DJ-1, a PTEN Inhibitor, is a Positive Regulator of Cancer Cell Motility

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Rawan M. Yassine

ABSTRACT

DJ-1 is a 20-KDa protein that was first identified as an oncogene product responsible for a subset of familial Parkinson’s disease; hence its name PARK 7. Previous studies showed that DJ-1 negatively regulated the tumor suppressor gene PTEN expression; thus promoting the phosphorylation of the PI3K/Akt signaling pathway, and activating cell proliferation and transformation. Therefore, DJ-1 can be used as an indication of cancer metastasis and can be a potential therapeutic target. However, the localization, detailed mechanism and the role of DJ-1 in cellular motility are still not fully understood. Therefore, our study showed that DJ-1, at normal cellular condition, is present in the nucleus, mitochondria, golgi apparatus and plasma membrane of astrocytoma (SF268) cells. Upon its stimulation with EGF, DJ-1 localize outside the nucleus, leading to an increase in cellular motility in lung, brain, and breast cancer cells by activating the PI3K pathway. We also showed that this activation is carried out by inhibiting PTEN and thus regulating the RhoGTPases mainly Rac which is known to be involved in cellular motility.

Keywords: DJ-1, PTEN, PI3K, Cancer, Motility, Rho-GTPases, Rac.
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LIST OF ABBREVIATIONS

WHO: World health organization

NSCLC: Non-small cell lung cancer

SCLC: Small cell lung cancer

LCT: Lung carcinoid tumour

PtdIns: Phosphatidyl-inositol

PPI: Phosphorylated Phosphatidyl-inositol derivatives

PI3K: Phosphatidylinositol 3 kinase

ABD: Adaptor binding domain

RBD: Ras binding domain

C2: Protein Kinase C homology 2

SH3: Src homology 3

BH: Breakpoint clustered homology

SH2: Src homology 2

iSH2: inter Src homology 2

RTK: Receptor tyrosine kinase

GPCR: G-protein-coupled receptor

IRS1: Insulin receptor substrate 1

PIP2: Phosphatidyl 4,5-bisphosphate

PIP3: phosphatidylinositol (3,4,5)-triphosphate

PH: Pleckstrin homology

PI-3-P: Phosphatidylinositol 3-phosphate
PI: Phosphoinositol
VPS34: Vacuolar protein sorting 34
PTEN: Phosphatase and tensin homolog deleted on chromosome ten
Akt: Acutely transforming retrovirus
PKB: Protein kinase B
PDK1: Phosphoinositide-dependent kinase-1
mTOR: Mammalian target of rapamycin
FOX: Forkhead box
MDM2: Mouse double minute 2 homolog
TSC2: Tuberous sclerosis 2
Rheb: Ras homolog enriched in brain
GTPase: Guanosine triphosphatase
Rho: Ras homologous member
Rac: Ras-related C3 botulinum toxin substrate
Cdc42: Cell division cycle 42
GDP: Guanosine diphosphate
GTP: Guanosine triphosphate
GEF: Guanine Nucleotide Exchange Factor
GAP: GTPase Activating Protein
GDI: Guanine Nucleotide Dissociation Inhibitor
DH: Dbl homology
PDGF: Platelet-derived growth factor
EGF: Epidermal growth factor
RhoA: Ras homologous member A
ROCK: Rho-associated coiled coil-containing protein kinase

PAK: p21 activating kinase

CRIB: Cdc42/Rac interactive binding

DMEM: Dulbecco's Modified Eagle Medium

FBS: Fetal Bovine Serum

siRNA: Small interfering Ribonucleic Acid

GFP: Green Fluorescent Protein

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

PVDF: Polyvinylidene fluoride

PBS: Phosphate Buffered Saline

ECL: Enhanced Chemiluminescence

BSA: Bovine Serum Albumin

GST: Glutathione S-transferase

RBD: Rhotekin binding domain

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Chapter 1

1. LITERATURE REVIEW

1.1. Cancer

1.1.1. Background

Cancer, a multifactorial disease, is considered to be the primary cause of mortality worldwide. In 2012, it reported around 8.2 million deaths according to the world health organization (WHO). This complex group of diseases, that affects a varied range of cells and tissues in an organism, is caused by nonlethal genetic damage that might be acquired, inherited or both. In spite of its prevalence, cancer is considered a rare frequency disease since it is due to the clonal expansion of only a single precursor cell that has acquired a genetic mutation allowing it to escape the normal cell cycle. Moreover, the likelihood of developing cancer depends on the gene being mutated, mutations in other genes as well as environmental factors (National Cancer Institute, 2014).

Tumour progression is initiated by a series of genetic mutations that alters the properties of the cell causing defects in its regulatory circuits; thus, disrupting homeostasis and allowing it to proliferate rapidly leading to the formation of a tumour (Szabo & Merks, 2013). These defects are known as the “Hallmarks of cancer” (Figure 1) that include the ability of the cell to: self-proliferate, evade growth suppressors, resist apoptotic signals, replicate uncontrollably, secret proangiogenic signals, invade and metastasize, evade the immune system, and recruit healthy cells to form a “tumour microenvironment” (Hanahan & Weinberg, 2000, 2011).
More than hundred different types of cancer have been identified. Most of them are named based on the organ or cell type of origin. They can be grouped into five categories including carcinoma; cancer starting in cells of the epithelial tissue, sarcoma; cancer starting in cells of the connective tissue, leukaemia; cancer starting in cells of the hematopoietic system, lymphoma and myeloma; cancers starting in cells of the immune system and finally central nervous system cancers; cancers starting in cells of the brain and spinal cord (National Cancer Institute, 2014).

1.1.2. Lung tumours

Lung cancer is considered to be one of the leading sources of mortality among all cancer types, with 1.8 million new cases identified in 2012 (World Cancer Research foundation, 2012). Lung Cancer is subdivided into three main classes, with around 85% accounting for non-small cell lung cancer (NSCLC), 15% for small cell lung cancer (SCLC) and less than 5% for lung carcinoid tumour (LCT) (American Cancer Society, 2014). Smoking is the main environmental factor contributing to the development of...
lungenkarzinome sowie andere ursächliche, genetische und epigenetische Faktoren (Haixiang et al., 2014). Ein Raucher in der ganzen Lebenszeit hat 20-mal höhere Wahrscheinlichkeit, an Lungenkarzinom zu erkranken im Vergleich zu einem Raucher in der ganzen Lebenszeit (Alberg & Samet, 2003). In Anbetracht der überragenden Fortschritte bei der Diagnose, Detektion und Behandlung von Tumoren, wird Lungenkarzinom meist erst bei einer fortgeschrittenen Stadienebene festgestellt, was zu einer schlechten Prognose und Überlebensrate führt mit nur 7% der Patienten fünf Jahre überlebend (Haixiang et al., 2014 & Parsons, 2010).

1.1.3. Gehirntumoren

2,42% aller Krebstermine weltweit sind mit Gehirntumoren (Siegel, Naishadham, & Jemal, 2013) assoziiert. Von 100,000 Menschen werden jährlich 6,4 mit Gehirntumoren diagnostiziert, was mit einer höheren Häufigkeit bei Männern verglichen mit Frauen (Homer et al., 2011) einhergeht. Gehirntumorzellen proliferieren schnell, um eine Masse von Krebsgewebe zu bilden, das die primären Gehirnfunktionen wie Gedächtnis, Sinneswahrnehmung und Muskelkontrolle behindert, ferner die normalen Körperfunktionen. Diese Tumoren werden aufgrund des Gehirntissuetyps, aus dem sie ursprünglich entwickelt wurden, benannt. Viele Risikofaktoren beitragen zur Entwicklung von Gehirntumoren, wobei genetische Faktoren die überwiegende Rolle spielen sowie Umweltfaktoren wie chemische und biologische Karzinogene (Wrensch et al., 2002).
1.1.4. Breast tumours

Breast cancer is considered to be one of the most frequent types of cancer worldwide, accounting for 14.1% and 6.8% of all cancer occurrences and deaths respectively. It is also considered to be the second primary cause of cancer mortality and the most commonly diagnosed type of tumour in women (Siegel, Naishadham, & Jemal, 2013). Inspite of the tremendous development in the diagnosis and treatment of metastatic breast cancer, deaths from this tumour remains elevated due to the emergence of therapy resistant cancer cells which will hinder the therapy used (Stockler et al., 2000). Therefore, current treatments cannot fully cure metastatic breast cancer yet. This type of cancer develop from a series of mutations that occurs due to genetic or enviromental factors (Al-Hajj et al., 2003). Such factors include tobacco, fat and alcohol consumption, obesity, oral contraceptives, radiation and family history.

Moreover, the risk of breast cancer formation increases with age and decreases after menopause (McPherson, Steel, & Dixon, 2000). Therefore, a better understanding of the effects of these mutations and early detection are the base of new therapeutic strategies and treatment of this disease.

1.2. The PTEN/PI3K pathway

1.2.1. The PI3K pathway components

1.2.1.1. PI3K

The plasma membrane of mammalian cells contains a relatively high amount of phospholipids known as phosphatidyl-inositol (PtdIns) but only few amounts of its
phosphorylated PtdIns derivatives (PPI). Phosphoinositide kinases, such as
Phosphoinositide 3 kinases (PI3Ks) produce PPI by the addition of phosphate groups to
inositol-glycero-phospholipids. Different phosphoinositides are selectively
phosphorylated depending on the individual PI3K member involved (Voskas et al.,
2014). Therefore, the PI3Ks is a family of proteins implicated in an intricate cascade of
signal transduction pathways that is involved in the regulation of cellular growth,
proliferation, metabolism, differentiation, migration, motility and survival as well as
vesicle trafficking and glucose homeostasis (Castillo et al., 2014 ; Engelman et al.,
2006).

*Classes of the PI3K pathway*

The PI3K family is subdivided into three classes: class I, II and III. Most of these
members are bounded to regulatory subunits that govern the signals modulating its
function (Cantley, 2002).

Class I PI3K:

Class I PI3K is the most studied member and is related to oncogenesis
(Markman, 2010). It is a heterodimer formed from a catalytic and a regulatory subunit,
known as an adaptor. The catalytic subunit is known as p110 and it constitute four
isotypes: isotype alpha encoded by PIK3CA gene, beta encoded by PIK3CB gene,
gamma encoded by PIK3CG and delta encoded by PIK3CD (Reifet et al., 2004). P110-
alpha is involved in insulin-dependent signalling, p110-beta in the aggregation of
platelets and insulin signalling and p110-gamma and delta in the activation of
lymphocytes, degranulation of mast cells and chemotaxis (Williams et al., 2009). All
these isotypes share similar domain configuration. They are made up of an amino
terminal adaptor binding domain (ABD) that interacts and binds the regulatory subunit, followed by a Ras binding domain (RBD) where Ras-GTP binds and interacts in order to stimulate PI3K and Ras driven signalling pathways, a protein kinase C homology 2 (C2) domain that possess the ability to bind to membrane phospholipids, a helical domain and finally a carboxyl terminal kinase domain (Walker et al., 1999). The most common regulatory subunit is known as p85 and is associated with the catalytic subunit p110. There are three isoforms of this regulatory subunit: p85-alpha (p85 alpha, p55 alpha, and p50 alpha isoforms) encoded by PIK3R1, p85-beta encoded by PIK3R2 and p55-gamma encoded by PIK3R3 (Reif et al., 2004). p85 alpha, is made up of several linked protein-protein interaction domains, starting with a Src-homology 3 (SH3) domain at the N terminal, followed by a breakpoint clustered homology (BH) domain, then two Src-homology 2 (SH2) domains at the C-terminal with an inter-SH2 (iSH2) domain in between. The catalytic subunit p110 binds primary to iSH2 domain of the regulatory subunit (Dhand et al., 1994) (Figure 2).

**Figure 2: The Catalytic and Regulatory Subunits of Class I PI3Ks.** This illustration shows the different isotypes and domains of the catalytic and regulatory subunits that makeup class I PI3Ks. Source: (Vanhaesebroeck et al., 2010)
Class I PI3K is also subdivided into class IA and class IB according to the catalytic and regulatory subunits they possess (Vanhaesebroeck et al., 1997). Class IA are activated downstream of receptor tyrosine kinases (RTK) and consists of the catalytic subunit p110 alpha, beta or delta linked to the regulatory subunit p85 alpha, p85 beta, p55 alpha, p55 gamma or p50 alpha. On the other hand, class IB are activated downstream of G-protein coupled receptors (GPCR) and consists only of the catalytic domain p110 gamma linked to the regulatory subunit p101, p84 or p87 (Backer, 2008; Dbouk & Backer, 2010). One of the regulatory subunits functions is linking the catalytic subunit to upstream signals so that they interact with receptor tyrosine kinases (RTK) and G-protein coupled receptors (GPCR). When these receptors are not stimulated, the regulatory subunits stabilize and suppress the catalytic subunit activities (Luo et al., 2005). Once a ligand binds to receptor tyrosine kinase, PI3K gets activated either through the direct binding of its SH2 domain of the regulatory subunit to the auto-phosphorylated phosphotyrosines of RTK or through the indirect activation by phosphorylating tyrosine residues on scaffolding proteins for instance insulin receptor substrate 1 (IRS1) which in turns activates PI3K(Manning and Cantley, 2007). The catalytic subunit, p110, is then activated by releasing the inhibition of the regulatory subunit, p85. Once the PI3K gets activated it localizes to the cellular membrane where p110 adds a phosphate group to phosphatidylinositol-4,5-bisphosphate (PIP2) at the D3 position of the inositol ring to produce phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Castillo,2012). PIP3 once formed, induces the recruitment and binding of a subclass of proteins harboring a pleckstrin homology (PH) domain to the inner plasma membrane of the cell. This high affinity of PH domain proteins to PIP3 result in the initiation and activation of multiple downstream signaling cascades (Voskas et al., 2014). The PH
domain was initially recognized as a 100 to 120 amino acid sequence that repeats twice in pleckstrin, a platelet protein, and binds with high affinity and specificity to phosphoinositides (Haslam et al., 1993; Mayer et al., 1993). PI3K might also be stimulated by Ras, activated Ras binds directly to its catalytic subunit (p110) leading to its activation (Cantley, 2002). Moreover, the catalytic subunit p110 beta was found out to be activated downstream of GPCR (Katso et al., 2001).

Class II PI3K:

Class II PI3K doesn’t have a regulatory subunit and is made up of three isoforms which are: PI3KC2-alpha, beta, and gamma. All these isoforms can be activated downstream of RTK, cytokine receptors and integrin receptors. Class II PI3K phosphorylates phosphatidylinositol 3-phosphate(PI-3-P) into phosphatidylinositol 3,4-bisphosphate (PI-3,4-P_2) and phosphoinositol (PI) into PI-3-P (Engelman et al., 2006). Studies have shown that this class of PI3K might play a role is the reorganization of actin cytoskeleton and cell adhesion during wound healing and cell migration in non-immune cell system (Domin et al., 2005); however these roles has been underestimated due to the limited availability of pharmacological tools against the different isoforms of this class (Domin et al., 1997; Virbasius et al., 1996).

Class III PI3K:

Class III PI3K, which is also known as vacuolar protein sorting 34 (VPS34), was first identified in Saccharomyces cerevisiae, a budding yeast (Herman & Emr, 1990). Its mammalian homologue, hVPS34, is encoded by PI3KC3 gene and is associated with tyrosine kinase p150 regulatory subunit. It was shown to be involved in the regulation of
autophagy, vesicular trafficking of membrane proteins to lysosomes and regulation of mTOR signalling pathway by cross talking with classI PI3K (Backer, 2008).

1.2.1.2. PTEN

PTEN, Phosphatase and Tensin homolog deleted on chromosome TEN, was initially identified in 1997 as a tumor suppressor that is mutated and lost in various types of cancer. It was shown to be mutated in 31% of glioblastoma, 6% of breast cancer, and all prostate cancer cell lines and xenografts tested (Li et al., 1997). It is made up of a tensin like domain linked to a catalytic domain. The catalytic domain possesses a protein as well as a phospholipid phosphatase activity that hydrolyses phosphates on position 3 from phosphoinositides. The tumor suppressor activity of PTEN depends on both its phospholipid phosphatase activity, encoded by its catalytic domain, as well as its ability to bind to membrane lipids, encoded by its C2 domain (Maehama and Dixon, 1998; Stambolic et al., 1998). Therefore, PTEN functions in reversing PI3K activity by dephosphorylating PIP3 into PIP2 on the plasma membrane (Stambolic et al., 1998). Mutations of PTEN commonly take place in its phosphatase domain (Eng, 2003), indicating its essential role as a main player in regulating PI3K signaling. And any changes in PTEN level of expression or function greatly impact the PI3K pathway (Carracedo and Pandolfi, 2008). Studies have shown that PTEN interferes with different processes including cell cycle progression, cellular migration, apoptosis, and metabolism, by down regulating the PI3K/Akt pathway and decreasing the phosphorylation of Akt substrates (Stambolic et al., 1998). It also inhibits cellular invasion, metastasis and differentiation (Nogueira et al., 2010; Waite et al., 2002).
addition to its phospholipid phosphatase activity at the plasma membrane, PTEN also play an important role in DNA damage in the nucleus (Ming and He, 2012).

1.2.1.3. Akt/Protein kinases B (PKB)

Once PI3K gets activated and phosphorylates PIP2 to PIP3 at the plasma membrane, proteins harboring a PH or a PH-like domains get recruited to the inner plasma membrane where they transmit there signal (Engelman et al., 2006). One of these proteins is protein kinase B (PKB) which is also known as Akt. Akt is a pro-survival kinase that was first recognized in 1991 as a weak oncogene (Bellacosa et al., 1991). Three mammalian isoforms where recognized for PKB which are PKBα, β and γ also known as Akt 1,2 and 3 respectively. All these isoforms share a similar amino acid sequence and are made up of three functional domains: an N-terminal domain containing a PH domain, a C-terminal domain containing a hydrophobic motif and a kinase domain (Scheid & Woodgett, 2003). Moreover, Akt, mainly Akt1, possesses two activating phosphorylation sites at amino acid Thr308 and Ser473. Upon PIP3 formation, Akt binds to it through its PH domain; however it remains inactive and its activation requires two additional phosphoinositide-binding proteins known as PDK1 and mTORC2. PDK1, phosphoinositide dependent protein kinase 1, is required for PIP3 dependent phosphorylation of Akt, it binds to PIP3 recruiting Akt to the plasma membrane where it induces its conformational change permitting its phosphorylation at Thr308. Additional phosphorylation of Akt takes place at Ser473 embeded in the hydrophobic motif by mTORC2, mammalian target of rapamycin complex 2, leading to the full activation of the kinase Akt (Sarbassov et al., 2005). Activated Akt phosphorylates and mediates
various downstream substrates that play a role in growth, survival, metabolism, proliferation, angiogenesis and glucose homeostasis (Manning & Cantley, 2007).

Akt leads to cellular survival by inhibiting proteins that are involved in apoptosis such as BAD and BAX which are pro-apoptotic members of the Bcl2 family (Engelman et al., 2006; Cantley, 2002). Moreover, it reduces the production of cell death stimulating proteins by inhibiting FOX (forkhead box) transcription factors. It also phosphorylates MDM2 (mouse double minute 2 homolog) proteins which leads to the inhibition of the tumor suppressor gene p53 (Duronio, 2008). In addition to that, Akt also phosphorylates tuberin also known as TSC2 (tuberous sclerosis 2) which inhibits the TSC1/TSC2 dimer activity and thus activates the GTP binding protein rheb (Ras homolog enriched in brain). Once rheb is activated it stimulates mTORC1 which in turn increases the p70-S6 kinase activity leading to increased protein synthesis and cellular proliferation (Engelman et al., 2006). Activation of S6 kinase by mTORC1 also leads to the phosphorylation and inhibition of the adaptor protein insulin receptor substrate 1(IRS1) thus, inhibiting PI3K activation. Therefore, S6 kinase plays a role in PI3K negative feedback regulation (Carracedo and Pandolfi, 2008) (Figure 3). Therefore, due to the different downstream functions of Akt, the PI3K/Akt pathway is considered to be the official backbone of PI3K signaling in spite of other important pathways taking part as well.
1.2.2. PI3K pathway in cancer

Different types of cancers have been highly associated with alteration in the PI3K pathway. PI3Ks, mainly class I, has been identified to be mutated and amplified in different types of tumours (Samuels et al., 2004). PI3Ks class I are the only class that plays a role in cancer, there is no evidence that links the other classes, class II and class III, to tumorigenesis. This is probably due to the different functions of the three classes where only class I leads to the production of PIP3 that is an essential component and regulator of cellular growth and replication (Zhao and Vogt, 2008).
Activating mutations in PI3Ks takes place mostly within the gene, PIK3CA, that codes for the catalytic subunit p110 alpha and leads to different types of cancer (Samuels et al., 2004; Samuels and Ericson, 2006). These single nucleotide substitution mutations, take place in one of three so called “hot spots”; two of which are found within the helical domain (E542K and E545K) in exon 9 and the third in the kinase domain (H1047R) in exon 20 (Wong et al., 2010). Mutations of the helical domain block the inhibitory interaction of the SH2 domain of the regulatory subunit p85 with the catalytic subunit p110 alpha, while mutations in the kinase domain constitutively activate PI3K activity (Huang et al., 2007). These mutations promote a gain of function in the catalytic subunit, leading to high levels of PIP3, activating Akt signaling and leading to cellular transformation in vitro as well as in vivo (Samuels et al., 2004; Ikenoue et al., 2005; Bader et al., 2006). Moreover, they result in the constitutive activation of the PI3K pathway in the lack of growth factors stimulation; thus removing the usual required interaction between PI3K and phosphorylated tyrosine on RTKs and/or adapters (Berns et al., 2007). It was shown that 30% of common cancer such as colon, breast, prostate and endometrium carcinoma possess these mutations and express constitutive high levels of PI3K downstream signaling molecules such as phosphorylated Akt, S6K, 4EBP and GSK3b which are all involved in cellular transformation (Ikenoue et al., 2005; Isakoff et al., 2005; Zhao et al., 2005). Moreover, additional rare oncogenic mutations that are widely spread over all domains of p110 alpha with the exception of the RBD were identified. These rare mutations also lead to a gain of function mechanism (Gymnopoulous et al., 2007). Mammary epithelial cells and fibroblast that express the mutated PI3KCA gene resulted in growth factor independent proliferation, apoptosis resistance and transformation (Ikenoue et al., 2005; Isakoff et al.,
Moreover, in vivo studies showed that transgenic mice with lung specific stimulation of the mutant kinase domain p110 alpha develop lung adenocarcinoma (Engelman et al., 2008). In addition to all these activating mutations, amplification of the PI3KCA gene is also observed in various tumors including ovarian cancer, however how this amplification activates PI3K and leads to cancer is still vague (Shayesteh et al., 1999).

Mutations in PI3Ks also take place in the gene, PIK3R1, that codes for the regulatory subunit p85. These mutations are seen in different human cancers such as glioblastomas, ovarian and colorectal cancer (Mizoguchi et al., 2004). They lead to either truncation or frame shift deletions in the iSH2 domain of the regulatory subunit p85 that was proved to interact with the C2 domain of the catalytic subunit p110. Therefore, these mutations constitutively activates PI3K signaling by removing the inhibitory effect of the regulatory subunit p85 on the catalytic subunit p110 (Huang et al., 2007).

PDK1 is rarely mutated in human cancer, however; amplification and overexpression of PDK1 was observed in 20% of breast cancers (Hunter et al., 2006; Brugge et al., 2007). Mutations in PKB/Akt isoforms was proved to play crucial roles in oncogenesis leading to tumor initiation, development, and metastasis (Stambolic and Woodgett, 2006; Hemming and Restuccia, 2012). Different types of cancers such colon, breast, ovarian, head, neck, pancreas and gastric were found to overexpress Akt1 and 2 (Liu et al. 2009). An oncogenic mutation in the lipid binding PH domain of Akt1 (E17K) was reported in melanoma, colorectal, breast, skin and ovarian cancers (Carpten et al., 2007; Davies et al., 2008). This amino acid substitution increase Akt phosphorylation by growth factor independent manner that leads to its constitutive association with the plasma membrane even in the absence of PIP3 (Yuan and Cantley, 2008). A similar
mutation was also found in Akt3 in melanoma tumors and cell lines (Davies et al., 2008). Moreover, mutations in the kinase domain of Akt2 were also found in colorectal cancer and amplification of this isoform was also observed in some human tumors (Parsons et al., 2005; Cheng et al., 1996).

Since its discovery, PTEN was identified as a gene that is highly mutated on chromosome 10 and lost in various cancers (Li et al., 1997; Steck et al., 1997). Its expression was found out to be tightly controlled at the transcriptional and post-translational levels (Salmena et al., 2008). PTEN loss is caused by genetic and epigenetic factors. Genetic mutations of PTEN usually take place either in its catalytic domain (C2) or within ubiquitinylation sites. Most of these somatic mutations result in the truncation and loss of function of the PTEN protein leading to cancer development and progression (Eng, 2003; Trotman et al., 2007). However, missense mutations that abolish its phosphatase activity were also discovered (Han et al., 2000). In addition to genetic mutations, PTEN inactivation was also found to occur due to transcriptional repression and epigenetic silencing of this gene, mainly by methylation of its promoter (García et al., 2004; Goel et al., 2004). PTEN inactivation results in the loss of its lipid phosphatase activity which will lead to the accumulation of PIP3 and the uncontrolled activation of the PI3K/Akt signaling pathway causing cellular growth and survival (Haas-Kogan et al., 1998). Inactivation of this gene was found in melanoma, glioblastoma, endometrial, kidney, bladder, prostate and colon cancer and under expression of this gene was also found in leukemia, lung, breast, liver and thyroid cancer (Hollander et al., 2011). Hence, the discovery of the tumor suppressor, PTEN, was the key that linked the PI3K pathway with human cancer. Mutations that lead to PI3K pathway activation are the most common in human cancers and several inhibitors for
Akt, PKB, and mTOR are undergoing clinical trials to be used in the treatment of cancer (Liu et al., 2009).

1.3. Cellular motility

Different biological processes in animals such as immune surveillance, embryonic morphogenesis, tissue hemostasis and wound healing depends on an intricate and highly coordinated process known as cell migration (Sivamani et al., 2007). This process is highly regulated and once it becomes abnormal, it leads to the development of several diseases including atherosclerosis, chronic inflammatory diseases and cancer (Norman and Hickey, 2005). Therefore, understanding the mechanisms underlying cell migration is of great therapeutic importance. Cell migration or motility is divided into several stages. In order for cells to migrate, they adhere to their surrounding environment, orient themselves toward a specific direction and polarize along an axis of movement. The direction of movement is usually toward an external stimulus such as a chemoattractant. Once the direction is determined, forward movement takes place using leading edge protrusions made up of lamellipodia and mainly filopodia that extend by actin polymerization and then gets stabilized by adhesive contacts at the leading edge of the cell. This forward movement of the leading edge is accompanied by the simultaneous retraction of the rear end were actin filaments disassemble and adhesive contacts detach (Figure 4). These mechanisms involve polarized cytoskeletal dynamics as well as broad spatial and temporal coordination (Cain and Ridley, 2009; Ananthakrishnan & Ehrlicher, 2007).
Efficient cell motility requires a variety of protein interactions such as adhesion molecules, actin binding proteins and adaptor proteins. All these protein interactions have been extensively studied and signal transduction pathways that modulate cell motility have been conducted. Actin remodeling was found out to be mediated mainly by the Rho family of small GTPases and PI3K was found out to be involved in determining cell polarity, defining the leading edge and cell migration (reviewed in Cain and Ridley, 2009). Therefore both the Rho family of GTPases and PI3K signaling pathways are required for efficient cellular motility through positive feedback loops that exists between the two (Fenteany & Glogauer, 2004).
1.3.1. The Rho family of small GTPases and their role in cellular motility and cancer

Actin cytoskeleton is best known to be regulated by the Rho family of small GTPases which also play a role in a variety of cellular functions such as cell cycle regulation, vesicular trafficking and transcriptional reprogramming. Twenty different Rho GTPases ranging between 20 and 40 KDa were identified in mammals. All of these GTPases binds GTP and most of them exhibit a GTPase activity that allows them to act as molecular switches and cycle between an active GTP-binding and inactive GDP-binding states (Boettner & Van Aelst, 2002). Their downstream activity is determined by their nucleotide binding status which is regulated by three groups of proteins: Guanine nucleotide exchange factors (GEFs) that stimulating GDP release and increase the active GTP-bound status of Rho GTPases, GTPase-activating proteins (GAPs) that stimulate the intrinsic GTPase activity and increase the inactive GDP-bound status of Rho GTPases and finally guanine nucleotide dissociation inhibitors (GDIs) that prevent the membrane targeting, interaction and activity of Rho GTPases with downstream effectors by adhering to Rho GTPases C-terminal prenyl group (Schmidt and Hall, 2002; Bernards, 2003; DerMardirossian and Bokoch, 2005) (Figure 5). Rho, Rac and Cdc42 are the most studied members of Rho GTPases and they were seen to regulate cytoskeletal dynamics of cell migration at different stages such as polarity, adhesion and protrusion (Takai, Sasaki, & Matozaki, 2001; Vega & Ridley, 2008).
Cellular motility is a crucial step that leads to tumor cells invasion and metastasis (Lauffenburger & Horwitz, 1996). As previously discussed, the first step in cellular motility is determining the direction of movement which has been shown to be accomplished by cdc42. Cdc42 was shown to be pivotal for sensing the direction of movement during chemotaxis, regulating actin cytoskeleton and maintaining cell polarity by regulating the establishment of finger-like membrane extension known as filopods at the leading edge of the cell (Allen, Zicha, Ridley, & Jones, 1998; Nobes & Hall, 1999; El-Sibai et al., 2007; Tang et al., 2008). Moreover it was also shown to be important in lamellipodia formation (El-Sibai et al., 2007). Cdc42 also induce Rac dependent lamellipodium extension (Hall, 1998). On the other hand, Rac was shown to activate the Arp2/3 complex which prompts the formation of lamellipodia and membrane ruffles at the leading edge of the cell. It also inhibits cofilin which stabilizes actin filaments and it activates PIP 5-kinase which leads to the production of PIP2 and inhibit actin capping proteins. After the extension of lamellipods, Rac induces the association of small punctate assemblies formed behind the front of the lamellipodia known as focal complexes that stabilizes the lamellipodia of migrating cells by binding.
to the extra cellular matrix (Condeelis, et al., 2001; Nobes & Hall, 1995; Rottner, Hall, & Small, 1999). These focal complexes don’t ensure enough contractility needed for motility unless they mature into focal adhesions by the act of RhoA signaling pathway (Kaverina, Krylyshkina, & Small, 2002). Focal adhesions are larger is size and less persistent and they provide the cells with the mechanical strength needed in order for the cell body to contract and slide over the ECM leading to cellular motility (Gupton & Waterman-Storer, 2006). The contraction of the cell body depends on actomyosin contraction that is regulated by Rho as well (Mitchison & Cramer, 1996; Ridley & Hall, 1992). The turnover of focal adhesions is very important since their accumulation due to increased Rho activation constrains cellular motility by strengthening the binding of cells to the ECM (Cox, Sastry, & Huttenlocher, 2001). Rho and Rac were proven to have antagonistic functions while establishing cellular polarity; Rac acts on the leading edge, while RhoA acts on cell sides and the rear end of the cell (Ridley et al., 2003).

Various human cancers show an altered expression in the family of Rho GTPases. Since Rho GTPases are involved in actin polymerization and cell motility, and since they are abnormally regulated in cancer cells, it is most likely that they play a role in tumor cells invasion. Studies showed that the regulation of cellular motility by Rho GTPases leads to cancer metastasis in some model systems. Melanoma cell metastasis in a mouse model was shown to be stimulated by increased Rho expression (Clark, Golub, Lander, & Hynes, 2000). Moreover, cdc42 activity was shown to be crucial for protrusion formation and motility of MTLn3 carcinoma cells (El- Sibai, et al., 2007). Therefore studying the specific roles of Rho GTPases in cellular motility might be of great importance in cancer therapy by inhibiting tumor metastasis and invasion.
1.3.2. The role of PI3K in cellular motility

The PI3K pathway was shown to be involved in cellular motility and migration in various cell types. It regulates cell migration either directly, through the binding of proteins to their lipid products, or indirectly, through cross talking with other pathways involved in motility, mainly Rho GTPase signaling (Cain and Ridley, 2009). PI3Ks and their lipid products were reported as intermediates that associate chemo-attractant signals with downstream components involved in chemotaxis responses and cellular polarity (Ward, 2004; Vanhaesebroeck et al., 2005; Oak et al., 2007). Moreover, studies have shown that the PI3K pathway plays a crucial role in the regulation and activation of Rho GTPases by recruiting and activating Rho GEF that in turn activates the different Rho GTPases. Rho GEF includes a PH domain linked to a DH (Dbi homology) domain that is required for GEF activation (Schmidt & Hall, 2002). This PH domain directly affects the DH domain catalytic activity (Zheng, Zangrilli, Cerione, & Eva, 1996). When the PH domain is bound to PIP2 it strongly associates with the DH domain and inhibits the GEF’s activity. However, once PI3K phosphorylates PIP2 to PIP3, PH binding to PIP3 weakens the interaction between the PH and DH domain leading to GEF activation and thus the activation of Rho GTPases and the increase in cellular motility (Bustelo, 2000; Das, et al., 2000).
1.4. DJ-1/PARK7

DJ-1 (CAP1/RS/PARK7) was first recognized in 1997 as a 20 KDa protein which has a sequence that is conserved among both prokaryotic and eukaryotic organisms (Nagakubo et al., 1997; Fang et al, 2010; Hod et al, 1999; Wilson et al, 2003; Miyajima et al, 2010; Sitaram et al, 2009). It was first identified by Nagakubo as a protein that cooperate with H-ras and transforms mouse NIH3T3 cells (Nagakubo et al., 1997) and was later identified as an oncogene product responsible for a subset of familial Parkinson’s disease; hence its name PARK7. Deletions or some mutations in DJ-1 have been described to be associated with recessively inherited autosomal Parkinson's disease by a loss of function mechanism (Moore et al., 2005; Bandyopadhyay et al., 2004). DJ-1 is ubiquitously expressed in cells and tissues and it was shown to localize in the nucleus, cytoplasm and mitochondria (Zhang et al., 2005; Canet-Aviles et al., 2004). However, its exact function is still unclear and several diverse cellular roles have been related to it (Cookson, 2003).

Many studies have shown that DJ-1 participates in different cellular biological processes such as the regulation of androgen receptor signaling (Niki et al., 2003; Takahashi et al., 2001), infertility (Welch et al, 1998; Okada et al, 2002), RNA-protein interactions (Hod et al., 1999) oxidative stress (Canet-Aviles et al., 2004; Mitsumoto and Nakagawa, 2001; Mitsumoto et al., 2001; Takahashi-Niki et al., 2004), transcriptional regulation (Ishikawa et al., 2010) and apoptosis (Hod, 2004). It was also shown to be highly expressed in multiple tumour tissues and thus involved in tumorigenesis, by affecting several cellular processes such as proliferation, differentiation, cell cycle arrest and autophagy (Gonzalez et al., 2009; lev et al., 2006) (Figure 5).
Figure 6: DJ-1 Function and Relation to Disease. DJ-1 is a multifunctional protein and excess activation or loss of its function triggers the onset of numerous diseases, including Parkinson’s disease and cancer. Source: (Ariga et al., 2013)

The DJ-1 human gene is made up of seven exons mapped to chromosome 1p36.2-36.3, where numerous chromosomal abnormalities related to cancer have been described (Taira et al., 2001; van Duijn et al., 2001). It was proved to be a biomarker for breast cancer since it is strongly expressed in about 50% of breast cancer patients (Le Naour et al., 2001). Moreover, high DJ-1 levels have also been reported in different tumours such as that of the lungs, ovaries, esophagus, thyroid, pancreas, and brain, and also in leukaemia, indicating that abnormally expressed DJ-1 levels, under certain circumstances, may play a role in the initiation and/or progression of cancer (Hinkle et al., 2011; Liu et al., 2010). In 2005, studies showed that DJ-1 negatively regulated the expression of the tumour suppressor, PTEN, thus promoting the phosphorylation of the PI3K/Akt signalling pathway, and activating cell survival, proliferation and transformation. Therefore, DJ1 can be used as an indication of cancer
metastasis and can be a potential therapeutic target. However, the detailed mechanism of DJ-1 localization and its role in cellular motility are still not fully understood.

In this study, we sought to determine the exact cellular location and localization pattern of DJ-1 upon EGF stimulation. In addition we wanted to investigate the effect and mechanism of DJ-1 on cancer cell motility.
Chapter 2

2. Materials and methods:

2.1. Cells and culture conditions

The cells used in the present study were the human lung adenocarcinoma epithelial cell line (grade IV) A549, the human adult glioblastoma cell line (grade IV) SF268 and the human breast carcinoma cell line (grade III) MDA-MB-231. All cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 100U of penicillin/streptomycin at 37°C and 5% CO2 in a humidified chamber.

2.2. Antibodies and reagents

The following primary antibodies were used in this study: Goat polyclonal antibody against DJ-1/PARK7 was purchased from Abcam (Abcam Inc., Cambridge, UK), rabbit monoclonal antibody against pan-Akt (Abcam Inc., Cambridge, UK) and rabbit monoclonal antibody against Akt1 phosphorylated at S473 (Abcam Inc., Cambridge, UK). Moreover, anti-goat, anti-rabbit and anti-mouse HRP-conjugated secondary antibodies were obtained from Promega (Promega Co., Wisconsin). Fluorescent secondary antibodies (AlexaFluor 488) were obtained from Invitrogen. To visualize the actin cytoskeleton, cells were stained with Rhodamine phalloidin (Invitrogen). DAPI was also used to stain the nucleus.
2.3. Cell transfection and small interfering RNA

Goat FlexiTubesiRNA for DJ-1/PARK-7 oligo-3 and oligo-6 were obtained from Qiagen (Qiagen, USA). The siRNAs used had the following target sequences: NM_001123377 and NM_007262. The cells were transfected with the siRNA at a final concentration of 10nM using a HiPerfect (Qiagen, USA) as described by the manufacturer. Control cells were transfected with siRNA sequences targeting GL2 Luciferase (Qiagen, USA). After 72 hours, protein levels in total cell lysates were analyzed by western blotting using the appropriate antibodies or the effect of the corresponding knockdown was assayed.

2.4. Western Blots

Whole cell lysates were prepared by scraping the cells with Laemmli sample buffer containing 4% SDS, 20% glycerol, 10% β mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl (pH 6.8). SDS-PAGE was carried out under standard conditions and proteins were blotted onto a PVDF membrane (Hod, Y et al. 2004). The membranes were then blocked with 5% non-fat milk for 1 hour at room temperature and then incubated with either primary antibody against DJ-1/PARK7 (Abcam Inc., Cambridge, UK) (1:1000 dilution), or against pan-Akt (Abcam Inc., Cambridge, UK) (1:1000 dilution) or against Akt1 phosphorylated at S473 (Abcam Inc., Cambridge, UK) for 2 hours. After washing and 1 hour incubation with secondary antibody (1:2000 dilution) for the respective antibodies. Blots were developed using chemi-luminescent reagent ECL detection kit from (GE
Healthcare). The levels of protein expression were compared by densitometry using the ChemiDoc XRS software.

2.5. Immunostaining

The cells were plated on cover slips, starved for 3 hours using L15 media and then stimulated with epidermal growth factor (EGF) for 0, 5 and 10 minutes. After stimulation, cells were fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.5% Triton-X 100 for 10 minutes. To reduce background fluorescence, cells were rinsed and then incubated for 10 minutes with 0.1 M glycine. For blocking, cells were incubated 4 times with 1% BSA, 1% FBS in PBS for 5 minutes. Samples were then stained with Rhodamine phalloidin (RP) and DAPI for 30 minutes then with primary antibodies for 2 hours followed with fluorophore-conjugated secondary antibodies for 1 hour. Fluorescent images were taken using a 60X objective on a fluorescent microscope.

2.6. Fractionation of membranous organelles

Cells were grown to confluent monolayer, washed twice with cold PBS and scraped on ice with lysis buffer containing 10mM Tris-Hcl, 10mM KCl, 1.5 mM MgCl2, 10 mM DTT. The lysate is then homogenized using a 21G needle and centrifuged at 1000g for 5 min in low speed centrifuge. The pellet containing the nucleus and cell debris rich fraction was collected and the supernatant aspirated carefully and spun at 5000g for 10 min in high speed centrifuge. The pellet containing the mitochondria rich fraction was then collected and the supernatant aspirated carefully and layer on top of a
40% sucrose solution cushion (containing: 40mM Tris HCL pH 7.4, 0.1mM MgCl2, 1mM EDTA, 10mM DTT). The solution was then spun down at 100,000 g for 1 hour in an ultracentrifuge and then the ER, GA and PM membranes will appear as a cushion at the interface above the 40% sucrose. The ER, GA and PM membrane rich interface is then aspirated carefully in circular movements using a glass Pasteur pipette and adjust to 40% sucrose using a solution of 60% sucrose. After that, these membranes were put in the bottom of a tube and a solution of 35% sucrose was layered carefully above them followed by another layer of 25% sucrose, and then another layer of 18% sucrose. The tube was then spun down at 100,000 g for 4 hours. The PM rich fraction was collected from the interface between 40-35% sucrose, the GA rich fraction between 35-25%, and the ER rich fraction between 25-18%.

2.7. Motility assay

Cells were plated on a 35mm petri dish and transfected with either si-luciferase or si-Dj-1 and the assay was performed 72 hours after transfection. For motility analysis, cells were imaged in DMEM (10% FBS) media, buffered using HEPES and overlaid with mineral oil on a 37°C stage. Images were collected every 60 seconds for 2 hours using a 20X objective lens on Zeiss Observer Z1 microscope. The speed of cell movement was then quantified using the ROI tracker plugin in ImageJ software and plotted in Excel.

2.8. Wound healing assay

Cells were grown to around 90% confluency on culture plates and then a wound was made on the monolayer using a sterile pipette tip. After wounding, the cells were
washed to remove debris and new medium was added. Phase-contrast images of the wounded area were taken at 0 and 48 hours after wounding. Wound widths were measured at 12 different points for each wound, and the average rate of wound closure was calculated in μm/hr.

2.9. RhoA/Rac/Cdc42 Activation Assay

Cells were plated on a 100mm petri dish and grown to 80 to 90% confluency. Cells were either transfected with si-DJ-1 or not. Then Pull down assay was carried out for Rac using PAK PBD protein agarose beads respectively according to the protocol provided by the kit’s manual (Cell Biolabs Inc., San Diego, CA 92126). Briefly, cell lysates were incubated with PAK1 PBD beads for 1 hour at 4 °C with gentle agitation. Then, the samples were centrifuged, and the pellet washed for several times. After the last wash, the pellets were resuspended with sample buffer and boiled for 5 minutes. GTP-Rac was detected by western blotting using the anti-Rac antibody provided in the kit. Total Rac was collected prior to the incubation with the PAK1 PBD beads and used as a loading control.

2.10. Statistical analysis

All the results reported represent average values from three independent experiments. All error estimates are given as the mean ± standard error of the mean (S.E.M.). Statistical differences were determined using one-way analysis of variance (ANOVA). P values less than 0.05 were considered significant.
Chapter 3

3. Results:

3.1. DJ-1 location and localization

We first wanted to study the location of DJ-1 protein in cells at normal cellular conditions. Therefore, the different organelles inside brain carcinoma (SF268) cells where fractionated using a discontinuous sucrose step gradient centrifugation, collected and then loaded into a western blot. The western blot was blotted using anti-DJ-1 antibodies. Results showed that DJ-1 at normal cellular condition is present in the nucleus, mitochondria, plasma membrane and golgi apparatus of astrocytoma (SF268) cells (Figure 7).

![Western blot of DJ-1 localization](image)

**Figure 7: DJ-1 Location Inside Brain Carcinoma Cells.** This is an image of a Western blot, blotted against DJ-1, showing DJ-1 presence in the nucleus, mitochondria, plasma membrane and golgiapparatus of astrocytoma cells.
We then wanted to further investigate the exact localization pattern of DJ-1 inside the cell before and after stimulation with EGF. Therefore, lung carcinoma A549 cells and astrocytoma SF268 cells were starved using L15 media for three hours and immunostained using anti-DJ1 antibody as well as DAPI stain to stain the nucleus and Rhodamine phalloidin to stain actin. Results showed that at 0 minutes, DJ-1 is completely present in the nucleus of both A549 and SF268 cells as indicated by Figure 7 A and B respectively. After 5 minutes of stimulation with EGF, DJ-1 was shown to exit the nucleus in both cell lines and remain in the cytoplasm after 10 minutes of stimulation (Figure 8).
Figure 8: DJ-1 Localization. Representative micrographs of DJ-1 localization after 5 min and 10 min upon EGF stimulation in lung carcinoma A549 cells (A) and glioblastoma SF268 cells (B) immunostained with anti-DJ-1 (left panels), Rhodamine Phalloidin and DAPI (middle panels). The two channels were merged in the right panels.
3.2. Role of DJ-1 in lung, brain and breast carcinoma cell motility

To assess the effect of DJ-1 on cellular motility, we started by knocking down DJ-1 in lung (A549), brain (SF268) and breast (MDA-MB-231) cancer cell lines, where approximately 53%, 46% and 46% knockdown was achieved in all cell lines respectively. The knockdown was determined by detecting the expression levels of DJ-1 by western blot in cells transfected with PARK-7 siRNA (oligos 3) compared to that in control cells transfected with the non-specific Luciferase siRNA (Figure 9B, D and F). DJ-1 knockdown resulted in the decrease of cancer cell motility in the three cell lines tested, as determined by time lapse assay in which images of the cells were taken every 60 seconds for 120 frames. The movies were then quantitated using ImageJ software, the average cellular speed was determined and the bar graphs representing cell motility were constructed. Quantitation results showed that DJ-1 knockdown significantly decreased cellular motility by 30%, 62% and 51% in lung (A549), brain (SF268) and breast (MDA-MB-231) cancer cells respectively (Figure 9A, C and E).
Figure 9. DJ-1 Effect on Cellular Motility. Knockdown of DJ-1 decreased cellular motility in A549(9A), SF268(9C) and MDA-MB-231 (9E) cells. Cells were transfected with luciferase control siRNA or DJ-1 siRNA, n=3, data are mean ± s.e.m. Quantitation of western blot to confirm DJ-1 knockdown in A549, SF268 and MDA-MB-231 respectively (9B, D and F).
To further assess the effect of DJ-1 on cellular 2D motility, we knocked down DJ-1 in lung (A549) cancer cells and studied its effect using the wound healing assay. Pictures were taken at 0 and after 48hrs and then assessed using Image J software (Figure 10A, B). Results showed a 2.4 folds decrease in cellular migration after 48 hours in cells transfected with siDJ-1 (2.02 μm/hr) compared to control cells (4.89 μm/hr) (Figure 10, B).

Figure 9: Wound Healing Experiment Upon DJ-1 Knockdown. Control cells were transfected with Luciferase, while treated cells were transfected with DJ-1 siRNA. Wounds were done after 72hr from transfection. A. Cells from the control and KD were grown in plates forming a monolayer, then wounded and directly pictured (left panel). After 48 hours, the pictures were taken at the same frame (right panel). B. Quantitation of the wound healing experiments was done as following: width of each wound was measured at 12 dissimilar points, and their average rate of the wound closure was calculated as μm/hr. (n = 3; mean ± SEM)
Recent studies have shown that DJ-1 is as a negative regulator of the tumour suppressor PTEN with stimulatory effects on PI3K-AKT/PKB signalling pathway (Sitaram et al, 2009; Yao et al, 2011; Fang et al, 2010). We wanted to elucidate this role of DJ-1 as a regulator of the PI3-Kinase pathway in lung carcinoma (A549).

Figure 11 shows the western blot of lung carcinoma cells treated with si-PARK7 and then blotted with anti-PTEN and anti-pPTEN antibodies. Figure 11B shows a 15% increase in p-PTEN levels upon DJ-1 knockdown.

Figure 11: DJ-1 Effect on pPTEN Activation in A549 Cells. Quantitation of western blot reveals a 15% increase in activated pPTEN levels for siDj-1 transfected A549 cells compared to control.
3.4. Effect of DJ-1 knockdown on RhoGTPases

We carried out a pulldown assay for Rac on A549 cell line that was treated with siDJ-1. Figure 12 shows the western blot of A549 cells treated with si-PARK7 then blotted with anti-Rac antibodies. Results showed that activated rac levels increased by 20% when DJ-1 is knocked down as compared to the total Rac levels (Figure 12B).

A.  

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Control</th>
<th>KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP Rac</td>
<td><img src="image" alt="" /></td>
<td><img src="image" alt="" /></td>
</tr>
<tr>
<td>Total Rac</td>
<td><img src="image" alt="" /></td>
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B.  

![Graph showing % Rac activity](image)

**Figure 12: DJ-1 Effect on Activated Rac Levels.** The quantitation of western blot show 20% increase in the activity of Rac for cells transfected with siDj-1 compared to the control; n=3, data are mean ±s.e.m.
Chapter 4

4. Discussion:

Studies have shown that DJ-1 is mainly located in the cytoplasm of cells as well as the nucleus and mitochondria (Zhang et al., 2005; Canet-Aviles et al., 2004). It was seen to translocate from the cytoplasm to the nucleus once exposed to growth factors (Nagakubo et al., 1997) and to the mitochondria and then to the nucleus in response to oxidative stress (Eunsung et., al 2009). However, the localization pattern of DJ-1 in cancer cells is still not elucidated. Moreover, since DJ-1 is known to be a regulator of the PI3K pathway and since different PI3K regulators localize to the plasma membrane of cells, we expected DJ-1 to localize in a similar manner. Therefore, we aimed in these experiments, first to study the location of DJ-1 in cancer cells at normal cellular conditions and then to study its localization pattern and to test whether it translocates to the plasma membrane of cancer cells in order to inhibit the tumor suppressor, PTEN.

Our results showed that DJ-1 is present in the nucleus, mitochondria, golgi apparatus as well as the plasma membrane of astrocytoma cells under normal conditions (Figure 6). Hence, confirming our expectations that DJ-1 is present at the plasma membrane of cells in order to inhibit PTEN and activate the PI3K pathway. Immunostaining results regarding DJ-1 exact localization pattern, showed that DJ-1 is primarily located in the nucleus of both lung (A549) and brain (SF268) carcinoma cells after starvation and then it translocates to the cytoplasm once the cells are stimulated with EGF for 10min (Figure 7). However, the localization of DJ-1 to the plasma membrane was not seen in this experiment. Longer stimulation with EGF is needed in order to further validate our hypothesis. Moreover further experiments are needed to
elucidate the exact detailed mechanism of DJ-1 localization from the nucleus and its correlation to cancer.

Studies regarding the involvement of DJ-1 in cellular motility concentrated mainly on its role in normal cells such as mitochondrial or sperm cells motility and not on cancer cells. A study on astrocytes showed that DJ-1 knockdown reduced mitochondrial motility primarily in the cellular processes of these cells (Larsen et al., 2011). Another study on asthenozoospermia patients proved that DJ-1 concentration is positively correlated with sperm motility (An et al., 2011). Our results on different carcinoma cell lines are in accordance with the recent reports on normal cells and reflected a possible positive role of DJ-1 in cancer motility. In lung, brain and breast carcinoma, DJ-1 knockdown caused a significant decrease around 30% in lung cancer cell line and around 50-60% in brain and breast cancer cell lines motility as determined by the effect on cell speed and net speed migrated in the time-lapse assay (Figure 8). This indicates that DJ-1 positively regulates lung (A549), brain (SF268) and breast (MDA-MB-231) cancer cell motility. To further confirm our results, we tested the effect of DJ-1 knockdown on cellular 2D motility using wound healing assay. Results showed that DJ-1 knockdown in lung (A549) cancer cells decreases the rate of wound closure by 2.4 folds compared to control cells; thus decreases cellular motility (Figure 9). Therefore, this result further validates our conclusion and proves that DJ-1 is an oncogene that increases cellular motility in cancer cells.

After proving that DJ-1 increases cancer cell motility, we wanted to investigate the cellular pathway by which it does so. Previous studies have shown that the PI3K pathway is involved in cellular motility of various cell types and that it is mutated in various cancers (Cain and Ridley, 2009; Samuels et al., 2004). Moreover, DJ-1 was
shown to be a negative regulator of the tumor suppressor PTEN (Sitaram et al, 2009; Yao et al, 2011; Fang et al, 2010), which is a negative regulator of the PI3K pathway (Carracedo and Pandolfi, 2008; Georgescu, 2011; Iwanami et al, 2009). Therefore, we aimed at studying the association of DJ-1 with the PI3K pathway and how it leads to cellular motility. When we knocked down DJ-1 in lung (A549) carcinoma cells, the result was almost a 15% increase in pPTEN level compared to PTEN (Figure 10). This means that DJ-1 leads to the decrease of PTEN phosphorylation and by doing so, it activates the PI3K pathway. Therefore, DJ-1 inhibits the inhibitor of the PI3K pathway and thus; is a positive regulator of it that leads to increase in cellular motility.

Efficient cellular motility requires the cooperation of both the PI3K pathway and the Rho family of GTPases through positive feedback loops that exists between the two (Fenteany and Glogauer, 2004). Since DJ-1 was proven to activate the PI3K pathway and since RhoGTPases are known to be activated downstream of PI3K, we wanted to study the effect of DJ-1 on Rho GTPases to further understand its role in cancer cell motility. Therefore, we knocked down DJ-1 in lung carcinoma cells and we determined the levels of activated Rac inside them by doing a pull down assay using PAK1 PBD agarose beads that binds to activated Rac and we compared them to total Rac levels inside the cells. Results showed that when DJ-1 is knocked down activated Rac levels increase by 20% compared to total Rac inside the cells which indicates that the absence of DJ-1 increases activated Rac levels. This result might seem contradicting at first knowing that Rac acts on the leading edge of the cell and helps in the formation and extension of lamellipodia that allows the cells to move and the formation of focal complexes that allows the cell to adhere to the ECM and move (Condeelis, et al., 2001;
Nobes & Hall, 1995; Rottner, Hall, & Small, 1999). Thus increased activated Rac levels theoretically must increase cellular motility; however this is not always the case since the motility cycle depends on a cycling activation and inactivation of Rho GTPases (Lambrechts, Van Troy, & Am, 2004). Moreover, a moderate small variation in total Rac1 activity was shown to serve as a switch that controls the general intrinsic pattern of cellular migration, where over expression of active Rac completely inhibited cell migration (Pankov et al., 2005). Studies have also shown that the activation of FAK/PI3K/Rac1 actin pathway leads to a decrease in breast cancer and melanoma cellular motility by controlling actin reorganization (Kallergi et al., 2007). Therefore, Rac over expression leads to a decrease in cellular motility by activating the persistence of focal complexes and keeping the cells attached to the extracellular matrix.

This study showed that DJ-1 activates the PI3K pathway and regulates Rho GTPases increasing cellular motility. Therefore, it can be used as an indication of cancer metastasis and can be a potential therapeutic target.
Chapter 5

5. Conclusion

This study showed that DJ-1 localizes from the nucleus to the cytoplasm; however the exact localization pattern requires further studies in order to determine how this movement affects cancer cells motility. Our results also showed that DJ-1 increases cellular motility by activating the PI3K pathway and regulating RhoGTPases, more studies could be done to look at DJ-1 effect on RhoA and cdc42 as well. Moreover, DJ-1 effect on cellular adhesion, invasion and metastasis could be further investigated in depth.


