrinsic SH2 domain, the binding of adaptor proteins (like Crk, CrkL, Dok1,

Grb2 and Nck1) also helps deliver their bound proteins to ABL catalytic site. By interacting with both substrate and Abl kinase, adaptor proteins serve to "bridge" and therefore promote processive phosphorylations of the substrate.

Abl kinase binds a wide range of proteins and is mainly involved with cytoskeleton remodeling. The conserved calponin homology (CH)-type F-actin binding domain found at the C-terminus of Abl kinases, the G-actin binding domain in Abl1 and the (I/L)WEQ (talinlike) F-actin binding domain in Abl2, all indicate that both Abl1 and Abl2 bind G-actin and also mediate the formation of F-actin bundles (Van Etten et al., 1994; Wang et al., 2001). In addition, the binding of Cas- and Crk-families proteins to Abl1 has also been reported to regulate cellular attachment and motility (Chodniewicz and Klemke, 2004). Thus far, the interactions between CrkII or CrkL and Abl family kinase have been known to limit the activity of Crk proteins via tyrosine phosphorylation (Feller et al., 1994; Ren et al., 1994; Sriram et al., 2011). In the case of CrkII, Abl-phosphorylated Y221 in CrkII limits its binding ability to p130Cas, an interaction crucial for cell motility and survival (Kain et al., 2003). Moreover, Abl has been shown to indirectly regulate the turnover of focal adhesions and cytoskeletal rearrangement via phosphorylation of CrkII and CrkL (Antoku and Mayer, 2009).

Despite strong evidence on the transforming activity of Abl kinase family, several mouse xenograft studies found contradicting roles of Abl. At least in the breast cancer-derived cell lines used in these studies, ABL1 seems to also exert a tumor suppressor role (Allington et al., 2009; Gil-Henn et al., 2013; Noren et al., 2006). For instance, the CrkII-Abl complex has been proposed to mediate the tumor-suppressing signal of the EphB4 receptor (Noren et al., 2006). In our previous study to uncover the significance of Crk SH3 domain binding partners, we found that the down-regulation of Abl in CrkI-transformed mouse fibroblasts (either through gene knockdown or Imatinib treatment) enhances anchorage independent growth (Zheng et al., 2010). Although

our results are consistent with these xenograft studies, it is unclear if the same mechanism(s) is responsible across these different cell types and what are the associations between them.

The presence of three nuclear localization signals (NLS (Wen et al., 1996)) and one nuclear export signal (NES (Taagepera et al., 1998)) in Abl1 (Figure 1.3) signifies the ability of Abl to relocate into the nucleus. Moreover, the DNA-binding domain identified at the C-terminus of Abl1 (Miao and Wang, 1996) also suggests that it can directly interact with DNA. Indeed, Abl1 binds 14-3-3 proteins and is involved in DNA repair and apoptosis responses in the nucleus (Yoshida et al., 2005). Combining with the above-mentioned observations, it appears that Abl's negative regulation of tumor growth depends on its SH2 domain, kinase activity and nuclear localization ability (Sawyers et al., 1994).

Ras small GTPase

Evidently, Ras small GTPase plays a critical role in the transformation of Crk proteins in fibroblast models. First, the expression of a dominant-negative Ras mutant in vCrk-transformed fibroblasts reverses the tumorigenesis of v-Crk (Greulich and Hanafusa, 1996). Then, our knockdown study found that the knockdown of son of sevenless (Sos, a Ras activator; see below), a known Crk SH3 binding target, severely compromises the transforming activity of CrkI (Zheng et al., 2010).

Ras was named after rat sarcoma after its identification as a viral oncogene that induces tumor formation in mice and rats (HARVEY, 1964; Kirsten and Mayer, 1967). It was later learned that as a small GTPase, Ras proteins hydrolyze guanine triphospohate (GTP) into guanine diphosphate (GDP). Like other G proteins, Ras was later discovered to function as a molecular switch depending on the binding GTP or GDP. Overall, Ras GTPases are regulated by activators known as guanine-nucleotide exchange factors (GEFs), and deactivators known as GTPase activating proteins (GAPs). GEFs catalyze the release of GDP from Ras, which is quickly

replaced by the more available GTP and thereby switching it "on"; GAPs bind to Ras and greatly enhance its intrinsic GTPase activity to convert GTP into GDP, effectively switching it "off". Excessively activated "on" Ras GTPases (either through mutation or overexpression) have been well-studied over the years and they are widely accepted as oncoproteins. In fact, Ras is one of the most commonly mutated genes in human cancer (Prior et al., 2012). GTP-bound Ras undergoes a conformation rearrangement of two highly flexible segments, named switch I (G2 motif) and switch II (G3 motif) to orientate itself into an accommodating structure for the catalytic Mg^{2+} and reactive water molecule in order to hydrolyze the bound GTP. In addition, the reorganization of switch I and switch II also provides interaction surface for its effectors (Vetter and Wittinghofer, 2001).

Decades after the discovery of the first Ras protein, today the Ras superfamily has expanded tremendously and now consists of over 150 small GTPases, which can be divided into six subfamilies: Ras, Rho, Ran, Rab, Arf and Kir/Rem/Rad. Within the Ras subfamily, 13 known members can be further divided into five subgroups: Ras, M-Ras, R-Ras, Rap and Ral (Ehrhardt et al., 2002). Among the five subgroups, the Ras subgroup is best studied and it comprises the p21 Ras or classical Ras proteins: H-Ras, N-Ras and K-Ras (which has two alternative spliced forms named K-Ras4A and K-Ras4B). Due to the immense and complicated background on the Ras superfamily, we will hereafter use the more defined Ras subgroup as an example for the general understanding of these small GTPases. Moreover, the Ras subgroup has been previously implicated to be essential for Crk-induced transformations.

Post-translational modifications (PTMs) are critical for the trafficking and localization of p21 Ras GTPases and thus, directly affect their signaling activities. These modifications include processes like farnesylation, proteolysis, methylation, palmitoylation, phosphorylation, peptidyl-prolyl isomerization, ubiquitylation, nitrosylation, ADP ribosylation and glucosylation. Several of these PTMs are needed for a fully functional Ras GTPase (Figure 1.4) (Ahearn et al., 2012).

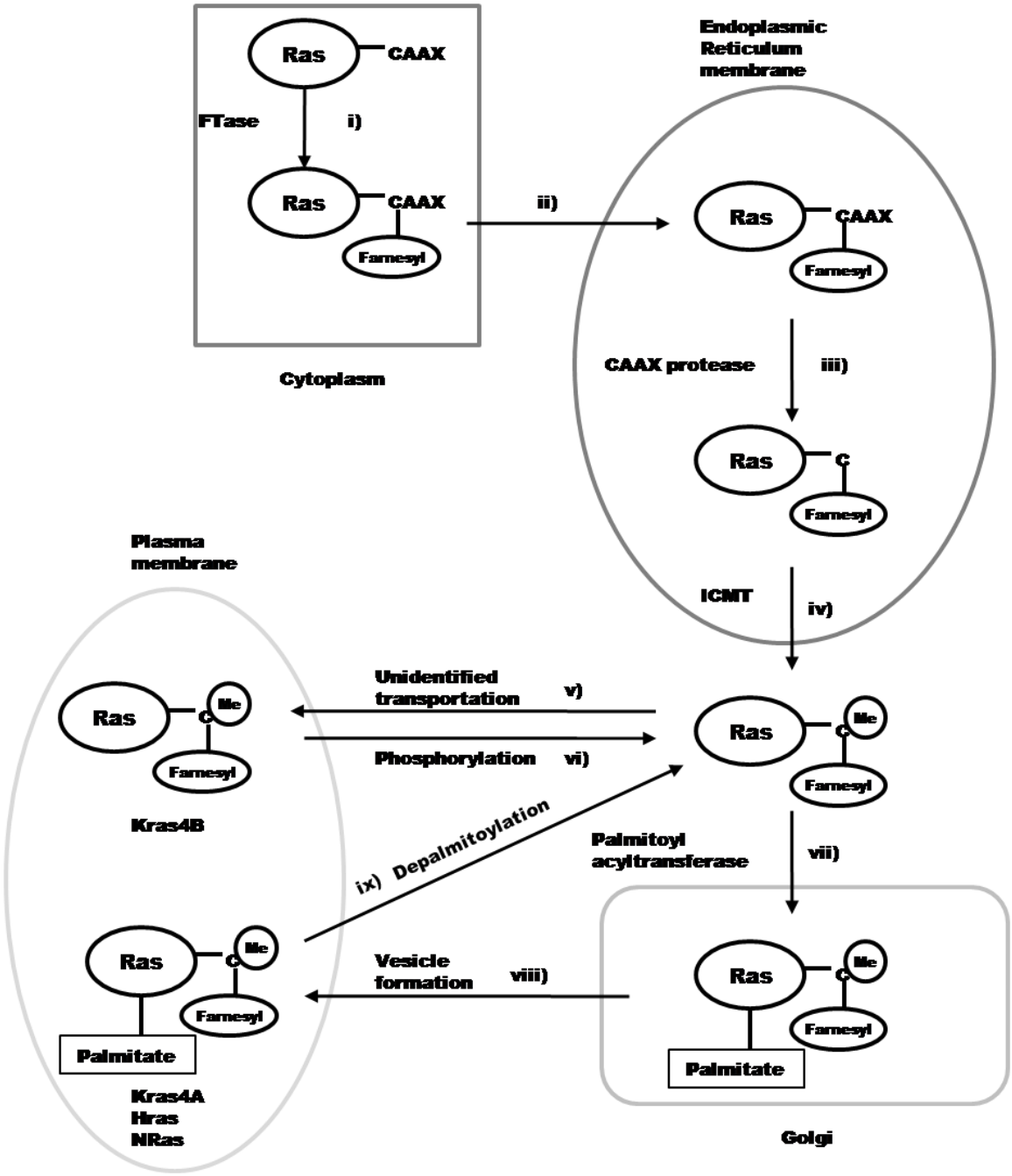

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Figure 1.4: Post-translational modifications of Ras subfamily for membrane targeting. (Figure modified from Ahearn *et al*., 2012)

i) Newly synthesized Ras proteins are farnesylated by farnesyltransferase (FTase) in the cytosol. ii) Ras proteins are transported onto the membranes of the endoplasmic reticulum (ER) iii) The last three C-terminal residues (AAX) of Ras proteins are removed via –AAX protease. iv) Isoprenylcysteine carboxylmethyltransferase (ICMT) methylates the C-terminal carboxylic acid of Ras proteins. v) Here forth, KRas4b deviates from other members and is directly transported to the plasma membrane. vi) Detachment of KRas4B from the membrane is regulated via a poorly understood pathway. vii) NRAS, HRAS and KRas4A are transported to the cytosolic face of the Golgi apparatus and are palmitoylated. viii) From the Golgi, they are transferred to the plasma membrane by the formation of vesicles. ix) Detachment of NRas, HRas and KRas4A are regulated by depalmitoylation.

Briefly, newly translated Ras GTPases proteins (H-Ras, N-Ras, K-Ras4A and K-Ras4B) in the cytosol undergo farnesylation at the cysteine residue at the C-terminal CAAX motif (via farnesyltransferase) before being transported onto the endoplasmic reticulum (ER). Once on the ER, farnesylated Ras GTPases are further processed with CAAX protease (known as Ras-converting enzyme1, RCE1) to remove the –AAX residues and the previously farnesylated cysteine will also be methylated by isoprenylcysteine carboxylmethyltransferase (ICMT).

Following the methylation, KRas4B deviates from the others and proceeds directly to the plasma membrane through a poorly understood pathway involving cytosolic chaperones. As for the remaining H-Ras, N-Ras and K-Ras4A, they exit the ER and move to the cytosolic face of the Golgi apparatus for palmitoylation by DHHC domain-containing 9-Golgi complex-associated protein of 16kDa (DHHC9-GC16) and will remain bound to the Golgi membrane. Then, through the formation of vesicles, they are transported and anchored to the plasma membrane for further activation. From the plasma membrane, KRas4B can be discharged through phosphorylation of Ser181 (by Protein kinase C, PKC) while the detachment of H-Ras, N-Ras and K-Ras4A requires depalmitoylation. Other modifications include ubiquitylation for the trafficking of H-Ras to and from endosomes; S-nitrosylation for the enhancement of guanine nucleotide exchange rate. The most significant role of all the above-mentioned PTMs is to relocate Ras GTPases to specific cellular compartments for activation/deactivation and signal transduction. For more details, please refer to the review article by Ahearn *et al.* (Ahearn et al., 2012).

GEF and GAP proteins control the activation of Ras GTPases. GEFs trigger the release of GTP by sterically displacing the Mg^{2+} and restructuring the nucleotide binding site of the GTPase. This rearrangement weakens nucleotide binding affinity and promotes the exchange from the pool of available nucleotides. However, Ras GTPases do not discriminate between GDP or GTP (Bos 07Cell) and the binding of GTP seems to rely on the relatively higher abundance of GTP (10- to 50-fold higher than GDP (Bos et al., 2007; Trahey and McCormick, 1987). In

mammalian cells, all identified Ras-GEFs can be classified into three classes, each with a modular architecture and sharing a REM-CDC25 tandem module comprising the catalytic domain (Figure 1.5). Due to the ubiquitous expression of Sos1/2 (with a few known exceptions) and the more tissue-specific expression of the other two classes of Ras-GEFs, RasGRF and RasGRP (in neuronal cells and leykocytes (Dower et al., 2000; Ebinu et al., 1998; Fernández-Medarde and Santos, 2011; Guerrero et al., 1996; Kawasaki et al., 1998; Pierret et al., 2000; Ruiz et al., 2007)), Sos 1/2 are most likely the main activators for Ras in most cells. That being said, our current understanding on the Ras-GEFs are not fully defined and requires more investigation.

On the other hand, GAPs accelerate the Ras GTPase activity by contributing a conserved arginine finger to the active site of Ras GTPases (Ahmadian et al., 1997; Kötting et al., 2008). The arginine finger in GAPs stimulates structural rearrangement of the GTPases, promoting the coordination of a water molecule for the nucleophilic cleavage of the gamma-beta-phosphoanhydride bond (Scheffzek et al., 1997; Vetter and Wittinghofer, 2001). Crystallography studies have revealed that the binding of GEFs and GAPs to Ras GTPases are mutually exclusive, as both regulators have large interacting surfaces that overlap the switch I and II motifs (Boriack-Sjodin et al., 1998; Scheffzek et al., 1997). There are five classes of Ras-GAP proteins currently identified (Figure 1.5). Among the five classes of GAPs, only Neurofibromin and DAB2IP have so far been shown to regulate Ras activities in cellular oncogenesis, and the roles for the remaining three classes are still unclear. Oddly, despite being a robust deactivator of Ras GTPases, RasGAP (also known as the p120GAP or RASA1) knockout studies showed no distinctive evidence that support increased cell proliferation (as a consequence of over-reactive Ras GTPases). Indeed, somatic mutation of RasGAP in human cancer is rare, and it was suggested that its roles in mitogenic signaling might not be as straightforward as previously thought (Hennig et al., 2015).

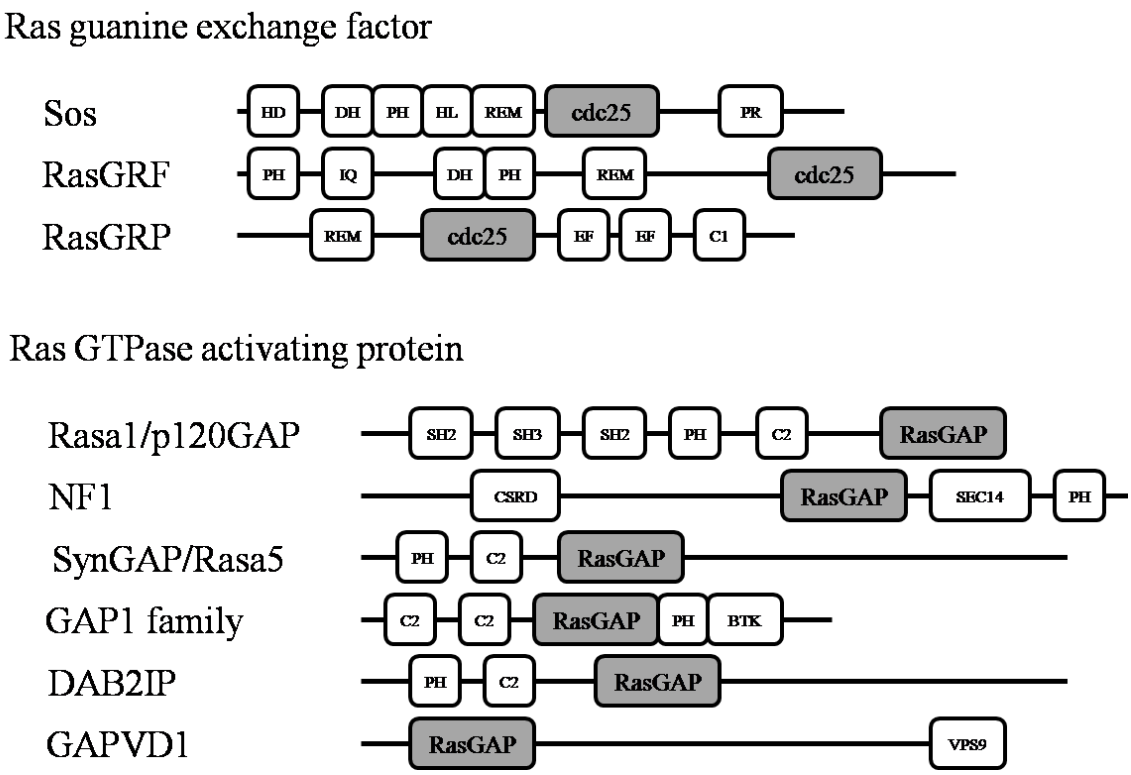


Figure 1.5: Domain structure of Ras-GEFs and Ras-GAPs. (Figure modified from Hennig *et al*. 2015)

Linear domain structure of confirmed members in theRas-GEF and Ras-GAP families. Domain nomenclature according to SMART database (http://smart.embl-heidelberg.de/): BTK: Bruton's tyrosine kinase Cys-rich motif; C1: DAG-binding C1 domain; C2: Protein kinase C conserved region 2; CDC25: catalytic GEF domain; CSRD: cysteine and serine rich domain; DH: Dbl homology; EF: Ca2+-binding EF hand; IQ: calmodulin-binding motif; HD: histone domain; HL: helical linker; PH: Pleckstrin homology; PR: Proline-rich region; RasGAP; catalytic domain of GTPase activating protein for Ras; REM: Ras exchanger motif; SEC14: Lipid-binding domain; SH2: Src homology 2 domain; SH3: Src homology 3 domain; VPS9: Domain present in VPS9 protein

The downstream effectors of the Ras subfamily can be either GEFs and GAPs for other Ras superfamiliy members, or protein or lipid kinases. To date, the best known Ras effector is Raf kinase (a classic MEKK, see below) and to a lesser extent, Ral guanine nucleotide dissociation stimulator (RalGDS), phosphatidyolinositol 3-kinase (PI3K), Rin1, T lymphoma invasion and metastasis protein 1 (Tiam1), Af6, Nore1, PLC_{ε} and PKCζ (Rajalingam et al., 2007). Raf family serine/threonine kinases consist of Raf-1, A-Raf, and B-Raf and they all interact with GTP-bound p21 Ras through their Ras binding domain (RBD). Raf-1 has been shown to be most efficiently activated by K-Ras4B, followed by K-Ras4A, N-Ras and H-Ras (Voice et al., 1999).

The overall downstream signaling pathways of activated Ras GTPase are diverse and have been implicated to affect overall cell proliferation, apoptosis, cytoskeletal rearrangement and other cellular responses (Ehrhardt et al., 2002). Different members of the Ras subfamily have been shown to have their distinctive functions, and besides differences in their affinities to regulators or effectors, subcellular localization plays a major role in their functionalities as well.

Mitogen activated protein kinases

The Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases, and generally form the third tier of a three-kinase cascades module. Immediately upstream of the MAPK family in the second tier is a highly specific kinase family known as the MAPK/Erk kinases (MAPKK or MAP2K or MEK), which activates MAPKs by dual phosphorylation of serine/threonine and tyrosine residues in the MAPK. And upstream of MEK, a first-tier group of kinases known as the MEK kinases (MAPKKK or MAP3K or MEKK) activate MEKs via serine/threonine phosphorylation (Figure 1.6). To date, examples of MAP kinases include extracellular signal-regulated kinases 1 and 2 (Erk1/2), c-JunN-terminal kinases/stress-activated protein kinases (Jnk/SAPK; hereafter simply as Jnk), and p38, along with Erk3 isoforms, Erk5, Erk7, NLK, MOK and other lesser known kinases (Pearson et al., 2001). For our current study,

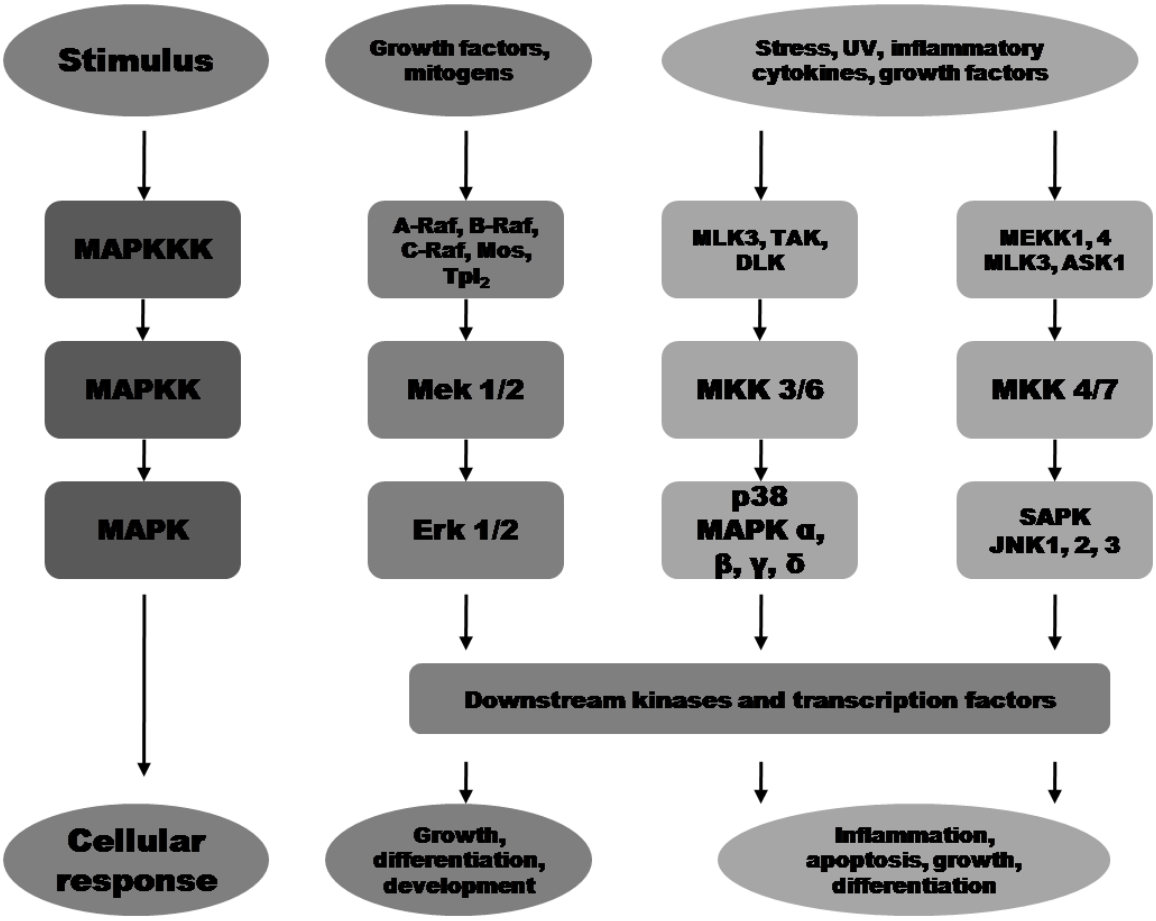


Figure 1.6: Mitogen-activated protein kinase signaling cascades.

Representatives of the classic three-tier MAPK signaling cascade.

we will only be focusing on the Erk1/2 and Jnk kinase pathways, as they have both been implicated in Crk-induced cell transformation.

As the classical and best characterized MAPKs, Erk1 and Erk2 are closely related (with an overall 85% homology and even higher in the substrate binding regions (Boulton et al., 1991; Boulton et al., 1990), and both are ubiquitously expressed across different tissues at varying levels. They are generally activated by serum, growth factors, cytokines, certain stresses, ligands for G protein-coupled receptors (GPCRs), and others stimuli. Activation of Erk1/2 requires a sequential dual phosphorylation of threonine and tyrosine (Ferrell and Bhatt, 1997), and it has been shown that both phosphorylation targets are separated by a glutamate residue, resulting in a TEY motif in the activation loop (Payne et al., 1991). The full activation of Erk1/2 has been reported to amplify their kinase activities by more than 1000-fold (Robinson et al., 1996).

Erk1/2 are substrates for their respective MEKs, MEK1/2 (Ahn et al., 1991; Crews et al., 1992; Kosako et al., 1992; Nakielny et al., 1992; Seger et al., 1992; Wu et al., 1993; Zheng and Guan, 1993). As the second tier of the three-part phosphorylation cascade, the activity of MEK requires dual phosphorylation as well. And while a single phosphorylation alone produces a significant degree of activation, their activity has been shown to increase by more than 7000-fold when both serine/threonine residues are phosphorylated (Alessi et al., 1994; Mansour et al., 1996). Moreover, subsequent biochemical experiments on MEK1 found that the deletion of its N-terminal sequence and substitution of the two phosphorylation sites with acidic residues render it constitutively active (Mansour et al., 1996). Combining with the specificity of MEK1/2 to Erk1/2, the MEK mutants have been very useful to study the role of Erk1/2 cascades in signal transductions (Mansour et al., 1994; Whalen et al., 1997).

In the Erk1/2 cascade, Raf isoforms and Mos are the MEKKs that phosphorylate and activate MEK1/2 (Dent et al., 1992; Force et al., 1994; Kyriakis et al., 1992), in particular the Raf

family kinases comprised of A-Raf, B-Raf and Raf-1 (or c-Raf) (Hagemann and Rapp, 1999). Three conserved regions, termed CR1, CR2 and CR3, are found in each Raf isoform and CR3 contains the kinase domain. Expression-wise, Raf-1 is ubiquitously expressed; A-Raf is mainly found in urogenital tissues; B-Raf is expressed highly in neuronal tissue and testis. The studies on Raf-1 demonstrated that its activation is mostly Ras-dependent (Leevers et al., 1994) but a Ras-independent regulation of Raf-1 in T cells has also been identified via deletion of the Ras-binding region (corresponding to CR1 and CR2 at the amino terminus) (Whitehurst et al., 1995). While Raf-1 is primarily activated by H-, K- and N-Ras (Hamilton and Wolfman, 1998), Rap1 appeared to be the main activator for B-Raf (Vossler et al., 1997; Zwartkruis et al., 1998). Over the years, the activation and stabilization of Raf-1 have been shown to be precisely regulated by protein-protein interactions, phosphorylation and subcellular localization. These multiple levels of regulation of Raf-1 is achieved through binding and interaction with other proteins such as small GTPases, PKC (Kolch et al., 1993), Pak3 (King et al., 1998), p50 (Stancato et al., 1993), heat shock protein (Hsp90) (Stancato et al., 1997) and 14-3-3 proteins (Fantl et al., 1994; Freed et al., 1994).

Apart from the Erk1/2 MAPK cascade, Jnk is another MAPK subfamily that has been studied intensively. Following the identification of a 54-kDa form of Jnk protein from cycloheximide–treated rat liver cells (Kyriakis and Avruch, 1990), similar proteins were later isolated through affinity absorption to a c-Jun fusion protein as UV-responsive protein kinases (Hibi et al., 1993). Shortly thereafter, several studies found at least 10 alternatively spliced forms encoded by 3 independent genes and these closely related kinases (with more than 85% similarity in the core catalytic domains) were eventually grouped under the Jnk subfamily (Dérijard et al., 1994; Gupta et al., 1996; Kyriakis et al., 1994). Like other MAPKs, the activation of all Jnks requires dual phosphorylation of threonine and tyrosine in the activation loop (separated by a proline residue; forming a consensus TPY motif). Alternatively known as stress-activated protein

kinases (SAPKs), Jnks are mostly stimulated in response to cellular stress, pro-inflammatory cytokines, as well as serum, growth factors and transforming agents (Kyriakis and Avruch, 2001).

MEK4 and MEK7 are two known MEKs that phosphorylate Jnk (Holland et al., 1997; Lin et al., 1995; Yamauchi et al., 1999). However, unlike the highly specific MEK1/2, both MEK4 and MEK7 have been shown to phosphorylate p38 as well (Meier et al., 1996). Interestingly, MEK4 and MEK7 each appears to preferentially phosphorylate tyrosine and threonine (respectively), and has been suggested that they both work cooperatively in Jnk activation (Lawler et al., 1998; Lisnock et al., 2000).

The main phosphorylation targets for MAPKs are transcription factors like Fos, Jun, activating transcription factors (ATF) and Maf (Shaulian and Karin, 2002). Once activated by various stimuli, pErk and pJnk translocate into the nucleus and phosphorylate these transcription factors and therefore, promote their dimerization and activation (Cavigelli et al., 1995; Hill et al., 1994; Karin, 1995). In particular, the Jun and Fos transcription factors have been best characterized and we will use them as examples to describe the overall understanding of MAPK-regulated transcription factors. The Jun family transcription factors (Jun, JunB and JunD) are known to homodimerize as well as heterodimerize with members of the Fos family (Fos, FosB, Fra-1 and Fra-2), but in contrast, Fos family transcription factors only dimerize with members of the Jun family (Zenz and Wagner, 2006). Despite both Fos and Jun families having high structural homology, their various dimers have been found to have unique DNA binding specificity, and is believed to be an important feature in regulating gene expression (Chang and Karin, 2001). These transcription factors serve as converters for various extracellular stimuli (such as growth factor, hormones, pathogens, physical and chemical stresses etc.) into specific gene expression to elicit appropriate cellular responses. Studies have shown that MAPK-regulated transcription factors ultimately stimulate a diverse range of cellular responses including differentiation, proliferation, survival, apoptosis and others.

Jun and Fos proteins are known positive regulator for cell proliferation and cell cycle progression of fibroblasts, as demonstrated via antisense RNA inhibition experiments (Shaulian and Karin, 2001) as well as knockout studies (Brown et al., 1998; Brüsselbach et al., 1995). However, on the opposite end, Fos and Jun activation have also been closely associated with cell death and apoptosis (Devary et al., 1991; Karin, 1998; Smeyne et al., 1993). These conflicting roles of MAPK-activated transcription factors are complicated and are most likely affected by the cell types and signaling background.

Finally, many other proteins have been described to interact with members of the MAPK cascades protein for precise cellular responses. In general, their interaction are mostly likely to i) facilitate effective serial activation of the MAPK cascade; ii) restrict signal reception for specificity; iii) regulate output signal through localization (Pearson et al., 2001).

Thesis overview

Since the overexpression of CrkI was first shown to transform fibroblast cells, the mechanism behind its oncogenesis is still largely unknown. In addition, our previous attempt to understand how the SH3 binding partners of CrkI influence cell transformation uncovered that the inhibition of Abl kinase (either through inhibitory drug, Imatinib or shRNA knockdown) further enhances CrkI transformation. Due to the popularity of Imatinib in various combination cancer therapies, we are concern if Imatinib may promote the growth of human cancer cells, especially when coupled with the overexpression of Crk proteins. Here, I attempt to uncover the underlying mechanism(s) for Imatinib-enhanced CrkI tumorigenesis. Moreover, using a panel of 60 human tumor-derived cell lines (known as the NCI60), I will investigate the possibility of Imatinib-promoted growth and how this may be associated to our fibroblast model.

In my thesis project, I will first address how the inhibition of Abl kinase enhances CrkI transformation in chapter 2. And in chapter 3, I will investigate other phenotypic changes that

might have contributed to the enhanced growth of CrkI-transformed NIH3T3 in suspension, and also to determine if Imatinib will have a similar effect on human cancer cell lines. Chapter 4 will describe the materials and methods used in my study and chapter 5 will be a summary and possible future directions will be discussed.

Chapter 2

Identification of Dok1 as a negative regulator for CrkI-induced cell transformation

Attribution: This chapter contains work published as: Ng KY, Yin T, Machida K, Wu YI, Mayer BJ. 2014. Phosphorylation of Dok1 by Abl family kinases inhibits CrkI transforming activity. Oncogene. Epub ahead of print.

All works performed in Figure 2.21, including the plasmids generation, cell transduction and live cell imaging were carried out by Yin T and Wu YI. All other experiments were done by Ng KY.

Abstract:

The Crk SH2/SH3 adaptor and the Abl nonreceptor tyrosine kinase were first identified as oncoproteins, and both can induce tumorigenesis when overexpressed or mutationally activated. We previously reported the surprising finding that inhibition or knockdown of Abl family kinases enhanced transformation of mouse fibroblasts by CrkI. Abl family inhibitors are currently used or are being tested for treatment of human malignancies, and our finding raised concerns that such inhibitors might actually promote the growth of tumors overexpressing CrkI. Here, we identify the Dok1 adaptor as the key effector for the enhancement of CrkI transformation by Abl inhibition. We show that phosphorylation of tyrosines 295 and 361 of Dok1 by Abl family kinases suppresses CrkI transforming activity, and that upon phosphorylation these tyrosines bind the SH2 domains of the Ras inhibitor p120 RasGAP. Knockdown of RasGAP resulted in a similar enhancement of CrkI transformation, consistent with a critical role for Ras activity. Imaging studies using a FRET sensor of Ras activation revealed alterations in the localization of activated Ras in CrkI-transformed cells. Our results support a model in which Dok1 phosphorylation normally suppresses localized Ras pathway activity in Crk-transformed cells via recruitment and/or activation of RasGAP, and that preventing this negative feedback mechanism byinhibiting Abl family kinases leads to enhanced transformation by Crk.

Introduction

Like other adaptors, Crk proteins function in signaling by mediating the formation of multiprotein complexes through their modular protein-binding domains (Bell and Park, 2012) and transformation by v-Crk and CrkI requires the binding activity of both the SH2 and SH3 domains (Mayer and Hanafusa, 1990; Zheng et al., 2010). The N-terminal SH3 domain of Crk binds proline-rich peptides with PxxPxK motifs (Knudsen et al., 1994; Tanaka et al., 1994) and the major binding partners include Abl family tyrosine kinases (Ren et al., 1994; Wang et al., 1996), and small G protein guanine nucleotide exchange factors including Sos (Matsuda et al., 1994), DOCK180 (Hasegawa et al., 1996) and C3G(Tanaka et al., 1994).

In our previous work to identify the Crk SH3 binding partners essential for transformation of NIH3T3 cells by CrkI, we found that the activity of Abl family kinases antagonized the transforming activity of Crk (Zheng et al., 2010). Knocking down expression of Abl and its close relative Arg, or inhibiting Abl family kinases with Imatinib (a clinically prescribed Abl kinase inhibitor), both led to increased tumorigenicity of CrkI overexpressing cells in vitro (assayed by anchorage-independent growth) and in vivo (assayed by injection of cells into nude mice).

Based on mouse xenograft models, several publications also reported a tumor suppressor role for Abl family kinases. However, the roles of Abl or Arg varies in these studies. For instance, Abl-Crk complex is required as an intermediate for the tumor suppressing pathway of EphB4 (Noren et al., 2006) but in the transforming growth factor-beta (TGF-β) responsive metastatic epithelial cells (MEC), Abl itself serves as an antagonist for TGF-β (Allington et al., 2009). In addition, in the epidermal growth factor (EGF) receptor and Src kinase signaling pathway, Abl2 appears to limit cell proliferation but promote cell invasiveness at the same time (Gil-Henn et al., 2013).

Taken together with the oncogenic role of Abl in CML, these studies add to the complicated roles of Abl kinase in tumorigenesis. The outcome of Abl kinase inhibition depends on the interactions between Abl and whatever activated pathways that are either promoting or inhibiting tumorigenesis. And because of this ever increasing level of uncertainty, we are determined to investigate the growth limiting mechanism of Abl kinase in relation to overexpressed Crk using our fibroblast model.

Among the many Abl family kinase substrates, our preliminary data suggested Dok1 as the most credible candidate (Figure 2.2). Initially identified as a 62kDa Abl-phosphorylated protein that binds Abl and p120RasGAP protein (hereafter as RasGAP) (Carpino et al., 1997; Yamanashi and Baltimore, 1997), Dok1 has also been regarded as a tumor suppressor among its many roles in immunological signaling pathways (Mashima et al., 2009).

The current Dok family has seven members and their basic structure includes a pleckstrin homology (PH) domain, a phosphoryrosine-binding (PTB) domain and a multiple tyrosine residues consisting C-terminal end (example of Dok1 in Figure 2.7). Most of the functions of Dok proteins rely on the phosphorylation of these tyrosines and their interaction with phospho-tyrosine binding domains in various proteins (Mashima et al., 2009). Out of the seven members, Dok1, Dok2 and Dok3 are grouped into a single subgroup based on their primary structure and their somewhat overlapping functions. In immune cells, Dok1 and Dok2 are known to be the negative regulator of B-cell receptor (BCR)(Yamanashi et al., 2000) and T-cell receptor (TCR)(Yasuda et al., 2007) as well as the proliferation of myeloid cells (Niki et al., 2004; Yasuda et al., 2004). Although this subgroup of Dok proteins are mainly expressed and studied in hematopoietic cells, they are also express in other cell types, presumably carrying out non-immunological functions(Carpino et al., 1997; Hosooka et al., 2008; Smith et al., 2004; Yamanashi and Baltimore, 1997; Zhao et al., 2006).

In human cancer, the Dok1 gene is located at human chromosome 2p13 (Nelms et al., 1998), a region where rearrangements are frequently seen in different types of cancer malignancies (Inaba et al., 1991; Nelms et al., 1998; Yoffe et al., 1990). For example, a truncated, functionally defective form of Dok1 (due to frameshift mutation) has been identified in cases of chronic lymphocytic leukemia (CLL) (Lee et al., 2004). Moreover, the lost of Dok1 expression due to hypermethylated promoter region have also been reported in various primary human tumors (Balassiano et al., 2011; Lambert et al., 2011; Saulnier et al., 2012).

Experimentally, several knockout studies have been used to understand the overall significance of Dok family proteins in cancer development. These studies resulted in varying outcomes but all agreed on the tumor suppressor role of Dok proteins. For some, the knockout of Dok1 and Dok2 causes post-natal development of myeloproliferative disorder (Mashima et al., 2009; Niki et al., 2004; Yasuda et al., 2004) and in others, the triple knockout of Dok1, Dok2 and Dok3 causes tumors formation post-natal, either as lung tumor (Berger et al., 2010) or histyocytic sarcoma (Mashima et al., 2010). Collectively, these knockout studies emphasize on two important features of the Dok proteins: First, Dok1, 2 and 3 are essential universal tumor suppressors and second, they have a significant degree of overlapping functions.

Over-reactive Ras signaling is well associated with human cancers(Prior et al., 2012) and unlike the relatively weaker Crk, oncogenic Ras readily transforms cultured cells to a much higher degree. With RasGAP as the potent antagonist for Ras, one can logically relate how the binding of RasGAP to Dok1 may have contributed to the regulation of cell transformation. Indeed, Dok1 has been shown to be indispensable for the regulation of Ras-MEK (MAP-ERK Kinase) pathway in various cell type (Jones and Dumont, 1999; Nelms et al., 1998; Wick et al., 2001; Yamanashi et al., 2000; Yoshida et al., 2000; Zhao et al., 2001) but the detailed mechanisms remained elusive.

RasGAP bind phosphorylated tyrosines 295 and 361 of Dok1 via its SH2 domains (Songyang et al., 2001) but this binding alone is insufficient for the suppression of Ras-MEK pathway (Wick et al., 2001; Zhao et al., 2001). Instead, another pair of RasGAP-independent tyrosine residues (tyrosines 336 and 340) in Dok1 was later identified for the suppression of MEK but still, the exact mechanism(s) is still unclear (Shinohara et al., 2004).

Dok1 is recruited to the plasma-membrane via its N-terminal PH domain in the presence of cellular phosphatidylinositol 3-kinases (PI3K) and this membrane recruitment is essential for the suppression of MEK pathway (Zhao et al., 2001) and presumably tumorigenesis. In addition, the nuclear export signal (NES) within Dok1 protein controls the cytoplasmic localization (Lee et al., 2004) and the accumulation of Dok1 in cytoplasm (perhaps to be more readily accessible) is regulated by extracellular stimulations, such as exposure to growth factors and/or cell adhesion (Niu et al., 2006).

Here, we show that Dok1 is responsible for the enhancement of CrkI transformation upon Abl kinase inhibition. Our results suggest the existence of a general feedback control mechanism whereby Abl, Dok family proteins and RasGAP work together to locally downregulate Ras activity.

Results

Dok1 is the major Abl-dependent phosphoprotein in Crk-transformed cells

We first examined more closely how Abl inhibition affected the ability of CrkI-transformed NIH3T3 cells to grow in suspension, a hallmark of malignant transformation. Consistent with previous results (Zheng et al., 2010), we found a significant increase (up to 10-fold) in the number of colonies in the soft agar growth assay when cells were treated with the Abl inhibitor Imatinib (Figure 2.1). The stimulatory effect of Imatinib increased proportionately with concentration up to 10 μM then decreased slightly, presumably due to increased toxicity (the reported IC50 for Imatinib falls within the range of 0.4–1.5 μM (White et al., 2005)).

We reasoned that Abl inhibition exerted its effects on Crk transformation by altering tyrosine phosphorylation. To identify Abl-dependent phosphoproteins, lysates of control and CrkI-transformed cells (with and without Imatinib treatment) were immunoblotted with anti-phosphotyrosine (anti-pTyr) antibody. A prominent tyrosine-phosphorylated band of ~ 64 kDa was seen in CrkI overexpressing cells when compared with the controls, the phosphorylation of which was strongly reduced upon Imatinib treatment (Figure 2.2). On the basis of known substrates of Abl and the apparent molecular weight, we surmised that this phosphoprotein might be Dok1 (Carpino et al., 1997; Yamanashi and Baltimore, 1997). To test this, a lysate of Crk-transformed cells was serially immunoprecipitated with anti-Dok1 antibody. This treatment depleted the 64 kDa tyrosine-phosphorylated protein from the lysates, verifying its identity as Dok1 (Figure 2.3). Immunoblotting with a phosphospecific antibody recognizing pY362 of human Dok1 (pY361 in mouse Dok1) further confirmed the dependence of Dok1 phosphorylation on Abl activity (Figure 2.4).

Dok1 corresponded to the only prominent tyrosinephosphorylated band in CrkI-transformed cells that was Abl dependent. Somewhat paradoxically, Dok1 phosphorylation was increased in CrkI-transformed cells compared with normal control cells (Figure 2.2). Together,

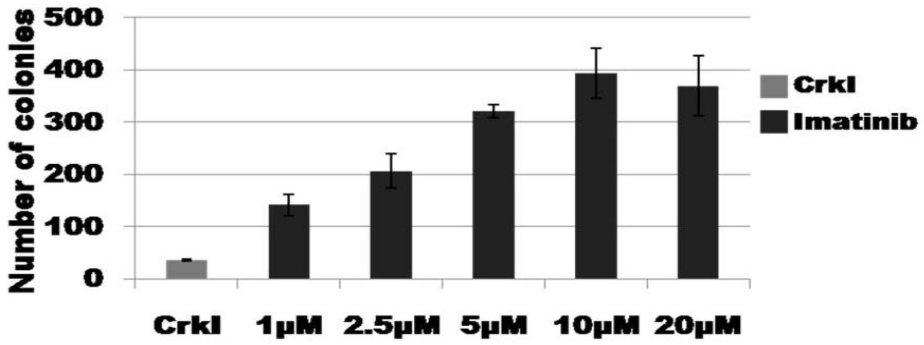


Figure 2.1: Imatinib enhances CrkI transformation.
Anchorage independent colonies formed by Crk1-tranformed NIH3T3 cells treated continuously with the indicated concentrations of Imatinib.

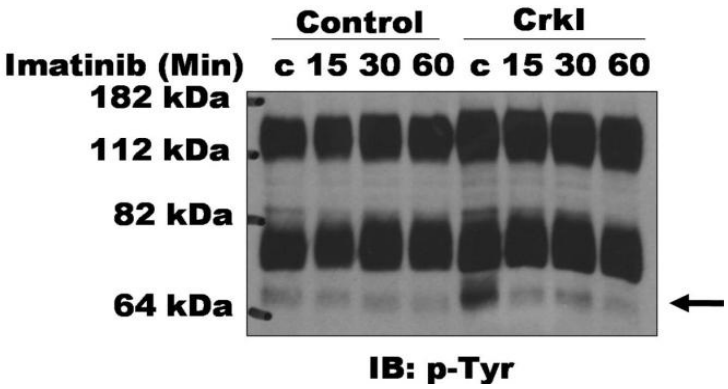


Figure 2.2: Changes in tyrosine phosphorylation in CrkI overexpression and Imatinib treatments. Serum-starved CrkI-transformed NIH3T3 cells treated with 20 μM Imatinib for indicated times were lysed and blotted with anti-pTyr. Phosphorylation of ~ 64 kDa band is decreased upon Imatinib treatment of CrkI-transformed cells (indicated by arrow). IB, immuno-blot; pTyr, anti-phosphotyrosine.

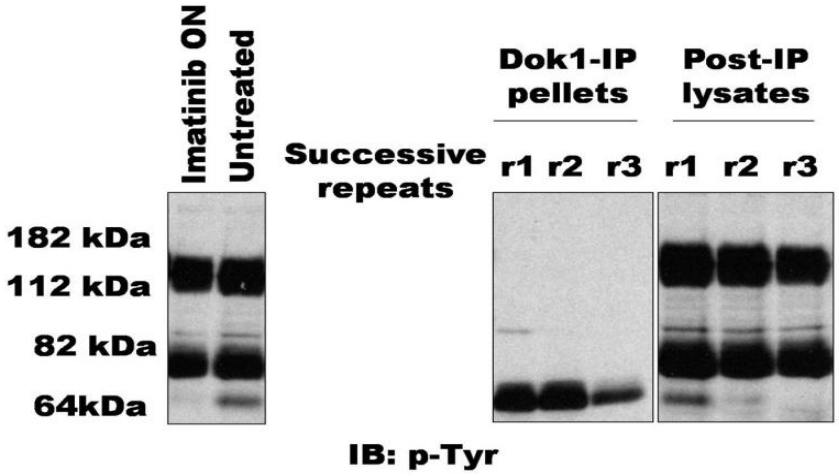


Figure 2.3: Identification of Dok1 as the phosphorylated ~64 kDa protein in CrkI overexpressing cells.

Lysates of CrkI-transformed NIH3T3 cells were serially immunoprecipitated using anti-Dok1 antibody. Left panel, whole cell lystates from cells treated with or without 2.5 μM imatinib; center and right panel, immunoprecipitate (IP) and supernatant (post-IP) fractions. ON, overnight incubation; successive rounds of immunoprecipitation indicated by r1, r2 and r3.

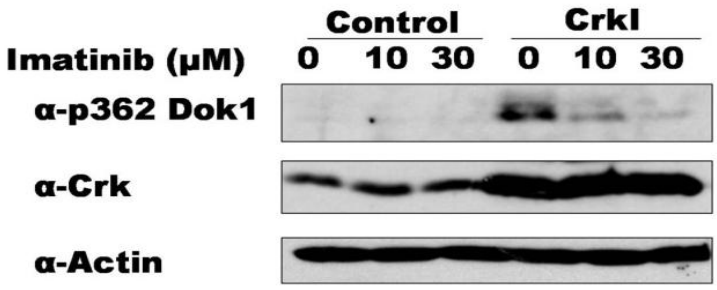


Figure 2.4: Verifying Dok1 phosphorylation in response to CrkI overexpression and Imatinib treatment.

CrkI-expressing or control NIH3T3 cells treated with indicated concentrations of imatinib were lysed and immunoblotted with phosphospecific Dok1 antibody (α-p362 Dok1). Immunoblotting with anti-Crk and anti-actin shown as controls.

these data suggest that tyrosine phosphorylation of Dok1 by Abl is induced by CrkI overexpression, and may act to partially suppress Crk-mediated transformation.

Phosphorylation of Dok1 regulates CrkI transformation

To test whether Dok1 is acting as a tumor suppressor protein in this system, we knocked down Dok1 expression in CrkI overexpressing cells using shRNA (Figure 2.5). We observed an enhancement of soft agar colony formation upon Dok1 knockdown, comparable to the effect of Abl knockdown or Imatinib treatment (Figure 2.6), implicating Dok1 as a crucial regulator of CrkI transformation.

To further probe the significance of Dok1 in suppressing CrkI-mediated transformation, the human homolog of Dok1 (hDok1) was used for rescue experiments. We used human instead of mouse Dok1 to avoid it being targeted for shRNA-mediated knockdown. The human homolog of Dok1 has an insertion at position 271, which shifts the amino acid numbering C-terminal to the insertion by +1 compared with mouse. For simplicity, henceforth the mouse amino acid numbering will be used unless stated otherwise.

In addition to the wild-type (WT) hDok1, we also generated mutant constructs to test the importance of several potential tyrosine phosphorylation sites. Shinohara et al.(Shinohara et al., 2004) reported that phosphorylation of tyrosines 259 and 361 was required for RasGAP binding, whereas phosphorylation of tyrosines 336 and 340 inhibited Erk activation through unidentified mechanism(s). Both Ras and its activator Sos1 were previously shown to have essential roles in Crk transformation (Greulich and Hanafusa, 1996; Zheng et al., 2010) and the MEK/ERK pathway downstream of Ras is well known to promote cell proliferation (Neuzillet et al., 2014).

Using site-directed mutagenesis, putative tyrosine phosphorylation sites were changed to phenylalanine. The resulting constructs were named according to the tyrosines mutated (Figure 2.7): M14 for mutation of sites 1 and 4 (Y295 and Y361), M23 for mutation of sites 2 and 3 (Y336 and Y340), M-all for mutation of all four sites, M1 for mutation of Y295 and M4 for

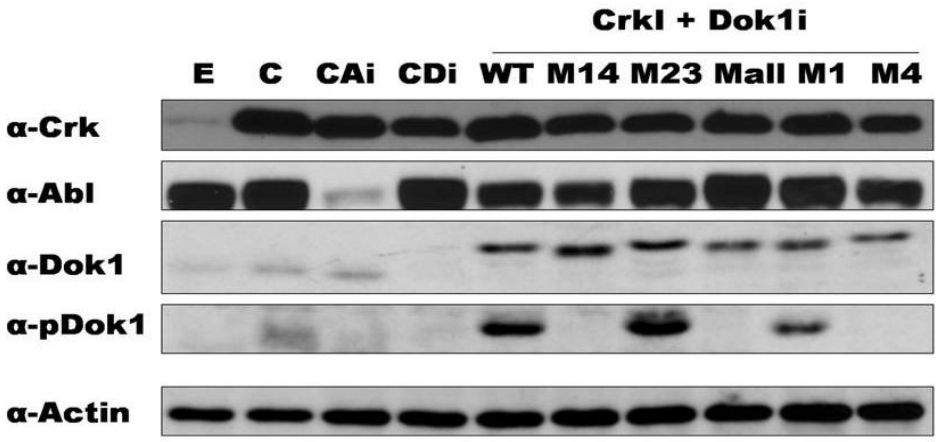


Figure 2.5: Knockdown and rescued Dok1 in CrkI overexpressing cells.
Dok1 knockdown and rescue with HA-tagged Dok1 constructs. NIH3T3 cell lysates were immunoblotted with antibodies indicated (αpDok1=phosphospecific Dok1 antibody). E, empty vector control; C, CrkI transformed; CAi, CrkI-transformed, Abl knockdown; CDi, CrkI-transformed, Dok1 knockdown; WT: wild-type Dok1; M14: Y295F and Y361F mutant Dok1; M23: Y336F and Y340F mutant Dok1; M-all: Y295F, Y336F, Y340F and Y361F mutant Dok1; M1: Y295F mutant Dok1; M4: Y361F mutant Dok1.

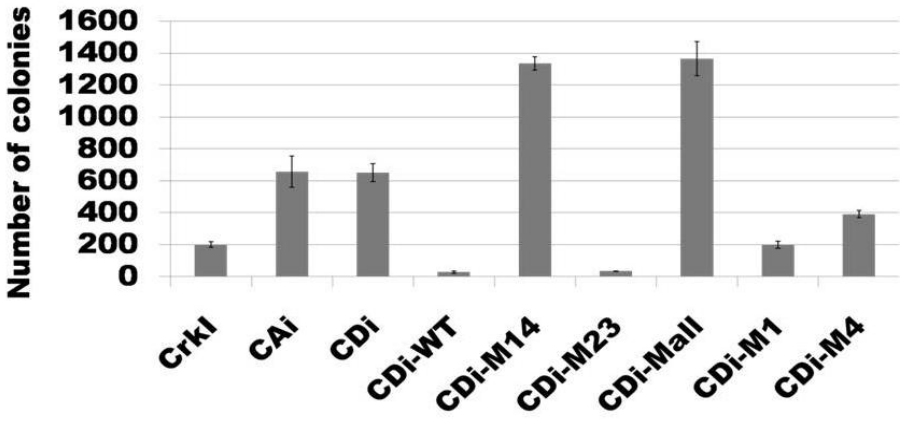


Figure 2.6: Colonies formation results in anchorage independent assay.

Anchorage independent colony formation results for cells in Figure 2.5. C, CrkI transformed; CAi, CrkI-transformed, Abl knockdown; CDi, CrkI-transformed, Dok1 knockdown; WT: wild-type Dok1; M14: Y295F and Y361F mutant Dok1; M23: Y336F and Y340F mutant Dok1; M-all: Y295F, Y336F, Y340F and Y361F mutant Dok1; M1: Y295F mutant Dok1; M4: Y361F mutant Dok1.

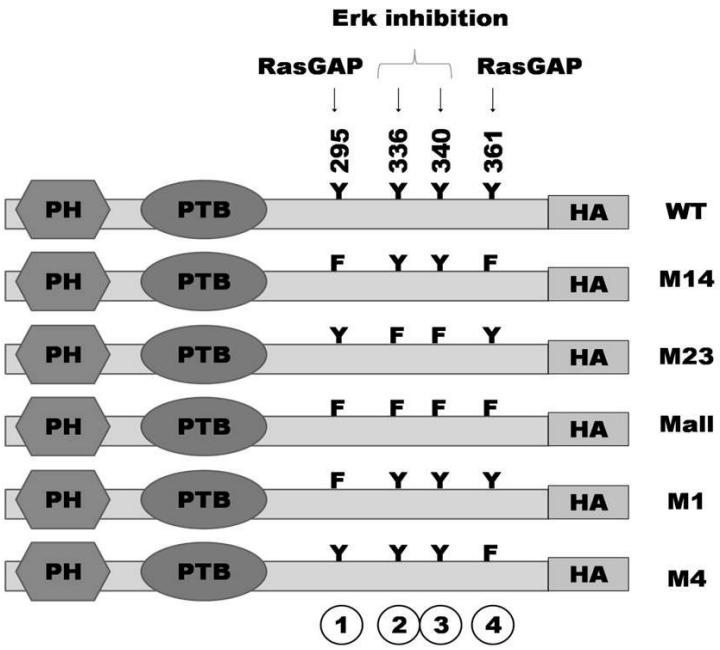


Figure 2.7: Dok1 constructs used in experiments.

Diagram of the human Dok1 cDNA constructs used, numbers at the bottom indicate positions of tyrosine phosphorylation sites mutated. PH, Pleckstrin homology domain; PTB, phosphotyrosine binding domain; Y, tyrosine; F, phenylalanine; HA, epitope tag; WT: wild-type Dok1; M14: Y295F and Y361F mutant Dok1; M23: Y336F and Y340F mutant Dok1; M-all: Y295F, Y336F, Y340F and Y361F mutant Dok1; M1: Y295F mutant Dok1; M4: Y361F mutant Dok1.

mutation of Y361. These constructs were re-expressed in the CrkI-transformed Dok1 knockdown cells (Figure 2.5) and cells assayed for anchorage independent growth in soft agar (Figure 2.6). Expression of the WT Dok1 or the M23 mutant rescued the phenotype (enhanced CrkI transformation) caused by Dok1 knockdown; in fact, transforming activity of rescued cells was even lower than cells expressing endogenous amounts of Dok1. On the other hand, knockdown cells expressing the M14 and M-all mutants showed an even greater increase in colony formation than seen with Dok1 knockdown alone. Expression of single mutants (M1 and M4) partially rescued the Dok1 knockdown phenotype. These results suggested that Y295 and Y361 work together to suppress CrkI tumorigenesis when phosphorylated. Dok1 knockdown followed by re-expression of WT or mutant Dok1 in control NIH3T3 cells yielded no colonies (data not shown), consistent with the effect of Dok1 tumor suppression being specific to cells transformed by Crk.

We also tested the effect of simple Dok1 overexpression in CrkI-transformed NIH3T3 cells (Figure 2.8). Consistent with its role as a putative tumor suppressor, overexpression of WT Dok1 and the M23 Dok1 mutant both suppressed CrkI transformation in the soft agar assay; by contrast, the M14 and M-all mutants enhanced CrkI transformation (Figure 2.9). This demonstrates that expression of Dok1 mutants that cannot be phosphorylated at sites 295 and 361 exerts a dominant-negative (pro-oncogenic) effect over endogenous Dok1. Surprisingly, overexpression of the M1 and M4 single mutants showed a greater suppression of transformation than seen for WT Dok1. Once again, these results were CrkI-dependent, as no colonies were seen in control 3T3 cells overexpressing Dok1 mutants (data not shown).

SH2 domain binding partners of tyrosine-phosphorylated Dok1

Most tyrosine-phosphorylated sites function in signaling by binding to the SH2 or PTB domains of effector proteins (Wagner et al., 2013). To assess what SH2 domains bind to the Dok1 sites implicated in suppressing CrkI-transformation, we carried out a dot-blot SH2 profiling assay (Machida et al., 2007) using purified glutathione S-transferase (GST)-SH2 or GST-PTB domain

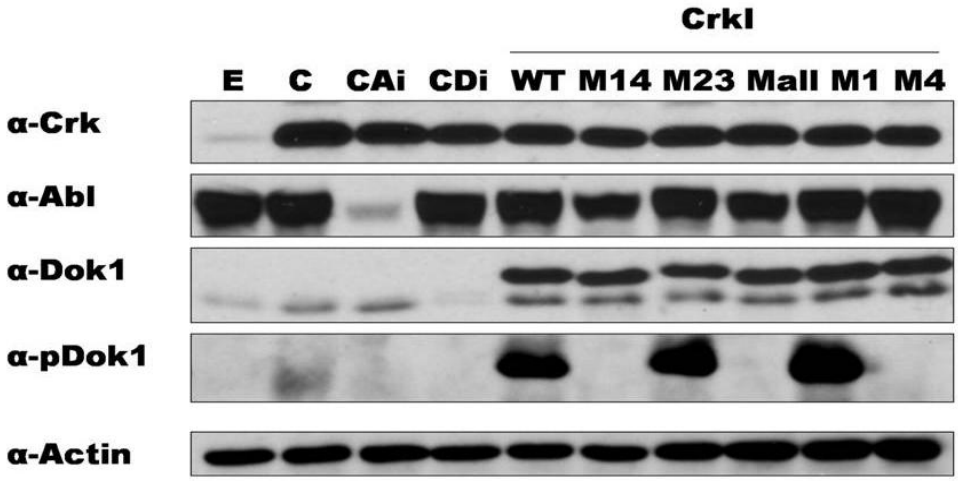


Figure 2.8: Overexpression of Dok1 in CrkI-transformed cells.
Overexpression of Dok1 in CrkI-transformed NIH3T3 cells. E, empty vector control; C, CrkI transformed; CAi, CrkI-transformed, Abl knockdown; CDi, CrkI-transformed, Dok1 knockdown; WT: wild-type Dok1; M14: Y295F and Y361F mutant Dok1; M23: Y336F and Y340F mutant Dok1; M-all: Y295F, Y336F, Y340F and Y361F mutant Dok1; M1: Y295F mutant Dok1; M4: Y361F mutant Dok1.

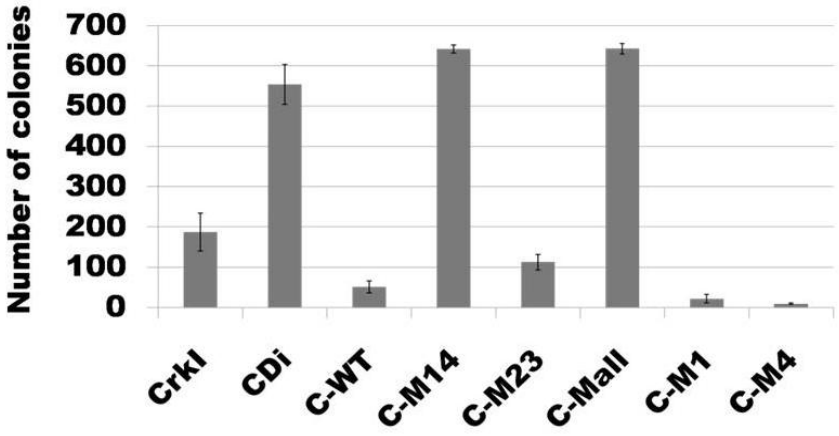


Figure 2.9: Colonies formation results in anchorage independent assay.

Anchorage independent colony formation results for cells in Figure 2.8. C, CrkI transformed; CDi, CrkI-transformed, Dok1 knockdown; WT: wild-type Dok1; M14: Y295F and Y361F mutant Dok1; M23: Y336F and Y340F mutant Dok1; M-all: Y295F, Y336F, Y340F and Y361F mutant Dok1; M1: Y295F mutant Dok1; M4: Y361F mutant Dok1.

fusion proteins to probe synthetic tyrosine-phosphorylated peptides corresponding to the four sites of interest in Dok1 (Figure 2.10 and Figure 2.11).

Of the 123 SH2 or PTB probes tested (Figure 2.12), a few showed strong binding to Dok1-derived phosphopeptides. Abl and Arg (Abl2) SH2 domains strongly bound to all four phosphorylated sites, but not the unphosphorylated control peptides (Figure 2.13). As expected, the SH2 domains of RasGAP (Figure 2.14) bound strongly to pTyr 295 and 361. The C-terminal RasGAP SH2 domain bound specifically to pTyr 295, whereas the N-terminal domain was less specific and bound to phosphopeptides corresponding to tyrosines 295, 340 and 361 (Figure 2.13) . SH2 domains of p85α, a subunit of phosphatidylinositol 3-kinase, also bound to phosphorylated sites in Dok1. The N-terminal SH2 domain of p85α bound pTyr 336, whereas a construct encompassing both SH2 domains bound pTyr 295, 336 and 361. Notably, none of the phosphopeptides tested bound strongly to the Crk SH2 domain (Figure 2.13). The unphosphorylated peptides did not bind appreciably to any of the SH2 domain probes, highlighting the specificity of our SH2 probes for tyrosine-phosphorylated peptides.

We also performed far-western blotting to probe Imatinib-treated cell lysates with RasGAP SH2 domains. The RasGAP SH2 probes bound to a band in Crk-transformed cells corresponding to phosphorylated Dok1; binding was abolished when Abl-mediated phosphorylation was inhibited with Imatinib (Figure 2.15). The relatively less specific RasGAP N-terminal SH2 domain showed the strongest difference in binding to Dok1 when cells were treated with Imatinib. This suggests that in vivo, Abl preferentially phosphorylates site 4 (Y361) relative to site 1 (Y295), as little binding was seen with the RasGAP C-terminal SH2 probe, which is highly specific to site 1. A similar pattern of binding was also observed in the control 3T3 cell lysates, albeit at a much lower intensity.

Role of RasGAP in regulating Crk transformation

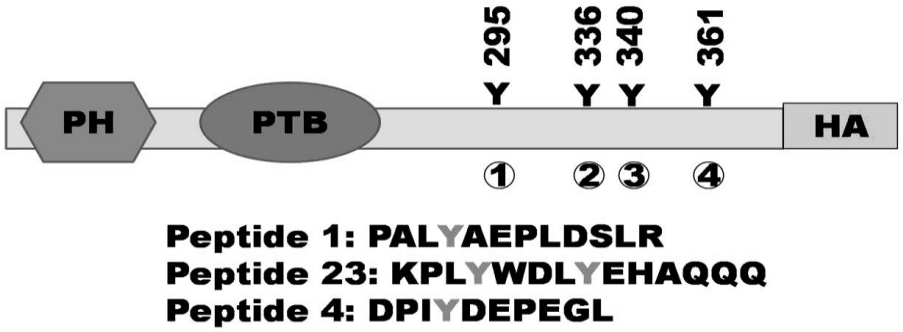


Figure 2.10: Tyrosine-containing peptides sequence based on their positions in Dok1. Phosphorylation sites on Dok1 are indicated; each site is numbered from 1 to 4. Sequences of corresponding synthetic peptides are indicated below. PH, Pleckstrin homology domain; PTB, phosphotyrosine binding domain; Y, tyrosine HA, epitope tag.

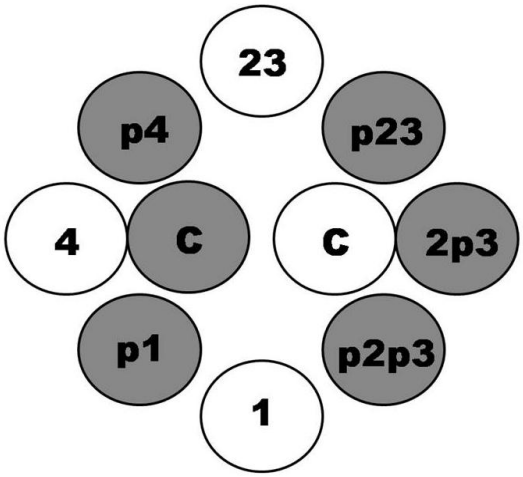
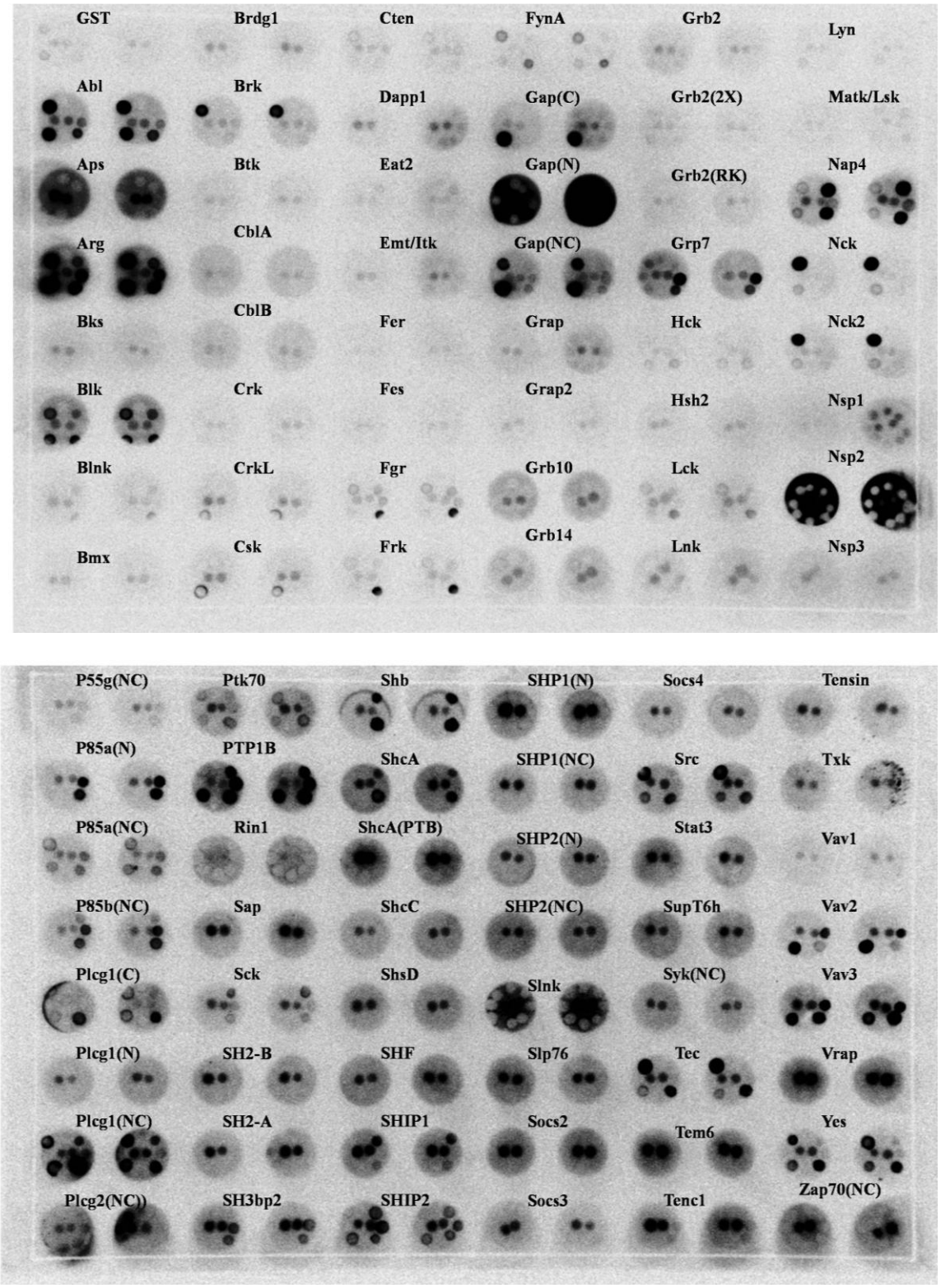


Figure 2.11: Peptides arrangements in SH2 domain dot-blotting assay.
Synthetic peptides were spotted to filters in the pattern shown. Gray circles and 'p' indicate tyrosine phosphorylation of corresponding site (for example, 'p1' denotes peptide in which site 1 is phosphorylated); c, control (whole cell lysates).



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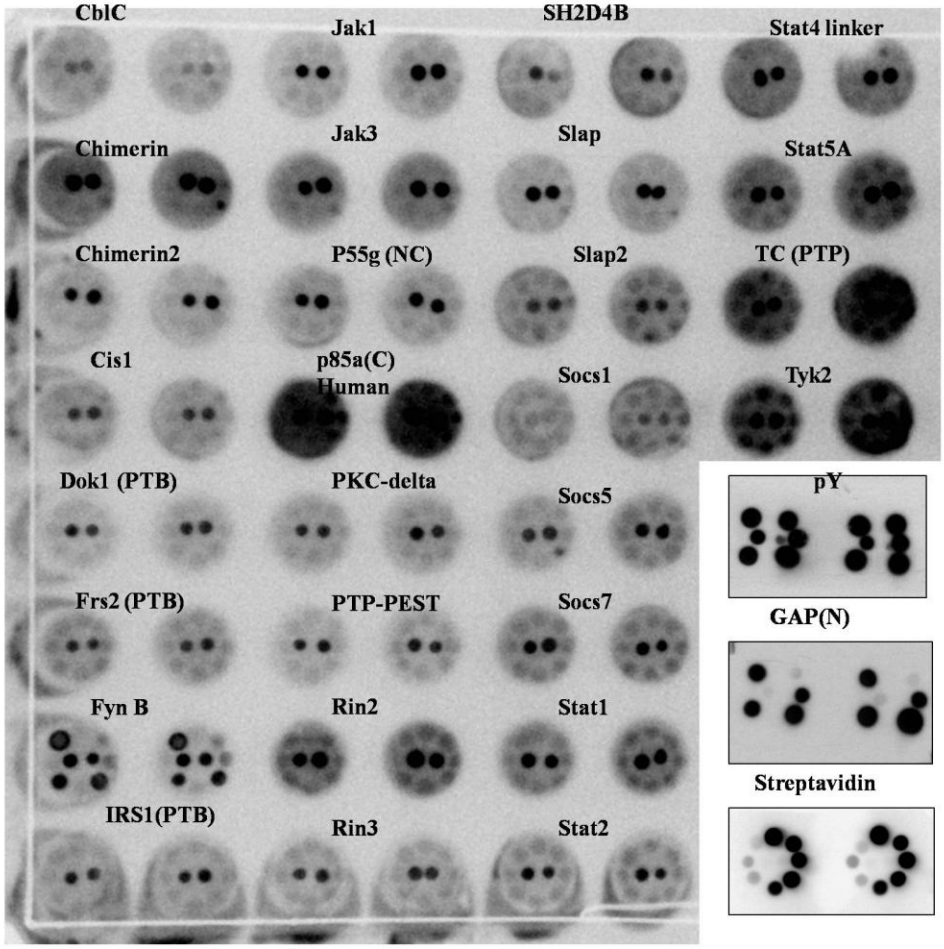


Figure 2.12: Results from SH2 domains binding assay.
Peptide-spotted filters were probed in duplicates with purified SH2 and PTB domains. A total of 124 probes were tested with varying results. pY: phosphorylated tyrosine antibody; N, N-terminal SH2 domain; C, C-terminal SH2 domain; NC, both N- and C-terminal SH2 domains; Streptavidin, Streptavidin antibody for spotting control.

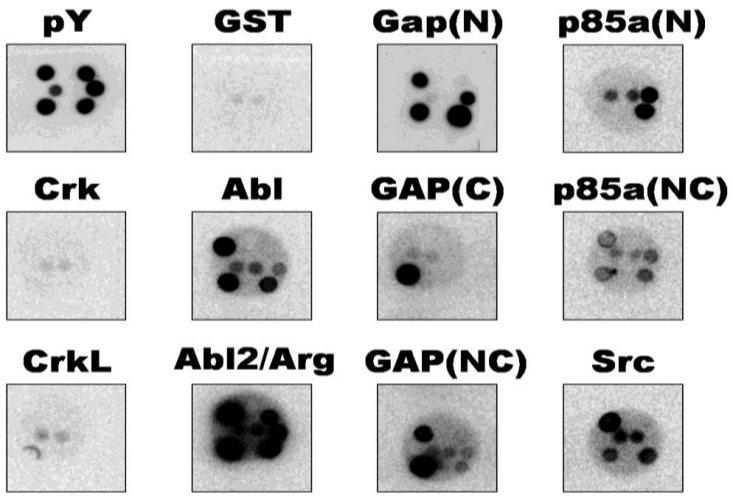
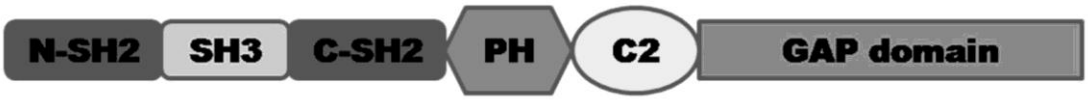


Figure 2.13: Selected results from SH2 domains binding assay.
Peptide-spotted filters were probed with purified SH2 and PTB domains. Binding results for selected domains are shown here. pY: phosphorylated tyrosine antibody; N, N-terminal SH2 domain; C, C-terminal SH2 domain; NC, both N- and C-terminal SH2 domains.



RasGAP domain structure

Figure 2.14: Domain structure of RasGAP.

RasGAP domain structure. N, N-terminal; C, C-terminal; SH2; Src Homology 2 domain; SH3, Src Homology 3 domain; PH, Pleckstrin Homology domain; C2, C2 domain; GAP, catalytic domain for GTPase activation.

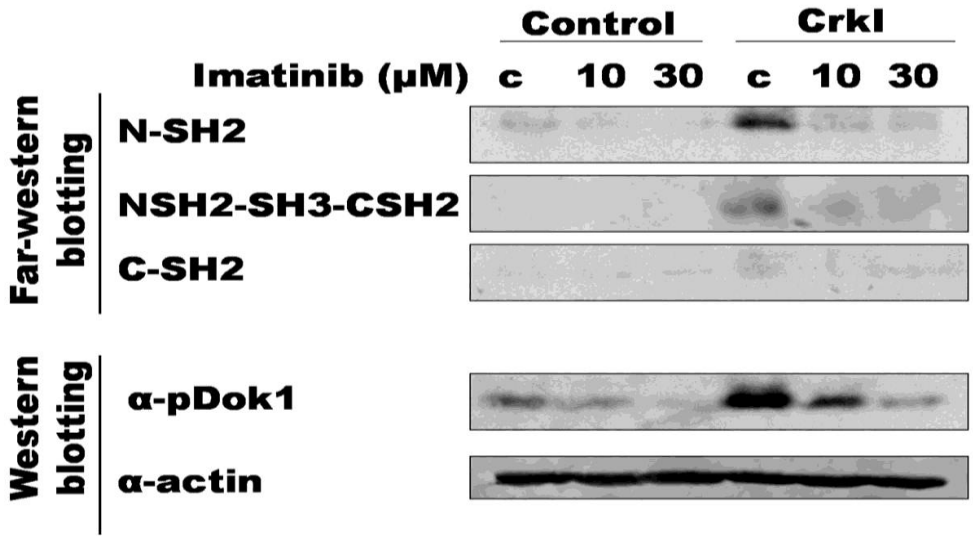


Figure 2.15: Far-western and immunoblotting results indicating phosphoDok1-dependent binding of RasGAP.

Far-western and immunoblotting of lysates from Imatinib-treated control and CrkI-transformed NIH3T3 cells. Top, lysates were probed with GST-RasGAP SH2 domain fusions; bottom, same lysates were probed with phosphospecific Dok1 antibody. C, untreated control lysates.

We next sought to confirm that RasGAP binds to phosphorylated Dok1 in our cell system by expressing HA-tagged hDok1 and immunoprecipitating with anti-HA antibody. To maximize the detection of phosphotyrosine-dependent interactions, cells were treated briefly with pervanadate (POV) before lysis to inhibit endogenous tyrosine phosphatases. Immunoblotting of anti-HA immunoprecipitates with anti-RasGAP antibody demonstrated association between RasGAP and WT Dok1; this binding was decreased, however, when tyrosines 295 and 361 were mutated (mutants M14 and M-all; Figure 2.16). These results are consistent with the results of phosphopeptide binding experiments (Figure 2.13 and Figure 2.15). The M1 and M4 mutants of Dok1 both associated with RasGAP to a similar extent as WT Dok1, demonstrating that, under these conditions, phosphorylation of either Y295 or Y361 alone is sufficient to mediate RasGAP binding.

Next we knocked down RasGAP in both the control and CrkI overexpressing cells (Figure 2.17). Loss of endogenous RasGAP in CrkI-transformed NIH3T3 increased the number of colonies (Figure 2.18), similar to what was observed in Abl and Dok1 knockdowns (Figure 2.6). This observation is consistent with a model in which Abl works through Dok1 and RasGAP to regulate CrkI transformation.

Ras activation in CrkI-transformed cells

Although the previous results all suggest a role for elevated Ras activity in Crk transformation, over many experiments we did not detect significant differences in total Ras or Erk activity in CrkI-transformed cells relative to control, or under conditions where Abl or Dok1 activity was manipulated (Figure 2.19). We therefore considered whether CrkI overexpression might induce localized differences in Ras activity that were not evident in total cell lysates. To study the spatiotemporal aspects of Ras activation, we employed a newly developed FRET sensor for activated Ras (dimerization optimized reporter for activation (Dora)-Ras) (Figure 2.20) and conducted live cell imaging of Ras activation during cell spreading on fibronectin. As Ras is

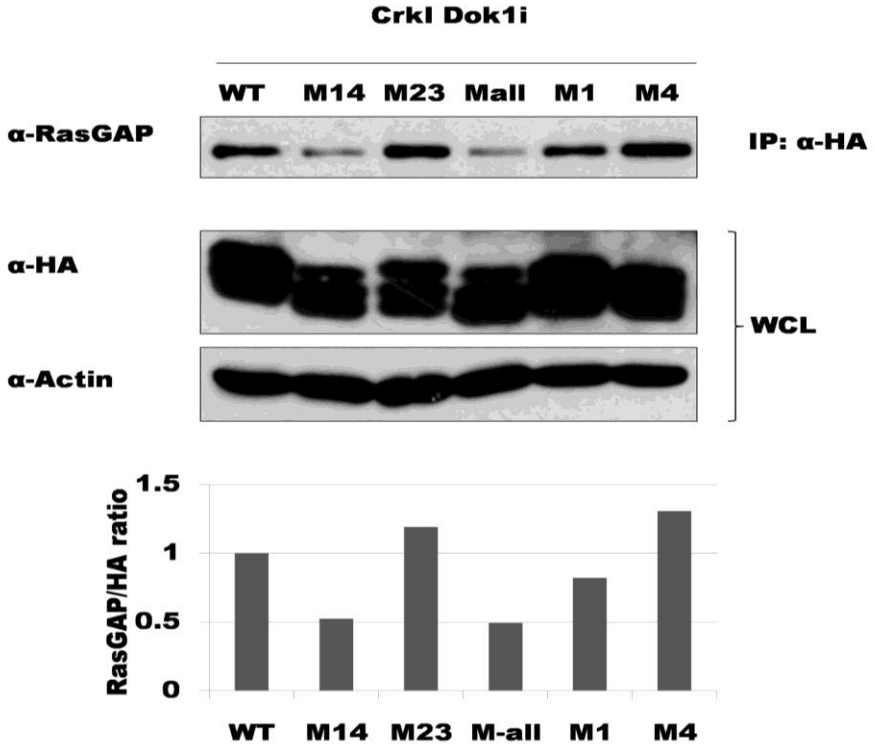


Figure 2.16: Tyrosines 295 and 361 in Dok1 were both required for *in vivo* for RasGAP binding. Lysates of CrkI-expressing Dok1 knockdown cells rescued with HA-tagged Dok1 mutants indicated were immunoprecipitated with anti-HA antibody before immunoblotting with anti-RasGAP antibody. RasGAP binding to Dok1 was decreased when tyrosines 295 and 361 were both mutated (M14 and M-all). Cells were treated with pervanadate before lysis to increase total pTyr levels. Bottom: bands from two independent experiments were quantified; average RasGAP/HA ratio was normalized to WT Dok1.

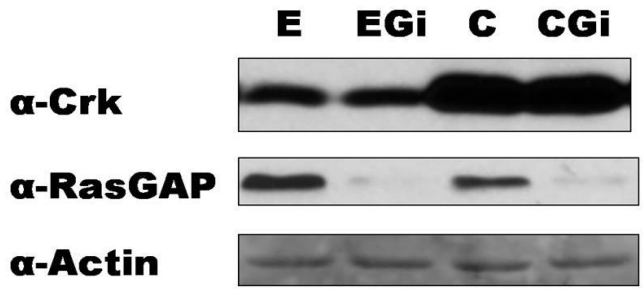


Figure 2.17: RasGAP knockdown in control and CrkI-transformed NIH3T3 cells.
Western blotting demonstrating knockdown of RasGAP. E, empty vector control NIH3T3 cells; EGi, RasGAP knockdown control cells; C, CrkI-transformed cells; CGi, CrkI-transformed, RasGAP knockdown cells.

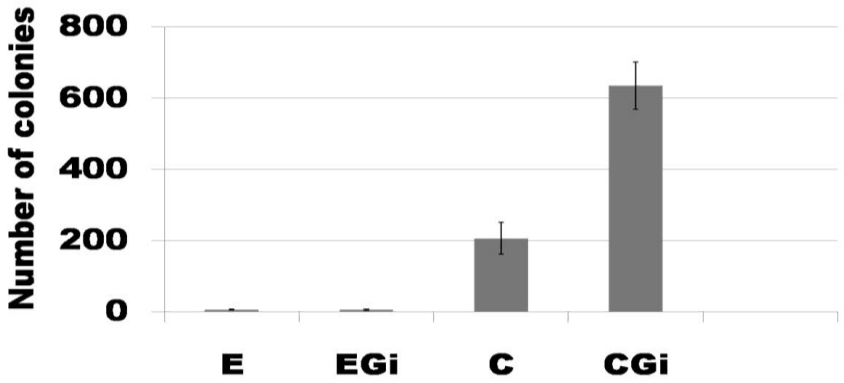


Figure 2.18: RasGAP knockdown enhances CrkI-transformed NIH3T3 cells.
Soft agar colony formation results for cells are shown in Figure 2.17. RasGAP knockdown increases the number of colonies in CrkI-transformed cells (C) but has no effect on the empty vector control cells (E). E, empty vector control NIH3T3 cells; EGi, RasGAP knockdown control cells; C, CrkI-transformed cells; CGi, CrkI-transformed, RasGAP knockdown cells.

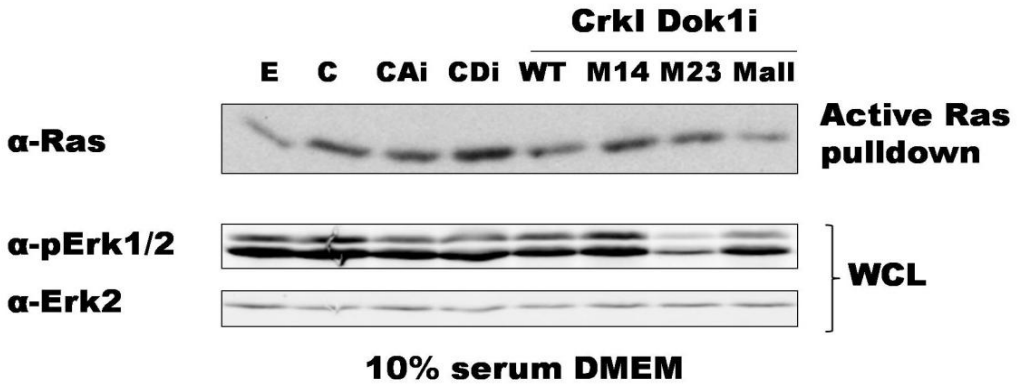


Figure 2.19: Ras-MAPK pathway activity is not strongly affected by CrkI or Dok1 expression. Activated Ras level was assayed in NIH3T3 cell lysates by pulldown using GST-tagged Ras binding domain (RBD) of Raf1. Phosphorylated Erk antibody detects both activated Erk1 and Erk2, while Erk2 antibody detects the overall Erk2 level and serves as loading control here. Minor differences in activated Ras or Erk levels seen in this experiment were not observed consistently in multiple independent experiments. E: empty vector control cells; C: CrkI-transformed cells; CAi: CrkI-transformed, Abl knockdown cells; CDi: CrkI-transformed, Dok1 knockdown cells; WT: wild type Dok1-expressing cells; M14: Y295 and Y361 mutant Dok-expressing cells; M23: Y336 and Y340 mutant Dok1-expressing cells; M-all: Y295, Y336, Y340 and Y361 mutant Dok1-expressing cells; WCL: whole cell lysate.

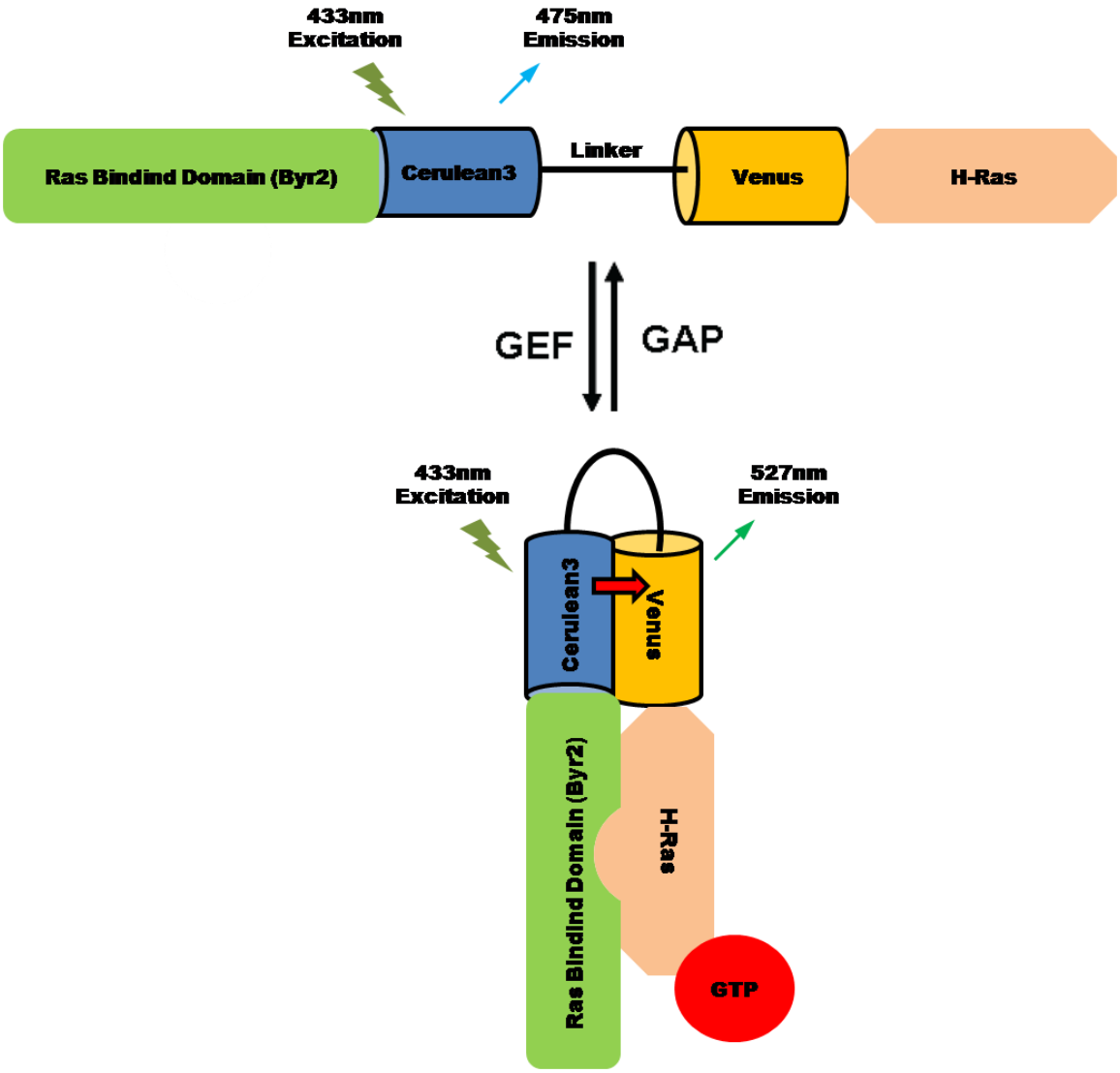


Figure 2.20: Dimerization optimized reporter for activation-Ras (Dora-Ras).
The Dora-Ras sensor contains an N-terminal Ras binding domain (Cys71-Ser161) (RBD) derived from Byr2, followed by a fluorescent protein FRET pair (Cerulean3-Venus), and an intact, wild-type H-Ras at the C-terminus. Upon activation of by Guanine exchange factor (GEF), the binding of GTP causes a conformational change in H-Ras and trans-binds the RBD domain. The close proximity of the FRET pair now allows the transfer of energy and emission of a 527nm wavelength fluorescent signal. On the other hand, GTPase Activating Protein (GAP) promote the hydrolysis of GTP to GDP in H-Ras and thus the disassociation from the RBD and emission of native 475nm wavelength.

activated on the plasma membrane, we used total internal reflection fluorescence (TIRF) excitation in our imaging studies to reduce imaging artifacts typically associated with wide-field imaging. Fifteen minutes after plating, cells transiently transfected with Dora-Ras sensor showed elevated ratio (FRET/CFP) values, indicative of Ras activation, in lamellipodial protrusions (Figure 2.21a). In contrast, cells transfected with a control construct (with a point mutation in the Ras binding domain that abolishes sensor response), showed no polarized distribution of ratio values (Figure 2.21b).

Crk is known to localize to adhesions by binding to phosphorylated paxillin and p130Cas (Chodniewicz and Klemke, 2004; Downey et al., 2008; Watanabe et al., 2009a). To relate sites of Ras activation to the location of adhesions, we co-expressed mCherry-tagged paxillin with the Dora-Ras sensor. In control NIH3T3 cells, Ras activity colocalized with nascent adhesions at the leading front, but diminished in the vicinity of mature focal adhesions as the cells spread (Figure 2.21c). In contrast, CrkI-transformed cells maintained elevated Ras activation while adhesions matured (Figure 2.21d). Strikingly, the spatial distribution of Ras activation often appeared bipolar or multipolar, and the cells developed multiple protrusions at an early stage of cell spreading. Thus we conclude that the localized distribution of activated Ras is dramatically different in CrkI-transformed cells compared with their normal controls.

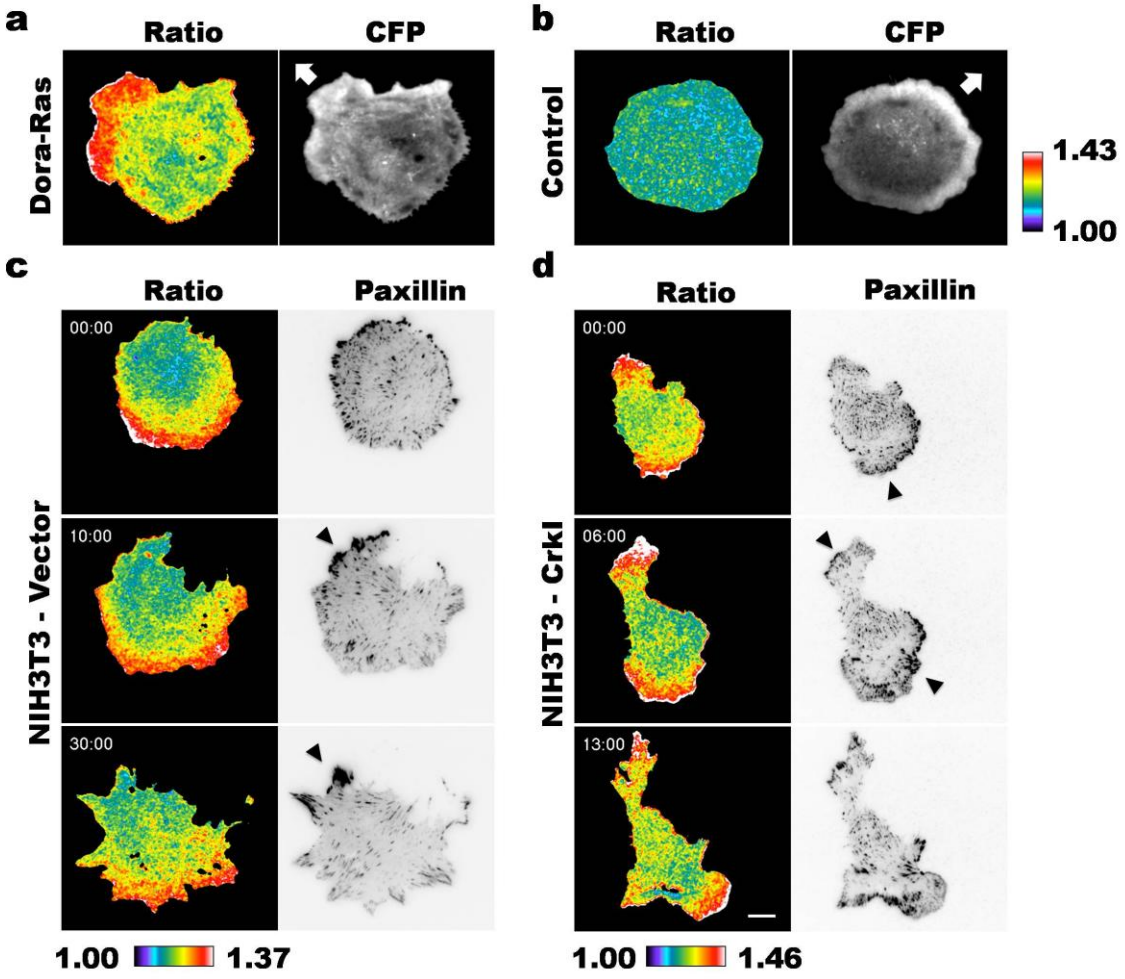


Figure 2.21: Ras activation is partially uncoupled from the turnover of adhesions in CrkI-transformed cells.

NIH3T3-vector (a–c, n=18 cells) or − CrkI (d, n=18 cells) cells were co-transfected with the Dora-Ras FRET sensor (a, c and d) or sensor control (b, n=9 cells) and mCherry-tagged paxillin. The cells were seeded onto fibronectin-coated coverslips and imaged with TIRF excitation 15 min after plating. The ratio (FRET/CFP) images were calculated and presented in pseudocolor based on the lookup table provided (a and b, right; c and d, bottom). CFP and mCherry intensities were illustrated in grayscale and inverted grayscale images, respectively. The arrows (a and b) indicate directions of cell movement and the arrowheads (c and d), clusters of focal adhesions. Scale bar, 10 μm.

Data by Wu's lab, CCAM, UCHC

Discussion

In Figure 2.22, we propose a model for how Abl regulates transformation through Dok1 phosphorylation. The overexpression of CrkI leads to its recruitment and clustering at membrane sites rich in tyrosine-phosphorylated proteins, such as focal adhesions. Crk in turn recruits its SH3 binding effectors such as Sos to these sites, leading to localized activation of Ras and ultimately resulting in cell transformation. At the same time, however, Crk triggers a negative feedback loop by recruiting Abl family kinases, which associate with and phosphorylate Dok1. Phosphorylated DokI recruits RasGAP, which counteracts the stimulation of Ras by Sos and thereby blunts Crk transformation. When Abl-mediated phosphorylation is inhibited (either by knockdown or Imatinib), the resulting decrease in Dok1 phosphorylation prevents it from recruiting RasGAP, thereby increasing local Ras activation and enhancing transformation. Interestingly, we have noticed that CrkI overexpression reproducibly causes a modest increase in endogenous Dok1 expression in addition to increased Dok1 phosphorylation (see Figures 2.5 and Figure 2.8), consistent with a negative feedback response to CrkI transformation.

While our data demonstrate a strong negative correlation between phosphorylated Dok1 and CrkI transformation, it is unclear what role this pathway has in other types of cell transformation. On one hand, we previously showed that Imatinib had little effect on the anchorage-independent growth of cells transformed by Src or Ras (Zheng et al., 2010). However, previous results showing that Dok proteins can negatively regulate signaling from the BCR and TCR, and that they exhibit tumor suppressor activity, suggest a more general role in feedback regulation of the Ras pathway. It is possible that the repressive role of Dok is particularly evident in Crk transformation because Crk is a relatively weak oncogene in mammalian fibroblasts.

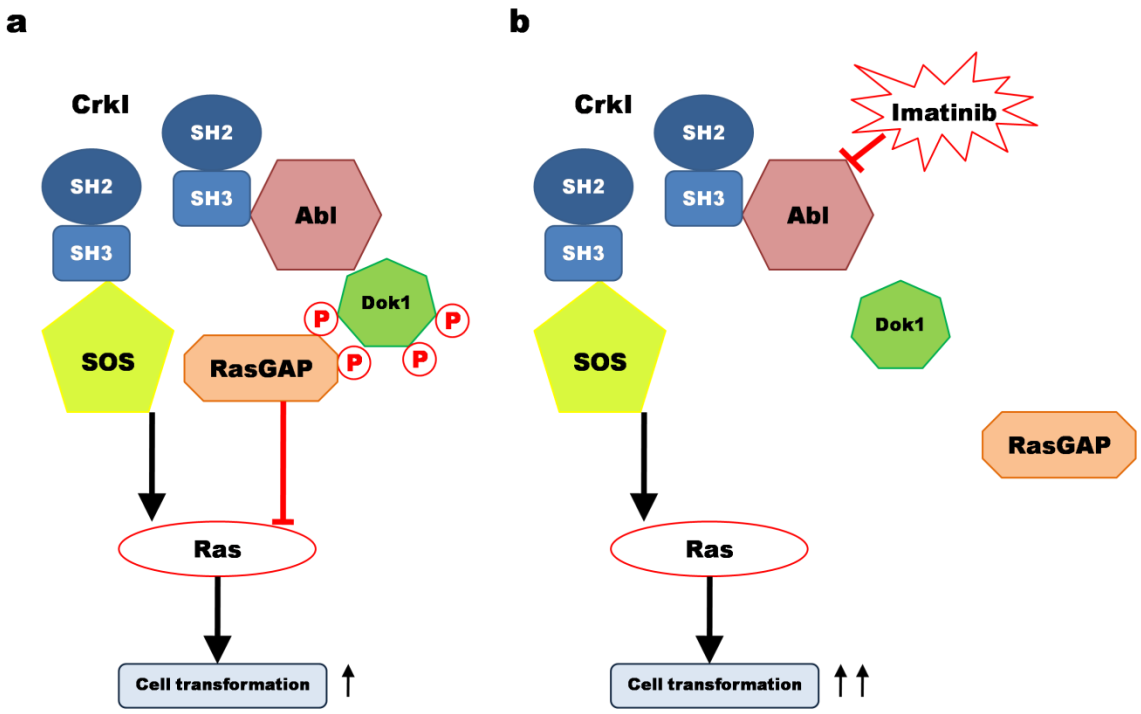


Figure 2.22: Phosphorylated Dok1 regulates CrkI transformation.
(a) CrkI (blue) overexpression nucleates formation of localized protein complexes, including those directly bound (Sos and Abl shown here) and indirectly bound (Dok1 and RasGAP). Sos serves as a crucial positive effector for CrkI transformation, whereas phosphorylated Dok1 acts as a negative regulator, likely by inhibiting the Ras pathway by recruiting RasGAP. The net output of the complex is relatively weak transformation. (b) When Abl family kinase activity is inhibited (by knockdown or inhibition), Dok1 is no longer highly phosphorylated and thus loses its ability to repress cell transformation, shifting the balance further toward strong transformation.

Greulich and Hanafusa (Greulich and Hanafusa, 1996) previously showed that dominant-negative Ras mutants blocked transformation by v-Crk, and we later showed that Sos1, the major guanine nucleotide exchange factor for Ras, is by far the most critical SH3 binding protein of those tested for CrkI transformation (Zheng et al., 2010). However, the specific role of Ras activation in Crk transformation remains enigmatic, as we and others have not seen a consistent or significant increase in the activity of Ras or its downstream effectors in Crk-transformed cells relative to controls (Akagi et al., 2000; Greulich and Hanafusa, 1996; Zheng et al., 2010). Our current results further implicate Ras activity in Crk transformation by demonstrating that phosphorylation of two sites in Dok1 that bind the Ras inhibitor RasGAP (tyrosines 295 and 361) is critical for suppressing Crk transformation. We show that Dok1 is highly phosphorylated in Crk-transformed cells, and that this phosphorylation is abrogated by the Abl inhibitor Imatinib, which stimulates Crk transformation. We also show that overexpression of Dok1 mutants that cannot be phosphorylated at the RasGAP binding sites strongly stimulates Crk transformation, whereas expression of WT Dok1 or Dok1 mutants in which these sites are intact strongly inhibits anchorage-independent growth induced by CrkI overexpression. Finally, we also show that RasGAP knockdown enhances CrkI transformation (Figure 2.18).

Interestingly, Dok1 knockdown cells rescued with Dok1 mutated at tyrosines 295 and 361 (M14 and M-all) were even more transformed than the Dok1 knockdown alone (Figure 2.6). A likely explanation is that the Dok1 mutants have a dominant-negative effect on the residual, endogenous Dok1 present in knockdown cells. The fact that simple overexpression of these Dok1 mutants had similar effects, even in the presence of unaltered endogenous Dok1, is also consistent with this idea. Phospho-dependent homotypic and heterotypic oligomerization between Dok1 and Dok2 is reported to be critical for their function (Songyang et al., 2001), so the presence of mutated Dok1 would likely disrupt the integrity of oligomers and compromise their normal ability to downregulate signaling.

One way to reconcile the apparent importance of Ras in Crk transformation with the lack of obvious elevation in total Ras activity in Crk-transformed cells is to suppose that Ras activation by CrkI is highly localized and therefore not apparent in whole cell lysates. Indeed, during cell spreading we noted striking differences in the localization of activated Ras in Crk-transformed cells relative to controls (Figure 2.21). In general, upon spreading, control cells rapidly formed a single leading edge where Ras activity was highest, whereas mature focal adhesions with the highest concentration of paxillin and Crk were mostly found along the sides and trailing edge of the cell, away from the areas of highest Ras activity. Others have previously noted that activated Ras is enriched in lamellipodia and the leading edge of polarized cells (Mochizuki et al., 2001; Yip et al., 2007). In CrkI-transformed cells, however, most cells formed multiple highly protrusive fronts with high Ras activity, which in some cases coincided with mature focal adhesions. This phenotype is consistent with the partial loss of a negative feedback loop that normally functions to repress Ras at focal adhesions after a brief burst of activity.

Our results suggest that CrkI overexpression has both positive and negative effects on cell transformation, through different sets of protein interactions. Inputs that interfere with some of these interactions will shift this equilibrium, pushing the cell toward one extreme or the other (normal vs tumorigenic). Consistent with this idea, Crk transformation of mammalian cells is relatively weak, presumably at least in part because of the negative feedback provided by phosphorylation of Dok1 by Abl. The ability of Crk to transform cells is likely to depend not only on the level of Crk overexpression, but also on the relative abundance of positive

effectors such as Sos, versus potential negative regulators such as Abl, Dok1 and RasGAP. Our results highlight the importance of understanding the role of negative feedback loops when considering therapies that incorporate Abl inhibition for the treatment of human tumors.

Chapter 3

Effects of Abl inhibition in Crk-transformed fibroblasts and human tumor cell lines

Attribution: Except for Figure 3.13, all data in this chapter were generated by Ng KY. Figure 3.13 was adapted from the on-line database of Developmental Therapeutics Program (DTP) in National Cancer Institute/ National Institute of Health (NCI/NIH)

Abstract

Tumorigenesis generally involves cells acquiring several growth advantages such as proliferation, migration, apoptosis resistance etc. We had previously identified an Abl-dependent phospho-Dok1 that negatively regulates CrkI transformation in NIH3T3 fibroblasts. Here, we address the phenotypic changes that resulted from the inhibition of the Abl/Dok1 signaling pathway. While our data indicate an increased cell proliferation rate following CrkI overexpression, we found no evidence to support the hypothesis that the subsequent disruption of Abl/Dok1 pathway further increases cell proliferation rate. Moreover, the two-dimensional migration rates of cells were not affected either by CrkI overexpression or Abl/Dok1 knockdown. As for the signaling pathways involved, the knockdown of Abl or Dok1 further activates the Jnk pathway and this emphasizes the role of Jnk in Imatinib-enhanced CrkI transformation. Taken together, our preliminary data suggest that the enhanced CrkI transformation following Abl downregulation is most likely linked to the dysregulation of small GTPases and enhanced Jnk phosphorylation. In addition to Dok1, we also found that Abl inhibition reduces the phosphorylation of tyrosine 221 in CrkII. But since the coexpression of unphosphorylatable CrkII (Y221F) and CrkI in NIH3T3 cells showed no significant advantage in the anchorage independent growth, we concluded that the increase of unphosphorylated CrkII has no immediate contribution to Imatinib-enhanced CrkI tumorigenesis. Finally, to understand if our Imatinib-enhanced CrkI transformation model may have any implication for human cancer, we selected eight cell lines from the NCI60 panel based on their Crk and Abl protein expressions for anchorage independent growth assay in the presence and absence of Imatinib. So far, we found one glioblastoma cell line, SF268 that showed increased colony size and number following the Imatinib treatment. Further research and confirmation are required in order to understand the underlying mechanism(s).

Introduction

Cell malignancy is often associated with cells that have undergone increased proliferation, enhanced migration and invasion, evasion of programmed cell death and/or acquiring other survival advantages (Hanahan and Weinberg, 2011). Although several Crk proteins have been shown to readily transform cultured fibroblasts via overexpression(Akagi et al., 2000; Greulich and Hanafusa, 1996; Iwahara et al., 2003; Matsuda et al., 1992; Zheng et al., 2010), the exact growth-promoting traits that confer cell transformation remain poorly understood. In the previous chapter, we described an Abl/Dok-mediated negative regulatory pathway that restricts the growth of CrkI-expressing cells in a mouse fibroblast model. However, the anchorage independent assay alone does not identify how CrkI and the subsequent inhibition of the Abl-Dok1 signaling induces and enhances transformation. In the other words, it is unclear if these colony-forming cells have gained an overall higher cell proliferation rate, bypassed anoikis regulation, or acquired some other growth advantages.

Most of the studies conducted in Crk proteins centered around their roles in focal adhesion sites due to their strong SH2-dependent binding affinity to p130Cas and paxillin (Birge et al., 1993; Downey et al., 2008; Gustavsson et al., 2004; Klemke et al., 1998; Sakai et al., 1994; Watanabe et al., 2009a; Yamada et al., 2011). Downregulation of Crk proteins in several primary human cancer cells eventually led to reduced cellular motility and invasiveness (Fathers et al., 2012; Kim et al., 2010; Linghu et al., 2006; Rodrigues et al., 2005; Watanabe et al., 2009b). The general consensus is that elevated Crk proteins in cancer cells confers higher metastatic potential and this has been correlated well with the anchorage-independent growth promoting phenotypes seen in several Crk-transformed fibroblasts models. However, even though the knockdown of Crk proteins in fibroblasts significantly reduces cell motility (Antoku and Mayer, 2009), it is unclear how the overexpression of Crk proteins affects the cell migration rate.

As for the downstream signaling proteins that control Crk transformations, several independent v-Crk and CrkI studies confirmed that Sos/Ras and C3G/Jnk signaling pathways are required for the tumorigenecity of Crk proteins (Greulich and Hanafusa, 1996; Tanaka et al., 1994; Tanaka et al., 1997; Zheng et al., 2010). However, given the complexity of protein signal transductions and the crosstalk between different pathways, we are still unable to connect and delineate a more precise and comprehensive signaling networks for the oncogenesis of Crk proteins. So far, we learned that following the overexpression of Crk proteins, Erk phosphorylations remain mostly unchanged but Jnk phosphorylations are highly elevated. Then, subsequent knockdown of Sos still has little effect on Erk (Zheng et al., 2010) but the knockdown of C3G or expression of a dominant-negative Sek1, a direct activator of Jnk, inhibited Jnk phosphorylation (Tanaka et al., 1997; Zheng et al., 2010) and all the above mentioned manipulations resulted in inhibited Crk transformation.

The observations above led us to hypothesize that Ras activation does not simply activate the canonical Sos/Ras/Erk pathway and that Jnk has a pivotal role in the signaling of oncogenic Crk proteins. Upstream of Jnk, preliminary studies showed that C3G, a Crk SH3 binding protein, binds and activates the Rap1 and R-Ras GTPases in v-Crk transformed fibroblasts (Mochizuki et al., 2000; Tanaka et al., 1997). The Ras superfamily consist of six subfamilies (Ras, Rho/Rac, Ran, Rab, Arf and Kir/Rem/Rad) and the Ras subfamily can be further divided into five subgroups (Ras, M-Ras, R-Ras, Rap and Ral). Rap1 and R-Ras each belongs to one of five Ras subgroups and within these five Ras subgroups, activators known as GTPase exchange factors (GEFs) and deactivators know as GTPase activating proteins (GAPs) bind and regulate these small GTPases across multiple subgroups. Biochemical studies found little specificity in the binding of these regulators (with a few exceptions) but in cells, more specific interactions do exist and they are mostly relying on the binding affinity between GEF/GTPase, GTPase/GAP and subcellular localization of these proteins (Ehrhardt et al., 2002).

Downstream of v-Crk, the expression of a dominant negative R-Ras (a C3G-activated small GTPase) morphologically reverses v-Crk transformation (Mochizuki et al., 2000). As for Rap1, it is first known as a tumor suppressor in KRas-transformed NIH3T3 cells (Kitayama et al., 1989) and it has been previously shown to suppress v-Crk transformation as well (Tanaka et al., 1997)(Tanaka et al., 1997)(Tanaka et al., 1997). Taken together with its higher affinity for B-Raf (Ohtsuka et al., 1996), Rap1 is believed to have little involvement in C3G-dependent Jnk phosphorylation. In addition, it has been reported that activation of Rac1, a small GTPase belonging to the Rho/Rac subfamily, is mediated by DOCK180 and it also activates Jnk in v-Crk tumorigenesis (Tanaka et al., 1997)(Tanaka et al., 1997)(Tanaka et al., 1997).

Apparently, Crk proteins stimulate the activation of various small GTPases, especially the Ras subfamily (which encompasses the Ras, M-Ras, R-Ras, Rap and Ral subgroups mentioned above). Although the upstream signal transductions of Sos/Ras and C3G/Jnk pathways appear independent, how the downstream signaling networks interact remains unexplored and interesting. Moreover, we think that the roles of Abl/Dok1 in regulating these small GTPases may have direct correlations with the suppression of Crk transformation.

The two isoforms of the Crk gene, CrkI and CrkII, differ from one another in terms of signal transduction. For instance, CrkI preferentially activates Rap1 whereas CrkII appears to favor Rac1 activation when cells were stimulated with PDGF (Antoku and Mayer, 2009). Since CrkI is essentially a shorter version of CrkII, the differences in their binding and activation patterns can only be attributed by the presence of tyrosine 221 and cSH3 domain in CrkII. While both v-Crk and CrkI readily transform cultured fibroblasts (Greulich and Hanafusa, 1996; Zheng et al., 2010), the transforming activity of CrkII has been ambiguous. Experimentally, overexpression of CrkII transforms mouse fibroblasts (NIH3T3) (Akagi et al., 2000) but not rat fibroblasts (3Y1) (Matsuda et al., 1992). A popular explanation is that the aforementioned self-regulating tyrosine 221 reduces CrkII signal transduction and thus limits its ability to transform

cells. However, as our understanding on the structure and regulatory elements found in CrkII cSH3 domain improves, tyrosine 221 may not be the only regulatory element that differentiates CrkII from CrkI and v-Crk.

In a study published in 2006, Noren *et al.* found supportive evidence that Abl-phosphorylated tyrosine 221 in CrkII serves as a critical intermediate protein complex for a tumor suppressor, the EphB4 receptor (Noren et al., 2006). They reported a strong correlation between the Abl-Crk pathway in regulating breast cancer cell proliferation, motility, invasion and the synthesis of matrix metalloprotease MMP-2. Moreover, relatively higher EphB4 activity has been associated with non-transformed mammary epithelial cells, and the disruption of the Abl-Crk signaling in MCF-10A (a non malignant human breast epithelial cell line) resulted in cell transformation. Additionally, consistent with the ambiguous roles of Erk in Crk signaling, their findings indicated no involvement of Erk as well. And finally, it is noteworthy that this study focused entirely on CrkII and thus may have overlooked the significance of CrkI in their system.

The success of Imatinib in Chronic Myelogenous Leukemia (CML) treatment raises the hope for its therapeutic effect in other human cancers, and in combination treatments. As a result, various studies and clinical trials that incorporated Imatinib have been conducted (Apperley et al., 2002; Hughes and White, 2013; Kilic et al., 2000; Reichardt et al., 2011; Sjöblom et al., 2001; Zermati et al., 2003).The National Cancer Institute has established a standard panel of 60 cancer cell lines known as the NCI 60 for the evaluations of various cancer drugs. Their Developmental Therapeutic Program (DTP) now provides a screening service for anticancer compound using these 60 different human tumor cell lines derived from leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate and kidney. The main purpose of this screening service is to obtain the dose response and to set up a pattern recognition algorithm in order to evaluate and to build a collective reference database. However, the screenings conducted by DTP measure the potency of a submitted compound via its ability to kill or stop the proliferation of cells. Based

on these data, the database provides a very reliable source of information for the efficacy and effectiveness of Imatinib, but a major caveat is that it focused on the ability of Imatinib to inhibit cell proliferation and thus any growth stimulatory effect might have been easily missed.

Results

Cell proliferation rates

To understand the general role of CrkI, Abl and Dok1 in regulating cell proliferation, we were interested to study if they regulate the proliferation of adherent cells as well as cells in suspension. Since we already had the data on suspended cell growth from the previous chapter, we utilized a cell viability assay to measure the growth rates of cells in 10% serum, monolayer culture conditions for comparison. In this assay, cell numbers were estimated indirectly via the presence of cellular metabolites and growth curves were obtained from measurements taken every other day for 11 consecutive days.

First, we found that the proliferation rate of attached NIH3T3 cells was clearly enhanced by CrkI overexpression, as previously reported (Zheng et al., 2010). However, the subsequent knockdown of Abl or Dok1 (CAi and CDi respectively) caused only a slight increase in cell proliferation(Figure 3.1), as opposed to the distinct differences seen in the soft agar assay. Likewise, re-expressions of various mutated Dok1proteins only resulted in minor changes in proliferation of monolayer cultures (Figure3.2), which again differed from suspended cell growth data.

Additionally, because Imatinib also targets the PDGF receptor and c-Kit kinases (albeit with weaker affinities), we wanted to address the significance of these targets in our system. We found that the PDGF receptor and c-Kit inhibitor, Ki 11502 completely suppressed the growth of both control and CrkI cells at a concentration that inhibits both kinases (according to the manufacturer's data). And when cells were treated with Imatinib, the proliferation of control cells was suppressed but that of CrkI-transformed cells was slightly increased (Figure 3.3 and Figure 3.4). Interestingly, when cells were plated at a higher density, the growth curve of CAi cells was comparable to those of Imatinib-treated CrkI cells, but the growth of CDi cells was significantly

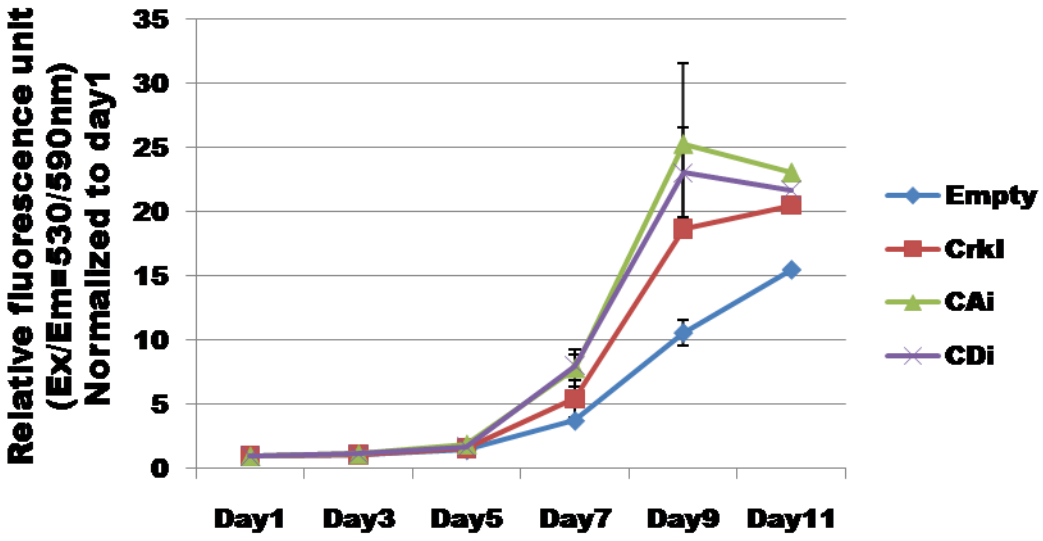


Figure 3.1: CrkI overexpression increases cell proliferation rate of adhered NIH3T3 cells. Under the attached condition, cells were plated at a standard density of 10 cells per well and the cell numbers were measured via a metabolites-sensitive fluorescence dye every other day for 11 consecutive days. The fluorescence units were normalized to day 1 and done in triplicate. E, empty vector control; C, CrkI transformed; CAi, CrkI-transformed, Abl knockdown; CDi, CrkI-transformed, Dok1 knockdown. Error bars represent standard deviation from three independent readings.

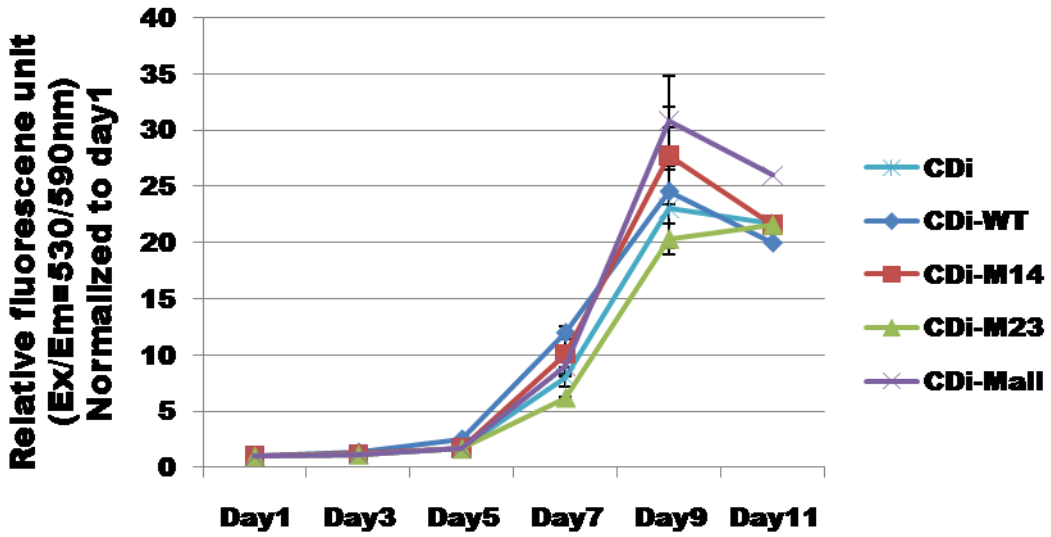


Figure 3.2: Insignificant changes in the growth curves of CrkI-transformed, Dok1 knockdown and recued cells.

Proliferation of adhered NIH3T3 cells was assayed as in Fig. 3.1. CDi, CrkI-transformed, Dok1 knockdown; WT: wild-type Dok1; M14: Y295F and Y361F mutant Dok1; M23: Y336F and Y340F mutant Dok1; M-all: Y295F, Y336F, Y340F and Y361F mutant Dok1. Error bars represent standard deviation from three independent readings.

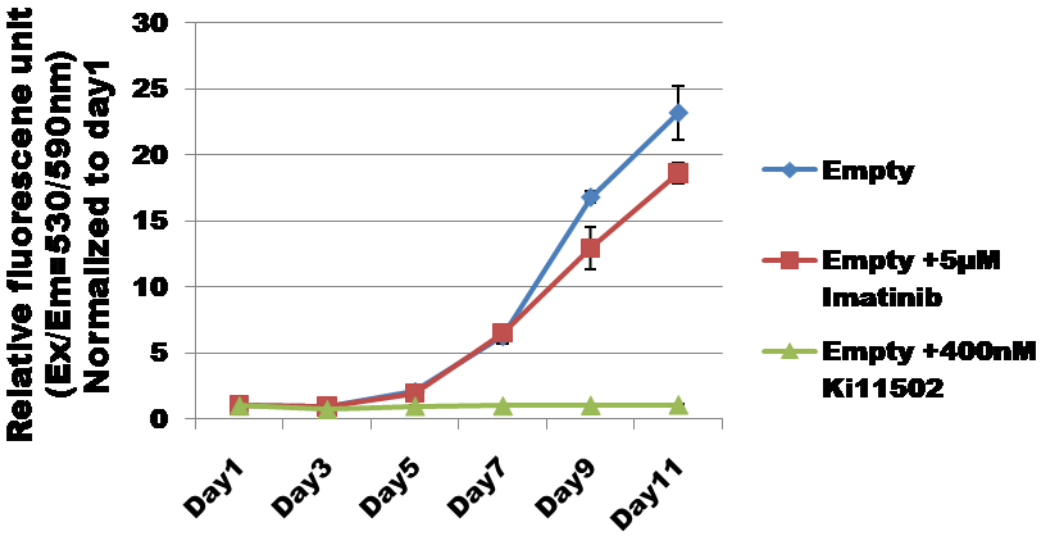


Figure 3.3: Imatinib and Ki11502 (a PDGFR and c-Kit inhibitor) suppress the growth of NIH3T3 cells at different degrees.

Cells were plated at a standard density of 100 cells per well in the presence or absence of respective kinase inhibitors on day 0, and proliferation was assayed as in Fig. 3.1. E, empty vector. Error bars represent standard deviation from three independent readings.

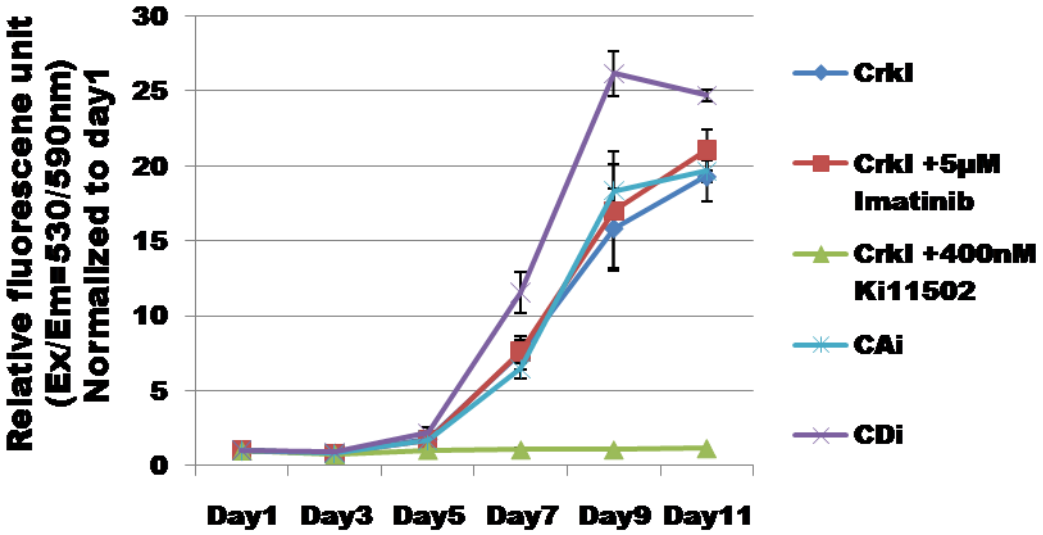


Figure 3.4: The growth rates of adhered CrkI-transformed NIH3T3 were i) suppressed by Ki11502 (a PDGFR and c-Kit inhibitor) ii) not affected by Imatinib or Abl knockdown and iii) further increased by Dok1 knockdown when seeded at a higher density.

Proliferation of adherent NIH-3T3 cells was assayed as in Fig. 3.1 but started with 100 cells per well instead. C, CrkI-transformed; CAi, CrkI-transformed, Abl knockdown; CDi, CrkI-transformed, Dok1 knockdown. Error bars represent standard deviation from three independent readings.

faster than that of CrkI cells. It is unclear how cell density affects the statistical discrepancies, but this may be caused by the combination of assay sensitivity and experimental variations. Naturally, further studies are needed to explore these effects.

We concluded that CrkI overexpression promotes overall cell proliferation, evidently in both adherent and suspended conditions. However, the growth promoting effects of subsequent knockdown and rescue manipulations seem more restricted to the suspended condition. The disparity between adherence vs. suspension suggests that the Abl/Dok1 signaling pathway are more likely to be involved in bypassing the normal inability of cells to grow in suspension, instead of altering the overall cell proliferation rate.

Cell motility

The most studied role for Crk proteins in cellular signaling is the regulation of cytoskeletal rearrangement and focal adhesions. To investigate if CrkI transformation and the subsequent knockdown of Abl or Dok1 affect cell motility, we performed a simple experiment known as the wound healing assay (also known as the *in vitro* scratch assay). It is a two-dimensional assay that measures the rate of cell movement in order to repopulate the introduced wound in monolayer culture condition (Liang et al., 2007).

As shown in Figure 3.5, we did not detect any significant changes in cell motility rate under the two-dimensional wound healing assay. The migration of cells across the plate appeared unaltered among the control, CrkI overexpressing, the following Abl knockdown, Dok1 knockdown and various Dok1 rescued cell lines. These observations suggest no significant motility advantage was acquired from CrkI overexpression and Abl/Dok1 knockdown under these conditions.

Erk, JNK and Caspase3 signaling pathways

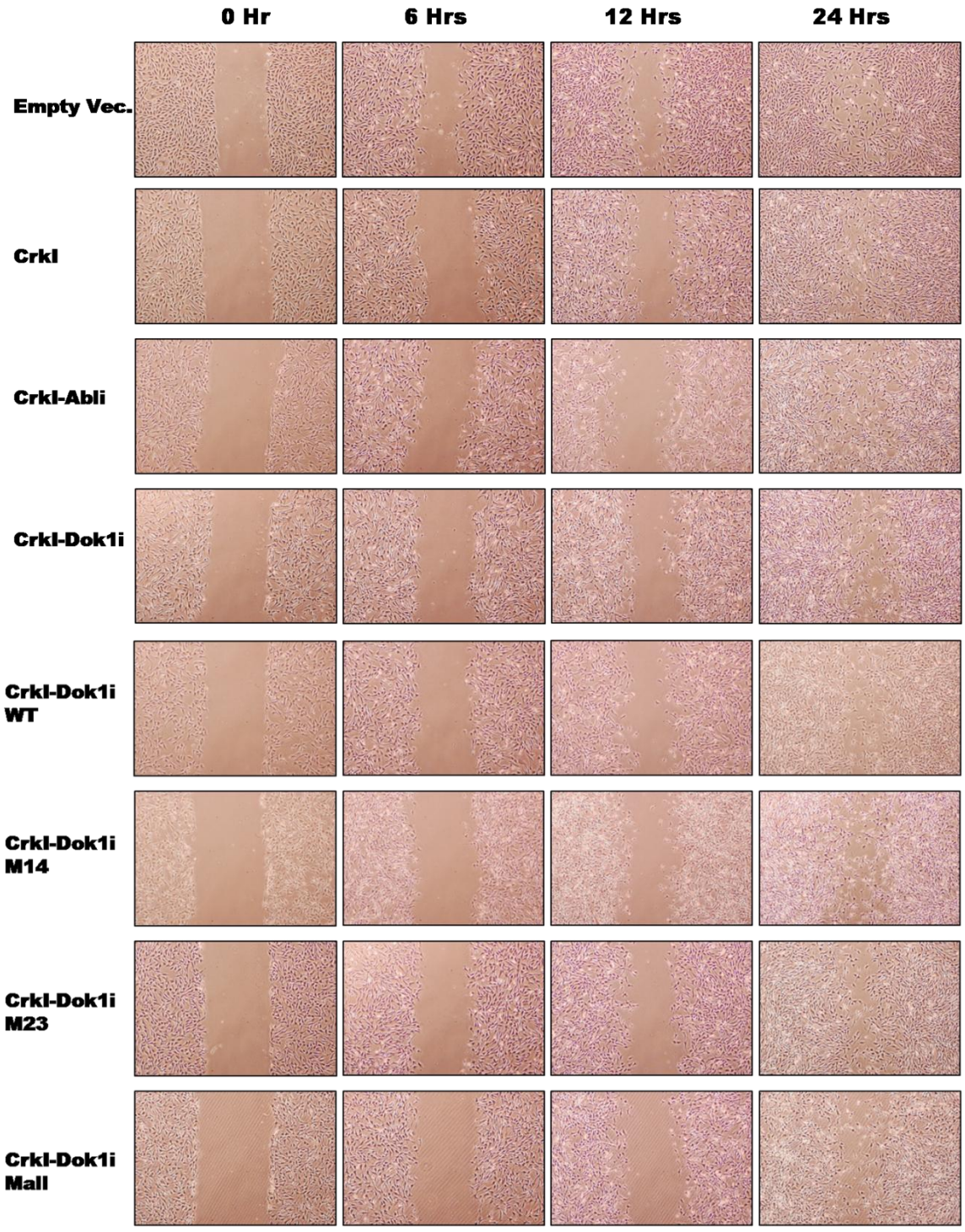


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Figure 3.5: Cell migration rates of NIH3T3 cells in wound healing assay over 24-hour period. Cells were grown to 95% confluent in 6-well plates and a "wound" was created by a clean 200µl pipette tip. The culture plates were then washed 3X with culture medium and photos were taken at 0 hr, 6 hrs, 12 hrs and 24 hrs after the wounding. E, empty vector control; C, CrkI transformed; CAi, CrkI-transformed, Abl knockdown; CDi, CrkI-transformed, Dok1 knockdown; WT: wild-type Dok1; M14: Y295F and Y361F mutant Dok1; M23: Y336F and Y340F mutant Dok1; M-all: Y295F, Y336F, Y340F and Y361F mutant Dok1.

In Crk-transformed fibroblast models, several key downstream signaling pathways have consistently been investigated, but their physiological significance still remains unclear. In particular, we are interested in the involvement of Erk and Jnk pathways in Crk transformation.

The reversal of v-Crk transformation via dominant negative Ras (Greulich and Hanafusa, 1996) first suggested the importance of Ras small GTPase in Crk tumorigenesis. And following that, our knockdown studies on how the two best known Ras regulators, Sos and RasGAP affect CrkI transformation (Ng et al., 2014; Zheng et al., 2010) corroborated the significance of Ras in the Crk signaling pathway. Yet, investigations of the canonical downstream Erk pathway (indicated by changes in Erk phosphorylation) failed to establish a direct correlation with Crk transformation (Greulich and Hanafusa, 1996; Zheng et al., 2010). To more carefully examine the role of Erk activity in Crk transformation, we compared the level of pErk under different serum conditions.

We plated identical sets of empty vector control (E), CrkI-transformed (C), CrkI-transformed with Abl knockdown (CAi) and CrkI-transformed with Dok1 knockdown (CDi) fibroblasts in i) overnight 0.1% serum, ii) 0% serum starvation for 2.5 hours and iii) normal 10% serum. Cells were then harvested and analyzed with anti-phospho Erk antibody to determine the level of Erk phosphorylation in each cell types and conditions. And consistent with several experiments conducted previously, overnight starvation and standard 10% serum demonstrated no significant changes among the cells. However, only the CDi cells showed a more persistent Erk phosphorylation when cells were serum-starved for 2.5 hours (Figure 3.6). Even though this observation correlated well with how Dok1downregulates pErk , it is unclear how cells eventually overcome the differences (as in overnight serum-starvation). These observations make a weak argument that the knockdown of Dok1 or Abl leads to relatively

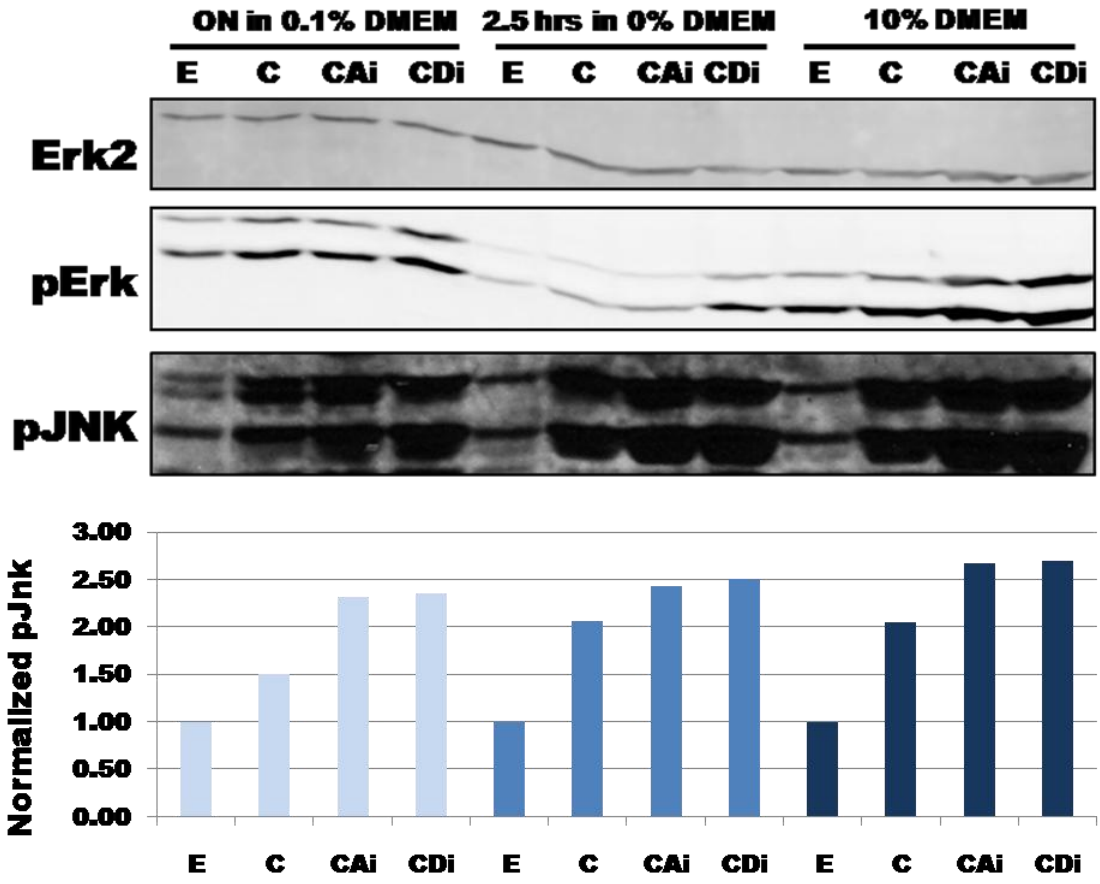


Figure 3.6: Dok1 knockdown in CrkI-transformed NIH3T3 temporarily sustains Erk phosphorylation but both Abl knockdown and Dok1 knockdown further increase Jnk phosphorylation under different serum conditions.

Cells were cultured under three serum conditions: i) overnight in 0.1% serum ii) 2.5 hours without serum and, iii) normal 10% serum. Cells were then lysed and the phosphorylation of Erk and Jnk were determined in western blots with their respective antibodies. The intensity of the lower pJnk bands were quantified by Image-J and all measurements were normalized to their respective control, E.

ON, overnight; E, empty vector; C, CrkI transformed; CAi, CrkI-transformed, Abl knockdown; CDi, CrkI-transformed, Dok1 knockdown.

sustained Erk signaling. In addition, how the CrkI-dependent Ras activation (Figure2.21) regulates cell growth in suspension remains ambiguous.

Unlike Erk, Jnk has been consistently reported to be activated in CrkI transformed cells, and this is yet another critical pathway capable of reversing Crk transformation (Tanaka et al., 1997)(Tanaka et al., 1997)(Tanaka et al., 1997). We found that JNK phosphorylation was elevated in CrkI overexpressing cells, and was further increased in the subsequent knockdown of either Abl or Dok1 (Figure 3.6). The increased Jnk phosphorylation coincided with increased colony numbers seen in anchorage independent growth assay and therefore, it will be interesting to understand how JNK's activation governs Crk transformation and more importantly, how the Abl/Dok1 signaling pathway is related to Jnk phosphorylation and what is their implication in cell transformation.

Finally, we first observed the increased level of cleaved-Caspase3 (an indicator for Caspase3 activation) in CrkI-overexpressing cells exposed to either UV-irradiation or hydrogen peroxide (Zheng et al., 2010). And now, we show that even in a standard 10% serum culture condition, the basal levels of cleaved-Caspase3 were higher in CrkI-overexpressing cells (Figure 3.7). Due to this unforeseen gain of apoptotic sensitivity in CrkI-overexpressing cells, we concluded that the enhanced growth of Abl or Dok1 knockdown cells in soft agar is not a result of increased apoptotic resistance. A possible explanation for this paradoxical observation is that CrkI overexpression simultaneously accelerated the rate of proliferation while also compromising apoptotic resistance to a lesser extent.

The role of CrkII phosphorylation in CrkI-induced transformation

Apart from the reduction of Dok1 phosphorylation as described in chapter 2, we also observed a reduction in CrkII phosphorylation when cells are treated with 2.5μM Imatinib (in both control

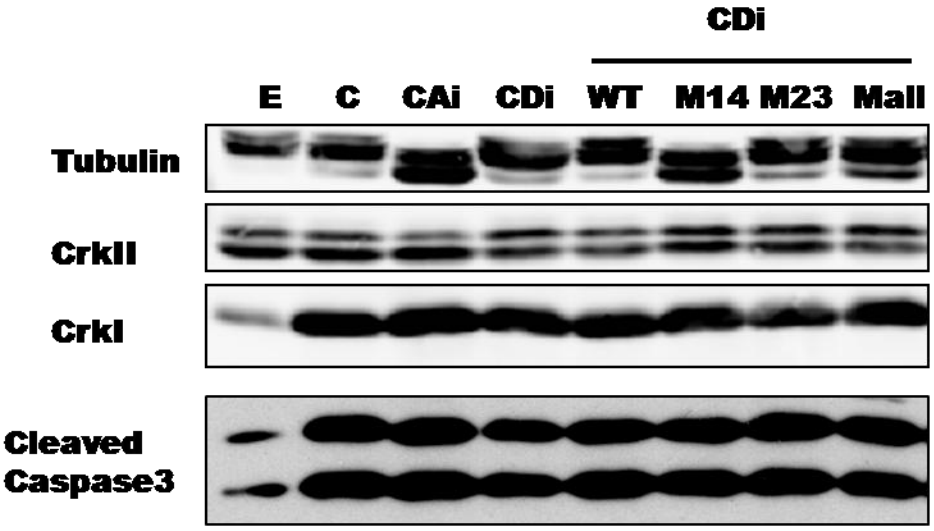


Figure 3.7: CrkI overexpression in NIH3T3 cells induces higher Caspase3 activation. Cells were cultured in normal 10% serum condition and harvested for standard western blotting to determine their basal level of cleaved Caspase3. E, empty vector control; C, CrkI transformed; CAi, CrkI-transformed, Abl knockdown; CDi, CrkI-transformed, Dok1 knockdown; WT: wild-type Dok1; M14: Y295F and Y361F mutant Dok1; M23: Y336F and Y340F mutant Dok1; M-all: Y295F, Y336F, Y340F and Y361F mutant Dok1.

and CrkI-transformed cells). More specifically, Imatinib reduces the phosphorylation of tyrosine 221 in CrkII (Figure 3.8), just as previously reported (Noren et al., 2006).

Because the phosphorylation of tyrosine 221 in CrkII has been proposed to be a self-inhibiting regulator (Rosen et al., 1995), we hypothesized that in addition to Dok1, the reduction in CrkII phosphorylation by Imatinib either amplifies or activates other transforming pathway(s). To test the hypothesis, we overexpressed either wild type CrkII or CrkII mutated at tyrosine 221 (Y221F) in CrkI-transformed cells, and established co-expressing cell lines with G418 (Figure 3.9).

In the soft agar assay, coexpression of neither CrkII nor Y221F in CrkI cells significantly increased the colony numbers like those of Imatinib-treated CrkI cells (Figure 3.10). In fact, coexpression of CrkII appeared to suppress CrkI transformation. In short, we found no supporting evidence and ruled out the possibility that dephosphorylation of CrkII contributed to CrkI transformation when Abl activity is suppressed.

Transforming activity between CrkI, CrkII and Y221 CrkII mutant

Despite being alternative spliced forms, CrkI and CrkII proteins have distinctive roles in signal transduction. One of the most significant differences is the presence of tyrosine 221 in CrkII, which induces a self-regulating mechanism (Figure 1.2) that has been thought to compromise the tumorigenicity of CrkII.

Due to the ambiguous CrkII transforming activity, and that coexpression of CrkII (but not its Y221F mutant) repressed CrkI transformation (Figure 3.10), we decided to compare the transforming activity of CrkI, CrkII and Y221F mutant CrkII. In addition to CrkI, we established cells overexpressing CrkII and Y221F-CrkII as well (Figure 3.11) but, after conducting the soft agar assay, we found no significant differences among the three overexpressed Crk proteins (Figure 3.12). It seems like at least in our NIH3T3 fibroblasts, CrkI and CrkII have comparable

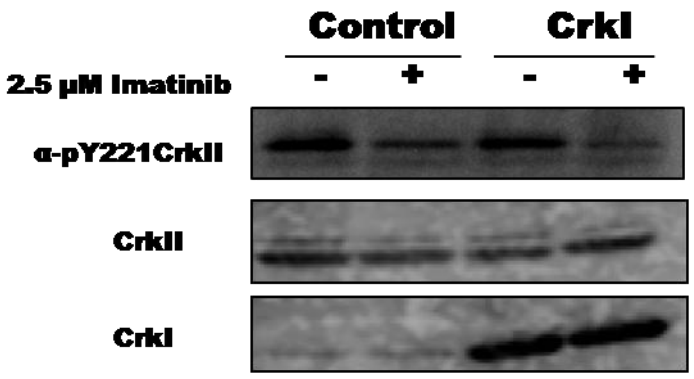


Figure 3.8: Imatinib reduces phosphorylation of tyrosine 221 in CrkII.
Cells were cultured in the absence or presence of Imatinib and the level of phosphorylated tyrosine 221 in CrkII was determined with an anti-phosphotyrosine 221 antibody.

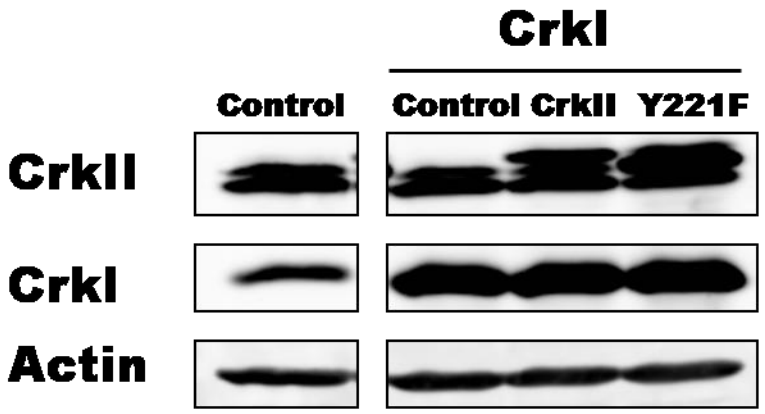


Figure 3.9: Coexpressions of CrkII and Y221F-CrkII in CrkI-transformed cells.
CrkI overexpressing NIH3T3 cells were co-infected with retroviruses carrying either wild type CrkII or mutant Y221F CrkII and cell lines co-expressing the respective CrkII were established after G418 drug selection. Both CrkII constructs were tagged with HA at the C-terminal end. Y221F, Y221F-CrkII mutant.

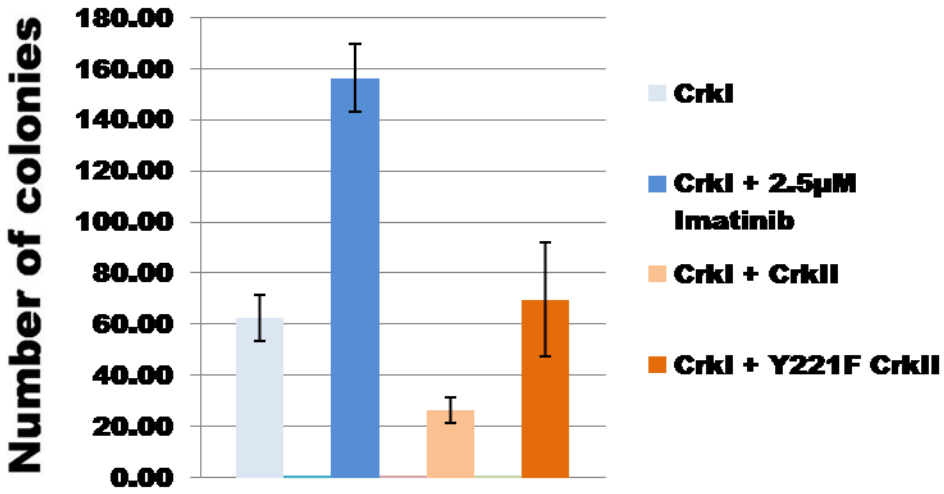


Figure 3.10: CrkII but not Y221F-CrkII suppresses CrkI-transformation in NIH3T3 cells. Established co-expressed cells were used for soft agar assay and the total colony numbers were compared to CrkI-transformed and Imatinib-treated CrkI cells.

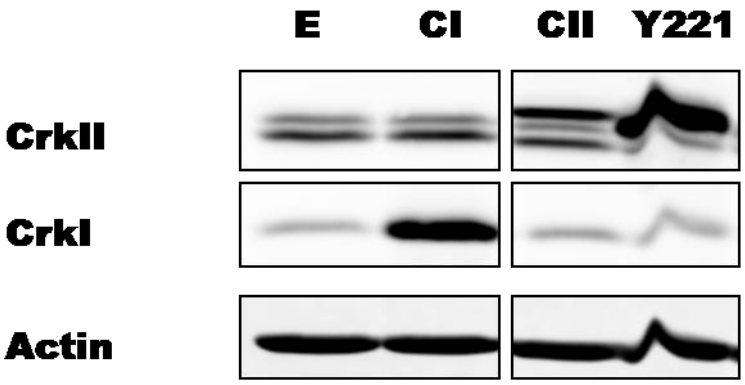


Figure 3.11: Overexpression of CrkI, CrkII and Y221F-CrkII in NIH3T3 cells. NIH3T3 cells were infected with retroviruses containing either CrkI, HA-tagged CrkII or HA-tagged Y221F mutant CrkII. Cells were then selected with their respective antibiotics (puromycin for CrkI and G418 for CrkII constructs) and subjected to standard western blotting to determine the expression of various Crk proteins. E, empty vector; CI, CrkI-transformed; CII, CrkII-transformed, Y221, Y221 mutant CrkII-transformed.

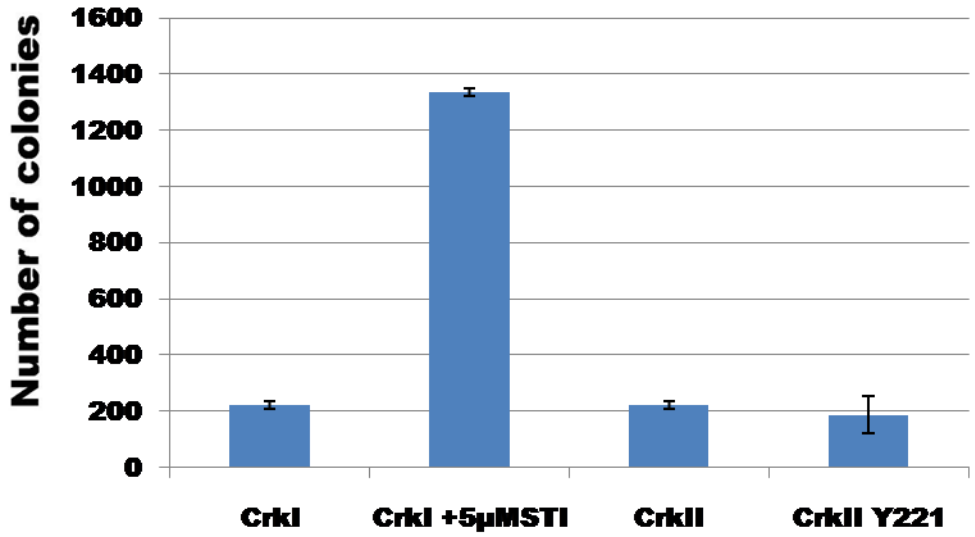


Figure 3.12: CrkI, CrkII and Y221-CrkII have comparable transforming activities in NIH3T3. The established cell lines from Figure 3.11 were subjected to soft agar assay and Imatinib-treated CrkI cells were included as a control. All empty vector controls yielded no colony in the same assay.

transforming activity and Y221 phosphorylation alone has a limited role in regulating the tumorigenicity of CrkII. Of course, with the aforementioned disparity in CrkII transformation, our observations may be limited to certain specific cell types and probably depends on the relative expression levels and assay conditions as well.

The effect of Imatinib in the NCI60 human cancer cell line

The suspension growth promoting phenotype of Imatinib observed in our CrkI-transformed cell model raised concerns about using Imatinib to treat non-CML human cancers, especially in those with elevated Crk expression. A previous study conducted by National Cancer Institute focused on the ability of Imatinib to inhibit cancer cell proliferation (Figure 3.13), and thus may have overlooked the growth-promoting effect. We therefore started our own investigation on Imatinib using the same panel of 60 established culture cell lines derived from human cancer tissues, the NCI60 cell lines.

After the cells were cultured in the presence and absence of Imatinib, we ran standard western blots to analyze the expressions of Abl, Dok1 and Crk proteins and to assess the extent of Abl kinase inhibition (Figure 3.14). While the expression of Abl, CrkI and CrkII varies among the 60 cell lines examined, none of the cell lines have detectable Dok1 protein. And based on the reduction of CrkII phosphorylation seen in several cell lines, the Imatinib concentration that we used was able to inhibit Abl kinase, an observation similar to those of NIH3T3 fibroblasts.

From the 60 cell lines, we then selected eight cell lines based on their Abl and Crk protein expression for soft agar assay. Specifically, we included cell lines that have relatively high Crk expression, and either higher or lower than average Abl protein level (to compare the significance of Abl). In addition, K562, a CML-derived leukemia line, was also included as a positive control for the effects of Imatinib.

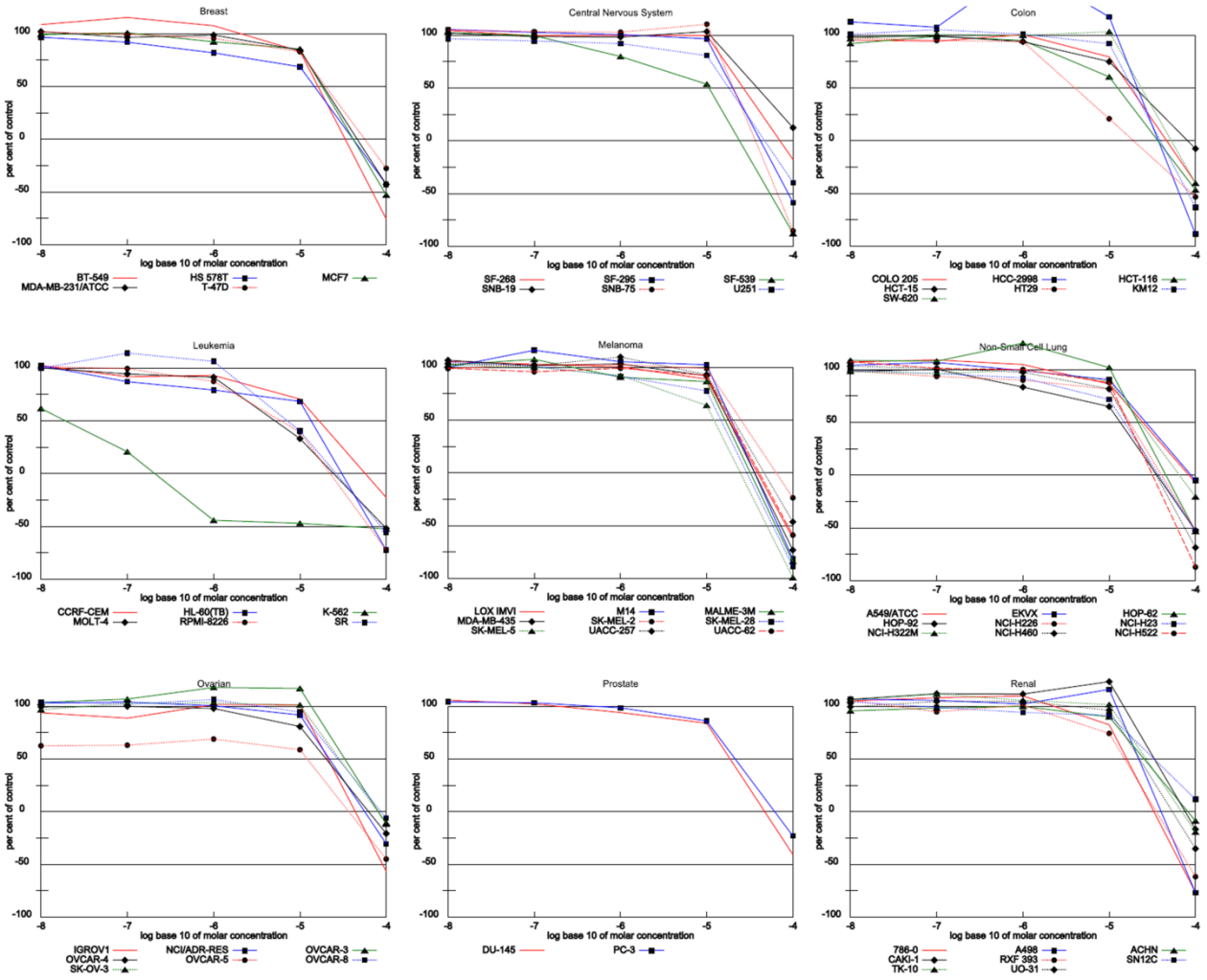


Figure 3.13: National Cancer Institute Developmental Therapeutics Program: Dose Response Curves for NSC 743414 (Imatinib).

Among the panel of 60 human cancer cell lines tested, only K562, a CML derived cell line, responded negatively to Imatinib treatment. Data/image obtained from: http://dtp.nci.nih.gov/dtpstandard/servlet/doseresponse?searchtype=NSC&searchlist=743414

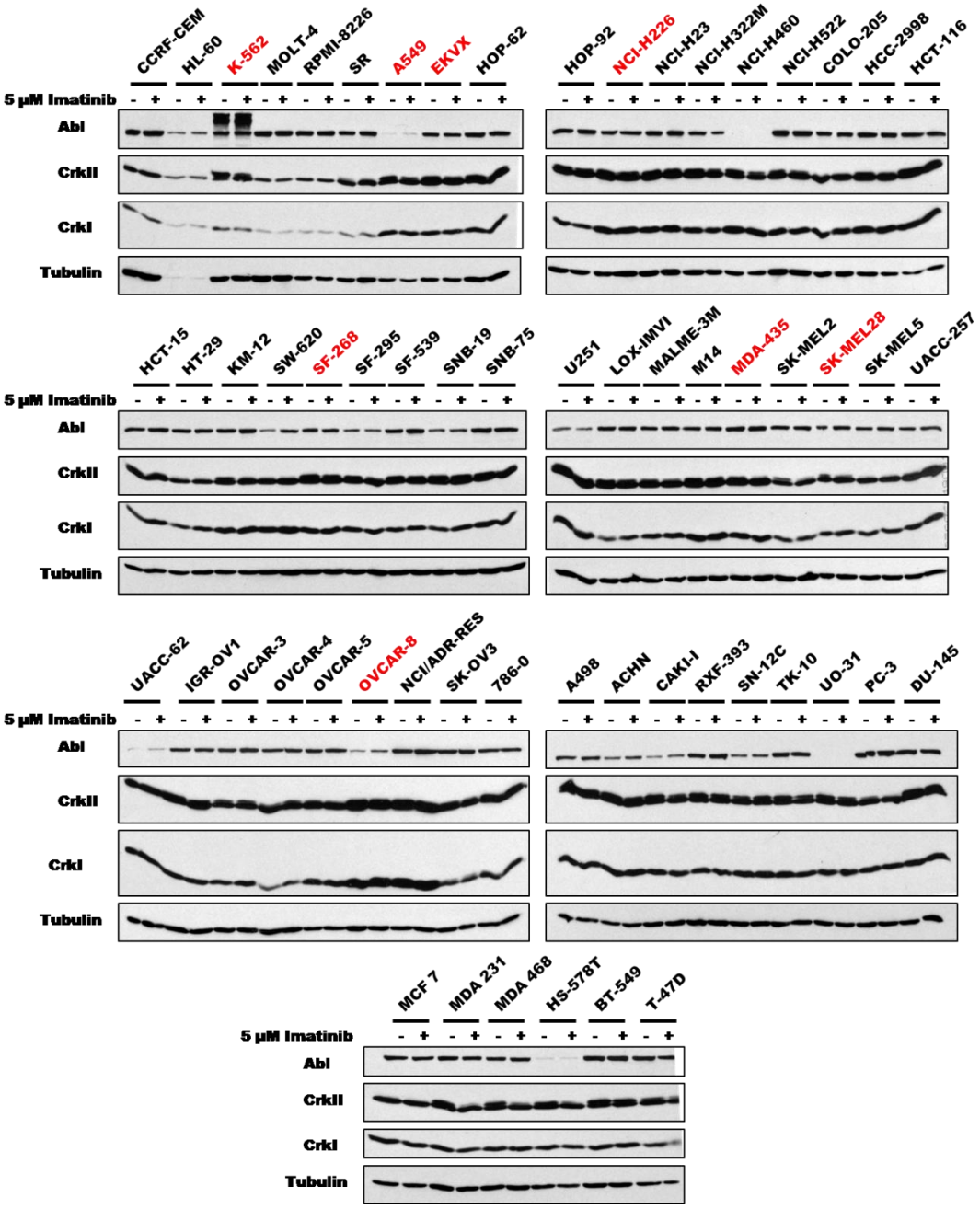
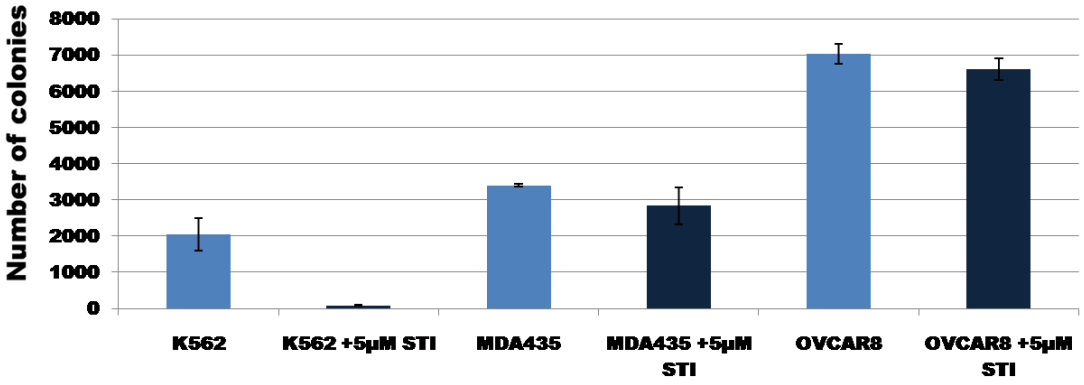


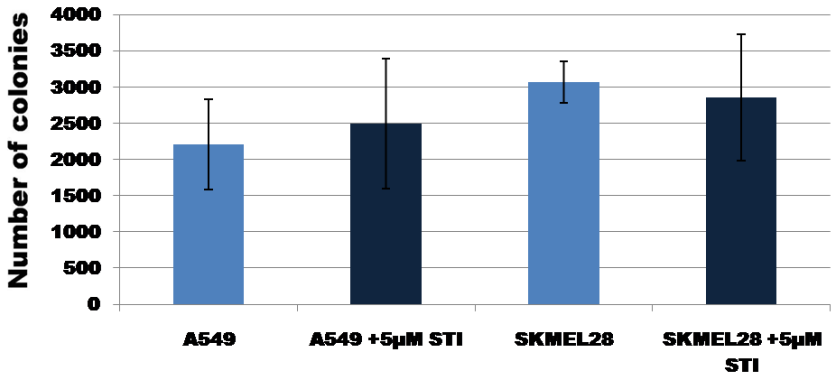
Figure 3.14: Expressions of Abl, CrkI, CrkII and actin in NCI-60.

The NCI60 cell lines were cultured in the presence and absence of Imatinib and standard western blots were used to detect the expression levels of Abl, CrkI and CrkII with their respective antibodies. Anti-Tubulin serves as consistency controls. Cell lines in red font were later selected for soft agar assay based on the expression of Crk proteins and Abl.

As expected, the human cancer cell lines varied in their growth and colony formation rates in the soft agar assay (Figure 3.15). In particular, the colony forming ability of CML-derived K562 was severely suppressed by Imatinib, as expected, but MDA 435, OVCAR8, A549, SKMEL28 EKVX and NCI H226 showed no significant changes.

Interestingly, Imatinib did enhance the colony growth of a glioblastoma cell line, SF268 and when plated at a lower density (10^4 cells per plate) the difference in colony numbers is even more pronounced and statistically significant (Figure 3.16). Morphologically, unlike our NIH3T3 fibroblast model, the Imatinib-treated colonies appeared larger and again, more prominent when seeded at a lower cell density (Figure 3.17). The changes in the colony morphology and numbers of SF268 suggested a more aggressive behavior resulted from Imatinib treatment, but the signaling pathways involved remain to be determined.





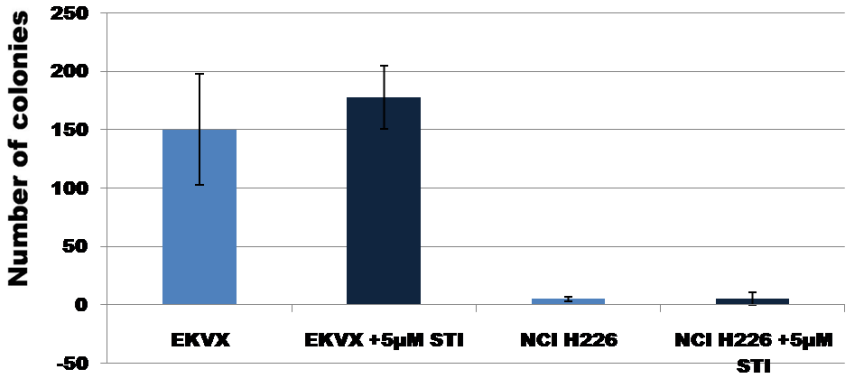


Figure 3.15: Imatinib suppressed colony formation of K562 but does not affect MDA435, OVCAR8, A549, SKMEL28, EKVX and NCI-H226 human cancer cell lines.
From the western analysis in Figure 3.14, eight cell lines were selected for soft agar assay with or without Imatinib. K562, an Imatinib-sensitive CML derived cell line, was used as a control.

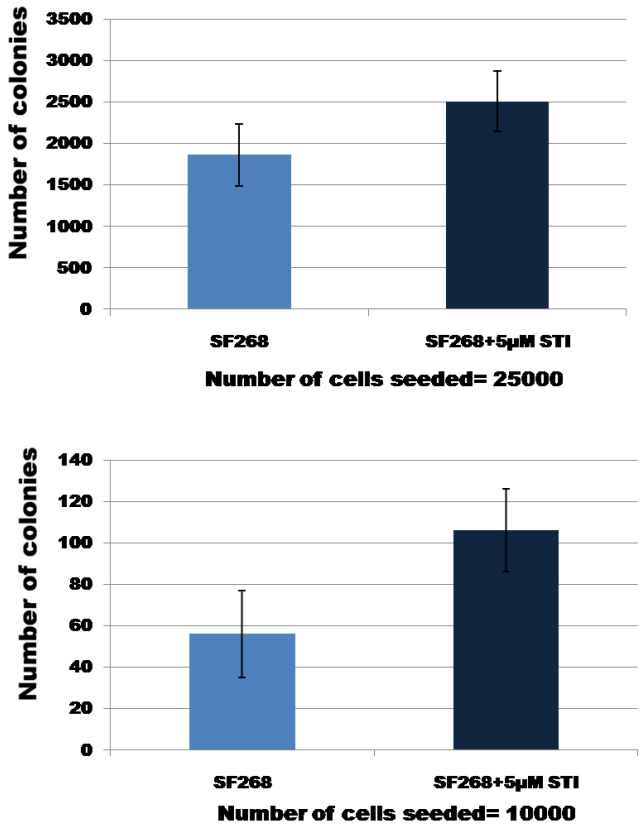


Figure 3.16: Imatinib enhances colony formation rates of SF268.

SF268 glioblastoma was subjected to soft agar assay in the absence or presence of 5µM Imatinib at different cell density (either 10000 cells per plate or 25000 cells per plate). Colonies were stained with Crystal Violet dye and the total colony numbers were determined via Image-J after 4 weeks.

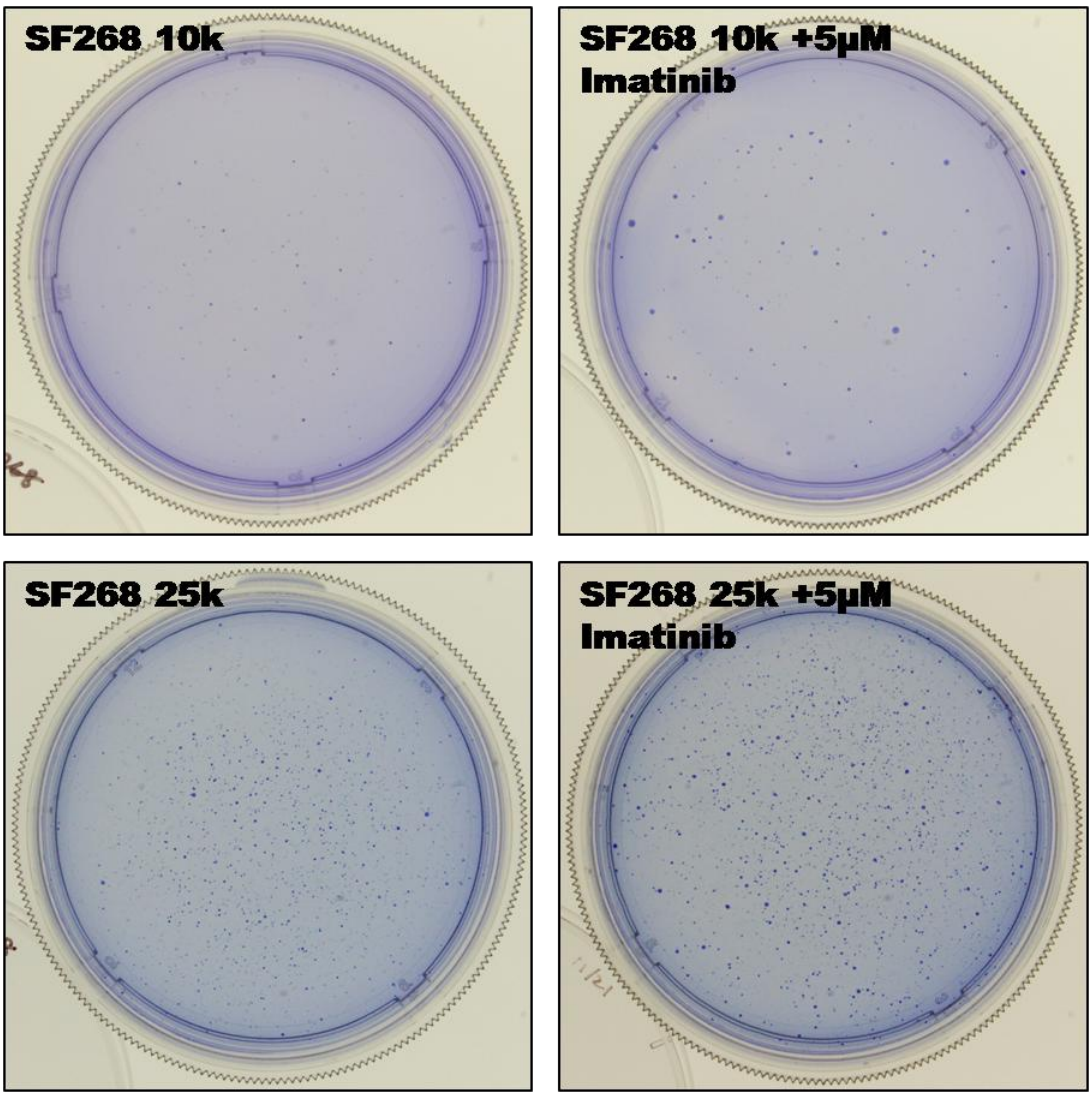


Figure 3.17: Imatinib increased the colony size and number of SF268.
SF268 glioblastoma was subjected to soft agar assay in the absence or presence of 5μM Imatinib at different cell density (either 10000 cells per plate or 25000 cells per plate). Colonies were stained with Crystal Violet dye and photos were taken after 4 weeks of incubation.

Discussion

The impact of CrkI, Abl and Dok1on cell proliferation and migration

Although the soft agar assay is a useful *in vitro* experiment to assess cell transformation, it does not distinguish the physiological changes that lead to growth advantage in suspension. By comparing the cell growth data from both attached and suspended cells, we concluded that CrkI overexpression increases the overall cell proliferation rate but the downstream Abl/Dok1 pathway has a less prominent role in regulating cell proliferation. Thus the increased colony numbers in the soft agar assay when the Abl/Dok1 pathway is dysregulated is can possibly be caused by enabling cells to overcome other suspension-specific growth regulatory mechanism(s).

As mentioned, some of the best-known functions of Crk proteins are associated with cytoskeletal rearrangement, and this can potentially relate to cell metastasis. However, as our data indicated, neither the overexpression of CrkI nor the subsequent Abl or Dok1 knockdown (and rescued variants) affected two-dimensional cell movement. Interestingly, a recent publication found that the knockdown of Dok1 in human glioma cells impaired their migration rates in a PDGG-BB-mediated chemotactic trans-well migration assay (Barrett et al., 2014). Since we saw no changes in our simple assay, we think more physiologically relevant assays like the trans-well cell migration assay may provide a better assessment in the metastatic potential of these cells.

The signaling pathways involved in Crk, Abl and Dok1 signal transduction

Thus far, Sos/Ras and C3G/Jnk signaling pathways have been reported to be critical for v-Crk tumorigenicity (Greulich and Hanafusa, 1996; Mochizuki et al., 2000; Tanaka et al., 1997). Indeed, our previous knockdown study further corroborated the importance of Sos and C3G GEFs as the two pivotal Crk SH3 binding proteins pertaining to CrkI cell transformation (Zheng et al., 2010). However, the exact relationship of Crk proteins to the canonical MAPK pathway is still ambiguous. In the previous chapter, we showed that during the spreading of mouse fibroblasts on

fibronectin, overexpression of CrkI lead to increased localized Ras activity in mature focal adhesion sites (Figure 2.21), but how this might affect the MAPK pathway is unclear.

We found that the knockdown of Abl or Dok1 further increases the level of pJnk, and this coincided with the increased colony numbers in soft agar assay. Given the complexity in Ras subfamily regulation, it is possible that the Abl/Dok1 signaling pathway somehow sequestered and suppressed some other Jnk-activating small GTPases; when either Abl or Dok1 were downregulated, the activation or redistribution of these small GTPases eventually leads to further amplification of Jnk phosphorylation.

On the other hand, the higher levels of cleaved Caspase3 in CrkI-overexpressing cells contradicted the general consensus that transformed cells tend to bypass programmed cell death. As stress-induced kinases, Jnks have been known to trigger both pro-survival and apoptotic responses in cells. However, it is still unclear whether the increased cleaved Caspase3 levels are a direct result of the elevated pJnk and furthermore, whether it ultimately leads to cell death or elicits other positive feedback responses related to cell transformation.

The variations between CrkI and CrkII signaling

In a mouse xenograft model of human breast cancer cells, Noren *et al.* concluded that Abl-phosphorylated CrkII regulates tumorigenesis downstream of EphB4 receptor tumor suppressor (Noren et al., 2006). Although we also observed the dephosphorylation of CrkII in Abl knockdown CrkI cells or when cells were treated with Imatinib, we did not find any supporting evidence that connects CrkII dephosphorylation to enhanced CrkI transformation in our system.

It appears that coexpression of CrkII (but not its Y221F mutant) suppresses CrkI tumorigenicity and thus decreases colony numbers formed in the soft agar assay. Compared to the mutated Y221F CrkII, phosphorylation of tyrosine 221 seems to trigger other unknown mechanism(s) that downregulate CrkI transformation. With both CrkI and CrkII sharing identical

binding partners, it is possible that Y221-phosphorylated CrkII affects the binding affinities and dynamics of CrkI in a competitive way. Other than that, we have not addressed the involvement of TGFβ signaling (as reported by Noren *et al.*) in NIH3T3 and how it may impact the role of CrkII phosphorylation here. Likewise, apart from suggested TGFβ tumor suppressor pathway, it will be interesting to know how Abl/Dok regulates cell transformation in the system used by Noren *et al*. Perhaps, the effects of unphosphorylated Y221 in CrkII described in these breast cancer cells may serve as an auxiliary signal to a more dominant tumor suppressing Abl/Dok1 pathway.

Since the discovery of cellular Crk proteins, there have been conflicting results on the tumorigenicity of CrkII (Iwahara et al., 2003; Matsuda et al., 1992). The phosphorylation of tyrosine 221 in CrkII was initially implicated as a self-inhibitory mechanism (Figure 3.12) and thus is expected to compromise the transforming activity CrkII. However, as more studies on the cSH3 domain of CrkII were conducted, it appears that the phosphorylation of tyrosine 221 alone may not be the sole regulator in CrkII signaling activity (Sriram et al., 2011). Indeed, when we compared the oncogenic potential of CrkII and its Y221F mutant, unphosphorylated tyrosine 221 conferred no significant advantage over the intact CrkII, and this observation undermined the purported self-limiting regulation of Y221. However, one caveat in our comparison here will be that the expression level of CrkI, CrkII and Y221F-CrkII were not precisely standardized and thus may have affected their respective transforming activity in the NIH3T3 model.

The effect of Imatinib in NCI60 human tumor cell lines

Among the eight chosen NCI60 cell lines, Imatinib enhances the growth of a glioblastoma cell line, SF268, in the soft agar assay. The increased colony size and number suggested that Imatinib promotes tumor growth and therefore partially validated our concerns over the adverse effect of Imatinib in human cancer. However, the increased colony size seen in

Imatinib-treated SF268 was not observed in our Imatinib-treated fibroblasts model. Together with the lack of detectable Dok1 protein in all the NCI60 cell lines, it is unclear how the signaling mechanism(s) in SF268 may be directly related to our fibroblast model. One possible connection between the two cell types is to look at the expression of Dok2 and/or Dok3 as well, for they have a significant degree of overlapping functions.

Apart from the Abl/Dok tumor repressive pathway, it is likely that the relatively higher CrkII expression in SF268 (Figure 3.15) could have contributed significantly to the cell growth regulation disrupted by Imatinib. As suggested by various studies using human breast cancer cell lines (Allington et al., 2009; Gil-Henn et al., 2013; Noren et al., 2006), several RTKs that are affected by Abl kinase activity may predominantly regulating the tumorigenesis of cells. For example, the role of Abl/CrkII in the tumor-suppressing pathway of EphB4 receptor (Noren et al., 2006) has not been addressed in our study so far.

Taken together, our data emphasize on the uniqueness and heterogeneity of various cancer cells and the need for personalized cancer treatment. Careful considerations for the suitability of kinase inhibitors should be based on the protein expression profile. Since, at least one human cancer cell line showed Imatinib-enhanced growth, we think further investigations are definitely required to understand its underlying mechanism(s), and hopefully this can better improve our understanding for future cancer drug development.

Chapter 4

Methods and materials

Cell culture

All cultured cells were propagated in a 37˚C incubator with 5% CO_2. All NIH3T3 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Mediatech) supplemented with 10% super calf serum (SCS) (Gemini Bioproducts). All HEK 293T cells were maintained in DMEM (Mediatech) supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproducts). All National Cancer Institute-60 (NCI-60) cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Gibco) supplemented with 10% FBS. Serum starvation was carried out with DMEM (Mediatech) supplemented with 0.1% serum or as indicated otherwise.

Anchorage-independent growth assay

Cells were trypsinized, counted and suspended in 10% FBS DMEM (all NIH3T3 derived cell lines) or RPMI 1640 medium (all NCI60 cell lines) with 0.3% Bacto agar (BD Bioscience) on top of a base agar layer (with identical medium but with 0.6% agar). A total of 2.5×10^4 cells (Figure 2.1, Figure 3.10 and Figure 3.15) or 5×10^4 cells (Figure 2.6, Figure 2.9, Figure 2.18 and Figure 3.12) or as indicated otherwise were seeded per 60mm plate. Plates were fed with 1ml of fresh medium every week. After 4 weeks, plates were stained with 0.005% crystal violet (Sigma-Aldrich), photographed and colony numbers were calculated using ImageJ (NIH). For Imatinib (LC Laboratories) treatment, drug was present at the indicated concentration both in the initial plating medium and in the medium used for weekly feeding.

Retrovirus production

Cesium chloride purified plasmids were co-transfected into freshly plated 293T cells with retrovirus packaging plasmids (namely the pMD.gag.pol and pMD.env plasmids). Transfections were carried out using conventional calcium phosphate precipitation methods (as described in (Sambrook and Russell, 2001)). Six hours later, the transfection media were replaced with collection media (10%SCS DMEM) and left over night. Retrovirus containing media were first

collected 16 hours later (and replaced with fresh media) and then every 4 hours thereafter. Retrovirus-containing media were then filtered with 0.45μm filter to remove any suspended packaging cells before applied to the cells for transduction. Additional media were kept frozen at -70° C for future usage.

Infection

NIH3T3 cells were seeded at low density (2×10^4 cells per 6-well plate) and incubated with the retrovirus-containing media (in the presence of 2μg/ml polybrene; Millipore) for at least 24 hours. Two days post-infection, untransduced cells were weeded out by the addition of corresponding antibiotics (1mg/ml Hygromycin B, 1mg/ml G418 or 1.2μg/ml puromycin respectively). Pooled infected cells were established after approximately 6 days later in selective media and maintained in normal 10% SCS DMEM.

Gene knockdown

Stable targeted gene knockdowns were performed using the shRNA retrovirus system. The 68 bp and 72 bp DNA inserts were first hybridized then cloned into pSUPER-hygro retrovirus vector (Oligoengine). The target sequences used for Abl/Arg, Dok1 and RasGAP knockdown were 5′-GAGTACTTGGAGAAGAAGA-3′ (Zheng et al., 2010), 5′-GGTAATGTTCTCCTTTGAA-3′ (modified from (Mihrshahi et al., 2009)) and 5′-AAGATGAAGCCACTACCCTATTT-3′ (Kunath et al., 2003). Plasmids were then amplified and purified using standard cesium chloride extraction methods (Sambrook and Russell, 2001).

Gene expression

Stable protein expressing cell lines were established using the MSCV retrovirus system. CrkI, CrkII and CrkII-Y221 constructs were cloned from previously described plasmids (Antoku and Mayer, 2009). Human Dok1 cDNA was generously provided by P. P. Pandolfi (Harvard

medical school) and the Dok1 variants were generated with PCR-based site directed mutagenesis. All cDNA constructs were cloned into either MSCVpuro or MSCVneo retroviral vector as indicated and plasmids purified with standard cesium chloride extraction methods (Sambrook and Russell, 2001).

Cell growth assay

The Visionblue (BioVision) cell viability assay uses a redox dye (Resazurin) which becomes highly fluorescent upon reduction by metabolically active cells and was measured with plate reader using an emission wavelength of 590-620nm (excitation wavelength = 530-570 nm). Cells were trypsinized, counted, seeded into 96-well plates and left in the incubator to settle overnight. On day 1 and every other day, 10µl of VisionBlue solution were added into wells (in triplicate) and incubated in the incubator for 1 hour. The numbers of viable cells were then estimated based on the readout of a plate reader and all signals were averaged and normalized to day 1. Growth curves were plotted after gathering all the data for 11 consecutive days.

Wound healing assay

Cells were first plated to ~95% confluence in standard 6-well plates and left to settle down overnight. The next morning, a clean p200 pipette tip (USA Scientific) was used to scrape off a straight "wound" and the plates were then washed 3X with standard 10% serum DMEM (to prevent reattachment) and left in fresh 10% serum DMEM. Photos were then taken at 0, 6, 12, 24 and 36hours later with Nikon D50 digital SLR camera mounted on a Nikon Eclipse TS100 inverted phase contrast microscope with Nikon Plan Flour 10x. N.A.=0.3 objective lens to assess cell motility.

Protein analysis

Except where stated otherwise, all cells were lysed in standard Kinase Lysis Buffer (KLB) (25mM Tris, 150mM NaCl, 5mM EDTA, 1% (v/v) Triton-X100, 10% (v/v) glycerol, 1mM Na_3VO_4, 10mM sodium pyrophosphate, 10mM β-glycerophosphate, 1mM NaF, 1mM phenylmethylsufonyl fluoride (PMSF), and 1mg/ml aprotinin, pH 7.4) for 10 minutes, and the lysates were centrifuged at 13200 rpm for 10 minutes at 4˚C. Protein concentrations were estimated using Bradford protein assay (Biorad) according to the manufacturer's manual. The concentration of cleared lysates were then standardized and lysates were mixed with 5× SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for Western blotting.

Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with TBST (0.05% (v/v) Tween-20, 150mM NaCl, and 10mM Tris-HCl, pH 8.0) containing either or 5% (w/v) non-fat milk or BSA (Sigma Aldrich; according the antibody's manual) for at least 1 hour. Antibodies with the appropriate dilutions were then added to fresh blocking buffer and applied to the membranes and incubated overnight at 4˚C. After approximately 16 hours, membranes were washed 3X with fresh TBST for 30 minutes (changing every 10 minutes) and secondary HRP-tagged antibodies (diluted in TBST with 5% (w/v) non-fat milk) were applied to the membranes for at least 1 hour at room temperature. Membranes were subsequently washed 3X with TBST for 30 minutes (changing every 10 minutes) and protein levels were detected via enhanced chemilumicescent reagents (GE Healthcare).

For immunoprecipitation, cells were first treated with 200μM pervanadate (POV) for 45 min before lysis. Equal amounts of lysates were precleared for 2 h with protein-A beads, then supernatant was incubated with anti-HA and protein-A beads overnight. The next day, beads were washed 4 × with a high-salt KLB solution (500mM NaCl) and once with standard KLB solution before addition of 5× sample buffer and subjected to standard western blotting.

Active Ras pulldown assay

Activated Ras GTPase pulldown assay was performed as previously described (Knaus et al., 2007). Sixty microgram of GST-Raf-1- RBD 1-149 (Plasmid 13338; Addgene) was first immobilized on GSH-Sepharose beads (GE Healthcare) at 4˚C overnight. Cells were lysed in GTPase lysis buffer (50mM Tris-HCl, 10mM $MgCl_2$, 200mM NaCl, 1%(v/v) Triton-X100, 5% (v/v) glycerol, 1mM Na_3VO_4, 1mM PMSF and 1mg/ml aprotinin, pH 7.5) for 5 minutes before centrifugation at 13,200 rpm for 10 minutes. 850μg of total lysates were then mixed with GST-fusion-protein-immobilized beads and incubated at 4˚C for 50 minutes. Then beads were washed 3X with GTPase washing buffer (25mM Tris-HCl, 30mM MgCl2, 40 mM NaCl and 1% (v/v) Triton-X100) and once with GTPase washing buffer without detergent. Together with the whole cell lysates as control, the pulldown fractions were then subjected to western blotting with anti-Ras antibody (MA1-012; Thermo Scientific).

SH2-phosphopeptide binding assay and far-western blotting

Biotinylated synthetic peptides were synthesized in both phosphorylated and unphosphorylated forms (Genescript), carefully spotted onto a gelatin-coated nitrocellulose membrane and fixed with 4% paraformaldehide for 5 min. The membrane was subjected to binding with a panel of GST-tagged pTyr binding domains as described in (Machida et al., 2007). Far-western blotting was carried out as described previously (Machida et al., 2007) using HRP-conjugated anti-GST antibody for detection.

Antibodies

pTyr (P-Tyr-100), phospho-CrkII (Tyr221; 3491), p-S473-Akt and p-Erk1/2, pT202/pY204 were from Cell Signaling (Danvers, MA, USA); Dok1 (A-3), pDok1 (Tyr362), Actin (I-19), Erk2 (D-2) and HA (Y-11) were from Santa Cruz Biotechnology (Dallas, TX, USA); CrkII (C-18) and Abl (8E9) were from BD Biosciences (San Jose, CA, USA); RasGAP (B4F8) was from Upstate Biotechnology (Lake Placid, NY, USA).

The procedures here forth were conducted in Yi Wu's lab in CCAM:

All works performed in Figure 2.21, including the plasmids generation, cell transduction and live cell imaging were carried out by our collaborator, Dr. Yi Wu's laboratory in Center for Cell Analysis and Modeling (CCAM, UCHC). The following methods and meterials are based on the information provided by them.

Construction of Dora-Ras

The Dora-Ras sensor is based on established design principles (Mochizuki et al., 2001; Pertz et al., 2006). It contains an N-terminal Ras binding domain (Cys71-Ser161) derived from Byr2, followed by a fluorescent protein FRET pair (Cerulean3-Venus), and an intact, wild-type H-Ras at the C-terminus. To improve the dynamic range, structural optimization was used to couple the dimerization of fluorescent proteins with the interaction between Byr2 and activated H-Ras (Scheffzek et al., 2001), hence the name dimerization optimized reporter for activation (Dora). The detailed characterization of the sensor will be described elsewhere (manuscript in preparation). As a control, a point mutation (R83E) was introduced in the Ras binding domain to disrupt Ras binding. The mutant sensor controls for potential alterations of fluorescence in the cell that are independent of Ras activation.

Live cell imaging and sensor data processing

Cells were seeded onto fibronectin (20 μg/ml) coated coverslips in phenol red- and vitamin-free Dulbecco's Modified Eagle's imaging medium (US Biological, Salem, MA, USA). Total internal refection fluorescence imaging was conducted on a customized Ti-E inverted microscope (Nikon, Tokyo, Japan) equipped with a multiline (440/515/594 nm) LMM5 laser merge module (Spectral Applied Research, Canada), a motorized XY stage (Ludl, Hawthorne, NY, USA), and a Stable Z-stage heater (Bioptechs, Butler, PA, USA). Images were acquired

through a 60 × 1.49 NA total internal reflection fluorescence objective (Nikon) on an iXon Ultra 897 EMCCD (Andor, Belfast, UK) under the control of MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). Biosensor data were processed using custom routines written in MetaMorph and MATLAB (MathWorks, Natick, MA, USA) for background subtraction, image segmentation, channel registration and ratiometric arithmetic as described previously (Hodgson et al., 2010).

Chapter 5

Summary and future directions

Summary

Unexpected Imatinib-enhanced anchorage independent growth has led us to the discovery a previously unreported Dok1-regulated inhibitory pathway for CrkI transformation. Upon validating the identity of Dok1 as an Imatinib-sensitive protein, our knockdown and rescue experiments confirmed that phosphorylated Dok1 (specifically, at sites Y295 and Y361) functions as a tumor suppressor in CrkI tumorigenesis. Furthermore, the overexpression of non-phosphorylatable mutant Dok1 (both Y295F and Y361F) showed a dominant negative effect in CrkI transformation. We next used both *in vitro* and *in vivo* systems to demonstrate that the binding of Dok1 to RasGAP, a deactivator for the Ras small GTPase, requires phosphorylated tyrosines 295 and 361.

Then, we showed that the knockdown of RasGAP in CrkI-transformed cells also causes enhanced anchorage-independent growth with the number of colonies comparable to that of Abl or Dok1 knockdown. This suggests that RasGAP is involved in the growth-limiting signaling pathway of Abl/Dok1. However, we were not able to observe any significant changes in the total Ras activation in the control, CrkI-transformed and the subsequent Abl or Dok1 knockdown cell lysates. Instead, we showed a localized dysregulation of Ras activation in CrkI-transformed cells through a FRET-biosensor that detects Ras activation. This observation corroborates the role of Ras in CrkI transformation, as concluded previously by several studies.

To investigate other phenotypic changes resulted from CrkI overexpression and the subsequent knockdown of Abl or Dok1, we measured the proliferation and migration rates of these cells in the monolayer culture condition. Other than the significantly higher proliferation rate of CrkI overexpressing cells (when compared to control NIH3T3 cells), the knockdown of Abl or Dok1only resulted in a subtle increase of cell proliferation rate. Moreover, the two-dimensional migration rates of cells were not affected either by CrkI overexpression or Abl/Dok1

knockdown. Due to the contrasting observations between the two culture conditions (monolayer versus anchorage independent), we hypothesize that culture condition plays an important role in our study and possibly, CrkI tumorigenesis.

Along with the decrease of Dok1 phosphorylation, we also observed a reduction in CrkII phosphorylation when cells were treated with Imatinib. However, when we co-expressed a non-phosphorylatable CrkII mutant (Y221F) in CrkI transformed cells, we found no significance differences in the anchorage independent growth. This ruled out the contribution of unphosphorylated CrkII in Imatinib-enhanced CrkI transformation.

We next assessed the activation status of MAPKs in relation to CrkI overexpression and the subsequent Abl or Dok1 knockdown. While Erk phosphorylation was unaltered, phosphorylated Jnk was highly elevated in CrkI-transformed cells and was further increased by the subsequent Abl or Dok1 knockdown. Previously, we have concluded that pJnk has an insignificant role in the oncogenic signaling of CrkI since it remains stably active even in the CrkI-transformed, Sos knockdown cell line (which is barely transformed). Taken together, we hypothesize that Abl inhibition may have escalated the significance of Jnk phosphorylation in CrkI transformation.

To assess if our mouse fibroblast model of Imatinib-enhanced CrkI oncogenesis has any implication on human cancer cells, we tested the effect of Imatinib in anchorage independent growth using several selected NCI60 human cancer cell lines. Among the eight cell lines we selected, only SF268, a glioblastoma cell line, showed increased colony number and size when cells were treated with Imatinib. However, the observed Imatinib response in SF268 cells was not correlated with any relative difference in Abl or Crk protein expression levels. Moreover, we were not able to detect Dok1 expression in any of the NC60 cell lines. For now, it is unclear whether or not the interaction between Abl/Dok1and CrkI is involved in Imatinib-enhanced

anchorage independent growth of SF268 and further investigation is required in order to understand the underlying mechanism(s).

Future directions

Other potential roles of Dok1in CrkI transformation

One of the signaling pathways that Dok1 has been known to regulate, but has not been discussed in our study, is the phosphoinositide 3-Kinase (PI3K) or Akt signaling pathway. Phosphorylation of Dok1 is highly elevated in CrkI overespressing NIH3T3 (Figure 2.1) and our *in vitro* phosphopeptide binding assay (Figure 2.13) confirmed the interaction between the two SH2 domains of p85α subunit of PI3K and the four phospho-tyrosines residues we examined. The PI3K pathway has been reported to be highly activated and plays a critical role in v-Crk induced transformation of chicken embryonic fibroblasts (CEF) (Akagi et al., 2000). This activation of Akt was later confirmed to be focal adhesion kinase (FAK) and Sos/H-Ras dependent (Akagi et al., 2002). In CrkI transformation, we have consistently observed an increased phospho-Akt as well (Zheng et al., 2010) but we have also ruled out its significance previously. This is mainly because the activation status of Akt remains unchanged even in the CrkI-transformed, Sos knockdown NIH3T3 cells (a cell line with severely-compromised CrkI transformation). However, since Abl-phosphorylated Dok1 has been identified as the negative regulator in our CrkI-transformed model and it was shown to interact with p85α subunit of PI3K, we are unsure how PI3K/Akt will be affected by Imatinib or Abl knockdown. It is possible that the previously insignificant role of PI3K is now activated by inhibition of Abl and have more influence over the oncogenesis of CrkI. By investigating the downstream targets of PI3K like the mammalian target of rapamycin complex 1 (mTORC1), ADP-ribosylation factors 6 (ARF6; a GEF for Rac GTPase) and others through protein analysis, we may be able to assess the involvement of PI3K in enhanced CrkI tumorigenesis.

Besides Abl kinase, insulin receptor (IR) (White, 1998), a receptor tyrosine kinase also phosphorylates Dok1 and causes it to bind RasGAP (Porras et al., 1992; Zhang and Roth, 1992). In 2001, two tyrosines residues in Dok1, Y361 and Y398 were identified as the direct targets of IR and their phosphorylation was shown to increase the binding of Nck (Y361) and RasGAP (Y361 and Y398) (Wick et al., 2001). Moreover, this study showed that mutated Y361 and Y398 decrease the inhibitory effect of Dok1 in Ras activation and reduce the activation of Akt, but had no effect on the MAPK pathway. While we are very certain that Y295 and Y361of Dok1 are required for RasGAP binding in our system, we should also investigate the significance of Y398 in strengthening Dok1/RasGAP binding and how it might play in suppressing CrkI transformation. Furthermore, we may also activate the IR kinase in our CrkI-transformed, Abl-knockdown cells to assess its ability to replace Abl as an activator of theDok1 regulatory pathway.

The cytoplasmic localization of Dok1 has been shown to be crucial for its tumor suppressor role. A previous study on chronic lymphocytic leukemia (LLC) found that a truncated Dok1 protein (deleted NES sequence) remains exclusively in the cell nucleus and loses its ability to inhibit cell proliferation and to promote cell spreading (Lee et al., 2004). A year later, the same research group reported that serum starvation and suspended culture condition favor nuclear localization of Dok1, while growth factor stimulation and cell adhesion promote the opposite (Niu et al., 2006). Their discovery on how suspension reduces Dok1's inhibitory role in cell proliferation via nuclear localization may be related to the inconsistency in our findings. We found CrkI-overexpressing, Dok1 knockdown cells (and the subsequent various Dok1-rescued cells) to show a clear differences in their proliferation rate using soft agar assay (suspended condition) but the differences were very subtle in adherent cell growth assays. It may be possible that culture conditions affected the localization of Dok1 proteins and thus provided a secondary regulation that caused the inconsistency. To verify if this is true, we may need to understand how

the nucleus versus cytoplasm localization of Dok1 affects its functionality. To address this issue, Dok1 fluorescence imaging and or nuclear-cytoplasmic cell fractionation of suspended and attached cells cultures should be performed.

Other roles of Abl kinase in our fibroblast model

In general, Jnk activation in cells often results in two opposing cellular responses, either enhancd cell proliferation or induction of programmed cell death, depending on the cell type and protein expression profile (Tournier, 2013). In our current study, CrkI overexpression appeared to increase Jnk phosphorylation and activate the apoptotic response. As mentioned, apoptosis may be the direct outcome of increased Jnk but it contradicts the growth promoting phenotype of transformed cells. Although we have limited understanding on how increased Jnk phosphorylation and apoptosis are related to Imatinib-enhanced CrkI transformation, we think Abl kinase may play a role in associating both observations.

Wild type Abl protein is predominantly located in the cytoplasm but relocates into the nucleus in response to DNA damage (Kharbanda et al., 1995; Yuan et al., 1997). According to Yoshida *et al.* the DNA-damaging agent adriamycin stimulates the activation of Jnk which then phosphorylates 14-3-3 proteins, a family of proteins with diverse functions (Yoshida et al., 2005). And because unphosphorylated 14-3-3 proteins (β, γ, ε, η, σ and ζ) form complexes with cytoplasmic Abl (Natsume et al., 2002) and retain Abl kinase in the cytoplasm by blocking Abl's NLS (Muslin and Xing, 2000; Tzivion and Avruch, 2002), they showed that increased Jnk activity promotes the release and subsequent nuclear localization of Abl. Interestingly, they also found that Imatinib-inhibited Abl1 kinase does not lose its association with 14-3-3 proteins and therefore remains in the cytoplasm (Yoshida et al., 2005).

Members of the 14-3-3 are expressed ubiquitously and bind phosphorylated proteins (Mhawech, 2005) but may have opposing functions in tumorigenesis, some as proto-oncogene (eg.

14-3-3γ) while others as tumor suppressors (eg. 14-3-3σ) (Radhakrishnan and Martinez, 2010). A recent overexpression study on 14-3-3γ or 14-3-3σ in NIH3T3 cells supported the involvement of PI3K and MAPK in their opposing roles in oncogenesis (Radhakrishnan et al., 2012). It would therefore be interesting to explore whether there are indeed auxiliary signals that link Jnk activation, Abl and 14-3-3 (especially on 14-3-3γ and 14-3-3σ) disassociation in our CrkI-transformed cells and more importantly, how significant are they in enhancing CrkI transformation.

Using human breast cancer cell lines, several studies have identified Abl or Arg as important mediators in suppressing tumor growth using mouse xenograft models (Allington et al., 2009; Gil-Henn et al., 2013; Noren et al., 2006). In one particular study (Noren et al., 2006), the binding of CrkII and Abl was proposed to mediate suppressive signals from the EphB4. Since the coexpression of CrkI and wild type CrkII (but not the CrkII Y221F mutant) resulted in suppressed colony formation (Figure 3.10), we wonder if the signaling pathways mentioned in these xenograft studies (ie. EphB4, TGFβ and EGF) play any role in our fibroblast model. We will first need to assess the importance of these receptors in NIH3T3 and how their activation level correlates with CrkI tumorigenesis. Then we will investigate how Imatinib affects their signal transduction in cell transformation.

The roles of small GTPases and MAPKs in Imatinib-enhanced CrkI transformation

The negative regulatory pathway of Abl-phosphorylated Dok1 suppresses CrkI transformation via RasGAP. However, how the recruitment of RasGAP leads to suppressed CrkI oncogenic signaling is still largely unknown at the moment. Moreover, the fact that RasGAP or Rasa1 knockout alone does not induce tumor development both *in vitro* and *in vivo* (Henkemeyer et al., 1995; Koehler and Moran, 2001; van der Geer et al., 1997) suggest a more complicated

interaction is at play. Ideally, we will be able to use the same Ras-FRET biosensor to visualize increased Ras activity in Crk overexpressing Abl, Dok1 or RASGAP knockdown cells.

Because GAP1 and SynGAP family proteins (two classes of RasGAP proteins) have been shown to activate both Ras and Rap1 GTPases (Kupzig et al., 2006; Sot et al., 2010), we are unsure if other small GTPases will be affected in our p120RasGAP knockdown cells as well. This may be caused by the activation of other RasGAP families (as a compensating response to the downregulation of p120RasGAP) or that p120RasGAP also have yet to identify non-Ras targets. In addition, even though our previous Sos knockdown study strongly corroborates the importance of Ras, we should not rule out other small GTPases, especially when we still do not understand RasGAP that well. Other active GTPase pull-down probes and FRET reporters, may reveal crosstalk between Ras and other small GTPase in CrkI transformation.

Based on our current understanding of CrkI transformation, MAPKs are most likely to have a significant role in CrkI tumorigenesis. In our previous study (Zheng et al., 2010), C3G and Sos, two GEFs for GTPases upstream of Jnk and Erk signaling cascades, respectively, have been shown to be the critical effectors of the SH3 domain of CrkI. While we previously concluded that Jnk activation has limited role in CrkI transformation, as it remained unaffected even in Sos knockdown cells (which severely compromises CrkI transformation), our most recent findings indentified much higher Jnk activation in both Abl knockdown and Dok1 knockdown cells. It is possible that the inhibition of Abl somehow results in activation of Jnk signaling pathway and thus, increasing the role of pJnk in Imatinib-enhanced CrkI transformation.

Integrin-mediated cell adhesion has been shown to activate C3G (de Jong et al., 1998) and induce Jnk phosphorylation in a Crk- and C3G-dependent manner (Dolfi et al., 1998). And since integrin modulates the attachment of cells to the extracellular matrix (ECM), the phosphorylation of Jnk and activation of C3G may be differently regulated in adhered and

suspended cells. Currently, all our protein analysis work is based on attached monolayer cells, and this raises the concern of whether or not this can accurately represent cells during anchorage independent growth. In contrast to the dramatic differences in colony formation rate among the various cell lines, we found only subtle changes when these cells were compared for their cell proliferation rate in monolayer culture. We hypothesize that the attachment of cells had contributed to Jnk phosphorylation and thus possibly the Abl/14-3-3 interaction and Abl-regulated apoptosis. If attachment increases basal phospho-Jnk levels, the effects of protein overexpression or knockdown may be masked considerably and this may explain the insignificant changes seen in monolayer cultured cells and the higher Caspase3 activity observed. To address this concern, we will investigate the status of Jnk and Dok1 phosphorylations in suspended colonies (since both are significantly changed in CrkI overexpression) and then, further investigate the differences in protein signaling and cellular responses between the two culture conditions.

For now, it is unclear how Erk1/2 affects the oncogenic signaling of CrkI. Although the expression of a dominant-negative Ras mutant has been reported to inhibit v-Crk transformation (Greulich and Hanafusa, 1996), total Ras activation and Erk phosphorylation are unchanged in CrkI-overexpressed cells as well as the Abl or Dok1 down-regulated CrkI-expressing cells (Figure 2.19). However, our cell imaging data did reveal an abnormally localized Ras activation in CrkI-overxpressing cells, and this suggests that the activation of Grb1/Sos/Ras/Erk is not as straight forward as previously thought. Recently, the work of Findlay *et al.* indicated that the various protein- and phospholipid-interacting domains in Sos and Grb2 were optimized to not have maximal binding affinity but to have temporal and spatial specificity for proper signal transduction (Findlay et al., 2013). It is possible that CrkI overexpression somehow shifts the spatial distribution of Sos and Ras and thus, disrupts the appropriate Grb2/Sos/Ras/Erk signaling pathway. Perhaps, by determining the *in situ* distribution of pErk, we may find changes in its

activation sites or even its nucleus trafficking dynamic that correlated with CrkI signaling and the effect of Abl inhibition.

Thus far, various cell-transforming signals (expression of oncoproteins, mitogenic stimulation and others) have been shown to increase the transcription of genes like the immediate-early genes (IEGs), which are critical for mitogenesis and tumorigenesis (Greenberg and Ziff, 1984; Herschman, 1991; Lau and Nathans, 1985; Meijne et al., 1997). To assess if Sos/Ras/Erk and C3G/R-Ras/Jnk pathways in CrkI-transformed cells have any direct gene expression effects via activation of transcription factors, we propose using RNAseq to screen for changes in mRNA levels that are correlated with CrkI transformation and Abl kinase inhibition (via Imatinib). By identifying the changes in gene expression, we may then work backward (using reporter plasmid with corresponding enhancer sequences) to indentify CrkI-responsive transcription factors associated with Erk and Jnk.

CrkII and CrkL

Along with the identification of the human Crk homolog proteins (CrkI and CrkII), the transformation potential of both isoforms were investigated in 3Y1 rat fibroblasts via overexpression and CrkII has been reported to possess no transforming activity (Matsuda et al., 1992). However, when the tumorigenicity of CrkII was investigated again with mouse fibroblasts, its overexpression transformed NIH3T3 cells successfully (Iwahara et al., 2003). Such an inconsistency highlights how differences in species origin, cell types, and protein expression influence the transformation potency of proto-oncoproteins. Here, our anchorage independent assay confirmed CrkII's tumorigenicity in mouse fibroblasts (NIH3T3). Furthermore, we found that the CrkII Y221 mutation conferred no advantage in this assay (Figure 3.12). We remain skeptical with this finding and more work will be required to rule out any other possibilities that might have affected this outcome. And because transforming ability of v-Crk in CEF requires

much higher level of expression (compared to chicken Crk proteins), we hypothesize that the efficiency of cell transformation by Crk family proteins is partly dosage-sensitive. If this is the case, we may be able to better assess of the role of Y221in the tumorigenecityof CrkII by carefully regulating its expression level.

So far, we have yet to determine the role of CrkL in Imatinib-enhanced CrkI transformation, but as the preferred Crk protein for BCR-ABL kinase (Sattler and Salgia, 1998; Senechal et al., 1996), it is very likely that its interactions will be affected in Imatinib-treated CrkI cells as well. Perhaps, by knocking-down and rescuing (with non-phosphorylatable Y251) CrkL, the role of CrkL in Imatinib-enhanced CrkI transformation can be revealed.

The growth promoting effect of Imatinib in human cancer cells and the mechanism involved

We initially selected several of the NCI60 human cancer cell lines to investigate the effect of Imatinib in their anchorage-independent growth, and have so far identified one that has Imatinib-enhanced suspended growth (SF268, a glioblastoma derived cell line). To obtain a more comprehensive understanding on how Imatinib affects the suspended growth of human cancer cells, we will first have to screen more cell lines. Apart from the remaining NCI60, we should also refer to the existing RNAseq database on human cancer cell lines and select those with higher Crk/Dok/Abl expression. It may also be interesting to asses those with transcriptional profiles similar to that of SF268, especially those derived from glioblastomas. With only SF268 alone, it is hard to draw any generalized conclusion on how Imatinib may have enhance human tumor growth, especially because our initial selection criteria (expression of Abl and CrkI/II) did not correlate with positive response to Imatinib as we had predicted. If more cell lines with Imatinib-enhanced growth are identified, we will need to identify similarities in their underlying cellular physiology using more high throughput assays such as RNAseq.

There are several phenotypic differences in anchorage independent assay between Imatinib-treated SF268 and CrkI-transformed fibroblasts. First, cellular density appeared to have a more profound effect on the size and number of colonies for SF268. And secondly, Dok1 is undetectable in SF268 (and the rest of the NCI60 cell lines). It appears that the Imatinib responses in SF268 and CrkI-transformed fibroblasts may involve different signaling proteins. Perhaps, it is the differences in cell types and species, hence the protein expression profiles that play a huge role in the responses of these cells. And since EphB4, EGF and TGFβ have been previously identified to be involved with the tumor suppressor role of Abl (Allington et al., 2009; Gil-Henn et al., 2013; Noren et al., 2006), we should also look into their roles in SF268 and CrkI-transformed NIH3T3.

So far, we have only confirmed that Dok1 is undetectable in NCI60 cell lines and that it is responsible for suppressing CrkI transformation in NIH3T3. However, mouse studies linking myeloproliferative disorder (Niki et al., 2004; Yasuda et al., 2004), lung tumor (Berger et al., 2010) and histiocytic sarcoma (Mashima et al., 2010) with Dok2 and Dok3 knockout, suggest that these proteins also havesome significant role in regulating tumorigenesis. That said, we first need to determine their expression level in the NCI60. If Dok2 and Dok3 proteins are indeed expressed and functional, we will need to investigate their role as Imatinib-sensitive tumor suppressors. It is plausible that the lack of Dok1 expression in these cells may be compensated for by its closest family members, Dok2 and Dok3.

In conclusion, from the unexpected observation of an Imatinib-enhanced anchorage independent growth of CrkI transformation, we discovered an Abl-driven growth inhibitory pathway that negatively regulates oncogenic CrkI signaling. Our findings emphasize the diverse roles of Crk proteins and how the localization and interaction between active signaling pathways

in cells can influence the effect of a supposedly growth-limiting drug. We have listed several suggestions for future studies above which will allow us to further investigate the role of CrkI in various signaling pathways and more importantly, to understand how Imatinib promotes tumor growth in CrkI transformation and possibly human cancer cells.

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