HPIP signaling in cell adhesion and migration: Potential role in tumour metastasis

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY
By

Suresh Bugide
(08LBPH10)

Supervisor
Dr. Bramanandam Manavathi, Ph.D.

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad-500046

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad 500 046
India

CERTIFICATE

This is to certify that this thesis entitled **"HPIP signaling in cell adhesion and migration: Potential role in tumour metastasis"** submitted to the University of Hyderabad by **Mr. Suresh Bugide**, for the degree of Doctor of Philosophy, is based on the studies carried out by him under my supervision. I declare to the best of my knowledge that this has not been submitted earlier for the award or diploma from any other University or Institution.

Dr. Bramanandam Manavathi, Ph.D.,

Supervisor

Head

Department of Biochemistry

Dean

School of Life Sciences

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad 500 046
India

DECLARATION

I hereby declare that the work presented in this thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of **Dr. Bramanandam Manavathi, Ph.D.** I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

Dr. Bramanandam Manavathi, Ph.D., Mr. Suresh Bugide

Supervisor

Date:

Place: Department of Biochemistry

University of Hyderabad

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad 500 046
India

DECLARATION

I, Suresh Bugide, hereby declare that this thesis entitled **"HPIP signaling in cell adhesion and migration: Potential role in tumour metastasis"** submitted by me under the guidance and supervision of **Dr. Bramanandam Manavathi, Ph.D.,** is a bonafide research work which is also free from plagiarism. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma. I hereby agree that my thesis can be deposited in shodganga/INFLIBNET.

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ACRONYMS

BRCA1	Breast cancer susceptibility gene 1
BSA	Bovine serum albumin
CAMs	Cell adhesion molecules
CC	Coiled coil domains
CD	Circular Dichroism
cDNA	Complementary DNA
CK	Cytokeratin
CLCA2	Calcium-sensitive chloride channel
CME	Clathrin-mediated endocytosis
DAPI	4’,6-diamidino-2-phenylindole
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
E2	Estrogen or 17β-estradiol
ECL	Enhanced chemiluminescence
ECM	Extra cellular matrix
EDTA	Ethylene diamine tetra acetic acid
EEA1	Early endosome antigen 1
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ErbB2	Avian erythroblastosis oncogene B2

ERK	Extracellular-signal-regulated kinases
FA	Focal adhesions
FAK	Focal adhesion kinase
G418	Geneticin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSK-3β	Glycogen synthase kinase-3β
GST	Glutathione *S*-transferase
GTP	Guanosine-5'-triphosphate
GTSE1	G2 and S phase-expressed-1
HEK293T	Human Embryonic Kidney 293T cells
HER2	Human epidermal growth factor receptor 2
HPIP	Hematopoietic PBX interacting protein
HRE	Hormone response element
IDC	Invasive ductal carcinomas
IgG	Immunoglobulin
ILC	Invasive lobular carcinoma
IMEM	Improved minimum essential medium
IPTG	Isopropyl β-D-thiogalactopyranoside
kDa	Kilo Dalton
LCIS	Lobular carcinoma *in situ*
M	Molar
MAPK	Mitogen-activated protein kinase
MAPs	Microtubule-associated proteins
MBC	Metastatic breast cancer
MEK	MAPK/ERK kinase

$MgCl_2$	Magnesium chloride
MLV	Multivesicular bodys
MMPs	Matrix metalloproteinases
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
OD	Optical density
PBS	Phosphate-buffered saline
PBX1	Pre B-cell leukemia homeobox protein
PCR	Polymerase chain reaction
PELP1	Proline, glutamic acid, and leucine-rich protein
PI3K	Phosphatidylinositol 3 kinase
PKCα	Protein kinase C alpha
PMSF	Phenyl methylsulfonyl fluoride
PR	Progesterone receptor
Rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute medium
RUFY3	RUN and FYVE domains protein 3
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SHC	SH-2-containing protein
Taq	Thermophilus aquaticus
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween-20
TNBC	Triple negative breast cancer

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CHAPTER I

General introduction

1.1 Introduction:

1.1.1 Breast cancer: Mammary glands are enlarged and modified sweat glands and made up of lobes and ducts. Mammary gland have the alveoli (hollow cavities) lined with milk-secreting cuboidal cells and surrounded by myoepithelial cells. Breast cancer is a tumor originating in the mammary gland. It is the most common cause of cancer deaths among women with 5,22,000 deaths in the year 2012 and the most frequently diagnosed cancer among women worldwide (Ferlay *et al*., 2015). It represents 12.7% of all cancer deaths and accounts for 23% of all newly diagnosed cancers in women worldwide (Ferlay *et al*., 2010). The estimated incidence of breast cancer has increased by more than 20%, while mortality has increased by 14% around the world since 2008.

1.1.2 Breast cancer facts in India

Shockingly, similar to developed countries, breast cancer is the most common cancer among women in India. In India, according to GLOBOCAN (WHO), 144,937 women were newly detected with breast cancer and 70,218 women died in the year 2012, more than any other country in the world (second: China – 47,984 and third: US – 43,909 deaths). So roughly, for every 2 women diagnosed with breast cancer, one lady is dying of it. Mortality rate is higher in India than western countries although number of new cases are less (one-third that of Western countries), which could be due to lack of awareness and delayed diagnosis of disease (Agarwal *et al*., 2008; Agarwal *et al*., 2009). Interestingly, the incidence of breast cancer in urban areas is relatively higher as compared to rural parts of the country which is mainly due to altered lifestyle, food habits, late child bearing, increased alcohol consumption and increased use of contraceptives. In India, unfortunately, the breast cancer incidence is more at an early age (30-

50) as compared to the western countries (50-70) and cancers in the young tend to be more aggressive, which demands early detection and better therapies.

1.1.3 Types of breast cancers: According to histology and invasiveness, breast cancers are classified into 3 subtypes.

(A) Ductal carcinoma in situ (DCIS): It is the most common noninvasive breast cancer in women. In DCIS, cells that lined the ducts have transformed into cancer cells. It is noninvasive, where cancer cells have not spread beyond the breast duct walls. It accounts for 18% of all newly diagnosed cancers in United States. Whereas in India it represents very low proportion of total disease as most of the breast cancer cases are detected in advanced stage (Agarwal *et al*., 2009). In some cases, DCIS may convert into invasive breast cancers.

(B) Invasive or infiltrating ductal carcinoma (IDC): It originates from the breast duct and spreads into surrounding breast tissues, and is further capable of metastasizing to other organs of the body through the blood and lymphatic system. This is the most common type of breast cancer, represents about 80% of breast cancers. About 8 of 10 invasive breast cancers are infiltrating ductal carcinomas. In United States, it is most frequently detected breast cancer with an estimated 1,80,000 cases every year (American Cancer Society).

(C) Invasive or infiltrating lobular carcinoma (ILC): It originates from the milk-producing glands (lobules) of the breast and will spread to other parts of the body. This is less common and represents about 1 in 10 breast cancers.

In addition to the above mentioned subtypes, there are less common types of breast cancers include, inflammatory breast cancer (1-3% of all breast cancers), medullary carcinoma

(3-5% of all breast cancers), metaplastic carcinoma, mucinous carcinoma, tubular carcinoma and papillary carcinoma.

As above classification of breast cancer lacks molecular features, and limits the ability to predict a response to newer targeted therapies. Recently, gene expression and microarray based studies identified several molecular subtypes of breast cancers that include, basal-like, HER2+, normal breast like, luminal subtype A, luminal subtype B and claudin-low (Perou *et al*., 2000; Sorlie *et al*., 2001; Prat *et al*., 2010). Most of the basal-like breast cancers (15-20% of all breast cancers) are triple negative. They are cytokeratin (CK5/6) and/or epidermal growth factor receptor (EGFR) positive, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) negative. These tumors tend to be very aggressive, high grade, and frequently have *p53* and *BRACA1* mutations. Basal-like tumors have poor overall survival due to lack of therapeutic options (Carey *et al*., 2006). Luminal A (40% of all breast cancers) is the most frequent subtype of breast cancer, they are ER positive, PR positive or negative, HER2, CK5/6 and EGFR negative. It shows a good prognosis and responds well to hormone therapy (Al Tamimi *et al*., 2010). Luminal B (20% of all breast cancers) type has low expression levels of ER and HER2 but they are highly proliferative (Malhotra *et al*., 2010). Her2 positive tumors are HER2 positive, ER, PR and EGFR negative. HER2+ tumors tend to be very aggressive and have poor prognosis. Claudin-low (12-14% of all breast cancers) breast cancers are low cludin-3/4/7, E-cadherin and ER negative (Malhotra *et al*., 2010).

1.1.4 Risk factors: The most important factors that will increase the risk of breast cancer are, age, personal and family history of breast cancer, mutations in breast cancer susceptibility gene 1

(*BRACA1*) and breast cancer susceptibility gene 2 (*BRACA2*) genes, late child bearing and no breast feeding (Freedman *et al*., 2005).

1.1.5 Screening and treatment: The best way to protect from breast cancer is through early detection. Breast cancer screening includes 3 methods: breast self-exams (monthly), clinical breast exams (every 3 years) starting from 20 years and mammographic screening (annually) starting at the age of 40 years which will help detection of breast cancer at early stages. Signs of breast cancer include a lump in the breast, a change in breast shape, dimpling of the skin and red scaly patch of skin. In developing countries like India, most of the breast cancer cases are diagnosed in late or advanced stage of disease (stage 3 or 4), makes treatment particularly challenging.

Treatment options for women diagnosed with breast cancer include surgery, radiation therapy, chemotherapy, hormonal therapy and targeted therapies. Cytotoxic drugs used for breast cancer chemotherapy include, anthracyclines, taxanes and 5-fluorouracil, and recently chemotherapeutic agents that have been developed are epothilones and ixabepilone (Nicolini *et al*., 2006; Alvarez *et al*., 2010). Hormone therapy either blocks ER or reduces circulating estrogen levels by inhibiting the aromatase enzyme. Tamoxifen is well known and widely used ER blocker (Jaiyesimi *et al*., 1995). The most common aromase inhibitors used for breast cancer treatment are letrozole, anastrozole and exemestane. Interestingly, letrozole and anastrozole were shown to have better therapeutic index compared to tamoxifen (Mouridsen *et al*., 2004; Thurlimann *et al*., 2004). However, chemotheraptic agent's use has been associated with many side effects like tiredness, fall of blood cell count, loss of appetite, weight gain and diarrhea. Trastuzumab is used as a targeted therapy for breast cancer, it is monoclonal antibody that

selectively binds to HER2 and blocks the proliferation of tumors that overexpress HER2 (Baselga *et al*., 1998; Nicolini *et al*., 2006). Recently developed HER2-targeting antibodies, such as pertuzumab and trastuzumab-MCC-DM1, have shown promising results in the treatment of metastatic breast cancer (Luu *et al*., 2011).

1.1.6 Breast cancer metastasis: The process of metastasis comprises of a series of steps that includes epithelial to mesenchymal transition (EMT), loss of cell adhesion, cell migration, new blood vessels formation, intravasation into blood or lymphatic vessels and extravasation. Failure to complete any of these processes will arrest the metastasis (Bissell *et al.*, 2011). Approximately 6-10% of new breast cancer cases are metastatic and it is estimated that 20-30% of all breast cancer cases will become metastatic. Metastatic breast cancer (MBC) defined as cancer occurring at sites distant from the breast and lymph nodes usually lungs, liver, bones and brain (Cardoso *et al*., 2010). The majority of deaths from breast cancer are due to metastasis of cancer to other organs in the body not because of primary tumor (Weigelt *et al*., 2005). Survival rate for MBC is approximately 18 to 30 months, although advances in the treatment for metastatic breast cancer have significantly improved in last decade (Cardoso *et al*., 2010; Pagani et *al*., 2010). Currently, detection of breast cancer metastasis relies on biopsies of affected organs, radiological evaluations, analysis of circulating tumor cells (CTCs) and serum tumor markers (Lacroix *et al*., 2006; Sun *et al*., 2011). Therefore, identification of novel genes and understanding of new molecular mechanisms of the breast cancer metastatic might improve clinical management of the disease. Hence there is urgent need to identify novel genes and proteins that play critical role in breast cancer metastasis and progression.

(A) Cell adhesion: Metastasis starts with the invasion of tumor cells into the surrounding tissue. The metastatic cancer cells must alter cell adhesion to detach from the primary site. Cell adhesion molecules have been involved in all steps of tumor metastasis, including detachment of tumor cells, intravasation into the blood stream, extravasation into distant sites, and formation of the secondary lesions (Makrilia *et al*., 2009). During metastasis, a tumor cell has to adopt enhanced plasticity in the interactions with neighboring tumor cells and other cell types within the tumor microenvironment. Cell adhesion molecules (CAMs) plays key role in such homotypic and heterotypic interactions. Based on the structure and sequence homology, CAMs are divided into different families: cadherins, selectins, integrins, immunoglobulin superfamily, and lymphocyte homing receptors (Gonzalez-Amaro *et al*., 1999; Rojas *et al*., 1999; Makrilia et al., 2009). The cadherin family has been known to play a large role in mediating cell-to-cell adhesion and plays key role in breast cancer metastasis (Li *et al*., 2011). Down-regulation of E-cadherin has been associated with progression and metastasis in breast cancer (Kowalski *et al*., 2003; Gould *et al*., 2006).

Tumor cells adhere to extracellular matrix (ECM) through integrins which are transmembrane receptors present on plasma membrane and interacts ECM components such as fibronectin, collagen, laminin and vitronectin (Mego *et al*., 2010; Li *et al*., 2011). Integrins regulate multiple biological processes, such as cell adhesion, migration, apoptosis, proliferation, invasion, and metastasis (Harburger *et al*., 2009). Several integrin subunits, such as α3, α5, α6, αv, β1, β3, αvβ3 and α6β4, are up-regulated and associated with breast cancer metastasis (Rathinam *et al*., 2010).

(B) Cell migration: For all multi-cellular organisms, cell migration is an essential and highly regulated process and plays a central role in development and maintenance of organisms. Cell

migration plays a key role in many biological processes, such as embryonic development, wound healing, tissue regeneration and immune response (Webb *et al*., 2002; Ridley *et al*., 2003). Deregulation of this process leads to serious consequences, including vascular disease, osteoporosis, tumor metastasis, chronic inflammatory diseases and mental retardation (Ridley *et al*., 2003). It is a multistep and dynamic process involves focal adhesion turnover, leading edge protrusion, tail retraction, generation of tractional forces and detachment (Ridley *et al*., 2003). This process is major extent regulated by integrins and proteins associated with focal adhesion formation like focal adhesion kinase (FAK), paxillin, talin, etc (Huttenlocher *et al*., 2011). Thus, understanding the molecular mechanisms underlying cell migration will provide effective therapeutic approaches for treating various diseases, particularly tumor metastasis.

To migrate, the molecular processes such as integrin-mediated adhesion formation and turn over, and adhesion assembly and disassembly are essential for optimum cell speed and directional migration (Huttenlocher *et al*., 2011). Integrins play crucial role in cell invasion and migration, not only for physically tethering cells to the extra cellular matrix, but also for receiving and sending signals that regulate these processes. Integrin mediated outside-in signaling is critical for cell migration. The inside-out signaling of FAK or SH-2-containing protein (SHC), these proteins signals through either Ras/Raf/MEK/ERK or through Ras-PI3K to activate small GTPases like Rac, Rho, and Cdc42 (Hood *et al*., 2002). Integrin α5β1, also known as fibonectin (FN) receptor induces angiopoietin-2 mediated breast cancer cell migration through integrin α5β1/integrin-linked kinase (ILK)/Akt and GSK-3β/Snail/E-cadherin signaling pathways (Imanishi *et al*., 2007). Apart from that integrin signaling, α5β1, α31β, and αvβ3 integrins, is also involved in the expression and activity regulation of matrix metalloproteinases (MMPs) and other extra cellular matrix (ECM) degrading enzymes to modulate breast cancer

cell motility (Rolli *et al*., 2003; Baum *et al*., 2007; Morozevich *et al*., 2009; Mitchell *et al*., 2010). Integrin αvβ3 is important mediator of breast cancer metastasis to bone and α6β4 contributes to the metastasis of breast cancer cells to lung through the calcium-sensitive chloride channel CLCA2 (Abdel-Ghany *et al*., 2001; Sloan et al., 2006).

1.1.7 Molecular players involved in cell migration: The past two decades have witnessed enormous progress in understanding cell migration and key molecules involved in this process. Many human diseases including cancer have been associated with altered adhesion and migration. Therefore, there has been strong interest in understanding the molecular mechanisms involved in these processes. However, understanding the mechanism of cell migration is challenging because it is the result of transient and quick, localized adhesion dynamics and signaling. Here, we are introducing some important molecular players involved in the cell migration.

(A) Focal adhesion kinase (FAK): In the early 90's, focal adhesion kinase (FAK), a non-receptor tyrosine kinase, was identified as major integrin-dependent tyrosine phosphorylated protein localized in focal adhesions (Guan *et al*., 1991; Kornberg *et al*., 1991). Numerous studies in the last 20 years have established FAK as a central mediator of integrin signaling as well as in cell migration and invasion. FAK is an essential regulator of cell migration as cells deficient in FAK showed poor cell migration and reintroduction of FAK into such cells restored cell migration in response to chemotactic factors (Ilic *et al*., 1995; Seig *et al*., 1999). Over expression and increased activity of FAK is found in several types of metastatic cancers and it is also one of the causes to make cancer cells escape from anoikis related cell death (Owens *et al*., 1995; Frisch *et al*., 1996; Golubovskaya *et al*., 2010). Upon localization to focal adhesions, FAK activates itself

by autophosphorylation at tyrosine 397 (Calalb *et al*., 1995; Schaller *et al*., 2010). Phosphorylation at this site is important for controlling of focal adhesion turnover (Hamadi *et al*., 2005). Recent report suggests that FAK phosphorylation at Y397 but not kinase domain is important for cell migration (Ritt *et al*., 2013). The bimolecular complex composing of FAK and Src phosphorylates variety of substrates like CAS, paxillin, PKL, CSK, etc., that are central to the regulation of processes such as cell adhesion and migration.

Recent studies show that focal adhesion dynamics play key role in cell migration. FAK is involved in maturation and turnover of focal adhesions (FA) (Ilic *et al*., 1995; Mitra *et al*., 2005). However, FAK deficiency has a greater effect upon focal adhesion disassembly than formation of focal adhesions (Ilic *et al*., 1995; Webb *et al*., 2004). FAK binds to Grb2 and recruits dynamin to FAs. This complex induces the internalization of integrins and thereby induces focal adhesion turnover (Ezratty *et al*., 2005). FAK appear to control focal adhesion turnover by modulating the activity of MAPK-calpain2 signaling pathway (Carragher *et al*., 2003). FAK is also shown to be inactivated by calpain-mediated proteolysis to desensitize the cell migration signal (Bhatt *et al*., 2002; Chan *et al*., 2010).

(B) Microtubules: The cytoskeleton, which mainly consists of actin filaments, microtubules and intermediate filaments, play an important role in cell migration. In particular, coordination between actin cytoskeleton and microtubules is essential for efficient cell adhesion and migration (Goode *et al*., 2000; Rodriguez *et al*., 2003). Microtubules are polymers of α- and β-tubulins, which bind in a head-to-tail fashion and form hollow tubes. The functions of microtubules are largely associated with microtubule-associated proteins (MAPs) and microtubule-associated motors (Etienne-Manneville *et al*., 2010). Motor proteins such as dyneins and kinesins transport proteins, vesicles, receptors, or mRNA from cell periphery to organelles or vice versa along the

microtubule track. Microtubules and their associating proteins can modify cell membranes, adhesive structures through various signaling pathways (Siegrist *et al*., 2007; Akhmanova *et al*., 2009). These properties, combined with directional extension of the microtubule network throughout the cytoplasm, allow microtubules to involve in crucial steps involved in cell migration such as focal adhesion assembly and disassembly. Microtubules play significant role in formation of cell protrusions and leading edge formation in migrating cells, and they are critical in achieving efficient directional migration over longer distances (Etienne-Manneville *et al*., 2013).

Microtubule assembly and disassembly is a dynamic process and it is crucial for cell division, cell polarity and cell migration. Through actin cytoskeleton, FAK has been shown to play critical role in focal adhesion formation, maturation and disassembly. Growing body of evidence support that highly polarized activity of cell migration is brought about by microtubules (Stehben *et al*., 2012). Treating of cells with microtubule poison drugs such as nocadazole revealed the significance of microtubules in focal adhesion disassembly and migration (Ezratty *et al*., 2005). Furthermore, microtubule motor protein, kinesin-1, has been implicated in regulating microtubule-induced focal adhesion disassembly (Krylyshkina *et al*., 2002). Whereas, G2 and S phase-expressed-1 protein (GTSE1), a microtubule TIP-end binding protein, regulates cell migration by modulating focal adhesion disassembly in an EB1, another microtubule TIP-end binding protein, dependent manner (Scolz *et al*., 2012). Similarly, ACF7, actin and microtubule binding protein, has been shown to play role in FA disassembly (Wu *et al*., 2008). Microtubules also influence Rho GTPases activity to promote cell migration through affecting the focal adhesion disassembly (Kaverina *et al*., 2011). Microtubule-mediated Rac activation may involve guanine exchange factors (GEFs) Sif and STEF (Tiam2), since STEF depleted cells have slower

focal disassembly rate and have enlarged focal adhesions (Rooney *et al*., 2010). However, the precise mechanism of microtubule-mediated focal adhesion disassembly is not well understood.

(C) m-calpain: m-calpain, also known as calpain2, is a member of the calpain family and it is calcium-dependent intracellular protease (Glading *et al*., 2002). First line of evidence of calpain role in cell migration derived from studies involving utilization of calpain inhibitors (Huttenlocher *et al*., 1997; Palecek *et al*., 1998). Recent studies have demonstrated role for calpains in several aspects of cell migration, includes cell spreading, chemotaxis, membrane protrusion, and focal adhesions formation and turnover (Franco *et al*., 2005). Furthermore, calpain2 is up regulated in some cancers and has recently been associated with breast cancer progression (Rios-Doria *et al*., 2004; Carragher *et al*., 2004; Wang *et al*., 2005). Calpain2 function is tightly regulated by its proteolytic activity, which must be controlled during cell migration.

Calpain-mediated proteolysis has emerged as a key signal-transducing mechanism that may regulate integrin- and growth factor-mediated signaling to control cell migration and focal adhesion disassembly. Calpain2 has been shown to regulate the turnover of focal adhesions by cleaving several focal adhesion proteins such as talin, paxillin, Src, FAK, tensin and α-actinin and resistant mutants of these substrates failed to regulate adhesion dynamics (Franco *et al*., 2004; Wozniak *et al*., 2004; Chan *et al*., 2010). Talin is a well-known substrate of calpain2, it cleaves the talin between the head and tail and thereby triggers the focal adhesion disassembly (Franco *et al*., 2004; Franco *et al*., 2005). FAK can interact with both ERK/MAPK and calpain2, and it might act as a platform where ERK/MAPK can activate calpain2, and subsequently it's recruitment to focal adhesions (Carragher *et al*., 2003; Glading *et al*., 2004). Furthermore, knockdown of calpain2 or inhibition of calpain2 led to diminished turnover of focal adhesions

and results in large and stable focal adhesion complexes (Bhatt *et al*., 2002; Franco *et al*., 2004). Interestingly, inhibition of calpains by calpastatin, a calpain-specific inhibitor, blocks microtubule-mediated adhesion turnover after nocodazole washout. This suggests the role of calpains in microtubule-mediated adhesion disassembly (Bhatt *et al*., 2002).

1.1.8 Focal adhesion dynamics: Extra cellular matrix (ECM) play key role in cell adhesion, migration, proliferation, survival, differentiation and cellular morphology. Attachment of cells to ECM induces clustering of integrins on the plasma membrane. Cytoplasmic portions of the clustered integrins acts as a platform for the recruitment of numerous proteins such as adaptor/scaffold and signaling proteins, and they form structures called focal adhesions (FAs) (Liu *et al*., 2000; Petit *et al*., 2000). Significant progress has been made in the last decade about understanding the molecular mechanisms that regulate the focal adhesion dynamics and signaling that involves in cell migration. Integrin-containing focal adhesion complexes are dynamic structures that undergo repeated cycles of formation and disassembly, this adhesion dynamics is crucial for cell migration (Webb *et al*., 2002). Directional cell migration requires focal adhesion dynamics with formation of nascent adhesions at the leading edge and the disassembly of focal adhesions at the rear edge (Webb *et al*., 2002; Broussard *et al*., 2008; Parsons *et al*., 2010). Near the leading edge, formation of nascent adhesions and stable, large mature focal adhesions are crucial for traction forces and formation of protrusions at the leading edge. In contrast, disassembly of focal adhesions is associated with rear-end retraction and detachment of cell body at rear edge. Therefore the turnover rate of focal adhesions predicts the speed of cell migration (Stehbens *et al*., 2012; Kim *et al*., 2013). Moreover, for effective cell migration, the spatial organization of focal adhesion proteins and rapid turnover is fundamental

and critical. Focal adhesion dynamics involve focal adhesion assembly and disassembly. Here we are discussing the mechanism of focal adhesion assembly and disassembly.

(A) Focal adhesion signaling: Focal adhesions are not only crucial for attachment and detachment of the cell from extracellular matrix, it also acts as dynamic signaling centers that will regulate multiple processes involved in cell migration and cancer progression. Because most of the focal adhesion proteins interacts with multiple potential interacting partners, this allows the cell to construct various signaling pathways leading to diverse cellular behaviors.

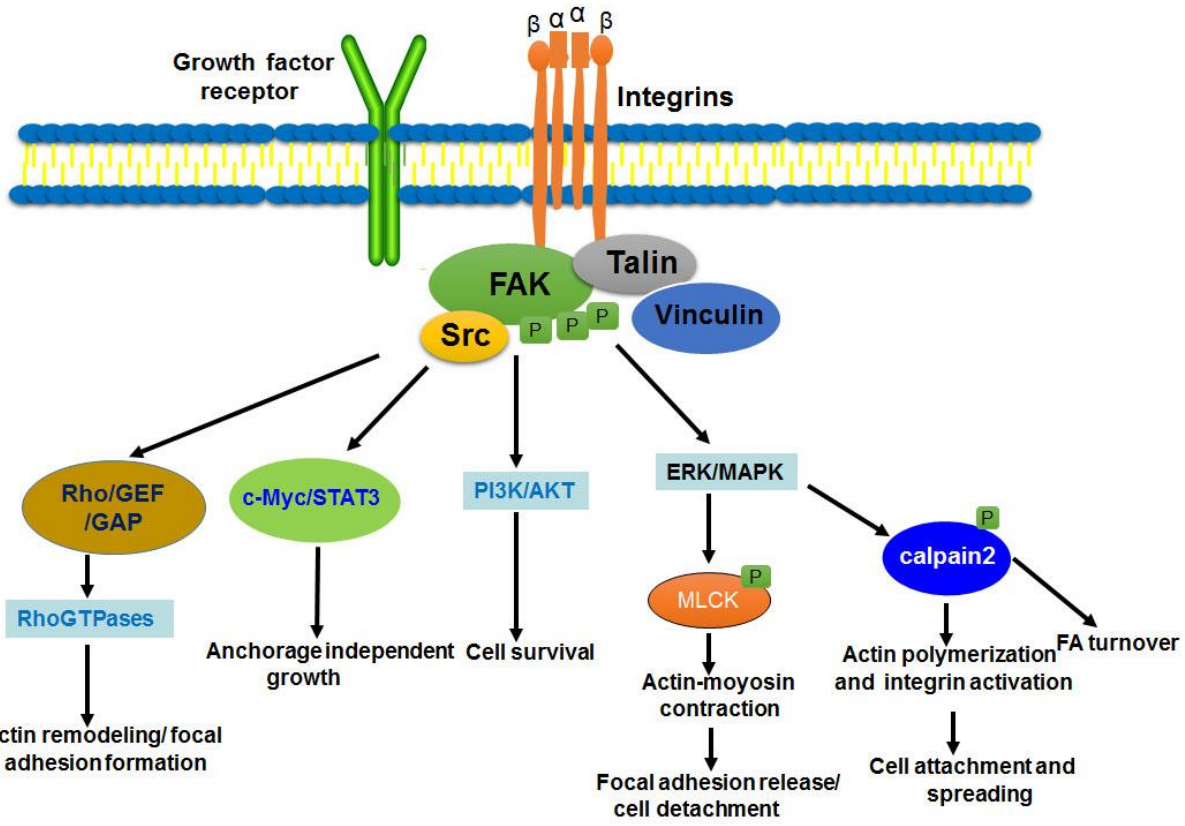


Figure 1: Role of focal adhesion signaling in various cellular activities: Integrin-mediated focal adhesion signaling regulate AKT/MAPK, calpian2 and small GTPases of the Rho families. Signals through FAK/Src not only regulate cell survival, adhesion and migration, but also control actin polymerization, calpian2 activation and cancer progression. FA, focal adhesions.

Major tyrosine kinases found in focal adhesions are Focal Adhesion Kinase (FAK) and Src, which activates several proteins to regulate focal adhesion dynamics and cell migration. Src/FAK signaling induces various downstream regulators, including ERK/MAPK, PI3K/AKT/mTOR, STAT3, and c-Myc, which has been shown to promote anchorage-independent growth, cell survival and promoting cell cycle progression (Golubovskaya *et al*., 2003; Westhoff *et al*., 2004) (Figure 1). Recent reports suggest that ERK/MAPK is recruited to focal adhesions and it is required for maximal activity of calpain2 enhance focal adhesion turnover and migration (Fincham *et al*., 2000; Carragher *et al*., 2003). FAK also regulates Rho/Rac GTPases both positively and negatively to control the actin polymerization/de-polymerization and actin stress-fiber assembly/disassembly (McLean *et al*., 20005**).**

(B) Focal adhesion assembly: The clustered integrins at ECM recruits numerous cellular proteins such as adaptor/scaffold and signaling proteins such as talin, paxillin, tensin, p130Cas, and α-actinin, zyxin, vinculin, integrin-linked kinase, Src, etc., to form focal adhesions (Lo *et al*., 2006). Focal adhesions, the best characterized of the cell-matrix adhesions, are comprised of clusters of integrin receptors and large complexes of signaling and structural molecules linked to the actin cytoskeleton. Focal adhesions are highly complex structures with over 80-100 different associated molecules, and the list of focal adhesion proteins continues to grow in the last few years (Geiger *et al*., 2009; Geiger *et al*., 2011). Depending on the cell type and its environment, they appear in different sizes, shape, number and molecular density (Kim *et al*., 2013). The initial structures of FAs called as the nascent FAs, these are immature and often short lived (Parsons *et al*., 2010). However, some of the nascent focal adhesions grow and form mature FAs that require actin stress fibers, and formation was regulated by the Rho and ROCK (Parsons *et al*., 2010). Focal complexes are another type of focal adhesions, and they are larger than nascent

adhesions, myosin II-dependent and require Rac activation (Ridley *et al*., 1992; Rottner *et al*., 1999). Focal complexes are very prominent in highly migrating cells like leukocytes that form less organized focal adhesions (Huttenlocher *et al*., 1995; Lauffenburger *et al*., 1996). Generally, the size of focal adhesions is inversely related to the speed of cell migration.

Several protein-protein interactions have been identified at focal adhesions. Because most of the focal adhesion proteins have numerous interacting partners, this allows the cell to construct various signaling complexes to affect the cell behavior. Tyrosine phosphorylation is one of the important signaling events occurring at focal adhesions (Kirchner *et al*., 2003). Tyrosine phosphorylation at the focal adhesion creates binding of SH2-containing proteins and regulates the subsequent activation of several proteins. Two of the major tyrosine kinases found in focal adhesions are Focal Adhesion Kinase (FAK) and Src, which bind to different partners to regulate focal adhesion dynamics and cell migration. Localization of FAK into focal adhesions enhances its autophosphorylation, i.e, phosphorylation at Y397 was enhanced by positive regulators of focal adhesion assembly proteins (Kwong *et al*., 2003; Schaller *et al*., 2010). In contrast, FAK phosphorylation at Y397 is also important for focal adhesion disassembly (Hamadi *et al*., 2005; Ritt *et al*., 2013). FAK also activates small GTPases like Rho by phosphorylating their exchange factors (Zhai *et al*., 2003). This is significant because small GTPases modulate focal adhesion formation. In addition to FAK, Src, another tyrosine kinase, targeting to FAs is crucial for focal adhesion turnover (Li *et al*., 2002). Upon activation, Src phosphorylates several focal adhesion proteins including FAK, p130Cas and paxillin (Frame *et al*., 2002). Besides these tyrosine kinases, tyrosine phosphatases like PTP-PEST also crucial for focal adhesion formation and turnover (Wozniak *et al*., 2004).

(C) Focal adhesion disassembly: Focal adhesions mediate interactions between ECM and migrating cells. Formation of FAs enhances the adhesion of cells to the ECM, whereas disassembly of FAs reduces adhesion to the ECM, therefore both are key for effective cell migration. Focal adhesion disassembly permit the cell to move forward and form focal adhesions at a new location (Petit *et al*., 2000; Parsons *et al*., 2010; Wehrie-Haller *et al*., 2012). Furthermore, focal adhesion disassembly is crucial for tail end retraction and detachment of cell at rear edge (Webb *et al*., 2002; Broussard *et al*., 2008; Parsons *et al*., 2010). Here, we are discussing some of the important proteins that are crucial for focal adhesion disassembly.

FAK and Src are both important regulators of focal adhesion turnover. FAK (-/-) fibroblasts have more stable, larger focal adhesions and slower migration rate (Illic *et al*., 1995), suggesting the role of FAK in focal adhesion disassembly. FAK also contributes to focal adhesion disassembly via activating of calpain2 trough ERK/MAPK pathway, and it also recruits calpain2 to focal adhesions (Carragher *et al*., 2003; Glading et al., 2004). Furthermore, FAK involve in dynamin-mediated integrin internalization and transport (Ezratty *et al*., 2005). Whereas Src directly phosphorylates several focal adhesion components that lead to reduction in focal adhesions which results in decreased cell adhesion (Wozniak *et al*., 2004). Recent studies established the role of microtubules in focal adhesion disassembly (Ezratty *et al*., 2005). Numerous microtubule interacting protein includes kinesin-1, dynamin, ZF21, GTSE1 and ACF7 has been implicated in regulating MT-induced focal adhesion disassembly (Krylyshkina *et al*., 2002; Ezratty *et al*., 2005; Wu *et al*., 2008; Nagano *et al*., 2010; Scolz *et al*., 2012). Recent studies also reveal the role of matrix degrading enzymes in focal adhesion disassembly, because more efficient way to trigger focal adhesion disassembly is to cut integrin attachment to the extra cellular matrix. Indeed, new findings indicate that the membrane type 1 matrix metalloprotease

(MT1-MMP or MMP-14) is targeted to focal adhesions and mediates ECM degradation and focal adhesion disassembly (Takino *et al*., 2006; Wang *et al*., 2012). MT1-MMP also required for fibronectin and integrin endocytosis (Shi *et al*., 2011).

1.1.9 Hematopoietic PBX interacting protein (HPIP): Hematopoietic PBX interaction protein, also known as pre B-cell leukemia homeobox interacting protein 1 (PBXIP1), was initially identified as a PBX1 interacting protein and act as a repressor for the PBX1 transcription factor (Abramovich *et al*., 2000). HPIP was located on chromosome 1 at position q21.3 and *HPIP* cDNA encodes a 731 amino acid containing protein that doesn't have homology with any known protein (Figure 2). Although the predicted molecular weight of HPIP is 80 kDa but it migrates at ~100 kDa in SDS-PAGE which could be due to posttranslational modifications of the protein. HPIP was shown to interact with different PBX family members such as PBX2 and PBX3 and block the binding of PBX-HOX complexes to DNA and thereby inhibits the transcriptional activity of E2A-PBX (Abramovich *et al*., 2000). Although HPIP is predominantly localized in cytosol but small amounts also found in the nucleus. The transcriptional repressive activity of HPIP towards PBX1 is primarily attributed to the presence of a nuclear export signal and two functional nuclear localization signals (Figure 2) (Abramovich *et al*., 2000). Interestingly, both HPIP and PBX1 have highest expression in early progenitor cells, e.g. CD34+ than in mature cells (Abramovich *et al*., 2000), suggests a role for HPIP in primitive stages of hematopoiesis. In support of this, recent studies revealed that HPIP is shown to regulate erythroid differentiation and display stem cell activity by modulating PI3K/AKT/GSK3β signaling pathway (Manavathi et al., 2012). Furthermore, the erythroid lineage-specific transcription factor, GATA1, binds to

the HPIP promoter and activates HPIP gene transcription in a CTCF-dependent manner in K562, a leukemia cell line (Manavathi *et al*., 2012).

HPIP has also been shown to involve in germ cell proliferation by inhibiting functional interaction between ERβ and Tex11 (Yu *et al*., 2012). It also interacts with microtubules and negatively regulates transcriptional activity of ERα in breast cancer cells (Manavathi *et al*., 2006). Recent studies also revealed that HPIP is phosphorylated by estrogen-activated kinase TBK1, and it leads to MDM2-dependent HPIP degradation in breast cancer cells (Shostak *et al*., 2014). In addition, HPIP is identified as a novel transcriptional target of p53 protein (Shostak *et al*., 2014).

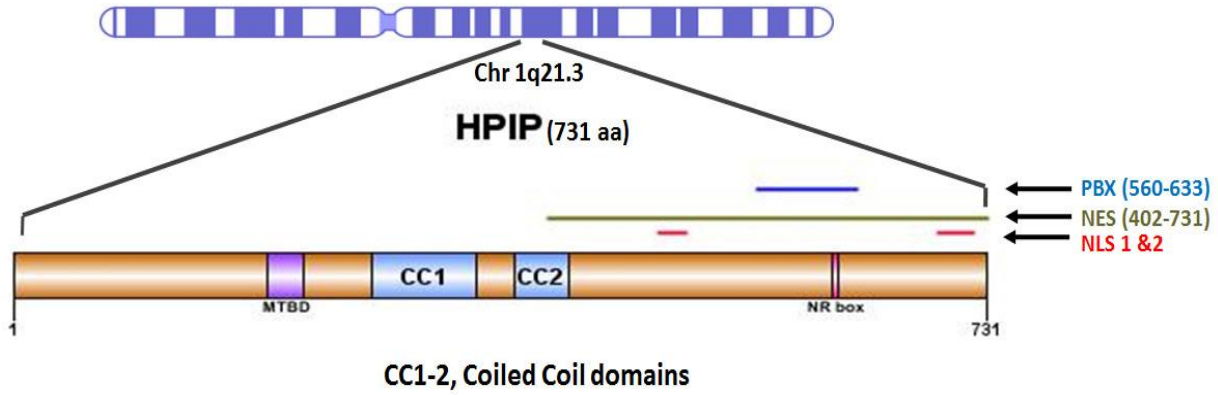


Figure 2: Chromosomal localization and physical map of HPIP. The distinct domains of HPIP (MTBD, CC1, CC2, NR box and PBX binding domain) are shown. MTBD, microtubule binding domain; CC1&2, Coiled coil domains 1&2; NR box, nuclear receptor interacting box. The boundaries of the domains were illustrated. Two putative nuclear localization signals (NLS) and one nuclear export signal (NES) are indicated.

In breast cancer cells, HPIP is reported to form a signaling complex involving microtubules, estrogen receptor alpha (ERα), PI3K and Src kinase that lead to the activation of AKT and ERK1/2 pathways in response to estrogen (Manavathi *et al*., 2006). The activated AKT

and ERK1/2 further impact on ERα phosphorylation and estrogen-responsive gene expression (Wang *et al*., 2008). Further, over expression of HPIP in breast cancer cells has been shown to promote adenocarcinomas in mice indicating the oncogenic nature of HPIP (Manavathi *et al*., 2006). Accumulating evidence support that HPIP controls various cellular functions such as cell proliferation, differentiation, migration, invasion and epithelial to mesenchymal transition (EMT) through the activation of divergent signalling pathways (Figure 3) (Manavathi *et al*., 2006; Manavathi *et al*., 2012; Xu *et al*., 2013; Pang *et al*., 2013).

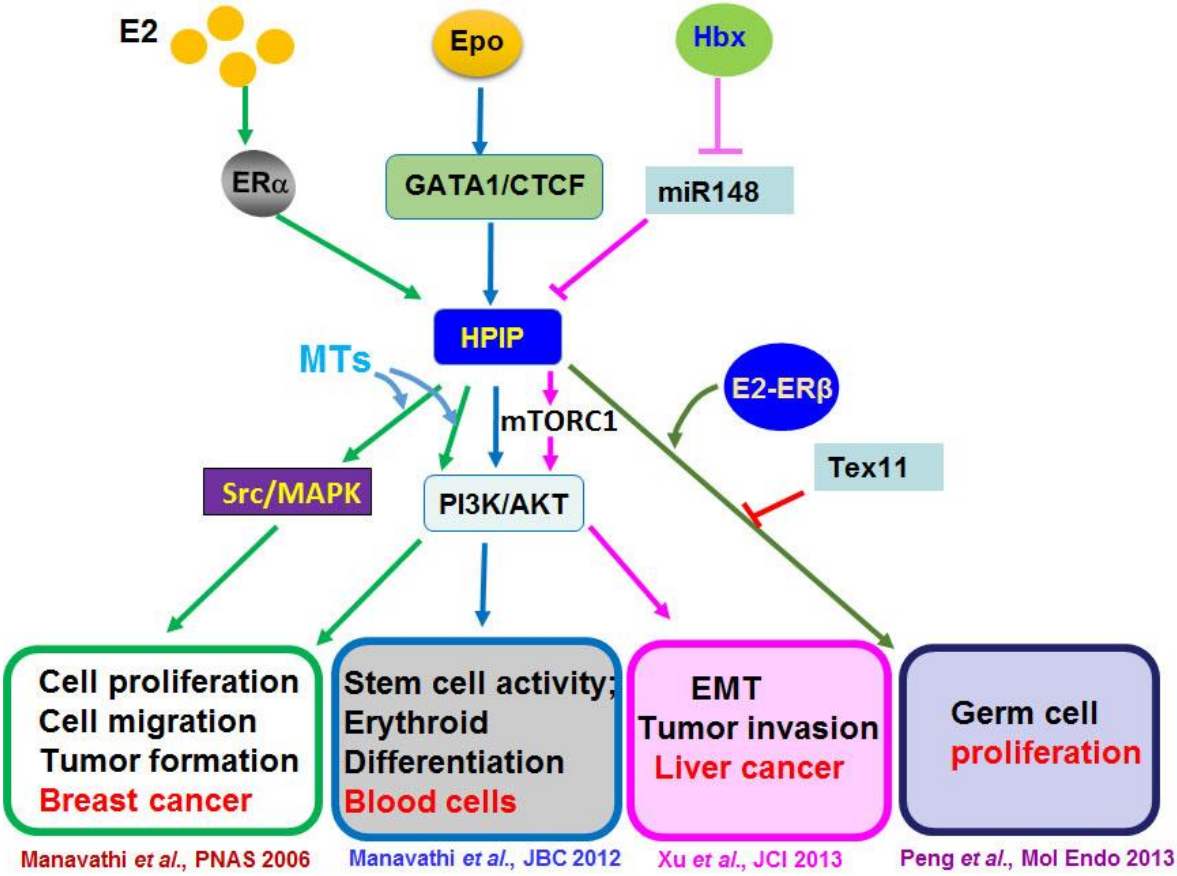
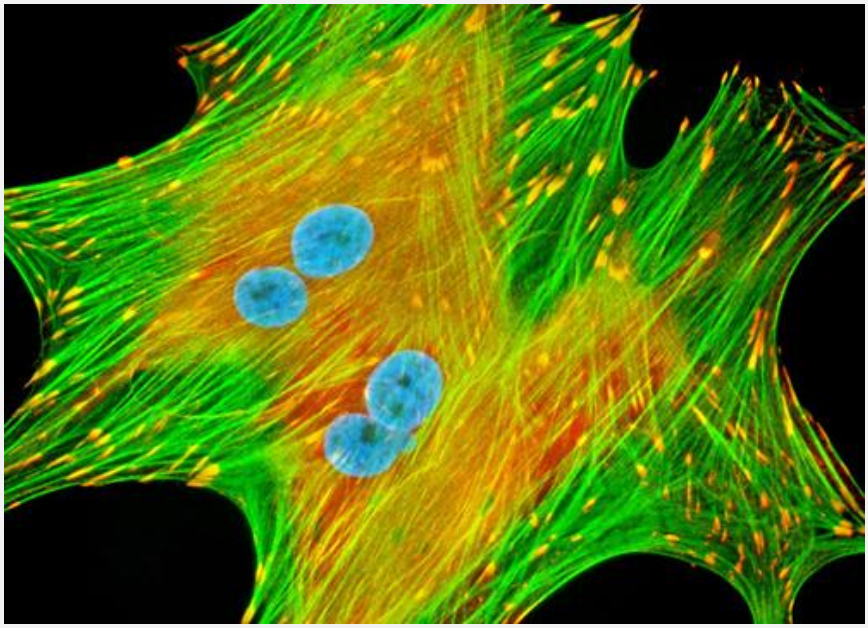


Figure 3: Role of HPIP-mediated signaling in various cellular functions**.** HPIP interacts with various signaling proteins such as microtubules, ER, ERbeta, Tex11, etc and activates different signaling pathways that control diverse biological functions. In addition, HPIP also acts through Epo signaling involving GATA1 and CTCF regulates erythroid differentiation.

Emerging studies showed HPIP over expression in several cancers that include, breast cancer (IDC), hepatocellular carcinoma (HCC), glioma, leomyosarcomas, oral carcinoma, colon cancer and gastric cancer (Manavathi *et al*., 2006; Xu *et al*., 2013; Van Vuurden *et al*., 2014, Okada *et al*,. 2015; Feng *et al*., 2015; Feng *et al*., 2015). These studies imply considering HPIP as a potential future therapeutic target for cancer treatment. However, the precise mechanism of HPIP-mediated cellular functions and its role in tumor development remain elusive. We believe that detailed understanding of HPIP function will provide us the information about its role in tumor growth, invasion and metastasis.

CHAPTER II

HPIP role in focal adhesion-mediated cell migration



2.1. Rationale, Hypothesis and objectives: Despite the convincing results of the role of HPIP in cell migration and invasion, much less was known about the mechanism that underlie in this process. Therefore, in this study we analyzed HPIP expression in human breast cancers, if any, and studied the mechanism by which it regulates cancer cell adhesion and migration.

To accomplish the above hypothesis, we framed the following objectives.

Objectives:

1. **To examine whether HPIP expression is associated with invasive breast cancers**
2. **To investigate the mechanism that underlie in HPIP-mediated cell adhesion and migration**
   - **(a) HPIP role in focal adhesion-mediated cell migration**
   - **(b) HPIP signaling in endosome-mediated cell migration**

2.2 INTRODUCTION:

Progression of epithelial cancer involves complex coordinated processes of highly regulated molecular events such as loss of cell adhesion, cell-cell and cell-extracellular matrix interactions resulting in cell migration and invasion (Desgrosellier *et al*., 2010). These processes are to a major extent regulated by integrins and proteins associated with focal adhesion formation like focal adhesion kinase (FAK), paxillin, talin and others (Hynes *et al*., 2002). Cells move by applying traction forces against the substratum at focal contacts, besides these focal adhesions also act as localized dynamic signaling centers that regulate cell migration. Such dynamic signaling centers involve focal adhesion turnover, leading edge protrusion, tail retraction and detachment (Lauffenburger *et al*., 1996; Ridley *et al*., 2003). Importantly, coordination between actin cytoskeleton and microtubules is essential for efficient cell adhesion and migration (Goode *et al*., 2000; Rodriguez *et al*., 2003).

FAK is an essential regulator of cell migration as cells deficient in FAK showed poor cell migration and reintroduction of FAK into such cells restored cell migration (Ilic *et al*., 1995; Sieg *et al*., 1999). Over expression and increased activity of FAK is found in several types of metastatic cancers (Weiner *et al*., 1993; Owens *et al*., 1995; Frisch *et al*., 1996; Golubovskaya *et al*., 2010). Upon localization to focal adhesions, FAK activates itself by autophosphorylation at tyrosine 397 (Calalb *et al*., 1995; Schaller *et al*., 2010). Phosphorylation at this site is important for controlling of focal adhesion turnover (Hamadi *et al*., 2005). Recent report suggests that FAK phosphorylation at Y397 but not kinase domain is important for cell migration (Ritt *et al*., 2003). FAK appear to control focal adhesion turnover by modulating the activity of MAPK-calpain2 signaling pathway and is also a substrate of calpain2 (Carragher *et al*., 2003).

Though actin cytoskeleton and FAK plays a major role in focal adhesion formation and maturation, growing body of evidence suggest that highly polarized activity of cell migration is brought about by microtubules (Stehbens *et al*., 2012). Cells treated with microtubule poison drugs such as nocadazole interferes with cell migration by affecting the focal adhesion disassembly and cell migration in a FAK-dependent manner (Ezratty *et al*., 2005). Furthermore, microtubule motor protein, kinesin-1, has been implicated in regulating microtubule-induced focal adhesion disassembly (Krylyshkina *et al*., 2002). However, to date only few reports are available to suggest the role of microtubule-binding proteins in focal adhesion turnover. Here we suggest a role for hematopoietic PBX interacting protein (HPIP), also known as pre B-cell leukemia homeobox interacting protein (PBXIP1), a microtubule binding protein, in focal adhesion turnover and cell migration.

HPIP was initially identified as a PBX1 interacting protein that regulates PBX1-mediated transcription functions and later its role in haematopoiesis has been determined (Abromovich *et al*., 2000; Manavathi *et al*., 2012). We previously reported that HPIP interacts with microtubules and plays a role in estrogen receptor signaling in cancer cells (Manavathi *et al*., 2006). Recent studies revealed that HPIP is involved in proliferation, migration and anchorage independent growth of breast cancer cells by activating the PI3K/AKT and Src/MAP kinase pathways (Manavathi *et al*., 2006; Wang *et al*., 2008). However, the precise mechanism of HPIP-mediated cell migration remains to be determined. Here, we report that HPIP directly interacts and activates FAK by increasing FAK phosphorylation at Y397 which is important for HPIP-mediated cell migration. Further we also show that HPIP promotes focal adhesion disassembly though MAPK-mediated calpain2 activation to ensure talin proteolysis that leads to focal adhesion turnover and thus cell migration.

2.3 Materials and methods:

2.3.1 Cell culture: MDA-MB231, MCF7, and HEK293T cells are purchased from National Center for Cell Science (NCCS), Pune, India. MDA-MB231 and HEK293T cells were grown at 37°C with 5% CO_2 in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA). Whereas MCF7 cells were grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/m streptomycin (Invitrogen, New York, USA).

2.3.2 Plasmids: pcDNA-HPIP, pEGFP-HPIP and pMNDUS-HPIP were from previous studies (Abramovich *et al*., 2000; Manavathi *et al*., 2006; Manavathi *et al*., 2012). pCMV-HPIP shRNAs and scrambled (control) shRNA plasmids were purchased from OriGene, USA. HPIP shRNA1: 5'-AAGGCTGAGCACTGGAAACATAAGAAGGA-3'; HPIP shRNA2: 5'-CTGCTGGACAAGCTGGCCAAGGAGAACCA-3'. Zyxin-DsRed2 and Paxillin-EGFP constructs were kind gifts from Dr. Elaine Fuchs, Rockefeller University, New York and FAK shRNAs from Dr. Samuel Aparicio, British Columbia Cancer Research Center, Vancouver, Canada.

Various C-terminal deletion mutants of HPIP were generated by polymerase chain reaction (PCR) amplification using pcDNA-HPIP as template with a common forward primer (fp) and three different reverse primers (rp). fp: 5'-GCCG*GGATCC*ATGGCCTCCTGCCCAGA-3' (*Bam*HI), reverse primer (rp) for HPIP(1-582): 5'-GCAGCC*CTCGAG*TGCCCGGTACTT-3' (*Xho*I), for HPIP(1-488): 5'-CTCAGC*CTCGAG*GTCTCTCTGCCA-3' (*Xho*I) and for HPIP(1-

327): 5’-GAGCCCG*CTCGAG*GGCTTCGCCCT-3’ (*Xho*I). Underlined are the restriction enzymes sites used for subcloning into pcDNA3.1 vector.

Similarly, FAK domains were generated by PCR using pCMV-HA-FAK as template using the following specific primers. FERM domain, forward primer: 5’-ATGC*GTCGAC*TATGGCAGCTGCTTACCT-3’ (*Sal*I) and reverse primer: 5’-ATGC*GGTACC*TCAAATCTCAGCATAATCATC-3’ (*Kpn*I); kinase domain (KD) forward primer: 5’-ATGC*GTCGAC*TATAGATGAAGAAGATACT-3’ (*Sal*I) and reverse primer: 5’-ATGC*GGTACC*TCAGGGGTCATAGGCCCAGC-3’ (*Kpn*I); FAT domain, forward primer: 5’-ATGC*GTCGAC*TAGCAGGCGGCCCAGGTTT-3’ (*Sal*I) and reverse primer: 5’-ATGC*GGTACC*TCAGTGTGGTCTCGTCTG-3’ (*Kpn*I). The PCR amplified fragments were ligated to pEGFP-C1 vector (Clonetech, California, USA).

2.3.3 Site-directed mutagenesis: RGD mutants of HPIP were generated using pcDNA-HPIP as template through PCR amplification by replacing glycine (G) at position 422 with alanine (A) using the following primer pairs: forward primer: 5’-GCCAGCCGCGCGGACCCAGCTCAT-3’ and reverse primer: 5’-ATGAGCTGGGTCCGCGCGGCTGGC-3’. HPIP-RGE mutant was generated by replacing aspartic acid (D) at position 423 with glutamic acid (E) using following specific primer pairs: forward primer: 5’-AGCCGCGGGGAACCAGCTCATGCT-3’ and reverse primer: 5’-AGCATGAGCTGGTTCCCCGCGGCT-3’.

2.3.4 Cell transfection and generation of stable clones: Day before transfection, cells were plated in DMEM medium in a 60 mm cell culture dish. Transfection of cells was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufactures protocol. Briefly, 1-

5 µg of DNA was mixed with Lipofectamine 2000 (1:1 ratio) in 100 µl of Opti-MEM medium and incubated for 20 min at room temperature. Medium was removed from culture dish and replaced with serum-free, antibiotic-free medium. Transfection mixture was then added to cells and medium was replaced with complete DMEM (+serum and +antibiotic) medium after 6 hrs. Post 48 hrs of transfection, cells were lysed in RIPA or NP40 lysis buffer.

MCF7 cells stably expressing HPIP were generated by transfecting pcDNA-HPIP using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer protocol. The next day, growth medium was removed and fed cells with medium containing increasing concentrations of G418 (G418 in a range of 0.1 mg/ml to 1.5 mg/ml) for 14-21 days. pcDNA vector stably expressing MCF7 cells were also generated in the same fashion to use as control. Similarly, HPIP knockdown in MDA-MB231 cells were achieved by transfecting pCMV-HPIPshRNA using Lipofectamine 2000 (Invitrogen, USA) followed by drug selection (0.1 µg to 1 µg/ml, puromycin) for two weeks. T7-HPIP was transfected into MDA-MB231-HPIP shRNA1 cells for HPIP rescue experiments. pCMV-Scrambled-shRNA vector stably expressing MDA-MB231 cells were also generated in the same fashion to use as control. Knockdown and overexpression of HPIP was confirmed by Westernblotting using anti-HPIP and anti-T7 antibodies.

2.3.5 Immunoblotting (Western Blotting): Cells were lysed in Radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM, Tris-Cl pH 7.4, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 1 mM PMSF and protease inhibitor cocktail (Roche, USA)) and were centrifuged at 10,000 rpm for 10 min to remove cell debris. To study phosphorylated proteins, cell lysates were prepared in RIPA buffer supplemented with phosphatase inhibitor cocktail (Sigma, USA). The protein concentration was determined by the Bio-Rad's RC-DC protein assay reagent kit, and equal amount of protein was loaded and resolved on a sodium dodecyl sulfate (SDS)-

polyacrylamide gel and transferred to nitrocellulose membrane (Pall, USA). Membranes were blocked with either 5% non-fat milk or 3% bovine serum albumin (BSA) prior to treatment with antibodies 4°C overnight, followed by three 15 min washes in Tris Buffer Saline (TBST) buffer. Following by secondary antibody treatment membrane was washed thrice with TBST. Membrane signals were detected using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) or Enhanced chemiluminescence (ECL) plus reagents (GE Healthcare, USA). Immunoblotting was performed using anti-HPIP, anti-Vinculin, anti-β-Actin, anti-β-Tubulin (Sigma, USA); anti-phospho-FAK-Y397, anti-FAK, anti-Talin, anti-calpain2 (Santa Cruz Biotechnology, USA); anti-phospho-tyrosine, anti-GAPDH, anti-phospho ERK1/2, anti-ERK (Cell Signaling Technology, USA); anti-T7 antibody (Novus Biologicals, USA); anti-GFP (Invitrogen, USA). If necessary, blots were stripped in stripping buffer (62.5 mM, Tris-Cl pH 6.8, 2% SDS and 10 mM β-Mercaptoethanol) at 55°C for 10-15 min and washed with TBS buffer, to reprobe with different antibodies.

2.3.6 Immunoprecipitation: For Co-IP, cells were lysed in NP-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA and 1X protease inhibitor cocktail). To study the interaction between FAK and HPIP by Co-IP, we lysed the cells in Triton X-100 lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 0.1% Triton X-100, 2 mM EDTA and 1X protease inhibitor cocktail). Immunoprecipitation with the indicated antibodies were carried out overnight at 4 °C followed by incubation with agarose A/G beads for 1 h. After thorough washing, protein complexes were dissolved in 2 × SDS loading dye and then subjected to western blot.

2.3.7 Cellular localization studies: Cells were cultured on cover slips and fixed with 4% formaldehyde for 20 min. The slides were washed thrice with TBS and permeabilized by prechilled acetone and methanol (1:3%) for 15 min. The fixed and permeabilized cells were washed three times with TBS (10 min each) and blocked with 3% bovine serum albumin (BSA) in TBS for 1 hr. Cells were probed with appropriate primary antibodies at 4°C overnight followed by three 15 minute washes in TBS buffer. Fluorescent labeled Alexa Flour secondary antibodies (Invitrogen, USA) were added and incubated at room temperature for 1 hr, followed by three 15 minute washes in TBS buffer. The cover slips were mounted on glass slide using prolong gold antifade reagent with DAPI (Invitrogen). Sixteen hour post incubation, edges of the cover slips was sealed by nail polish. Fluorescent images were captured by either fluorescence microscope (Olympus, Singapore) or DeltaVision Elite deconvolution microscope (GE Healthcare, Pittsburgh, USA).

2.3.8 Immunohistochemistry: Imunohistochemistry of breast invasive ductal carcinoma (IDC) samples was carried out by Diana David (Dr. Asha Nair lab) at Rajiv Gandhi Centre for Biotechnology (RGCB), India. Resected specimens from human breast tumors were fixed with 10% paraformaldehyde and embedded in paraffin blocks. After rehydration, five-micrometer sections of representative tumour blocks were subjected to antigen retrieval in boiling buffer (10 mM sodium citrate and 10 mM citric acid) for 10 min. Sections were then treated with protein-blocking solution for 30 minutes and incubated with HPIP antibody (1:100) (Bethyl laboratories, Montgomery, USA) for overnight at 4°C. After several rinses in PBS, the sections were incubated in biotinylated secondary antibody for 30 minutes. The bound antibodies were detected using Vecta Elite ABC staining kit (Vector Laboratories). The slides were rinsed in

PBS, exposed to diaminobenzidine, and counterstained with Mayer's hematoxylin. Expression of HPIP was measured depending on the intensity of immunoreactivity and scored as mild (+), moderate (++), and intense (+++).

2.3.9 Cell adhesion assay: Cell adhesion assay was performed using MDA-MB231 cells stably expressing control shRNA, HPIP shRNA1, HPIP shRNA2 or HPIP shRNA1 plus T7-HPIP. Cell adhesion assay was carried out in 24 well plates (Corning, USA). Culture plates were coated with fibronectin (5 µg/ml) (Sigma Aldrich, USA) for 2 hrs at room temperature and then washed twice with PBS to remove unbound fibronectin. Nonspecific binding sites were blocked with 0.5 mg/ml bovine serum albumin (BSA) and cells were trypsinized, and suspended in serum free DMEM medium. Approximately $3x10^4$ cells per well seeded in 24 well culture dishes were incubated for 30 min in humidified CO_2 incubator. No adherent cells were removed by washing with PBS and adherent cells were trypsinized and counted using hemocytometer. Images were taken under 10x magnifications using bright field microscope (Olympus, Singapore).

2.3.10 Cell migration assay: Wound healing assays were performed using MDA-MB231 cells stably transfected with control shRNA, HPIP specific shRNA1, HPIP shRNA2, HPIP shRNA1 plus T7-HPIP. Cells grown in 60 mm dishes as confluent monolayers were mechanically scratched with a 10 µl pipette tip to create a wound. Cells were then washed with PBS to remove cellular debris and the speed of wound closure was monitored as mentioned. Images were captured under 10X magnifications using bright field microscope (Olympus, Singapore) immediately after wound incision and at later time points. Wound closure was measured in

pixels. Apparently, 7-10 areas were measured in each experiment. Wound closure was converted into percentage and mean values were plotted in the graph (Microsoft Excel).

2.3.11 Focal adhesion disassembly assay: Focal adhesion disassembly assay was performed as described previously (Ezratty *et al*., 2005; Scolz *et al*., 2012). MDA-MB231 cells stably expressing either control shRNA or HPIP shRNA grown on fibronectin-coated glass cover slips were serum starved for 48 hours and then treated with 10 μM nocodazole for 4 hours. Post treatment, drug was removed by washing twice with PBS and cells were allowed to resume the polymerization of microtubules for various time points in growth medium. Cells were then fixed in 4% paraformaldehyde and images were captured by DeltaVision Elite deconvolution microscope (GE Healthcare, USA).

2.3.12 Live cell time-lapse microscopy imaging and quantification of adhesion dynamics: MCF7 and MDA-MB231 Cells were cotransfected with either control shRNA or HPIP shRNA1 along with DsRed-zyxin using Lipofectamine 2000. After 48 hours of transfection, the dynamics of DsRed-zyxin at focal adhesion points were monitored for 60 minutes using 100X objectives on a fluorescence microscope. When necessary, F14 (Santa Cruz Biotechnology, USA), a FAK-specific inhibitor was used at concentrations of 10 μM for 6 hr. Quantification of adhesion dynamics was performed as described previously (Chan *et al*., 2010; Webb *et al*., 2004). Rate constant measurements for disassembly (decreasing fluorescence intensity) of individual adhesions were determined from the slopes of trend lines fitted to semilogarithmic plots of fluorescence intensity ratios over time as described previously (Webb *et al*., 2004). Focal adhesion area was calculated using the formula $\pi d^2/4xl$ (d=width, l=length), from randomly selected 15-20 focal adhesions from 7-10 different cells.

2.3.13 *In vivo* phosphorylation: For *in vivo* phosphorylation, control shRNA and HPIP shRNA cells were incubated in phosphate-free DMEM medium for 12 hr in the presence of 250 μCi of [^{32}P]orthophosphoric acid per ml. At the end of the labeling period, the cells were washed and harvested in RIPA lysis buffer (50 mM, Tris-Cl pH 7.4, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 1 mM PMSF, phosphatase inhibitors and 1x protease inhibitor cocktail). The lysates were centrifuged at 10,000 rpm for 10 min at 4°C and protein estimation was done by the Bio-Rad's RC-DC protein assay reagent kit, and equal amount of protein was subjected to immunoprecipitation. Calpain2 immunoprecipitation was carried out at 4°C using calpain2 antibody. After centrifugation and washed thrice with RIPA lysis buffer, the immunoprecipitated calpain2 was resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and subjected to autoradiography.

2.3.14 *In vitro* calpain cleavage assay: Calpain cleavage assay was performed as described previously (Chan *et al*., 2010). HEK293T cells were transfected with pEGFP and pEGFP-HPIP plasmids and 48 hrs of post transfection, cells were lysed in NP40 lysis buffer. Immunoprecipitated GFP-HPIP from HEK293T cells was incubated in cleavage buffer (50 mM Tris-HCl pH 7.4, 137 mM KCl, and 1 mM $MgCl_2$) at different concentrations of purified calpain2 (Calbiochem, San Diego, USA) at 30 °C for 30 min in the absence or presence of $CaCl_2$ (1 mM). Cleavage reactions were stopped by the addition of 4X SDS sample buffer and reaction mixtures were subjected to SDS PAGE followed by Western blotting.

2.3.15 Bioinformatics and statistical analysis: Oncomine, a publicly available cancer data base (Compendia Biosciences, Ann Arber, MI), was used for gene expression analysis. To analyse HPIP expression in cancer samples, several breast cancer datasets were exported from

Oncomine. Box plot was used to show fold change of HPIP expression in invasive breast carcinomas vs normal tissues. For clinical data set survival analysis, association between HPIP expression and breast cancer patient survival was assessed by Kaplan-Meier plotter (Gyorffy *et al*., 2010) (www.kmplot.com/breast).

For reproducibility, all the experiments were performed 2-3 times. The results are expressed as means ±standard deviation, and differences between groups were analyzed by one-way ANOVA using sigma plot.

Plasmids used in this chapter

Name	Description	Insert size	Reference
BM 22	pcDNA3.1/T7-HPIP	2196 bp	Manavathi *et al*., 2006
BM 31	pEGFP-C1-HPIP	2196 bp	In this study
BM 37	pMNDUS/flag-HPIP	2196 bp	Manavathi *et at*., 2012
BM 67	pcDNA3.1/T7-HPIP (1-327)	979 bp	In this study
BM 68	pcDNA3.1/T7-HPIP (1-488)	1463 bp	In this study
BM 69	pcDNA3.1/T7-HPIP (1-582)	1746 bp	In this study
BM 189	pEGFPC1-FAK full length	3400 bp	In this study
BM 190	pEGFPC1-FERM domain	1200 bp	In this study
BM 191	pEGFPC1-FAT domain	1150 bp	In this study
BM 192	pEGFPC1-kinase domain	800 bp	In this study
BM39	pcDNA3.1/T7-HPIP (G422A)	2196 bp	In this study
BM40	pcDNA3.1/T7-HPIP (D423E)	2196 bp	In this study
BM250	pMIG/flag-mHPIP	2196 bp	In this study
BM 153	DsRed2-Zyxin	1716 bp	Kodama *et al*., 2003
BM 152	pEGFP-C1-Paxillin	1671 bp	Kodama *et al*., 2003
BM 188	pGEX4T1-GST-HPIP	2196 bp	In this study
BM251	pEGFP-FAK shRNA clones	-	Dr. Samuel Aparicio, Canada
BM 119	pGFP-V-RS-HPIP shRNA 1	-	OriGene, USA
BM 120	pGFP-V-RS-HPIP shRNA 2	-	OriGene, USA
BM 121	pGFP-V-RS-HPIP shRNA 3	-	OriGene, USA
BM 122	pGFP-V-RS-HPIP shRNA 4	-	OriGene, USA
BM123	pGFP-V-RS-control shRNA	-	OriGene, USA

2.4 RESULTS

2.4.1 HPIP is overexpressed in human breast infiltrative ductal carcinoma

Previous demonstration of HPIP influencing migratory properties in breast cancer cell lines led to the hypothesis that HPIP may play a role during breast tumor progression to invasive stages *in situ* (Manavathi *et al*., 2006). To test this hypothesis, we first analyzed the expression levels of HPIP in 53 primary breast tumors from public cancer transcriptome database (Finak *et al*., 2008) which showed a higher expression of HPIP in infiltrative ductal carcinoma compared to non-tumor tissues (Figure 4A). Consistent with this dataset, three other cancer datasets also showed similar results of HPIP expression in invasive/infiltrative ductal carcinoma (IDC) as compared to non-tumor tissues (Figure 4B-D).

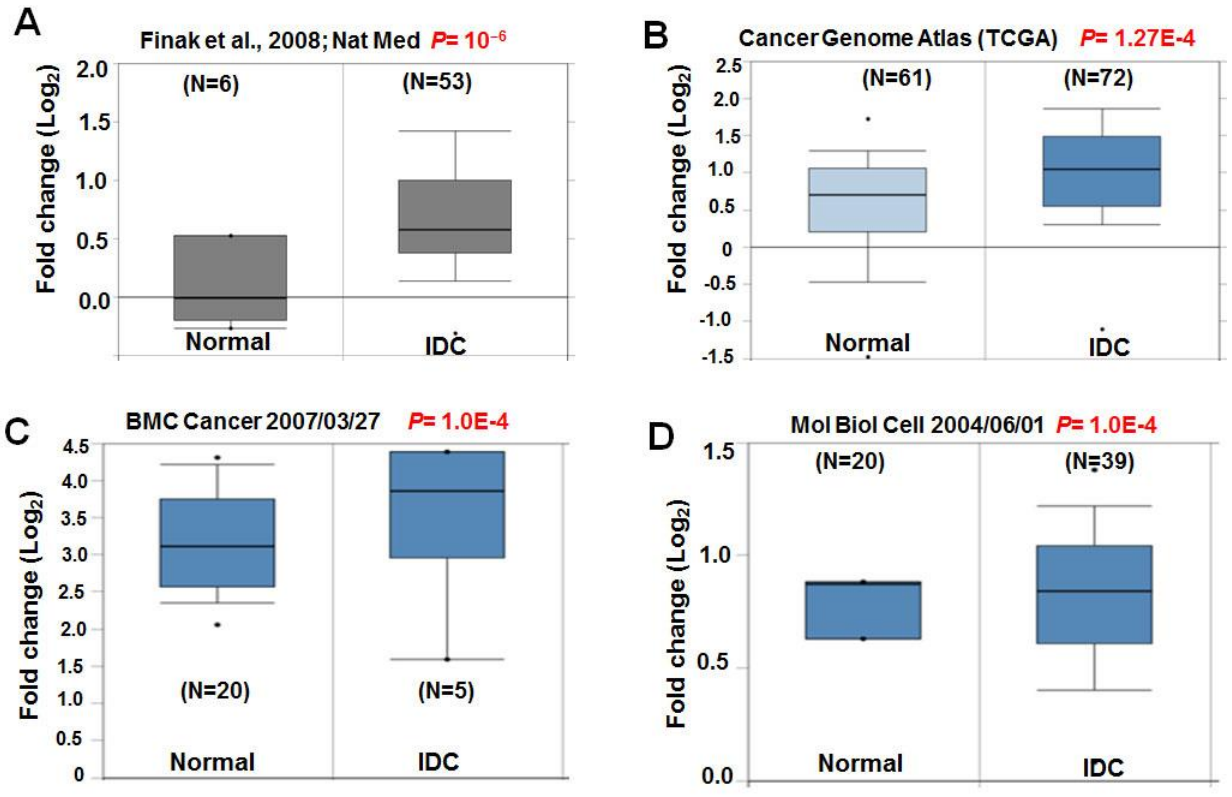
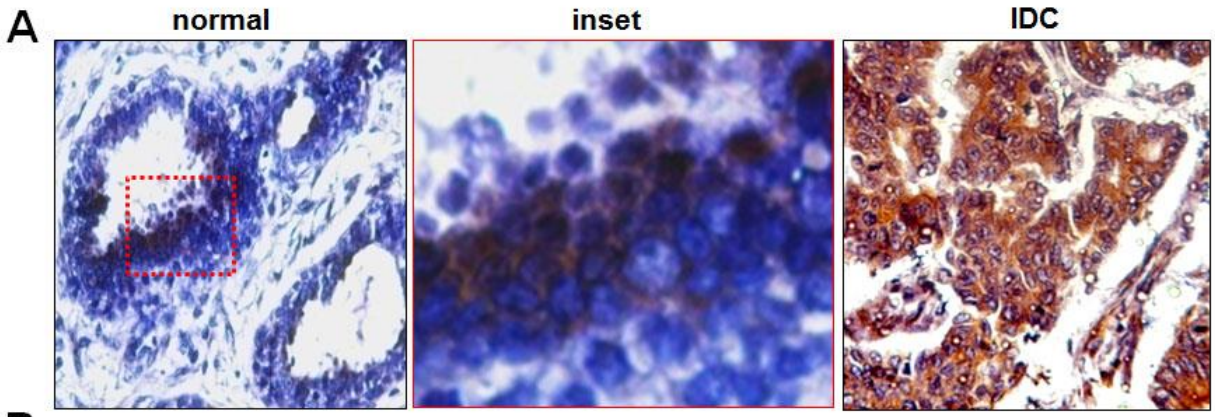


Figure 4. Expression of HPIP in primary breast tumors. (A) Oncomine microarray data was used to analyse HPIP expression (mRNA) in cancer (IDC) (N=53) vs. normal breast tissues (N=6). Samples were organized by fold change of HPIP mRNA from normal were graphed by a box plot. $^{*}P < 10^{-6}$. (B) PBXIP1/HPIP expression in The Cancer Genome Atlas (TCGA) breast dataset. 1. Breast normal (N=61), 2. Invasive breast carcinoma (N=76). *P*= 1.27E-4. (C) PBXIP1/HPIP expression in Turashvili breast dataset. 0. Normal (N=20), 1. Invasive ductal breast carcinoma (N=5). *P*= 1.0E-4. (Ref: BMC Cancer 2007/03/27). (D) PBXIP1/HPIP expression in Zhao breast. 0. Normal (N=3) 1. Invasive ductal breast carcinoma (N=39). (Ref: Mol Biol Cell 2004/06/01). *P*= 1.0E-4. N= number of samples.

We further analyzed primary infiltrative breast tumors by immunohistochemistry. In support of our hypothesis, we noted 100% (32/32 cases vs. 5/15 healthy primary tissues) of the infiltrative ductal carcinoma (IDC) tissue expressing either very high (78%, 25/32) to moderately high (22%, 7/32) levels of HPIP (Figure 5A, B). Both in the normal as well as in the tumors, HPIP expression was confined to cytoplasm while no expression was observed in the nucleus. In addition, 8 matched-sets of primary mammary carcinoma vs. surrounding healthy breast tissue by Western blot analysis. As shown in Figure 5C, 87.5% of mammary carcinoma had elevated expression of HPIP compared to their normal counterparts.

To understand the clinical relevance of this observation, we performed Kaplan-Meier analysis (Gyorffy *et al*., 2010) on breast cancer patients with molecular subtype defined as basal like breast cancers which are highly invasive. We observed a significantly poor overall survival (*P*= 0.041) in breast cancer patients with elevated levels of HPIP in cancerous tissue compared to normal controls (Figure 6). Together these results strongly support that elevated HPIP expression estimates poor patient survival and is positively correlated with invasive stages of cancer progression.



B

Breast tissue (n=no of samples)	HPIP expression intensity (cytoplasm)			HPIP in nucleus
	Mild (+)	Moderate (++)	Intense (+++)	
NORMAL (n=15)	5(33%)	0(0)	0(0)	0(0)
TUMOR - IDC (n=32)	0(0)	7(22%)	25 (78%)	0(0)

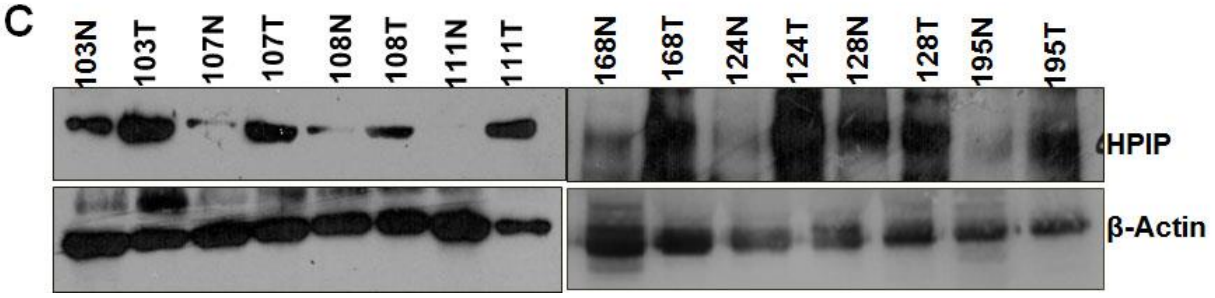


Figure 5. Expression of HPIP in invasive ductal carcinoma. (A) Representative pictures of IHC analysis of HPIP expression in cancer tissues (IDC) and adjacent normal healthy breast tissues. (B) Summary of immunohistochemistry (IHC) analysis of HPIP expression in normal vs. breast tumors. Number in parentheses refers to the percentage of samples either positive or negative. (C) Western blot analysis of HPIP expression in 8 matched-sets of infiltrative/invasive ductal carcinoma (IDCs) vs. adjacent normal breast tissues.

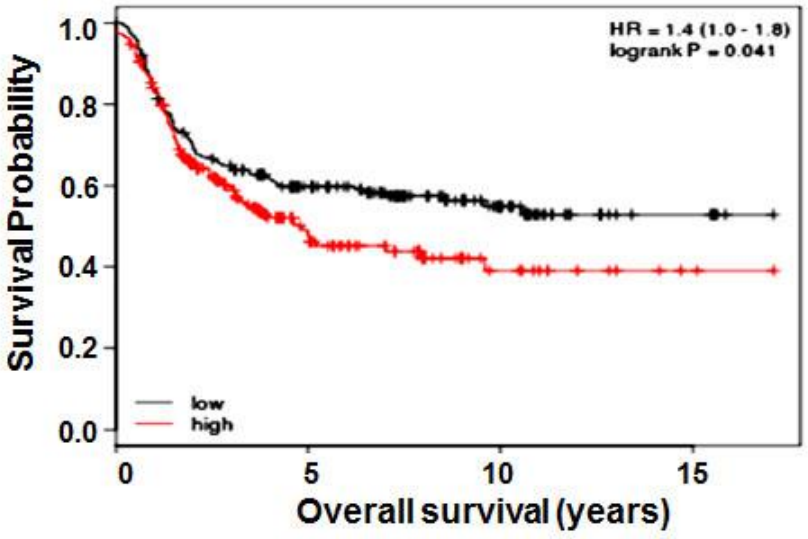


Figure 6. Kaplan–Meier plot of relapse-free survival of breast cancer patients expressing high or low HPIP in KM-plot database. Log-rank test p value is displayed (*P* =0.041). Red line: cases with high expression of HPIP, black line: cases with low expression of HPIP.

2.4.2 HPIP regulates cell migration by modulating cell adhesion behaviour

Previously the link between HPIP and cell migration has been reported involving estrogen signalling in breast cancer cell line, MCF7 (Manavathi *et al*., 2006). To confirm the functional link between cell migration and HPIP expression, we sought to determine the effect of HPIP knockdown on cell migration in MDA-MB231 cells, an ER-negative invasive breast cancer cell line. To address this issue, we first generated MDA-MB231 cell lines that stably expressing scrambled shRNA (control sh), HPIP sh1 or HPIP sh2. Interestingly, HPIP knockdown clones showed tightly packed colonies compared to control cells that showed characteristic mesenchymal-like (spindle shape) appearance suggesting possible changes in migratory function as a consequence of alterations in morphological features (Figure 7A). Cell adhesion assay performed on fibronectin-coated plates additionally showed that significantly increased proportion of HPIP knockdown cells adhered to the surface compared to control cells (Figure

7B, C). However, increased cell adhesion observed in cells which had been knockdown by HPIP sh1 was rescued following overexpression of T7-HPIP (Figure 7B, C). Further using cell migration assay, we observed a reduction in migration of HPIP knockdown cells as compared to control cells (Figure 8). However, forced overexpression of T7-HPIP in cells in which HPIP had been knockdown by HPIP sh1 restored cell migration (Figure 8). These results indicate that HPIP is a regulator of breast cancer cell migration, and it may partly involve in molecular mechanisms that suppresses cell adhesion.

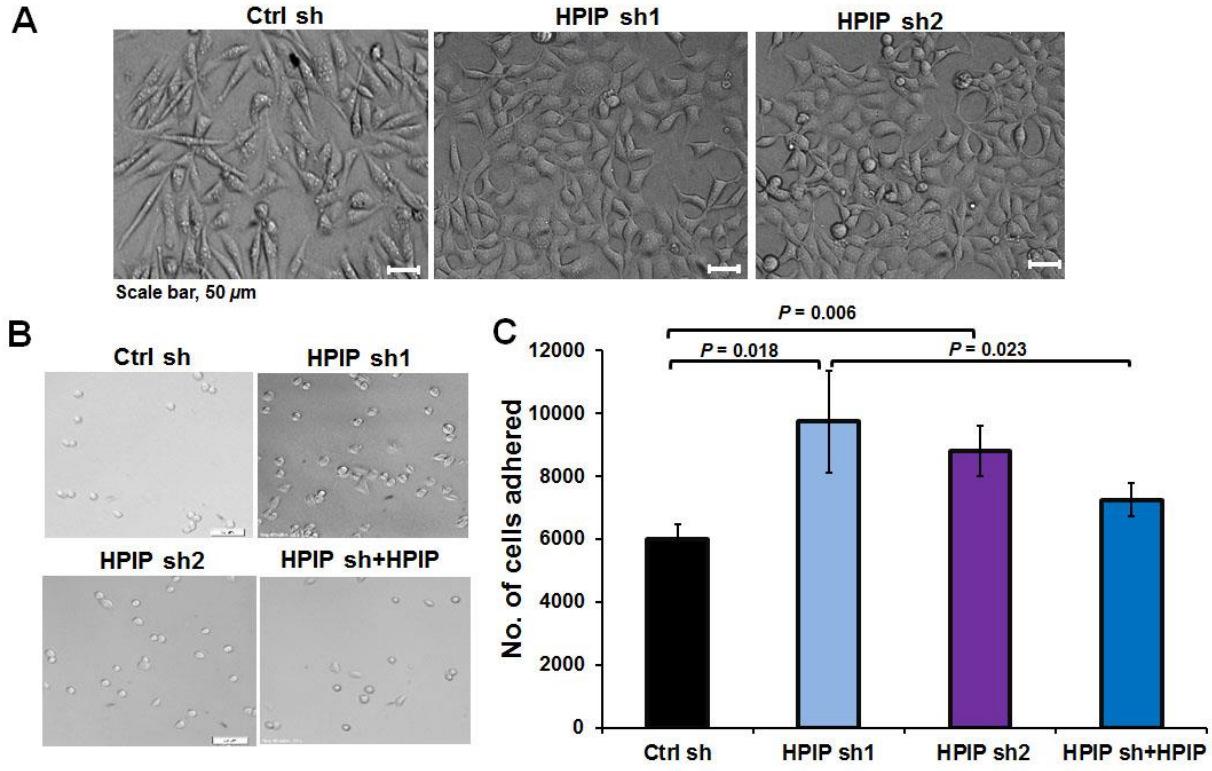


Figure 7. HPIP expression modulates cell morphology of breast cancer cells. **(**A) Representative bright field microscopy images (10x magnification) of MDA-MB231 cells expressing control sh, HPIP sh1 or HPIP sh2. (B) Effect of either HPIP knockdown (by HPIP sh1 or HPIP sh2) on the cell adhesion potential of MDA-MB231 cells or forced overexpression of T7-HPIP on the cell adhesion potential of MDA-MB231 cells in which HPIP had been

knockdown by HPIP sh1. Representative phase-contrast images showing the adherent cells that expresses either control sh, HPIP sh1, HPIP sh2 or HPIP sh1 plus T7-HPIP. (C) Quantification of cell adhesion assay.

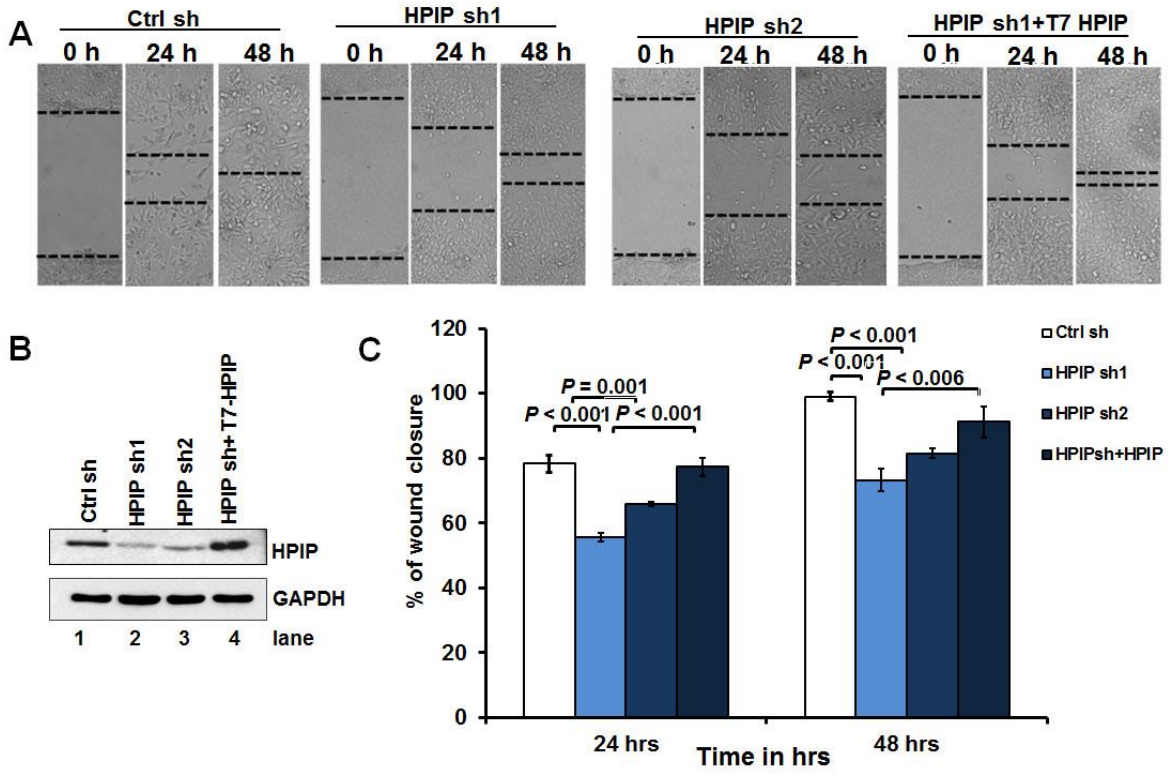


Figure 8. HPIP expression promotes migration in breast cancer cells. (A) Representative bright field microscopy images (10x magnification) of MDA-MB231 cells expressing control sh, HPIP sh1, HPIP sh2 or HPIP sh1+T7 HPIP after creating wound and later time points. **(**B) Western blot analysis showing either HPIP knockdown MDA-MB231 cells (lanes 2-3) or overexpression of T7-HPIP in MDA-MB231 cells in which HPIP was knockdown by HPIP sh1. (C) Quantification of the kinetics of wound closure assay showing the effect of either HPIP knockdown (by HPIP sh1 or HPIP sh2) on migration of confluent monolayers of MDA-MB231 cells or forced overexpression of T7-HPIP on migration of confluent monolayers of MDA-MB231 cells in which HPIP had been knockdown by HPIP sh1.

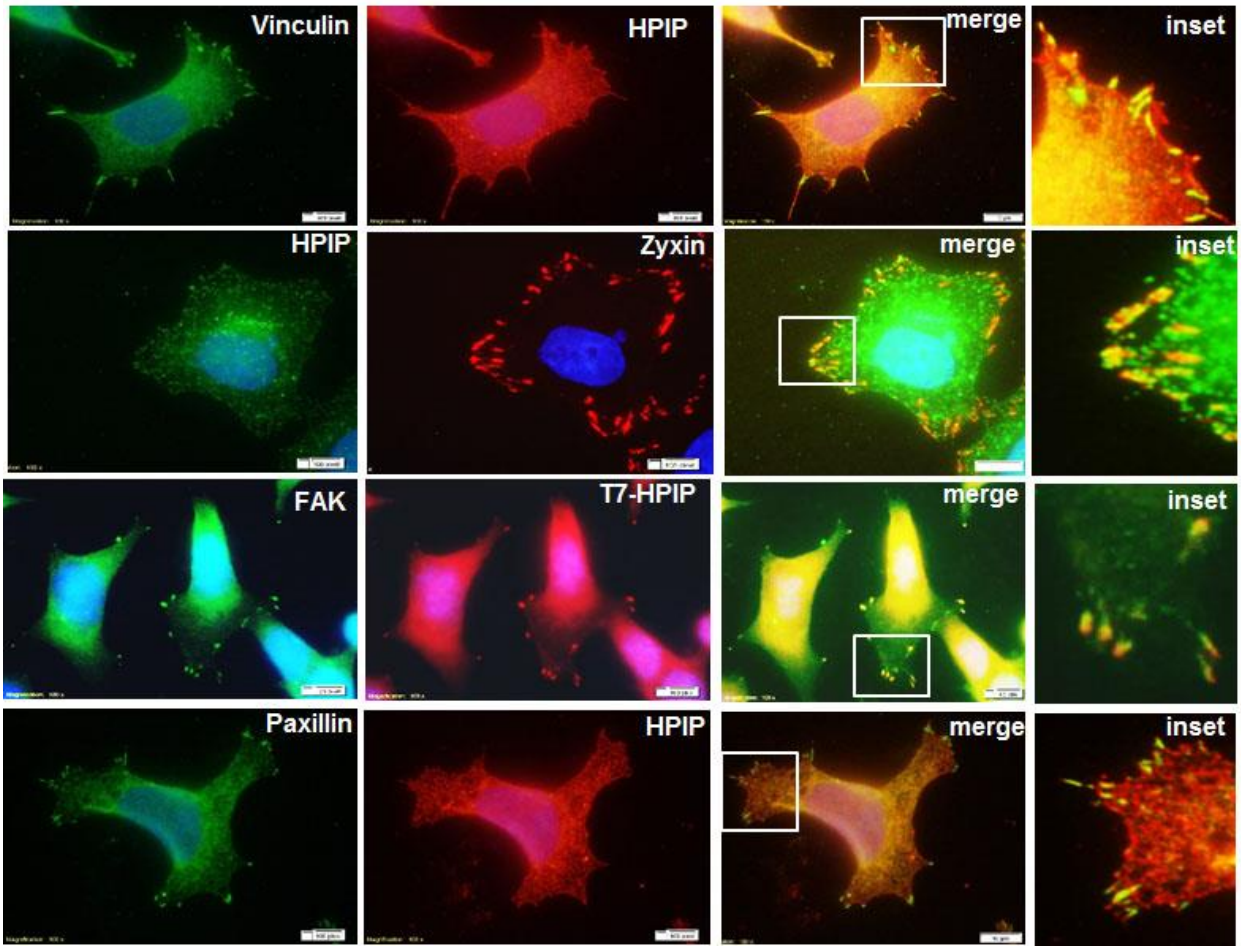


Figure 9. Colocalization of HPIP with focal adhesion proteins. **(**A) Immunofluorescence (IF) analysis showing focal adhesion localization of HPIP similar to focal adhesion proteins such as Vinculin, Paxillin, Zyxin and FAK in MDA-MD231 cells. DAPI (blue), nucleus. Representative 100x magnified immunofluorescent images are shown. Boxed areas are magnified in the insets. The white arrows indicate representative focal adhesion sites.

2.4.3 HPIP is a component of focal adhesions and activates focal adhesion kinase

Previously we have reported that HPIP functions as an adaptor/scaffold with multiple domains which facilitate to associate with various signaling proteins including microtubules (Manavathi *et al*., 2006). To investigate the molecular players involved in HPIP-dependent cancer cell migration, we first asked whether HPIP was localized in plasma membrane regions and if so,

whether they were colocalized with key cell adhesion molecules. Preliminary analysis with MDA-MB231 cells suggested that HPIP was expressed in cell surface in a punctuate fashion in regions that resembled focal adhesion sites and colocalized with vinculin, paxillin, zyxin or focal adhesion kinase (FAK) (Figure 9).

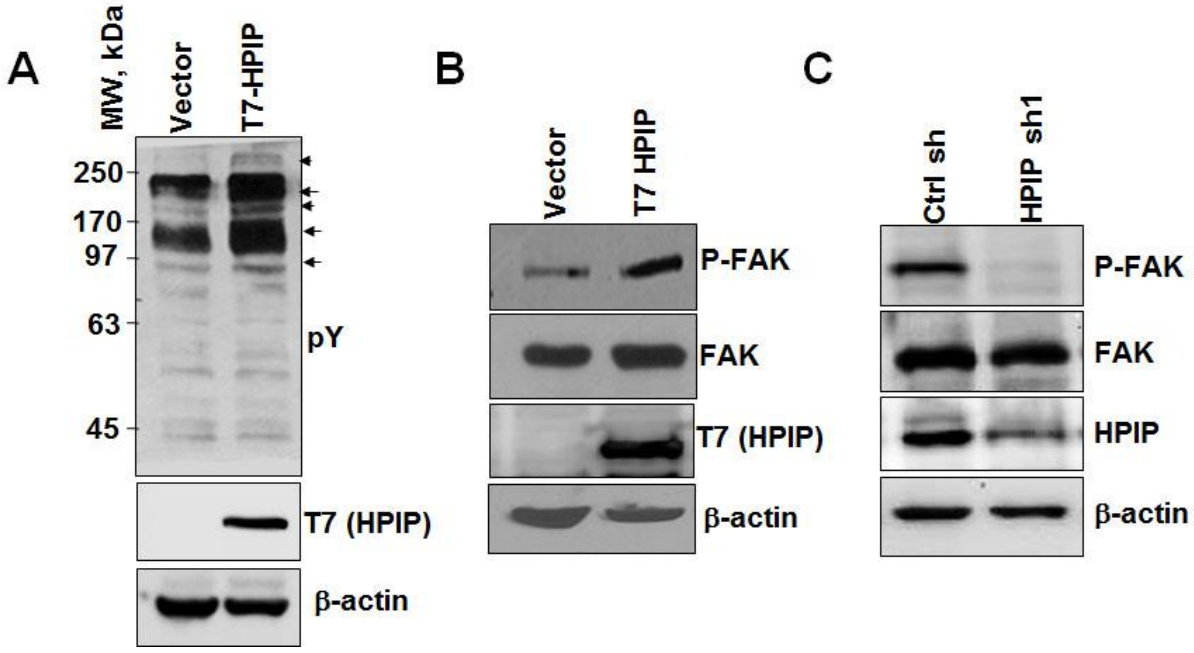


Figure 10. HPIP activates FAK in breast cancer cells (A) Western blot analysis of total lysates from either control vector or T7-HPIP transfected MCF7 cells. Membranes were blotted as indicated. pY, anti-phosphotyrosine antibody. (B) Similarly, Western blot analysis of total lysates from control sh or HPIP sh1-transfected in MDA-MB231 cells or lysates from either control vector or T7-HPIP transfected MCF7 cells. Membranes were probed with indicated antibodies.

One of the hallmark event in cell adhesion is the activation of focal adhesion proteins through tyrosine phosphorylation (Panetti *et al.*, 2002). Given the observation that HPIP is colocalised with focal adhesion proteins, we asked whether HPIP regulation of cell adhesion and migration involves tyrosine phosphorylation of these proteins. As shown in Figure 10A, an

increased tyrosine phosphorylation of proteins ranging from 80 to 200 kDa (indicated by arrows) detected by pan-phosphotyrosine antibody is observed in HPIP overexpressing cells compared to control cells. Activation of FAK by tyrosine (at position Y397) phosphorylation is an essential feature in various invasive and metastatic tumours (Owens *et al*., 1995; Weiner *et al*., 1993). Therefore, we next analysed the effect of HPIP expression on FAK activation. As shown in Figure 10B, ectopic expression of HPIP increased FAK phosphorylation at Y397 in MCF7 cell lines. Conversely, HPIP knockdown in MDA-MB231 cells reduced FAK-Y397 phosphorylation indicating that HPIP regulates FAK phosphorylation and therefore, HPIP-mediated cell migration may be dependent on FAK activation (Figure 10C).

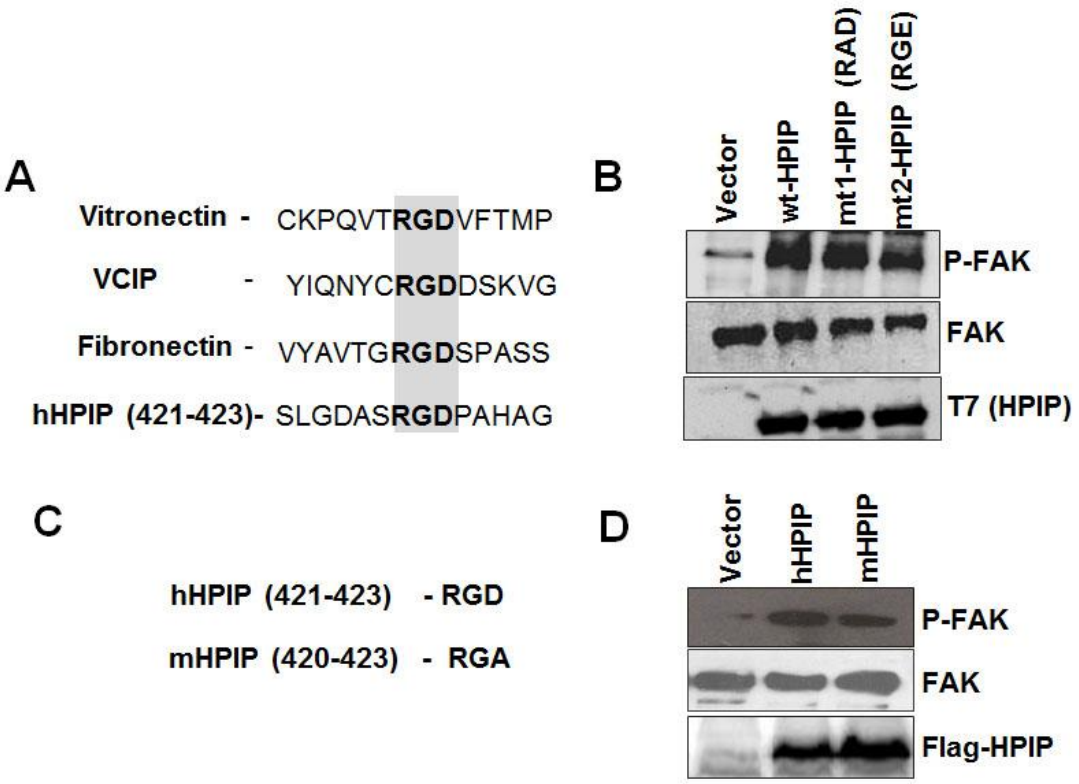


Figure 11. RGD domain is not involved in HPIP-mediated FAK activation. **(**A) Alignment of RGD motif of HPIP (amino acids 421 to 423) with consensus RGD motifs of known ECM proteins. (B) Effect of RGD mutations in

HPIP on FAK activation. Western blot analysis of total lysates from control vector, wild type HPIP (wt), mt1 (HPIP-RAD) or mt2 (HPIP-RGE) transfected in MCF7 cells. (C) Alignment of RGD domain of HPIP protein from different species (top). (D) Western Blot analysis showing the effect of either hHPIP or mHPIP on FAK activation in MCF7 cells. Membranes were probed with indicated antibodies (bottom). h, human; m, mouse.

Previous studies have shown that RGD domain activates the FAK through integrin signalling pathway (Schaller *et al*., 2010). Because HPIP contains a RGD domain at position 421-423, we verified its role on FAK activation (Figure 11A). As shown in Figure 11B, mutation of RGD domain did not affect FAK activation by HPIP. Similarly, mouse HPIP, which contain RGA in place of RGD found in human HPIP (Figure 11C), also activated FAK in MCF7 cells (Figure 11D). These results suggest that HPIP-mediated activation of FAK in cancer cells is not dependent on its putative RGD domain.

2.4.4. HPIP activates FAK through its direct interaction with kinase domain

To test whether FAK activation by HPIP was a direct consequence of interaction of HPIP with FAK, we performed co-immunoprecipitation using MDA-MB231 cell lysate. In Figure 12A, we show that HPIP was able to coimunoprecipitate with FAK in these cells. Next we mapped the domains involved in the interaction between HPIP and FAK. As shown in Figure 12B, none of the HPIP deletion mutants (C-terminus) showed interaction with FAK except full length HPIP (1-731 aa). Conversely, coimmunoprecipitation using GFP antibody (GFP-fusion FAK domains) demonstrated the involvement of kinase domain (KD) of FAK in binding to HPIP (Figure 12C-D). Consistent with the above results, HPIP deletion mutants lost their ability to activate FAK except full length HPIP (Figure 13A-B). Immunofluorescence analysis further supported that HPIP deletion mutants were unable to colocalize with FAK (Figure 13C). Together these results

suggest that C-terminal region spanning 583-731 amino acid region of HPIP is involved in interaction with kinase domain of FAK and is therefore important for FAK activation.

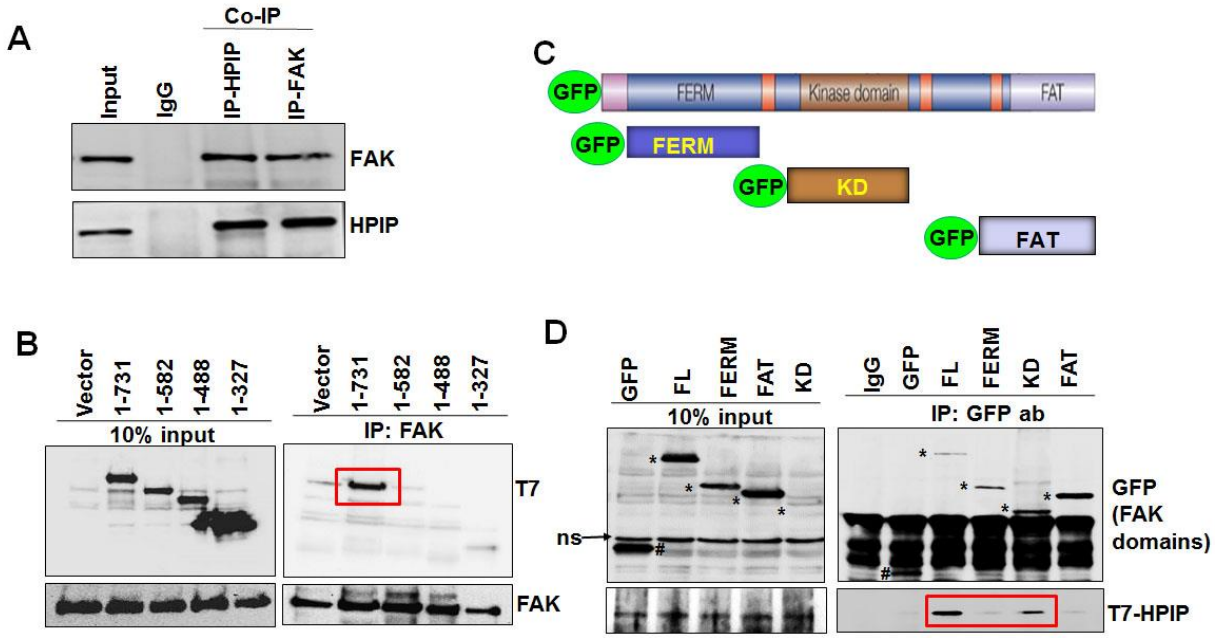


Figure 12. Mapping of domains involved in the interaction between HPIP and FAK. (A) Extracts from MDA-MB231 cells were immunoprecipitated with either HPIP or FAK antibodies and blotted as indicated. Cell extracts immunoprecipitated with IgG antibody was used as control. (B) Requirement of C-terminal of HPIP for binding with FAK. Extracts from HEK293T cells transfected with plasmid constructs encoding various mutant proteins (C-terminus) of HPIP (T7-tagged) along with full length HPIP (1-731 aa) were immunoprecipitated with FAK antibody and blotted as indicated (right). 10% of input lysates were blotted to verify the expression of indicated proteins (left). (C) Schematic representation of FAK domains. (D) Involvement of kinase domain (KD) of FAK in binding to HPIP. Extracts from HEK293T cells transfected with plasmid constructs encoding various domains of FAK (GFP-tagged) were immunoprecipitated with GFP antibody and blotted as indicated. The asterisks indicate corresponding FAK domains. #, GFP, ns, non-specific

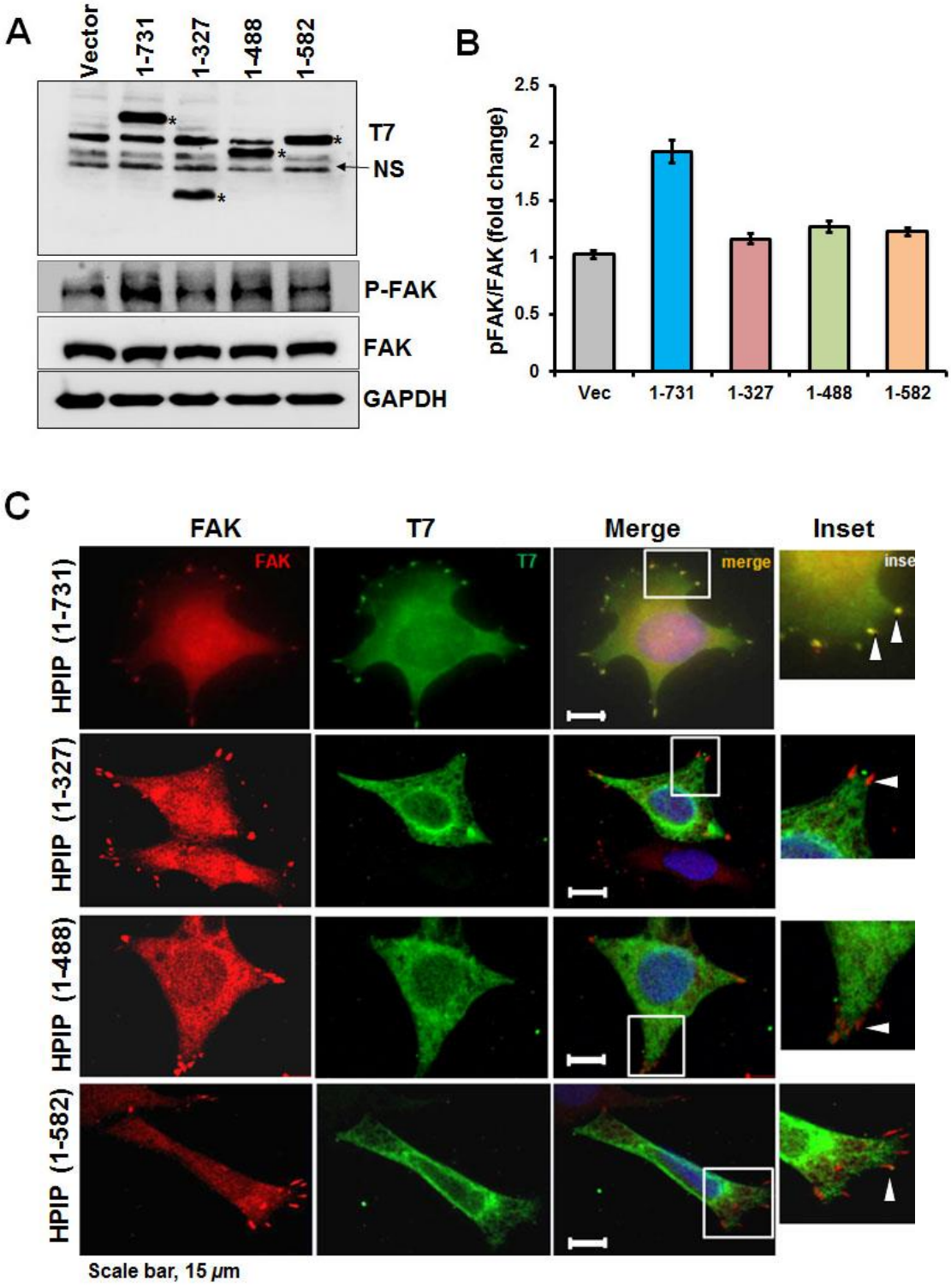


Figure 13. C-terminal region of HPIP is required for FAK activation and its localization to focal adhesions. (A) Extracts from MCF7 cells transfected with plasmids encoding various deletion (C-terminus) mutants of HPIP were

subjected to Western blotting and probed with indicated antibodies. The asterisks indicate corresponding C-terminal deletion peptides of HPIP. NS, non-specific protein bands. (B) Quantification of FAK activation (ratio between the intensities of pFAK and FAK protein bands) from three independent experiments is shown. (C) Immunofluorescence analysis showing localization of various deletion mutants of HPIP (T7 tagged) (green) and FAK (red) in MDA-MB231 cells. DAPI (blue) stains nucleus.

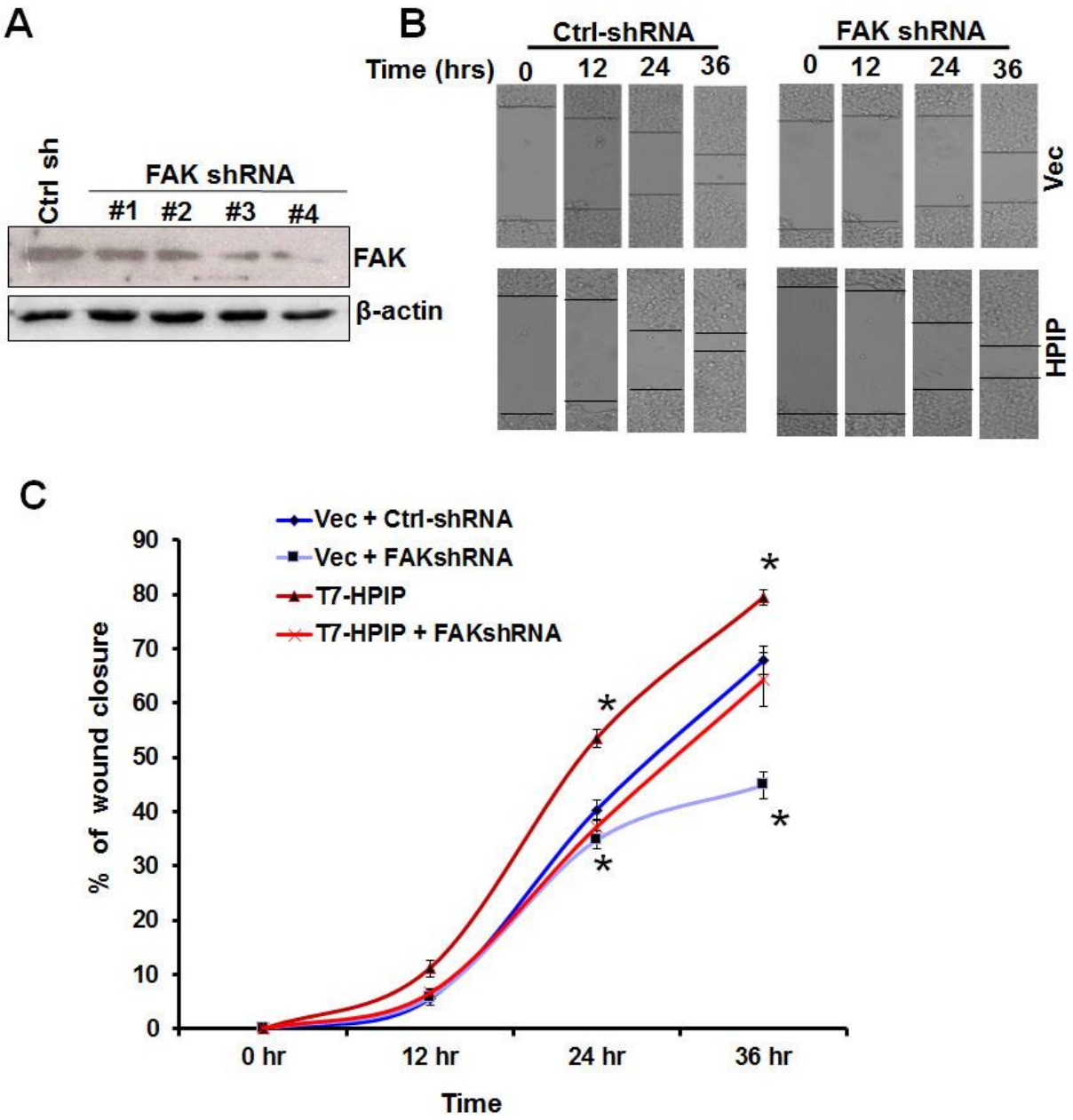


Figure 14: HPIP mediated cell migration requires FAK. (A) Western blot analysis showing the effect of various FAK shRNA (clones 1-4) on FAK knockdown in MDA-MB231 cells (top). (B) Effect of FAK knockdown by FAK sh4 on migration of confluent monolayers of stable clones of MDA-MB231 cells that expresses either control vector or T7-HPIP. Twenty four hours post transfection of FAK sh4, cells were subjected to wound closure assay. Representative phase-contrast images of the site at different time points as indicated after scratch wounding were presented. (C) Quantification of the kinetics of wound closure assay. P=0.025, samples between Vec+Ctrl sh and Vec+FAK sh4 at 24 h; P<0.001, samples between HPIP+Ctrl sh and HPIP+FAK sh4 at 24 h; P=0.001, samples between Vec+Ctrl sh and Vec+FAK sh4 at 36 h; P=0.002, samples between HPIP+Ctrl sh and HPIP+FAK sh4 at 36 h. h, hours.

Accumulating evidence support that FAK is a critical regulator of cell migration as enhanced FAK signaling promotes cell motility, whereas inhibition of FAK signaling impairs cell migration (Mitra *et al*., 2005). We hypothesized that HPIP-mediated cell migration requires FAK. To test this hypothesis, we measured cell migration in HPIP stably expressing MDA-MB231 cells under FAK knockdown conditions (Figure 14A). As shown in Figure 14B-C, overexpression of T7-HPIP enhanced cell migration in MDA-MB231 cells compared to control cells, however FAK knockdown reduced HPIP-induced cell migration suggesting that HPIP-mediated cell migration requires FAK.

2.4.5 Loss of HPIP results in defective focal adhesion turnover

To explore the possible mechanisms that underlie HPIP-induced FAK mediated cell migration, we first determined the focal adhesion turnover in MDA-MD231 cells. As shown in Figure 15A, HPIP knockdown had little or no effects on the number of focal adhesions but these cells showed larger focal adhesions compared to control cells (Figure 15B). Similar observations were also made in MCF7 cells (Figure 16A). As large focal adhesions at the cell periphery indicate defects in focal adhesion dynamics (Ilic *et al*., 1995; Wu *et al*., 2008), we monitored focal adhesion

turnover using high-resolution live cell time-lapse video microscopy following cotransfection of MCF7 cells with either control sh or HPIP sh1 and DsRed-Zyxin (Figure 16B). A movie of the focal adhesion dynamics measured using DsRed-zyxin as a tracking molecule show the assembly and disassembly of focal adhesions (Figure 16A). Live fluorescence imaging demonstrated that Zyxin-containing adhesions in HPIP knockdown cells were extended in duration by 20 min compared with control cells (control sh, 20 min, ± 3.823 vs. HPIP sh, 40 min, ± 4.661) (Figure 16C). From plots of DsRed-zyxin fluorescence intensities over time, we generated rate constants for net disassembly rates. As shown in Figure 16D, focal adhesion disassembly rate was significantly reduced in HPIP knockdown cells compared to control cells. Similar observations were being made in MDA-MB231 cells (Figure 17A-B).

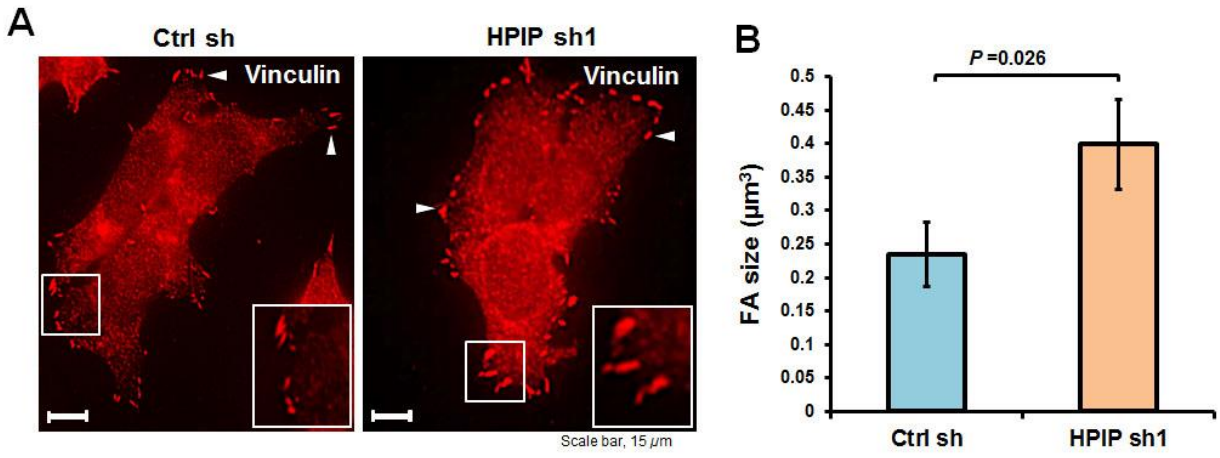


Figure 15. HPIP regulates focal adhesion (FA) size in MDA-MB231 cells. (A) Effect of HPIP knockdown on FA morphology in MDA-MB231 cells. Vinculin (red). Representative 60x magnified immunofluorescence images captured by DeltaVision Elite deconvolution microscope are shown. Boxed areas are magnified in the insets. The white arrows indicate representative focal adhesions. B) Quantification of the FA size from control sh or HPIP sh1 transfected MDA-MB231 cells.

To further confirm whether HPIP-induced FA disassembly is dependent on FAK, we monitored the FA disassembly time in MCF7 cells transfected with either control vector or Flag-HPIP along with DsRed-Zyxin and were treated with F14, a FAK-specific inhibitor. As shown in Figure 18A, D, overexpression of Flag-HPIP reduced the disassembly time as compared with control cells, however, FAK inhibition by F14 significantly reduced Flag-HPIP effects on FA disassembly.

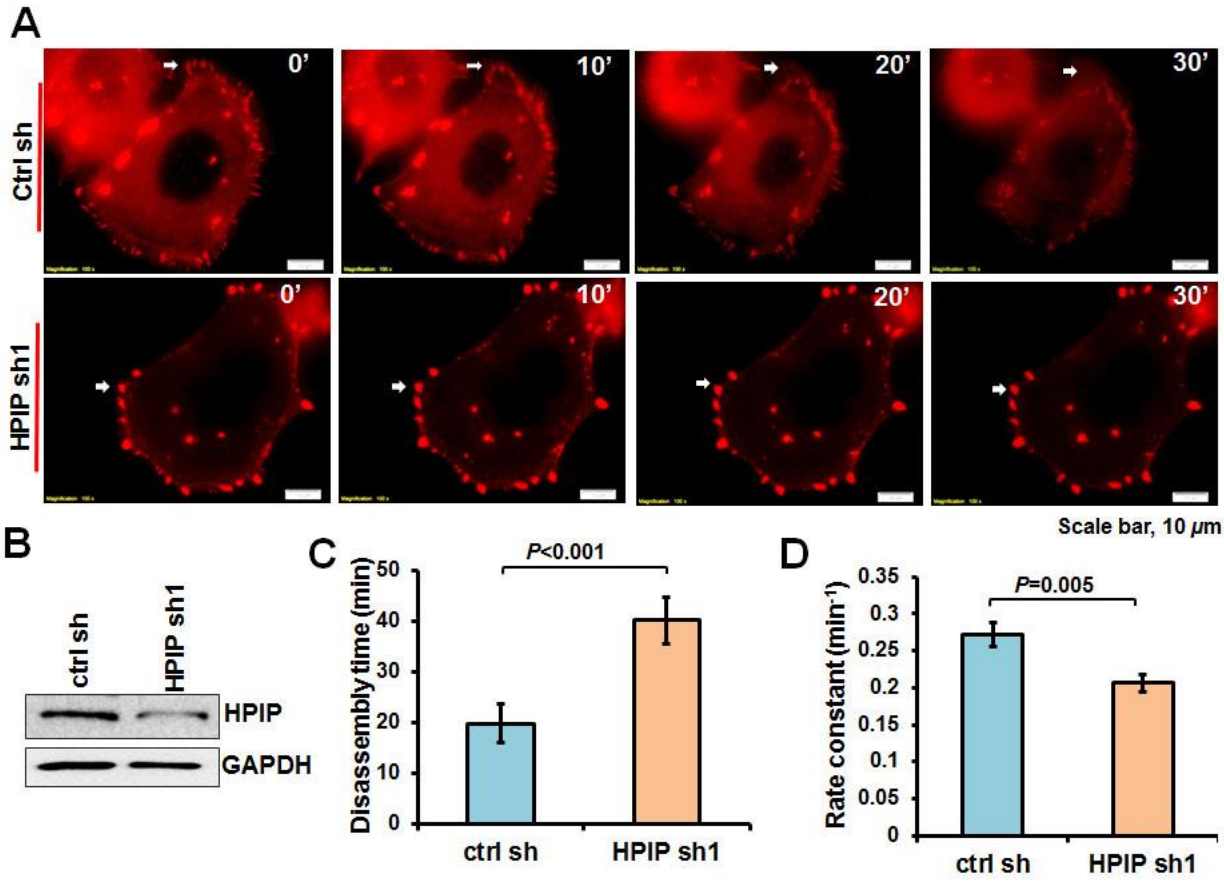


Figure 16. HPIP knockdown delays focal adhesion disassembly in MCF7 cells (A) Time-lapse video microscopy analysis showing the time elapsed between the appearance and dissolution of observed FAs. MCF7 cells cotransfected with either control sh or HPIP sh1 and DsRed-zyxin plasmids were subjected to time-lapse video fluorescence microscopy analysis. The white arrows indicate representative FAs undergoing disassembly as time

progresses. (B) Western blot analysis showing knockdown of HPIP in MCF7 cells. C) Quantification of FA disassembly time. We analyzed 4-5 cells for time-lapse FA disassembly analysis as described previously (Webb *et al*., 2004). (D) Rate constants for net FA disassembly were calculated from plots of fluorescence intensities of DsRed-zyxin as described under 'Material and Methods'. The results were expressed as means ±standard deviation, and differences between groups were analyzed by one-way ANOVA.

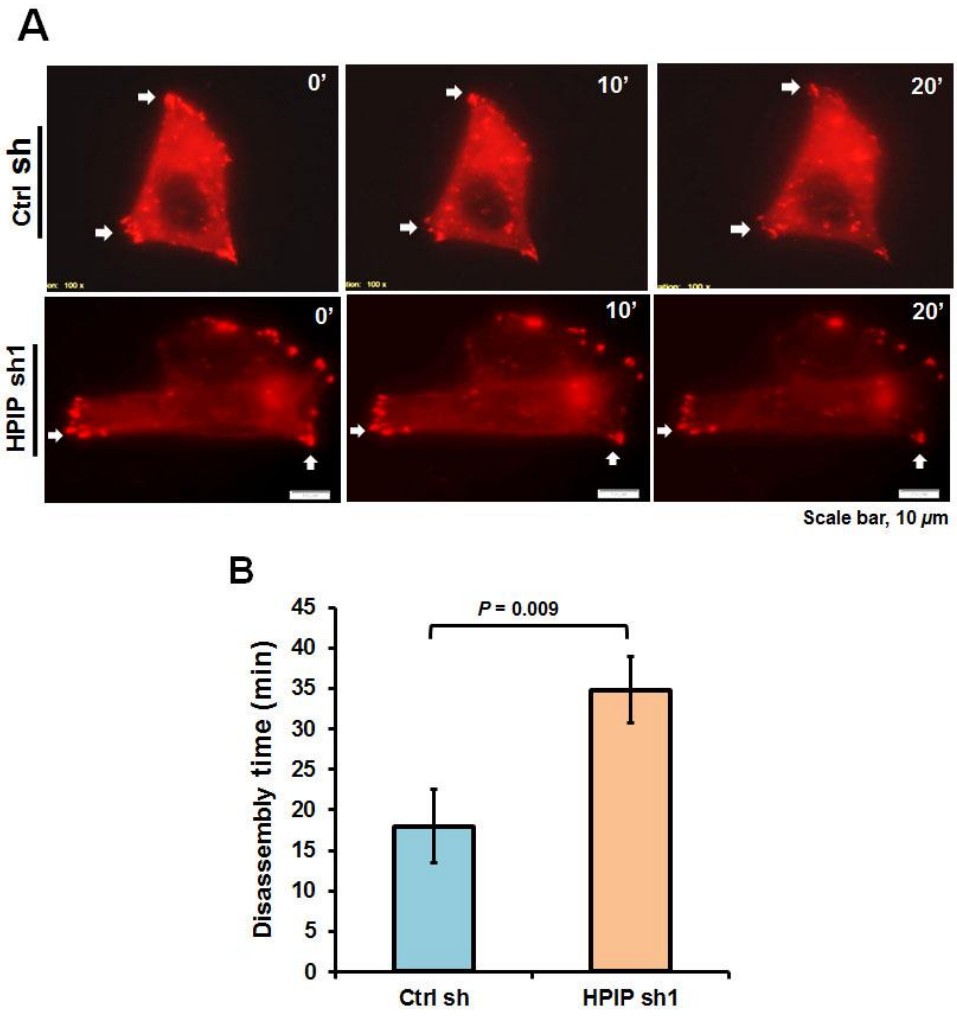


Figure 17. HPIP knockdown delays focal adhesion disassembly in MDA-MB231 cells. (A) Representative images of time lapse video microscopy analysis showing the time elapsed between the appearance and dissolution of an observed FAs in MDA-MB231 cells cotransfected with either control sh or HPIP sh1 and DsRed-zyxin plasmids. B) Quantification of focal adhesion disassembly time. We analyzed 4-5 cells for time-lapse FA disassembly analysis as described previously (Webb *et al*., 2004). The results are expressed as means ±standard deviation and differences between groups were analyzed by one-way ANOVA.

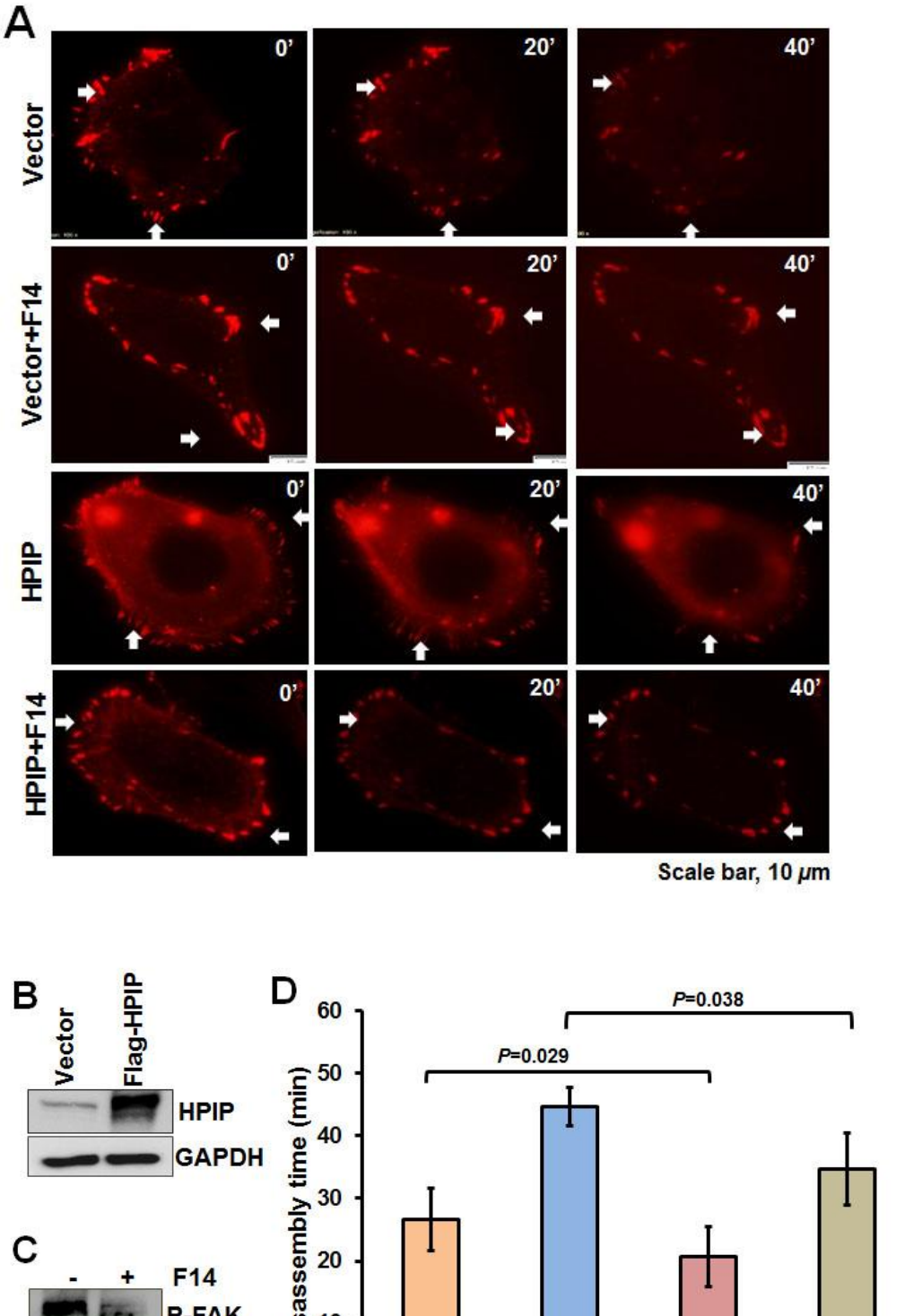

A
Vector
Vector+F14
HPIP
HPIP+F14
0'
20'
40'
Scale bar, 10 μm
B
Vector
Flag-HPIP
HPIP
GAPDH
C
-
+
F14
P-FAK
FAK
D
Disassembly time (min)
P=0.029
P=0.038
MNDUS
MNDUS+ F14
MNDUS HPIP
MNDUS HPIP+F14

Figure 18. HPIP-mediated focal adhesion disassembly requires FAK. (A) Representative images of time-lapse video microscopy analysis showing the time elapsed between the appearance and dissolution of observed FAs (Arrow denotes focal adhesions) in MCF7 cells becotransfected with either pMNDUS vector or pMNDUS-HPIP along with DsRed-zyxin plasmids and treated or untreated with F14, FAK-specific inhibitor (10 µM for 6 hr) (left). (B) Western blot analysis showing overexpression of Flag-HPIP (pMNDUS-HPIP) in MCF7 cells. C) Effect of F14 on FAK activation. Transfected cells were treated with (20 µm) F14 for 16 hrs and analysed by westernblotting using indicated antibodies. (D) Quantitation of focal adhesion disassembly time, 20-30 focal adhesions were analysed from 4-6 different cells as described previously (Webb *et al*., 2004). The results were expressed as means ±standard deviation, and differences tween groups were analyzed by one-way ANOVA.

2.4.6 HPIP modulates microtubule-dependent focal adhesion disassembly

Since HPIP is a microtubule binding protein (Manavathi *et al*., 2006) and microtubule dynamics together with FAK activation are critical for focal adhesion disassembly (Ezratty *et al*., 2005), here we speculated the role for HPIP in microtubule-dependent focal adhesion dynamics. As previously reported, serum starved cells had relatively few focal adhesions (FAs) (~6% of cells have more than 5 focal adhesions; (Figure 19A-B) (Scolz *et al*., 2012). In HPIP knockdown cells, we observed a dramatic increase in the number of FAs present as compared to control cells (Figure 19A-B) indicating a markedly reduced FA disassembly in HPIP knockdown cells. Next we examined whether HPIP promotes microtubule-dependent FA disassembly by microtubule regrowth assay in MDA-MD231 cells. Following nocodazole treatment, both control and HPIP knockdown cells accumulated FAs to a similar degree (Figure 19C-D). In contrast, following nocodazole washout and 30 minutes of microtubule regrowth, the number of cells containing FAs (> 5) was significantly reduced (from ~70% to 22%, ~3.1 fold) compared to HPIP

knockdown cells (~65% to 40%, ~1.6 fold) (Figure 19C-D), indicating that HPIP may control microtubule-dependent focal adhesion disassembly.

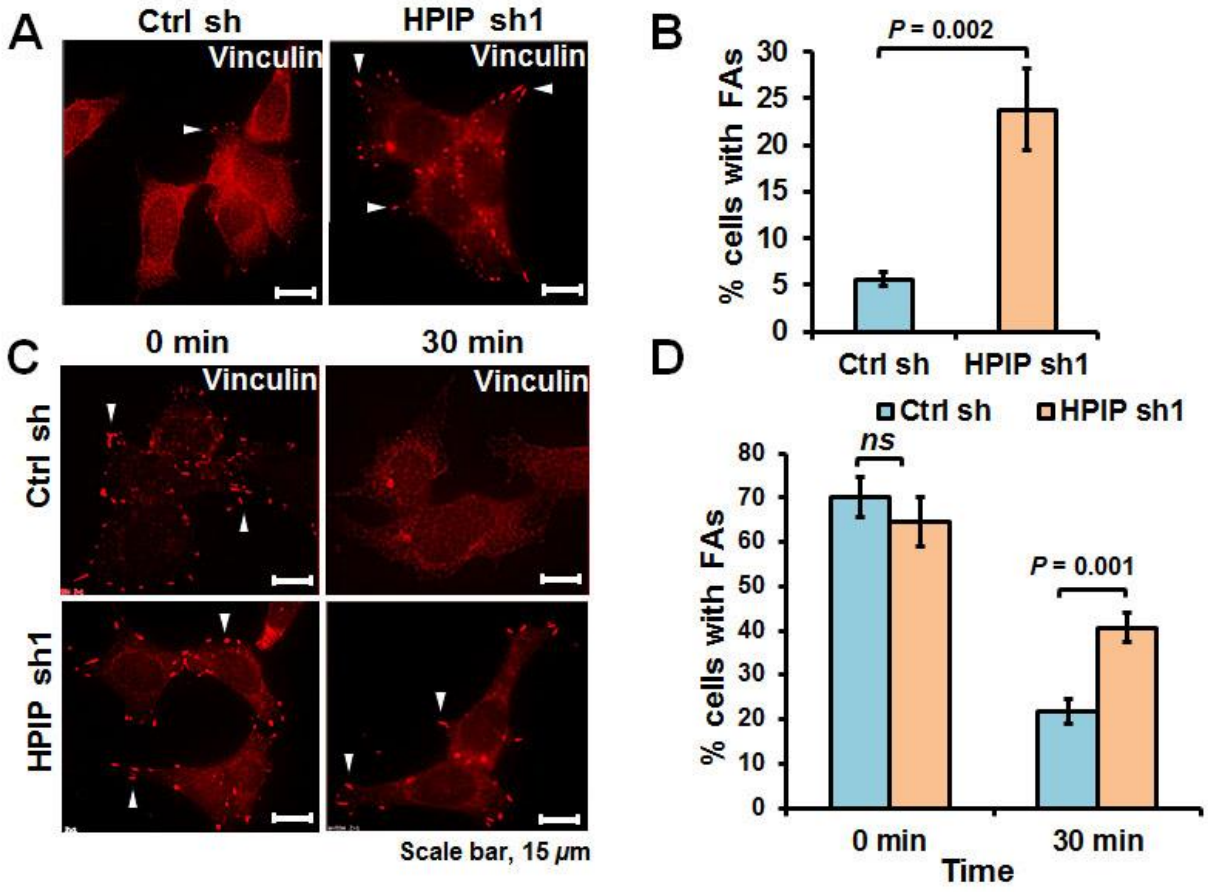


Figure 19. HPIP is required for microtubule-induced focal adhesion disassembly. (A) Effect of HPIP knockdown on focal adhesion number following serum starvation for 48 hours in MDA-MB231 cells that stably expressing either control sh or HPIP sh1. Representative immunofluorescence images of vinculin (red dots at cell periphery indicated by white arrow heads) in MDA-MB231 cells are shown. (B) Quantitative data of cells have more than 5 focal adhesions are counted and plotted as indicated. ns, no significance. (C) MDA-MB231 cells stably expressing either control sh or HPIP sh1 grown on fibronectin were left untreated or treated with nocodazole (10 μM) for 4 hours. The drug was then washed out and microtubules were allowed to regrow as indicated. Cells were fixed, immunostained for Vinculin and analysed by DeltaVision Elite deconvolution microscopy. (D) Cells have more than 5 focal adhesions are counted and plotted as indicated.

2.4.7 HPIP activates calpain2 through MAPK-mediated phosphorylation

Calpain proteases play crucial role in cell migration as they regulate focal adhesion disassembly by targeted cleavage of several focal adhesion proteins, including talin (Glading *et al*., 2002; Franco *et al*., 2004). Further it has been reported that calpain2 phosphorylation by MAPK is required for its activation (Glading *et al*., 2004). We have previously shown that HPIP activates MAPK in breast cancer cells (Manavathi *et al*., 2006). Based on these findings, we hypothesized that HPIP-mediated MAPK activation may also impact on calpain2 phosphorylation and its activation. Consistent with this notion, our *in vivo* phosphorylation assay showed decreased calpain2 phosphorylation upon HPIP knockdown (Figure 20A). As shown in Figure 20B, MAPK activation is decreased upon HPIP knockdown in MCF7 cells and as a result reduced talin proteolysis. Conversely, HPIP overexpression in MDA-MB231 cells enhanced talin proteolysis compared to control cells (Figure 20C). Together these results suggest that HPIP assist in calpain2-mediated talin proteolysis partly by calpain2 activation through MAPK signaling.

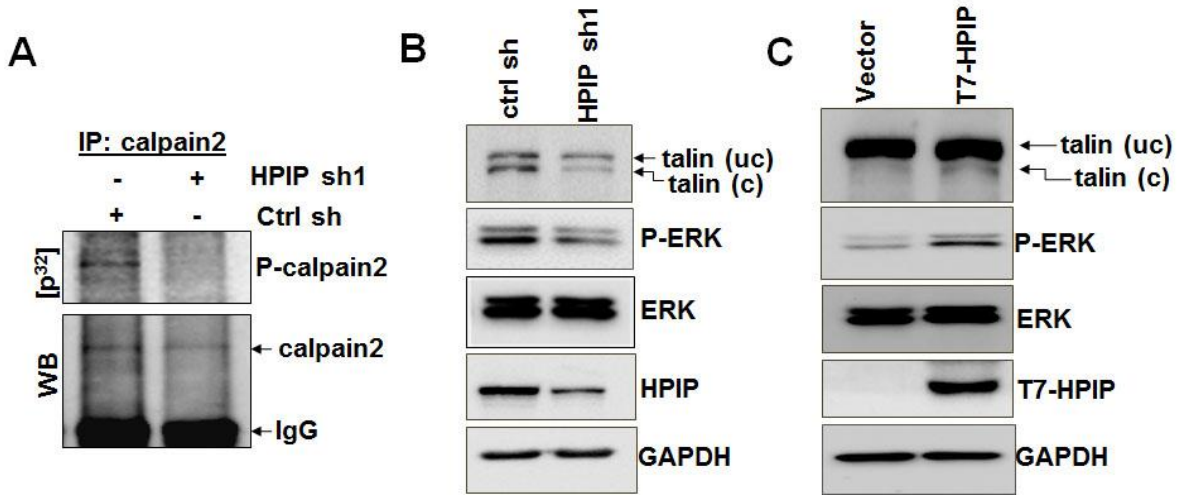


Figure 20. HPIP mediates calpain2 activation. (A) Effect of HPIP knockdown on *in vivo* phosphorylation of calpain2 in MDA-MB231 cells. Extracts of MDA-MB231 cells stably expressing either control sh or HPIP sh1

grown in phosphate-free DMEM containing [p^{32}] orthophosphate were subjected to immunoprecipitation using calpain2 antibody. Following Western transfer, membrane was exposed to phospho imager cassette and autoradiography was carried out. P-calpain2, phospho-calpain2 (B) Effect of HPIP knockdown on MAPK activation and talin proteolysis in MCF7 cells. Extracts of MCF7 cells stably expressing either control-sh or HPIP sh1 were subjected to Western blotting and probed with indicated antibodies. (C) Similarly, the effect of HPIP ectopic expression on MAPK activation and talin proteolysis in MDA-MB231 cells. Extracts of MDA-MB231 cells transiently expressing either control vector or T7-HPIP were subjected to Western blotting and probed with indicated antibodies. uc-uncleaved; c-cleaved.

2.4.8 HPIP is a substrate of calpain2

Interestingly, when cells were treated with calpain2 inhibitor ALLN, we noticed strikingly increased HPIP levels in MDA-MB231 cells (Figure 21A). Consistent with previously reported cell lines, ALLN also inhibited calpain2-mediated FAK proteolysis in MDA-MB231 cells (Figure 21B). Further coimmunoprecipitation analysis showed that HPIP interacts with calpain2 (Figure 21C, D) indicating HPIP could be a potential substrate for this protease. To check this possibility, we performed an *in vitro* calpain2 proteolysis assay using recombinant calpain2 as enzyme and immunoprecipitated GFP-HPIP (N-terminal tag) from HEK293T cell lysate as a substrate. As shown in Figure 22A (ponceau blot), calpain2 is capable of cleaving HPIP in the presence of 1 mM $CaCl_2$ thereby releasing a protein band at ~80 kDa (4^{th} lane, left). Immunoblot probing with HPIP antibody (the antigenic epitope is located in HPIP between 18–141 aa) did not detect HPIP (lane 4; middle), however probing with anti-GFP detected ~45 kDa protein band/s (MW of GFP, 27 kDa plus N-terminal HPIP, ~18 kDa; right). In consistent with these results, bacterial purified GST-HPIP was also proteolysed by calpain2 (Figure 22B) indicating that calpain2 binding site in HPIP is located at the N-terminus of the protein. Calpain2 cleavage site in HPIP was predicted using bioinformatics analysis, as expected cleavage site was located

in N-terminus region of HPIP protein (Figure 22C). Together these results suggest that HPIP is a substrate of calpain2 in cancer cells.

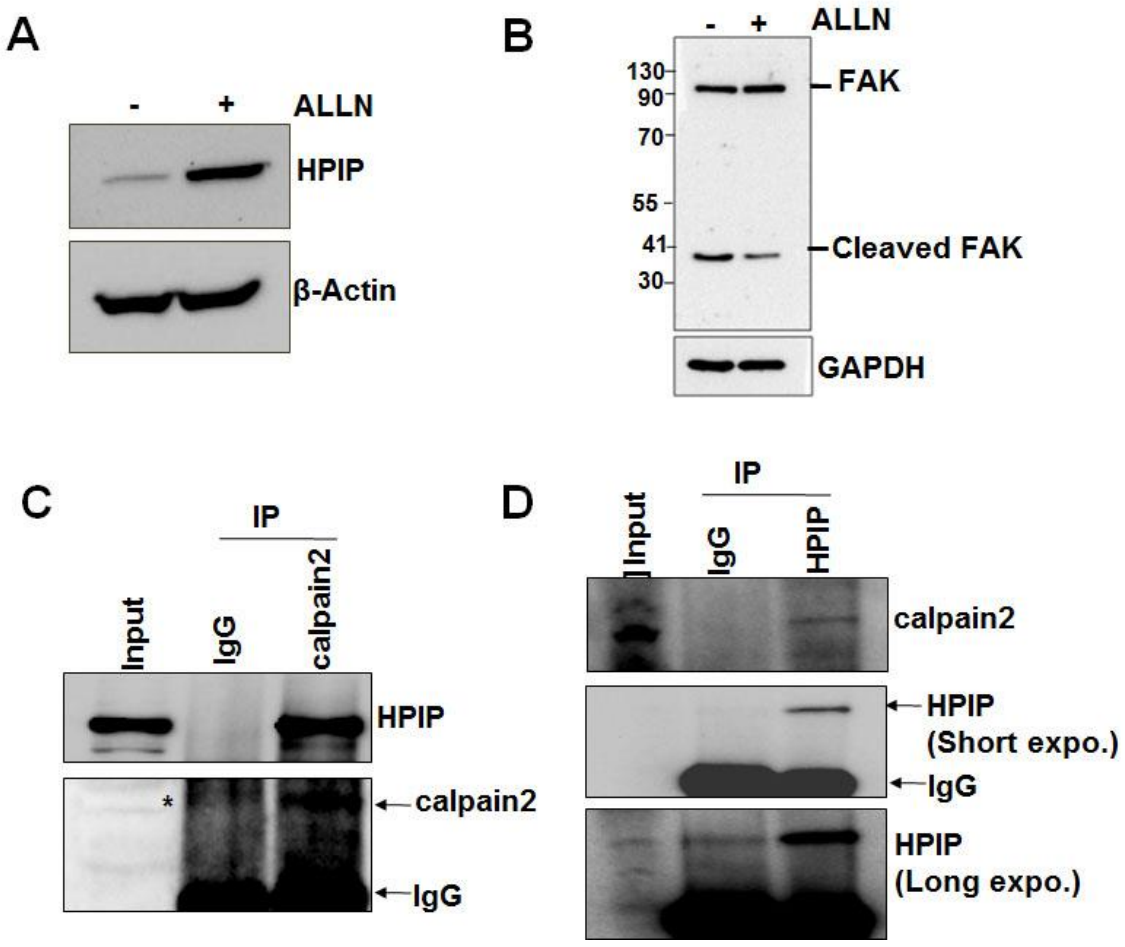


Figure 21. HPIP interacts with calpain2. (A) Western blot analysis showing the effect of calpain2 inhibitor, ALLN, on HPIP levels in MDA-MB231 cells. (B) Western blot analysis showing the effect of calpain2 inhibitor ALLN (20 µM, 18 hrs) on FAK proteolysis in MDA-MB231 cells. Anti-FAK antibody (Santa Cruz Biotechnology, USA; clone C-20, Cat No: sc-558, antibody specific to C-terminal FAK) could detect unproteolysed (~115 kDa) as well as proteolysed fragments (~35 kDa) of FAK in ALLN untreated sample (lane 1). (C) Coimmunoprecipitation (Co-IP) analysis demonstrating interaction of HPIP with calpain2 in MDA-MB231 cells. Extracts from MDA-MB231 cells were immunoprecipitated with calpain2 antibody and blotted as indicated. The asterisk indicates calpian2 protein

band. (D) Conversely, extracts from MDA-MB231 cells were immunoprecipitated with HPIP antibody and blotted as indicated.

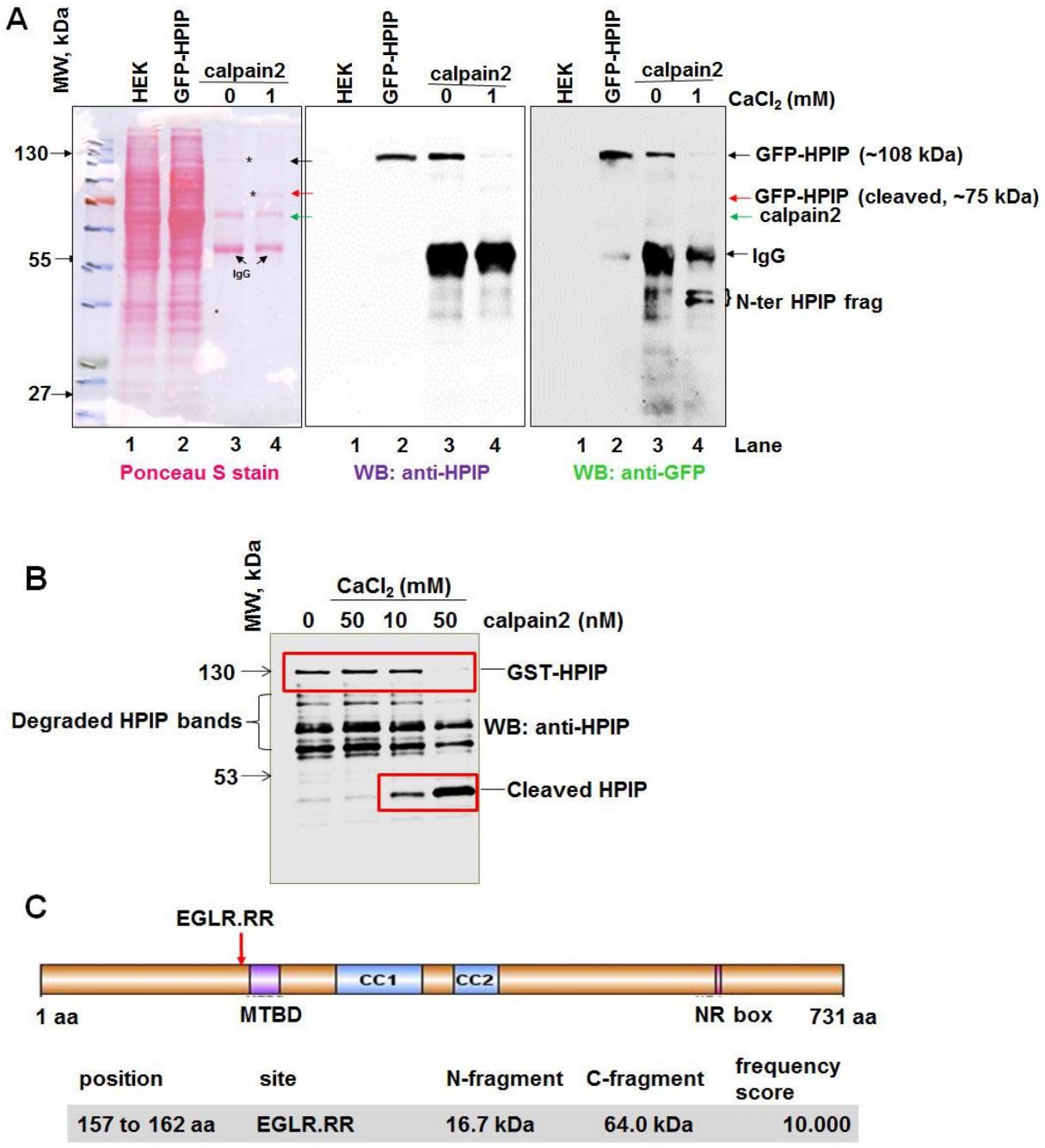


Figure 22. HPIP is a substrate of calpain2. (A) *In vitro* calpain2 cleave assay. Immunoprecipitated GFP-HPIP from HEK293T cells was incubated calpain2 in presence or absence of $CaCl_2$ (1 mM). After incubation at 30°C for 30

minutes, reaction mixtures were subjected to Western blot analysis and blotted as indicated. *, uncleaved GFP-HPIP; **, cleaved GFP-HPIP; ***, calpain2. (B) *In vitro* calpain cleavage assay showing the proteolysis of HPIP by calpain2. Bacterial-purified GST-HPIP (5 µg) was incubated in calpain cleavage buffer with the indicated concentrations of purified calpain2 at 30°C for 30 min in presence or absence of $CaCl_2$(1 mM). Western blot analysis detects approx. 45 kDa (GST, 27 kDa plus N-terminal HPIP, 18 kDa) protein fragment released from GST-HPIP (FL) (GST, 27 kDa plus full length HPIP, 80 kDa). The asterisk denotes degraded protein products of HPIP during purification/storage. (C) Bioinformatics analysis and prediction of calpain2 cleavage site in N-terminus region of HPIP. MTBD, microtubule binding domain, NR box, nuclear receptor box, CC, coiled coil domains.

2.5 DISCUSSION

Locomotion is one of the adaptive mechanisms for better survival of living organisms. It is a property that cancer cells also explore to spread to distant tissues for inhabitation and survival. We and others demonstrated that HPIP has the potential to regulate extranuclear genomic estrogen receptor alpha (ERα) signals by acting as a scaffold protein. It recruits cell survival signaling molecules such as Src and PI3K along with ERα to microtubule network resulting the formation of a 'signalosome' which further led to activation of AKT and ERK1/2 kinases. The activated kinases are attributed for the HPIP-mediated cell proliferation, migration, anchorage independent growth and invasion of cancer cells (Manavathi *et al*., 2006; Wang *et al*., 2008). However, the precise mechanism by which HPIP regulates the cell migration and invasion remains elusive. In our study, we discovered the regulatory mechanism by which it controls cell migration using breast cancer cell lines such as MCF7 and MDA-MB231 as model systems.

Our investigation identified the mechanism by which HPIP regulates cancer cell adhesion and migration. We established cell lines that altered their migratory behaviour in response to HPIP overexpression or knockdown. Using these cells we identified the following key regulatory mechanisms. 1) HPIP is recruited to focal adhesion sites where it regulates focal adhesion turnover and migration. 2) At focal adhesion sites, HPIP interacts with focal adhesion complex containing FAK using its C-terminal domain, activates FAK and regulates cell migration in FAK-dependent manner. 3) To mediate turnover of FAs through disassembly of focal adhesion complex, HPIP activates calpain2 through MAPK mediated phosphorylation that ensures the talin proteolysis. 4) Calpain2 scaffolding by HPIP leads to HPIP proteolysis (a novel substrate for calpain2) and thereby inhibition of FAK activation and desensitising FA disassembly signal (Figure 23).

The Oncomine data along with other data sets has revealed high levels of HPIP expression in advanced stages of breast cancer patients. Also our immunohistochemistry studies in IDC patient samples determined high HPIP protein levels in cancerous tissues as compared to the surrounding normal tissues. Further we found a positive correlation of elevated levels of HPIP expression with poor patient prognosis. These findings implied the intimate association of high level expression of HPIP with invasive phenotype and raised the possibility of its role in regulating cell motility and invasion. In support of this idea, the in vitro assays measuring the rate of cell adhesion and cell migration clearly indicated that increased levels of HPIP protein is commensurate with decreased affinity of the cells to bind to the substratum, change in cell morphology and increased cell migration in MDA-MB231 cells. Recently, human microRNA-148a (miR-148a), was reported in hepatocarcinoma cells and animal models to down regulate HPIP expression, and to inhibit the cell migration and invasion (Xu *et al*., 2013). Furthermore, methylation-induced silencing of miR-148a was shown in several cancers that include breast, colon, lung, neck and pancreatic cancers, and associated with tumor metastasis (Lujambio *et al*., 2008; Hanoun *et al*., 2010). The above results established a firm ground of evidence about the possible role of HPIP in cell migration. Also the increased HPIP expression pattern in small groups of tumor cells at the edge of IDC tissue sections clearly indicated its involvement in cell migration and dissemination. However, whether the role of HPIP in cell migration is because of PI3K/AKT/Src/MAPK nexus or if it has any other unique role needs to be established.

Interestingly, our localization studies of HPIP revealed its presence in the cell periphery at the sites resembling that of FAs. Furthermore, these studies revealed the engagement of HPIP with other key molecular players of FA such as Paxillin, FAK, Vinculin and Zyxin in FAs. These

result together point out to a novel role for HPIP in FAs dynamics which may be associated with its role in cell migration.

As the focal adhesion are dynamic in nature, the exact number and the precise stoichiometry of the proteins involved in FA formation is largely unknown. Focal adhesions regulate cell migration through their dynamic assembly and disassembly (Wozniak *et al*., 2004; Geiger *et al*., 2009). Particularly, the activation of FAK is crucial for cell adhesion and migration (Mitra *et al*., 2005). FAK Y397 phosphorylation has been identified previously as a marker of focal adhesion signal activation (Calalb *et al*., 1995; Schaller *et al*., 2010). We and others have showed that cellular levels of HPIP are able to modulate cancer progression by influencing cancer cell migration (Manavathi *et al*., 2006; Xu *et al*., 2013). The key molecular player regulating the FA dynamics is FAK and an intriguing question is that whether these two proteins bear any significant relationship. This question was addressed through domain mapping studies. Despite HPIP contained a typical integrin ligand binding domain i.e, RGD motif (R, arginine, G, glycine and D, aspartic acid), the C-terminal domain of HPIP, but not RGD, directly interacted with kinase domain of FAK, and increased autophosphorylation of FAK at Y397 residue which is the activation core of this mighty protein. In our study, by knockdown of endogenous HPIP expression, phosphorylation of FAK at Y397 was clearly suppressed and conversely overexpression of HPIP was associated with increased Y397 phospho-FAK. Further, all C-terminal deletion mutants of HPIP lost their ability to activate FAK and localization to focal adhesions suggesting that C-terminal region spanning 583-731 amino acids of HPIP was crucial for HPIP-mediated FAK activation and its localization to FAs. Hence, we are speculating that the interaction of HPIP with FAK may bring about the conformational changes in FAK resulting in activation of the kinase or may be this interaction open up the inhibitory domains that hinder the

activation of FAK in its native form. The inhibition of FAK using FAK-specific inhibitors or by knockdown approach led to decease in HPIP-mediated FA disassembly and cell migration indicating that HPIP-mediated cell migration requires FAK.

The studies with HPIP shRNA in breast cancer cell lines revealed that the size of the FAs was increased significantly upon HPIP knockdown. The increase in size of FAs is a clear indicator of deregulated kinetics of FA disassembly (Ilic *et al*., 1995; Wu *et al*., 2008). Further, Time lapse video microscopy analysis of MCF7 cell lines using zyxin as a FA marker further revealed that loss of HPIP expression lead to reduced motility, delayed FAs disassembly and decreased turnover rate. Focal adhesions regulate cell migration through a dynamic process that involves disassembly of the macromolecules. Several disassembly factors and mechanisms have been proposed to explain how the focal adhesion disassembly is mediated. For instances, ZF21 regulates cell migration by modulating FAK Y397 phosphorylation (Nagano *et al*., 2010). Whereas G2 and S phase-expressed-1 protein (GTSE1), a microtubule TIP-end binding protein, regulates cell migration by modulating focal adhesion disassembly in an EB1, another microtubule TIP-end binding protein, dependent manner (Scolz *et al*., 2012). Similarly, ACF7, actin and microtubule binding protein, has been shown to play role in FA disassembly (Wu *et al*., 2008). Interestingly, HPIP is also a microtubule-binding protein that directly interacts and activates FAK to modulate the microtubule-induced focal adhesion disassembly and cell migration. Further, *in vitro* microtubule polymerization assay indicated that HPIP promotes tubulin polymerization (data not shown).

Our investigations with nocodazole, a microtubule poison drug, washout followed by microtubule regrowth indicated that cells lacking HPIP decreased FA turnover and migration implying that HPIP is essential for microtubule-mediated FA turnover. Previous studies shown

that HPIP acts as a scaffold protein and recruits several proteins to the microtubule network Manavathi *et al*., 2006). Accordingly, binding of HPIP with microtubules may provide an ideal specified platform, wherein the rich amino acid diversity of HPIP acts as a 'protein hub' to several proteins to halt upon and perform their activities in a spatial and temporal manner. Together this data suggests that the HPIP-mediated focal adhesion disassembly could be partly through the activation of FAK and also the modulation of microtubule dynamics.

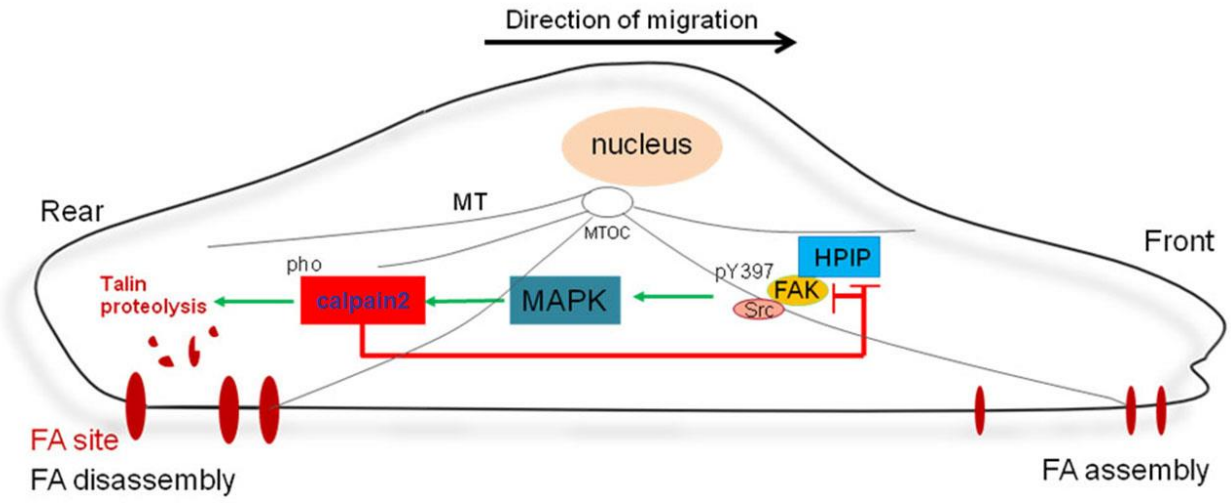


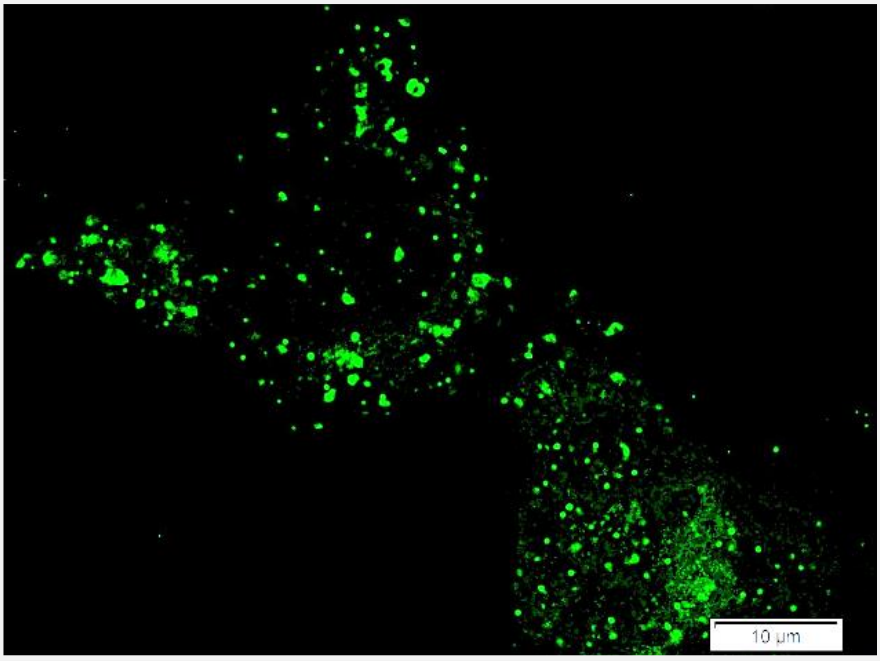
Figure 23. Model illustrating the regulation of cell motility by HPIP signaling. HPIP activates FAK through direct interaction. Src is recruited to FA site following FAK activation. HPIP promotes focal adhesion disassembly via activation of MAPK which subsequently trigger talin proteolysis though calpain2 resulting the focal adhesion disassembly at rear edge and cell migration. The activated calpain2 in turn cleaves HPIP and FAK to desensitize the cell migration signal suggesting a negative feedback mechanism. MT, microtubule; MTOC, microtubule originating center; CLPN2, calpain2. The black arrow indicates the direction of cell migration. Green arrows denotes the activation, whereas (-|) in red color indicates inhibition. FA assembly and disassembly takes place at the front and rear ends of the cell, respectively.

Another intriguing mechanism for HPIP-mediated cell migration here we show is that HPIP-mediated calpain2 activation through MAPK pathway. Because MAPK phosphorylation of calpain2 leads to its activation (Glading *et al*., 2004) and, we and others have shown the activation of MAPK by HPIP (Manavathi *et al*., 2006; Wang *et al*., 2008), we observed decreased calpian2 phosphorylation and talin proteolysis upon HPIP knockdown, implying that calpain2 activation through MAPK and subsequent talin proteolysis is partly dependent on HPIP (Figure 23). Several proteins that participate in focal adhesion disassembly including FAK, paxillin and spectrin have been identified as calpain2 substrates and resistant mutants of these substrates failed to regulate adhesion dynamics (Franco *et al*., 2004; Chan *et al*., 2010).

Interestingly we also found that HPIP is a new substrate of calpain2. HPIP proteolysis by calpain2 provides a negative feedback mechanism to desensitize the cell migration signal. The activation of calpain2 results in proteolysis of talin and initiation of disassembly events, at the same time cleavage of HPIP by calpain2 which may perhaps results in desensitization of cell migration signal. This mechanism provides the key insight into the role of HPIP wherein it may acts as an 'epicenter' for the convergence of different pathways and channelizes them to bring about a synergistic effect. Thus breakup of HPIP though calpain2-mediated proteolysis might lead to dismantling of the essential components of FA, their turnover and ultimately the cell migration. Further investigations are under way in our laboratory to identify the calpain2 cleavage site in HPIP. In conclusion, HPIP is shown to be an important player in focal adhesion turnover and cell migration. Further investigation of the additional mechanisms of HPIP and identification of new interacting proteins may serves as an attractive candidate for cancer therapy.

CHAPTER III

HPIP signaling in endosome–mediated cell migration



3.1 Rationale and Hypothesis: Previously we have reported that HPIP function as an adaptor/scaffold protein having multiple domains that integrate various signaling proteins including estrogen receptor, PI3K, Src and microtubules (Manavathi *et al*., 2006). After establishing the role of HPIP-FAK-calpain2 pathway in cell migration (Bugide et al., 2014), we sought to further understand the precise mechanism that underlie in HPIP-dependent focal adhesion signalling and cell migration. Therefore, we intend to identify novel interacting partners of HPIP. Through *in silico* analysis, we identified two coiled coil domains (CC1- 270-341 aa and CC2- 370-415 aa) in HPIP protein and they were well conserved across the species, indicating the importance of these domains in HPIP-mediated cellular functions.

Using CC1 and CC2 domains of HPIP, we identified RUFY3 is a HPIP interacting partner. Recent studies have implicated a role for RUFY3 in cell migration (Wang *et al*., 2015). Furthermore, RUFY3 has been shown to interact with Rab5, an early endosomal marker (Yoshida *et al*., 2010). Emerging studies showed a role for Rab5 in focal adhesion disassembly, cell migration and invasion (Torres *et al*., 2008; Torres *et al*., 2010; Diaz *et al*., 2014). Along with these reports, our immunofluorescence analysis revealed that HPIP forms cytoplasmic speckles like structures in OAW42 and MDA-MB231 cells which appear to be endosomes (Figure 24). This led us to investigate the additional mechanisms that underlie in HPIP-mediated cell migration involving RUFY3 and Rab5.

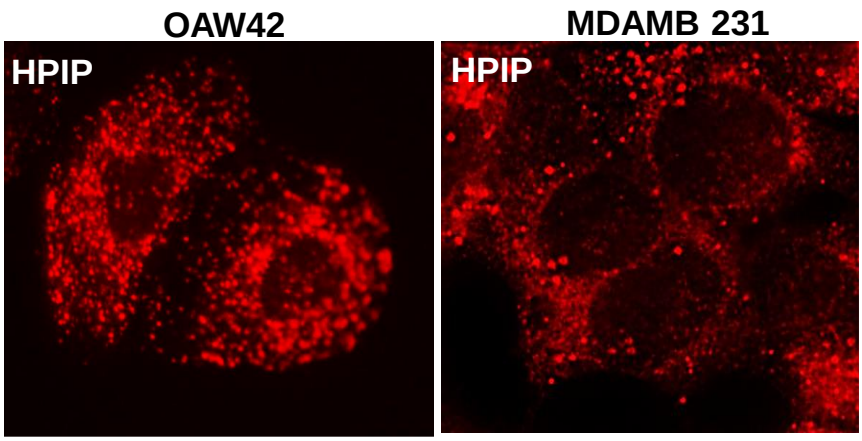


Figure 24. Immunoflourescence analysis showing endosomal like localization of HPIP (red) in indicated cancer cells.

3.2 Introduction

3.2.1 Endocytosis: Endocytosis is a process by which cells absorb molecules such as fluid, proteins, macromolecules, receptor–ligand complexes, lipids, nutrients, extracellular–matrix components, cell-debris, bacteria, viruses, etc, by formation of vesicles and vacuoles through membrane fission. It is a complex cellular program that involves in regulation of numerous pathways in the cell by sorting, recycling, storing, activating, silencing and degrading of substances and receptors. It is deeply associated with signaling, growth, cell dynamics, regulation, defense and it almost linked to all aspects of cell life and disease (Huotari *et al*., 2011). Furthermore, it plays key role in mitosis, antigen presentation, and cell migration (Doherty *et al*., 2009).

Over the years, numerous types of vesicular endocytosis have been discovered, in which clathrin-mediated endocytosis (CME) is the most extensively studied and well-characterized mechanism (Mills *et al*., 2007). CME mainly involves in transport of low density lipoprotein, nutrients, pathogens, transferrin, growth factors, antibodies, receptors, etc. Clathrin and other adapter proteins such as AP2 and epsin play pivotal role in this process. Other major endocytosis processes are phagocytosis and pinacocytosis, which are mainly involved in internalizing larger size of fluids and substances (larger than around 0.75 μm). Recent findings suggest the involvement non-classical endocytic pathways in lipid rafts and rafts associated proteins transport (Nichols *et al*., 2001; Pelkmans *et al*., 2001; Parton *et al*., 2007). Caveolae is the most common reported non-clathrin-endocytosis, which exist on the surface of many, but not all cell types (Parton *et al*., 2007). They consist of the cholesterol-binding protein caveolin (Vip21) with a bilayer enriched in cholesterol and glycolipids. It is cholesterol dependent process as depletion of cholesterol inhibits it.

After endocytosis, receptors enter into the early endosome where they are sorted, and they are either recycled back to the plasma membrane or degraded by late endosome/lysosomal pathway. Early edosomal markers such as Rab5 and early endosome antigen 1 (EEA1) are crucial for generation and maintenance of early endosomes (Christoforidis *et al*., 1999). Normally, Clathrin-dependent cargoes were recycle back to the plasma membrane through a rapid recycling pathways that requires Rab35 and Rab4, Rab10, Rab11, Rab22a (Grant *et al*., 2009). Some endocytic cargoes in the early endosome selected by ESCRT machinery will form multivesicular bodies (MLV) and transported to late endosomes and finally degraded through lysosome machinery.

3.2.2 Endocytosis and cancer: Endocytosis regulates growth factor signaling by internalization of target receptors to different fates: recycling to the plasma membrane or degradation, thereby contributes to the net signaling output of a cell (Di Guglielmo *et al*., 2003; Sigismund *et al*., 2008). Endocyte trafficking is regulated by the Ras associated binding (Rab) family of small GTPases, and they function as molecular switches of vesicular transport in cells (Chia *et al*., 2009; Stenmark *et al*., 2009). In recent years, more attention has been paid to the role of Rab GTPases in cancer progression, and various members of the Rab family proteins such as Rab11, Rab21 and Rab25 have been shown to be overexpressed in various cancer types including breast and ovarian cancers (Cheng *et al*., 2004; Pellinen *et al*., 2006; Stenmark *et al*., 2009; Subramani *et al*., 2010). Because Rab GTPases play key role in integrin trafficking and recycling, deregulation of Rab GTPases is closely associated with cancer development and progression (Cheng *et al*., 2004; Pellinen *et al*., 2006; Stenmark *et al*., 2009; Subramani *et al*., 2010). In addition to this, other endocytic proteins such as huntingtin interacting protein 1 (HIP1) and

caveolin-1 overexpression is also associated with cancer progression (Williams *et al*., 2005; Lanzetti *et al*., 2008).

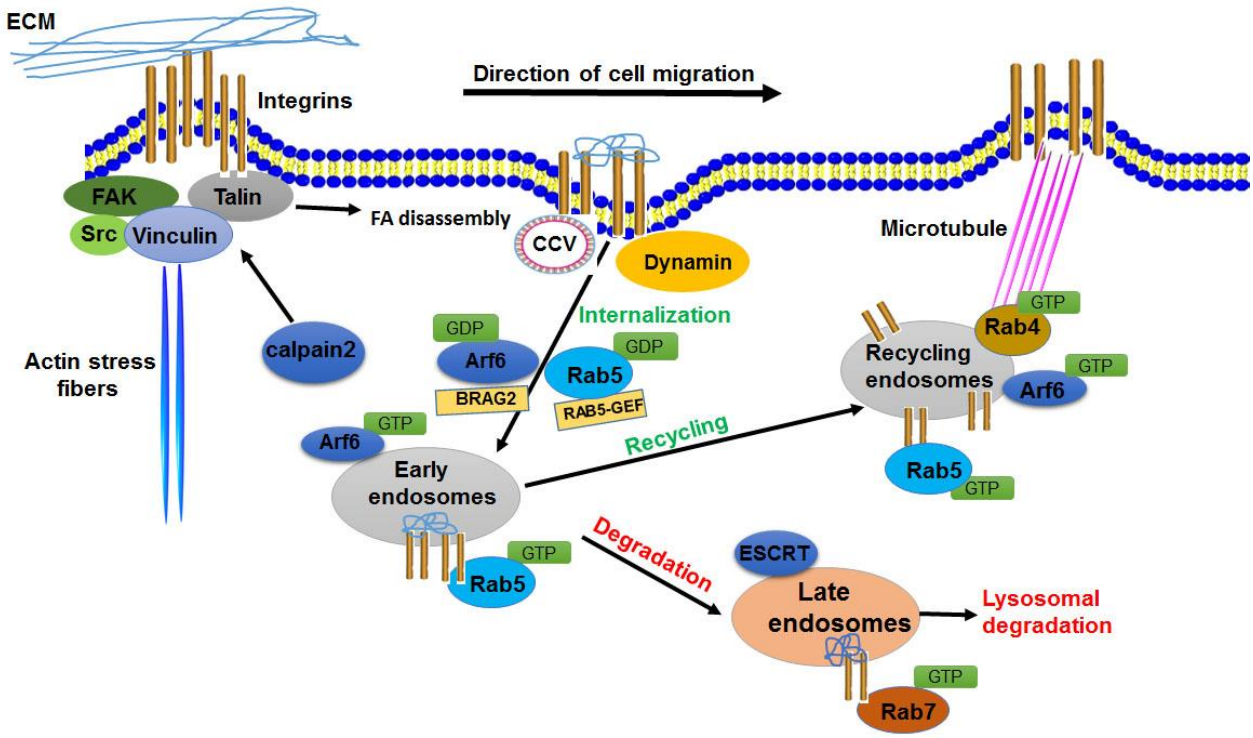


Figure 25: Illustrated model for endosome-mediated integrin trafficking: Integrin internalization and focal adhesion disassembly is a coupled process. Post focal adhesion disassembly, integrins were internalized by either dynamin or CCV mediated endocytosis. Rab5 and Arf6 is activated by GEFs, and activated Rab5 play key role in endosome formation. Internalized integrins have different fates, by growth factor stimulation they will recycle back to plasma membrane by short/long loop recycling process that involves activated Rab5, Rab11, Rab21 and Rab4. Increased recycling of integrin to the plasma membrane has recently been shown to be facilitated by Akt-GSK3β-pathway dependent stabilization of microtubules. Some integrins were degraded through Rab7 dependent lysosomal degradation.

Integrin trafficking and recycling by endocytosis is well known to regulate the cell migration of many cells (Figure 25), including fibroblasts and several cancer cell lines (Sabe *et*

al., 2003; Caswell *et al*., 2009). The endocytosis of integrins enhances the focal adhesion disassembly and detachment from extra cellular matrix (ECM) at the rear end, and thereby promotes cell migration (Ezratty *et al*., 2005). Several Rab GTPases are known to regulate endocytosis of integrins and intracellular trafficking (Caswell *et al*., 2008; Caswell *et al*., 2009). Rab5 and Rab21 interacts with β1-integrin and control its endosomal trafficking in MDA-MB-231 cells (Pellinen *et al*., 2006). The roles of Rab GTPases in tumorogenesis are closely related to integrin recycling. For example, Rab25 contributes to tumorogenesis by directing the integrin-recycling vesicles and thereby promotes cell migration (Caswell *et al*., 2007). In another study, Rab21 is shown to promote cell adhesion and migration through integrin endocytosis (Pellinen *et al*., 2006).

3.2.3 Rab5: Amongst the small Rab GTPase proteins, Rab5 is extensively studied in the context of endocytosis, as it participates in a variety of cellular functions such as early endosome fusion, vesicle formation, early-to-late endosome maturation and cell migration (Christoforidis *et al*., 1999; Rubino *et al*., 2000; Hoepfner *et al*., 2005; Rink *et al*., 2005). Therefore, RAB5 is considered as a master regulator of early endosome dynamics. Rab5 is located on chromosome 3 at position p24.3 and *Rab5* cDNA encodes a 215 amino acid residue protein that have close homology with other Ras and Rab family proteins. Rab5 has conserved GTP/GDP binding motif, mutations in this region influence nucleotide binding and GTP hydrolysis. Rab5 binding to GDP/GTP nucleotides brings up conformational changes in protein structure that will influence effector molecules binding to Rab5. Rab5 C-terminus CCXX has been shown to undergo isoprenylation, deletion of this domain completely abolishes Rab5 activity due to its inability to localize to membrane, indicating that isoprenylation is key for Rab5 membrane localization and biological functions (Figure 26) (Li *et al*., 1993). In addition to its role in

endocytosis, recent evidences implicate the role of Rab5 in other processes, such as cell adhesion and migration (Torres *et al*., 2011). Rab5 also regulates actin remodeling, as it recruits the Rac1-GEF (guanine exchange factor) factor TIAM1 to early endosomes, and this leads to Rac1 activation (Rac1-GTP) (Lanzetti *et al*., 2004; Palamidessi *et al*., 2008; Torres *et al*., 2010). Additional reports suggest a role for Rab5 in lamellipodia formation (Palamidessi *et al*., 2008; Torres *et al*., 2008; Torres *et al*., 2010).

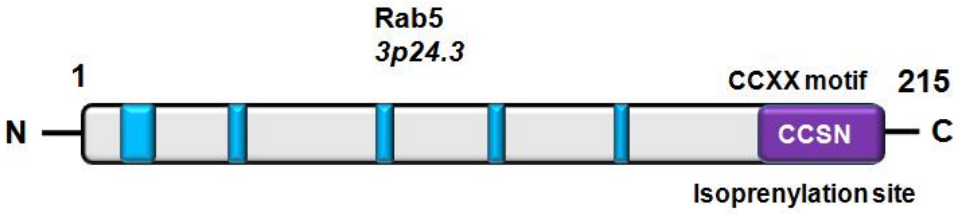


Figure 26: Schematic representation indicating the domain structure of Rab5 (215 aa). The functionally important domains like GTP/GDP binding domains (blue color) and CCXX motif are shown. Rab5 is located on chromosome 3p24.3. Isopneylation site, CCSN (CCXX motif), spans between amino acids, 212-215. GTP/GDP binding sites, span between amino acids, 27-35, 46-52, 75-79, 133-136 and 153-155 aa respectively.

Recent studies implicate a role for Rab5 in cell migration and invasion through the regulation of focal adhesion disassembly in tumor cells (Mendoza *et al*., 2013). Rab5 promotes focal adhesion disassembly by interacting with focal adhesion components such as Paxillin, Vinculin, FAK, and β1 integrin in migrating cells (Mendoza *et al*., 2013). Rab5 knockdown by Rab5-shRNA leads to a significant reduction in focal adhesion disassembly and cell migration, whereas reintroduction of wild type Rab5 but not Rab5 S34N (high affinity for GDP), a dominant negative mutant, recovered the migratory phenotype (Mendoza *et al*., 2013), suggesting that Rab5-GTP loading is important for focal adhesion disassembly and cell

migration. This is in support of previous reports indicating the key role for Rab5-GTP in cell migration (Palamidessi *et al*., 2008; Torres *et al*., 2010; Pellinen *et al*., 2006). Furthermore, the interaction of β1 integrins and Rab5 was also dependent on GTP loading, because wild-type Rab5 and the Rab5/Q79L (GTPase deficient, active conformation), but not Rab5/S34N (high affinity for GDP, inactive state), were found to co-immunoprecipitate with β1 integrin (Torres *et al*., 2010), suggesting that Rab5-GTP, but not Rab5-GDP, promotes FA disassembly. Noteworthy, a recent study showed that RN-Tre, which is a Rab-GTPase activating protein (GAP), inhibits cell migration which is based on its ability to delay focal adhesion dynamics (Palamidessi *et al*., 2013). Most importantly, the effect of RN-Tre on focal adhesion disassembly is mainly due to its GAP activity toward Rab5. In another study it was shown that, Rab5 is required for caspase 8-mediated Rac activation and cell migration (Torres *et al*., 2010). Caspase-8 promotes Rab5 mediated internalization and recycling of β1 integrins by increasing Rab5 GTP loading through interacting with P85 (Torres *et al*., 2008; Torres *et al*., 2010). Thus, it appears that p85α interaction with caspase-8 allows greater accumulation of Rab5-GTP-loading. It fits with a role of p85α as a Rab-GAP, when it is in free form, but not when bound to PI3K or caspase-8.

3.2.4 RUFY3: RUFY3, also known as single axon-related protein 1 (singar1)/KIAA0871/RPIPx, which contain RUN and coiled coil domains and located on chromosome four at position q13.3 (Figure 27). Recent studies implicated the role of RUFY3 in the formation of a single axon in developing neurons (Mori *et al*., 2007). Knockdown of singar1 (RUFY3) and its splicing variant singar2 by RNAi increases surplus axons formation in neurons in a PI3K-dependent manner (Mori *et al*., 2007). Subsequent studies revealed that it interact with constitutive active mutant Rab5Q79L but not with dominant negative Rab5S34N implying that this interaction is

GTP-dependent (Yoshida *et al*., 2010). In a recent study, RUFY3 was shown to interact with Fascin, an actin cytoskeleton protein and controls the actin filament organization at the axonal growth cones (Wei *et al*., 2014). Together these studies suggest an important role for RUFY3 in Rab5 and PI3K–mediated cellular functions.

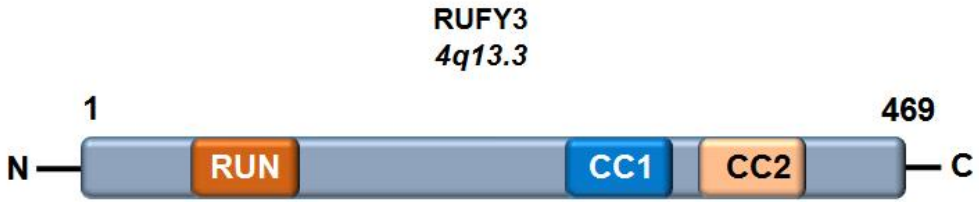


Figure 27: Physical map of RUFY3. The functionally important domains are shown. RUN domain, Coiled coil domain -1 (CC1) and -2 (CC2). Genomic location (4q13.3) and length of RUFY3 (469 aa) are also shown.

3.3 Materials and methods:

3.3.1 Plasmids: Using pBSKII-RUFY3 plasmid (a kind gift from Naoyuki Inagaki, Kagawa University, Japan) as template**,** *RUFY3* gene was amplified using following specific primers; forward primer: 5'- TAGCC*CTCGAG*CCACCATGTCTGCCCTGACGCCT-3' (*Xho*I) and reverse primer: 5'-GATCG*GGATCC*TAATGATGTTTTGGGATCAG-3' (*Bam*HI) and subcloned into pDsRed-N1 vector (Clonetech, USA). Full length and various domains of *RUFY3* gene were PCR amplified using pEGFP-RUFY3 as template with the following specific primers: *RUFY3* full length forward primer: 5'-GCAAA*GAATTC*ATGTCTGCCCTGACGCCTCCGACT–3' (*Eco*RI), reverse primer: 5'-GACCC*CTCGAG*CTAATGATGTTTTGGGATCAGTTT-3' (*Xho*I), RUN domain forward primer: 5'-GCAAA*GAATTC*TCTGACTACGCACCTCTCCAG-3' (*Eco*RI), reverse primer: 5'-GACCC*CTCGAG*CTATTCTCCTTTCATGCAGAAATT-3' (*Xho*I), coiled coil domain forward

primer: 5'-GCAAA*GAATTC*AACAGACATCTGAATGCTACT-3' (*Eco*RI), reverse primer: 5'-GACCC*CTCGAG*CTATTGCTCCAGCTGTTTAATGGT-3' (*Xho*I). The PCR fragments were then subcloned into pET28a vector.

GFP-Rab5 and GFP-EEA1 plasmids were kind gift from Dr.Marino Zerial, Germany (Max Planck Institute of Molecular Cell Biology and Genetics). Full length *Rab5* was cloned into pET28a vector (Novagen, USA) using the following specific primers: forward primer: 5'-GCAAA*GAATTC*ATGGCTAGTCGAGGCGCAACA-3' (*Eco*RI), reverse primer: 5'-GACCC*CTCGAG*CTAGTTACT ACAACACTGATTCCT-3' (*Xho*I).

Coiled coil 1 (CC1) and 2 (CC2) domains of HPIP were generated by PCR amplification using pcDNA-HPIP (full length) as template with the following specific primers. For HPIP-CC1, forward primer: 5'-GACATC*GGATCC*CTTCTGCTGGACAAGCT-3' (*Bam*HI) and reverse primer: 5'-CTAG*CTCGAG*TCAGCCCCGGACACAGTCGG-3' (*Xho*I); for HPIP-CC2 domain, forward primer: 5'-GACATC*GGATCC*GAACTCAGCTTCCTGAA-3' and reverse primer: 5'-CTAG*CTCGAG*TCACTGCAAGCTCCTCTCCA-3'. Respective amplified fragments were subcloned into pGEX4T1 vector (GE Healthcare, United Kindom) and clones were verified by restriction digestion.

The strategy followed to generate HPIP coiled coil mutants HPIPΔCC1 and HPIPΔCC2 is a two-step approach. The first step comprises of amplifying the regions immediately adjacent to the region to be deleted as two separate fragments and the second step involves the ligation of these two separate fragments into mammalian expression vector, pcDNA3.1C. The detailed procedure involving cloning of these deletion mutants is discussed hereunder.

Construction of HPIPΔCC1:

At first instance, we amplified the region from 1-810 bp of HPIP gene (fragment A) by PCR using HPIP full length forward primer (FP): 5'-GACA*GGATCC*TGATGGCCTCCTGCCCA-3' (*Bam*HI) and CC1 reverse primer (RP): 5'-CTAG*GATATC*ACCCATGTTTTGCAG-3' (*Eco*RV). This fragment was subcloned into pcDNA3.1C vector using the same restriction enzyme sites generated clone 1, i.e., HPIP (1-810). In the second step, the region from 1023 to 2196 bp (fragment B) was amplified by PCR using CC1 forward primer: 5'-GACA*GATATC*CCCAGATGGGGTGTG-3' (*Eco*RV) and HPIP full length reverse primer: 5'-CTAG*CTCGAG*TGCCCCGGTGGTGGTG-3' (*Xho*I). The two fragments amplified (A and B), lie immediately adjacent to the CC1 region to be deleted. The fragment B and clone 1 were digested with *Eco*RV and *Xho*I, and ligated to generate HPIPΔCC1 (Figure 28). The overall reading frame of the CC1 deletion mutant of HPIP was later confirmed by pyrosequencing.

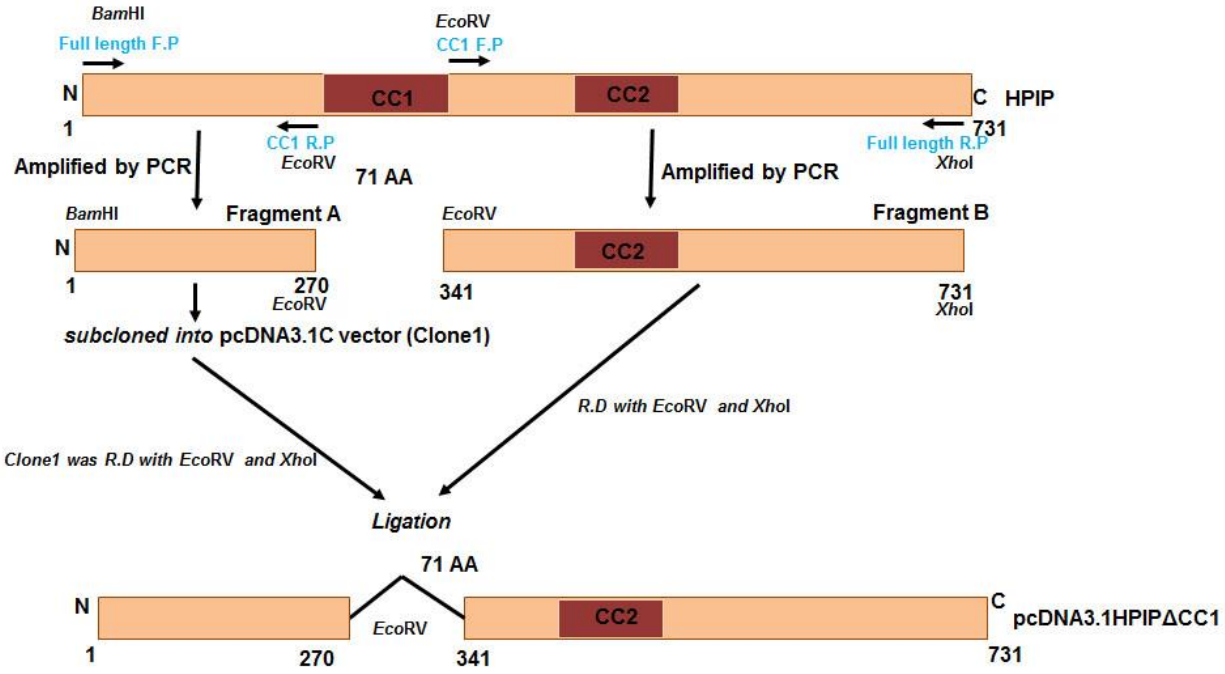


Figure 28: Cloning strategy for construction of HPIPΔCC1 clone. Cloning of HPIPΔCC1 is a two-step approach. In the first step, the region comprises of 1-810 bp which is immediately adjacent to the N-terminus of CC1 region was subcloned into pcDNA3.1C vector. In the second step the region from 1023 to 2196 bp was amplified ligated to clone 1 at *Eco*RV and *Xho*I sites generating HPIPΔCC1. Similar strategy was followed for generation of HPIPΔCC2 clone. R.D, restriction digestion.

Construction of HPIPΔCC2 clone:

To clone CC2 (2079 bp) deletion mutant of HPIP i.e., HPIPΔCC2, we followed similar approach used for HPIPΔCC1. The region of HPIP gene from 1-1100 bp (fragment A) was PCR amplified using HPIP full length forward primer: 5'-GACA*GGATCC*TGATGGCCTCCTGCCCA-3' (*Bam*HI) and CC2 reverse primer: 5'-CTAG*GATATC*GAG TGCCCCGGTGGTGGTG-3' (*Eco*RV) and the same was cloned into pcDNA3.1C vector, generating clone 1. The fragment B comprising the region between 1245-2196 was amplified using CC2 forward primer: 5'-GACA*GATATC*CCAGCCGCGGGGACC-3' (*Eco*RV) and HPIP full length reverse primer: 5'-CTAG*CTCGAG*TGCCCCGGTGGTGGTG-3' (*Xho*I). The rest of the procedure is same as described for cloning ΔCC1 mutant. The overall reading frame and the sequence of this mutant were confirmed by sequencing.

Construction of coiled coil double mutant of HPIP i.e., HPIPΔCC1-2:

To generate HPIP double mutant lacking both Coiled coil domains (CC1&2), we first digested pcDNA-HPIPΔCC1 plasmid with *Eco*RV and *Xho*I enzymes releasing a linear vector with 1-810 bp fragment of HPIP. In the second step, the region from 1023 to 2196 bp which lacked CC2 domain of HPIP was amplified using CC1 forward primer: 5'-GACAGATATCCCCAGATGGGGTGTG-3' (*Eco*RV) and HPIP full length reverse primer: 5'-CTAGCTCGAGTGCCCCGGTGGTGGTG-3' (*Xho*I) using pcDNA-HPIPΔCC2 as a template.

The amplified product was digested with *Eco*RV and *Xho*I, and ligated to HPIP (1-810 bp) linear vector as generated above to get HPIPΔCC1-2 double mutant (Figure 29). The overall reading frame and the sequence of this mutant were confirmed by pyrosequencing.

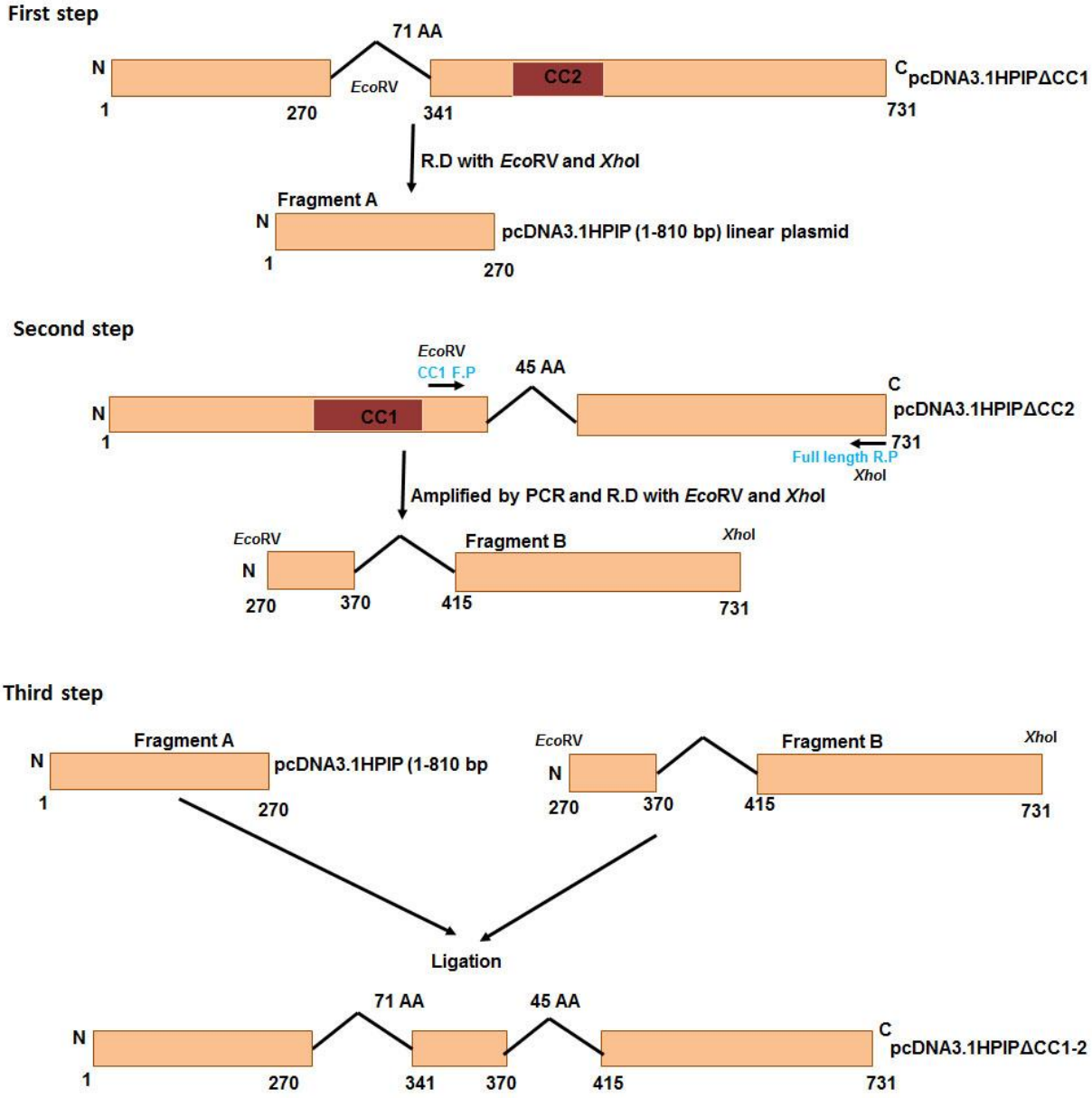


Figure 29: Cloning strategy for construction of HPIPΔCC1-2 clone. Cloning of HPIPΔCC1-2 is a three-step approach. In the first step, pcDNA3.1HPIPΔCC1 plasmid was digested *Eco*RV and *Xho*I enzymes to generate linear plasmid containing pcDNA3.1HPIP (1-810 bp). In the second step, the region from 1023 to 2196 bp was amplified using pcDNA3.1HPIPΔCC2 plasmid as a template. In the third step, amplified fragment and linear plasmid (pcDNA3.1HPIP1-810 bp) were ligated to generate the pcDNA3.1HPIPΔCC1-2.

3.3.2 Site-directed mutagenesis: Rab5 active mutant (Rab5Q79) was generated using pEGFP-Rab5 as template through PCR amplification by replacing glutamine (Q) at position 79 with a leucine (L) using the following primers: forward primer: 5'-GATACAGCTGGTCTAGAACGATACCAT-3' and reverse primer: 5'-ATGGTATCGTTCTAGACCAGCTGTATC-3'. The amplified plasmids were digested with *Dpn*I enzyme followed by tranasformation into *E.coli* DH5α cells and all clones were confirmed by DNA sequencing. GFP-Rab5 inactive mutant (Rab5S34N) was purchased from addgene, USA.

SiRNA transfection: MDA-MB231 cells were transfected with control siRNA, RUFY3 siRNA, Rab5 siRNA or both RUFY3 siRNA and Rab5 siRNA (Qiagen, USA) using Lipofectamine 2000 (Invitrogen, USA) according to the manufactures protocol. Briefly, 25 nm of siRNA was mixed with 10 µl of Lipofectamine in 100 µl of Opti-MEM medium and incubated for 20 min at room temperature. Medium was removed from culture dish and replaced with serum-free, antibiotic-free medium. Transfection mixture was added to the cells after 6 hrs, medium was replaced with complete DMEM (+serum and +antibiotic). Post 48 hrs of transfection, cells were lysed in RIPA or NP40 lysis buffer and analysed knockdown of proteins using Western blotting.

3.3.3 Expression and purification of recombinant GST-tag and His-tag proteins: *E. coli* BL21 (DE3) cells harbouring pET28a-RUFY3, pET28a-Rab5 or pET28a-RUFY3-CC were inoculated into Luria-Bertani broth (LB) medium supplemented with 50 µg/ml kanamycin and incubated at 37°C with shaking (200 rpm). Next day, 2% overnight culture was inoculated into 500 ml of LB medium supplemented with 50 µg/ml kanamycin. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce protein expression when the OD_{600} reached 0.6. After 3 hrs of incubation at 30°C, the culture was then harvested by centrifugation at 8,000 rpm for 20 min and the cell pellet was stored at −80°C until further analysis. Similarly, *E. coli* BL21 (DE3) cells harbouring pGEX4T1-HPIP, pGEX4T1-HPIP-CC1 or pGEX4T1-HPIP-CC2 were inoculated into LB medium supplemented with 100 µg/ml ampicillin and incubated at 37 °C with shaking (200 rpm). IPTG was added to a final concentration of 1 mM to induce protein expression when the OD_{600} reached to 1.2 OD but not at 0.6 OD as we noticed poor expression at this OD. After 3 hrs of incubation at 30°C, the culture was then harvested by centrifugation at 8000 rpm for 20 min and the cell pellet was stored at −80°C until further analysis.

The harvested cell pellet was resuspended in bacteria lysis buffer (50 mM Tris–HCl pH 8.0, 10% glycerol, 20% glucose and 1X protease inhibitor cocktail). Followed by sonication, the cell lysate was separated by centrifugation at 11000 rpm for 10 min at 4°C and the soluble fraction was collected. Supernatant was incubated with Nickel beads or Glutathione Sepharose beads (Clonetech, California, USA) for 90 min at 4°C, followed by four washes (each 30 min) with NP-40 lysis buffer (50 mM Tris–HCl pH 8.0, 10% glycerol, 1% NP-40 and 137 mM NaCl and 1X protease inhibitor cocktail). To check the protein purity, 20-30 µl of beads were loaded onto SDS PAGE.

3.3.4 Glutathione S-Transferase (GST) pull-down assay: *In vitro* transcription and translation of wild type HPIP (pcDNA-HPIP) and coiled coil deletion mutants of HPIP (pcDNA-HPIPΔCC1, pcDNA-HPIPΔCC2 and pcDNA-HPIPΔCC1&CC2) were performed using TNT kit (Promega Scientific, USA), where 1 µg template plasmid DNA was translated in presence of [^{35}S]methionine in a reaction volume of 50 µl. Translation and product size were verified by analyzing 2 µl of the reaction mixture in a SDS-PAGE followed by autoradiography. The GST pull-down assays were then performed by incubating equal amounts of His-Rab5, His-RUFY3 or His-RUFY3-CC beads with 10 µl of *in vitro* translated [^{35}S]-labeled protein in 500 µl of binding buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10% glycerol, 0.1% Nonidet P-40) for 90 min. Post incubation at 37°C for 1 hr, beads were washed five times with GST binding buffer followed by eluting the proteins using 2X SDS buffer, and finally separating the proteins on SDS-PAGE. The interacted proteins were then visualized by autoradiography (Thyphoon scanner, USA).

3.3.5 Dimer exchange assay: For formation of heterodimer on exchange between differently tagged proteins, His-Rab5 or His- RUFY3 or His-RUFY3-CC and GST-HPIP-CC domains 1, 2 (HPIP-CC1 and HPIP-CC2) were incubated together in a ~1:2 molar ratio at room temperature for 30 min in 500 µl volume of binding buffer (150 mM NaCl, 50 mM Tris buffer, pH 8.0). As previously described (Li *et al*., 2013), samples were flash-frozen in liquid nitrogen to capture the resulting association state, and then thawed on ice. After beads were thoroughly washed with binding buffer or NP40 buffer, the bound proteins was eluted by 2X SDS sample buffer and subjected to Western blotting using His tag antibody.

3.3.6 Rab5 activation assay: Rab5 activation assay was performed using MDA-MB231 cells stably transfected with control shRNA, HPIP-specific shRNA1 or HPIP shRNA2 or HPIP shRNA1 plus T7-HPIP. Cells were treated with PI3K-specific inhibitor LY294002 as necessary. Rab5 activation kit was purchased from Abcam, USA, and assay was done according to manufacturer's protocol. After removing the culture medium, cells were washed twice with ice-cold PBS, and lysed in ice-cold 1X assay/Lysis buffer (0.5 – 1 mL). Culture plates were kept on ice for 20 minutes followed by scraping the cells with a cell scraper. Cell lysates were cleared by centrifugation for 10 minutes (12,000 x g at 4°C) and total ~1-2 mg of total protein was used for assay. Rab5-GTP was immunoprecipitated using anti-active Rab5 antibody for 1 hr at 4°C followed by 3 washes with 1x assay Lysis buffer. After brief centrifugation, the bound proteins were eluted by 2x SDS loading dye and then subjected to Western blotting with respective antibodies.

Plasmids used in this study

Name	Description	Insert size	Reference
BM 50	pGEX4T1-HPIP-CC1	220 bp	In this study
BM 51	pGEX4T1-HPIP-CC2	120 bp	In this study
BM 199	pcDNA3.1/His-HPIP-ΔCC1	1979 bp	In this study
BM 198	pcDNA3.1/His-HPIP-ΔCC2	2079 bp	In this study
BM 200	pcDNA3.1/His-HPIPΔCC1-2	1859 bp	In this study
BM 81	pEGFPC1-RUFY3	1400 bp	In this study
BM 189	pDsRedN1-RUFY3	1400 bp	In this study
BM 190	pEGFPC3-EEA1	4236 bp	Marino Zerial, Germany
BM 191	pEGFPC3-Rab5	646 bp	Marino Zerial, Germany
BM 192	pEGFPC3-Rab5-Q79L	646 bp	In this study
BM 205	pCDNA-EGFP-Rab5-S34N	646 bp	Sun *et al*., 2010
BM 206	pEGFPC3-Rab7	693 bp	Marino Zerial, Germany
BM 27	pcDNA3.1/His-HPIP	2196 bp	In this study
BM 185	pET28a-His-RUFY3	1400 bp	In this study

BM 182	pET28a-His-Rab5	646 bp	In this study
BM 115	pGEX4T1-HPIP	2196 bp	In this study
BM 119	pGFP-V-RS-HPIP shRNA 1	-	OriGene, USA
BM 120	pGFP-V-RS-HPIP shRNA 2	-	OriGene, USA
BM 121	pGFP-V-RS-HPIP shRNA 3	-	OriGene, USA
BM 122	pGFP-V-RS-HPIP shRNA 4	-	OriGene, USA
BM 123	pGFP-V-RS- control shRNA	-	OriGene, USA

3.4 Results:

3.4.1 Identification and characterization of coiled coil (CC) domains of HPIP:

HPIP contains three distinct functional domains, the N-terminal microtubule binding domain, two carboxy-terminal nuclear localization signals and one nuclear export signal (Abramovich *et al*., 2000). Our *in silico* analysis identified two highly conserved coiled coil domains (CC) in HPIP such as CC1, 270-341 aa and CC2, 370-415 aa (Figure 30A).

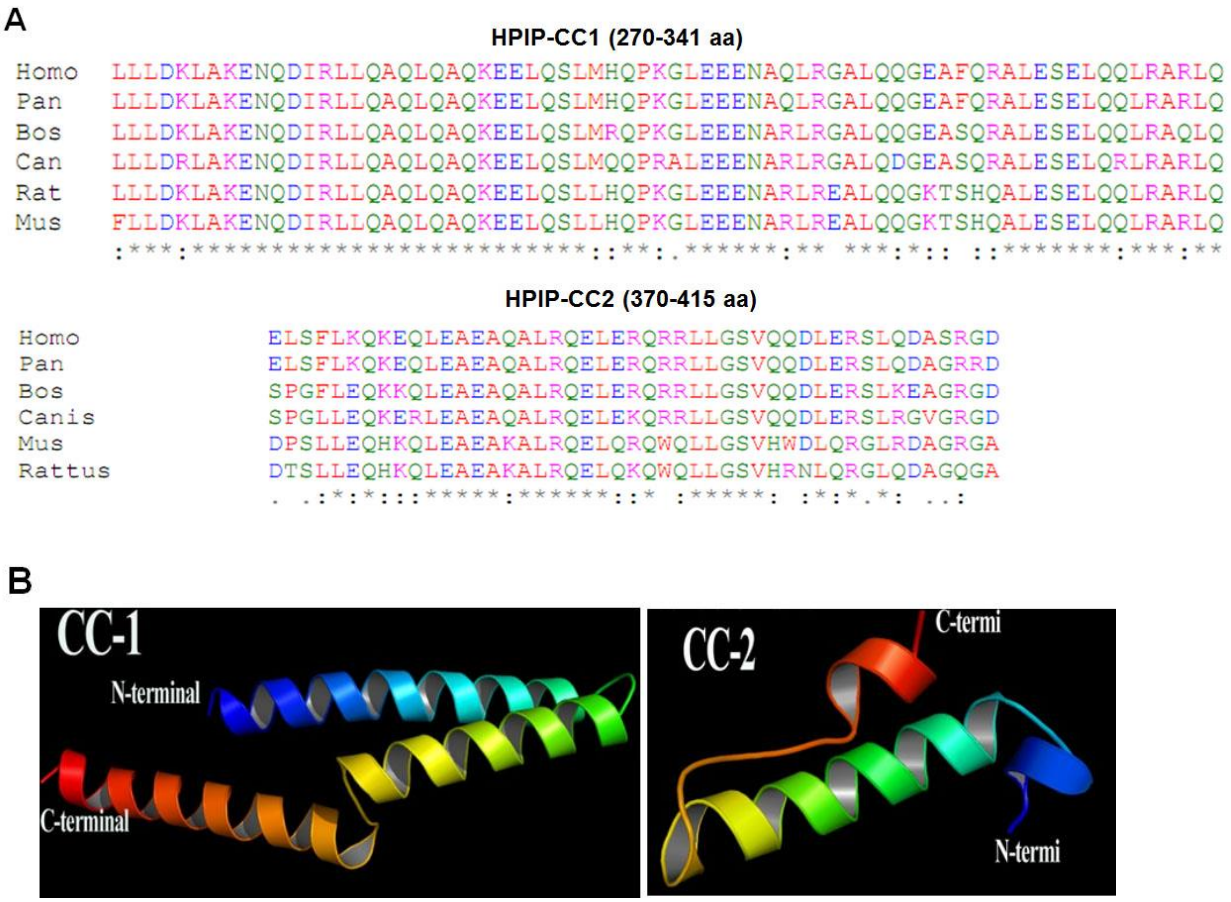
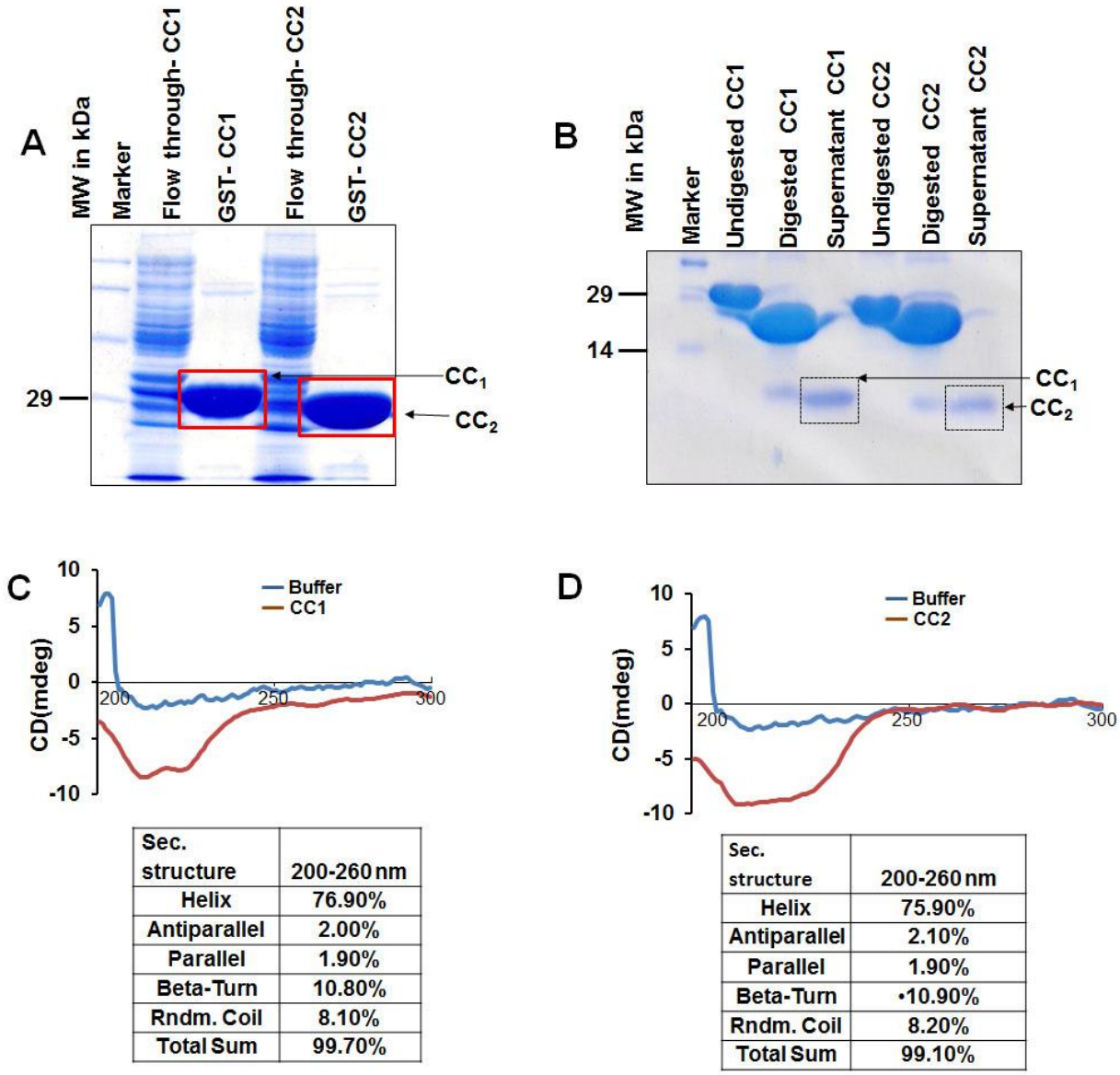


Figure 30. Identification of coiled coil domains in HPIP. (A) Multiple alignment of coiled coil domains of HPIP using Clustal W. (*) Indicates conserved amino acids in all species and (:) indicates partially conserved amino acids. Alignment scores, HPIP-CC1 is 97% and HPIP-CC2 is 93%. (B) Predicted secondary structures of coiled coil domains using I-TASSER server.

Coiled-coil proteins are estimated to comprise 3–5% of the genomic complement suggesting their involvement in diverse biological activities (Wolf *et al*., 1997; Newman *et al*., 2000). For example, coiled coil domains are involved in transcriptional activation (Renzon *et al*., 2001), membrane sensors (Surette *et al*., 1996) and dimerization/oligomerisation of proteins (Li *et al*., 2012). *In silico* studies on HPIP protein revealed that both CC1 and CC2 domains form perfect coiled coil structures comprising of helices throughout their length that are energetically feasible (Figure 30B). To further characterize these domains, both CC1 and CC2 domains were subcloned into pGEX-4T1 vector and expressed as recombinant GST tagged proteins in *E. coli* BL21 (DE3). Later, GST tag was removed by digesting the GST-fusion proteins with thrombin treatment (Figure 31A-B). The purified CC1 and CC2 domains were subjected to Circular Dichroism (CD) analysis for secondary structural analysis. The secondary structure of purified coiled coil domains of HPIP was determined using CDNN 2.1, software. This study revealed that CC1 domain of HPIP comprises of 76.9% of α-helix, 13.9% of β-sheet (including parallel and anti-parallel) and 8.10% of random coil (Figure 31C), which is in close agreement with previous reports (McNamara *et al*., 2008; Li *et al*., 2012). Similarly, the secondary protein conformation of CC2 domain of HPIP was found to be 75.9% α-helix, 14.0% β-sheet (including parallel and anti-parallel) and 8.20% random coil (Figure 31D). Taken together, these results suggest that HPIP has two coiled coil domains that are well conserved across the species and these coiled coil domains present a unique architecture to the HPIP protein that is crucial for the interaction of HPIP with other cellular components and thus its cellular functioning.



Sec. structure	200-260 nm
Helix	76.90%
Antiparallel	2.00%
Parallel	1.90%
Beta-Turn	10.80%
Rndm. Coil	8.10%
Total Sum	99.70%

Sec. structure	200-260 nm
Helix	75.90%
Antiparallel	2.10%
Parallel	1.90%
Beta-Turn	•10.90%
Rndm. Coil	8.20%
Total Sum	99.10%

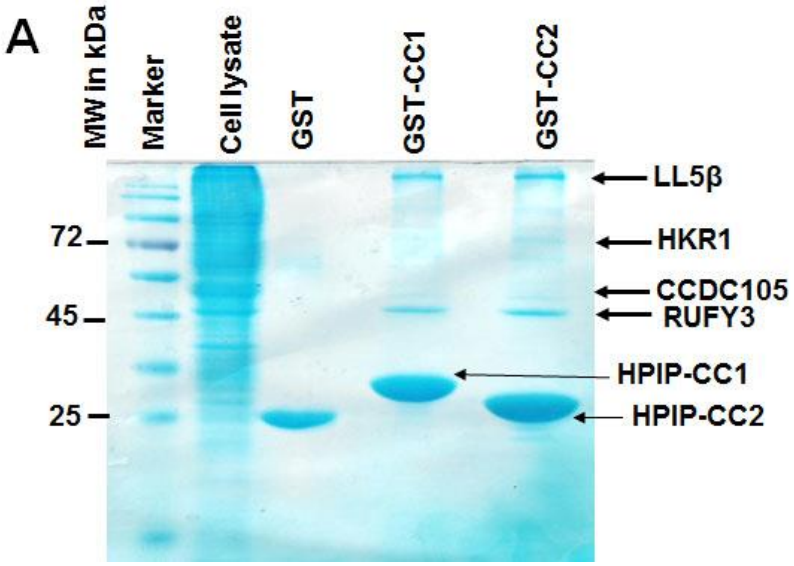
Figure 31. Characterization of coiled coil domains of HPIP. (A) SDS-PAGE analysis showing the expression and purification of recombinant GST-CC1 and GST-CC2 domains of HPIP from *E.coli* BL21 (DE3). To check the protein purity ~25 µl of beads were ran on SDS-PAGE. (B) Purified GST-CC1 and GST-CC2 proteins were subjected to thrombin digestion overnight to remove GST tag and purified domains were collected after brief centrifugation and purity was analyzed on SDS-PAGE. (C, D) Purified CC1 and CC2 domains in phosphate buffer saline (PBS) were used for CD spectra analysis at 20°C at a scan rate of 60°C/h in a 2 mm path-length cuvette (upper panel). Protein secondary structures were analyzed using CDNN2.1 software (lower panel).

3.4.2 Identification of novel HPIP-interacting proteins in cancer cells:

Protein complexes play pivotal role in many biological processes. The scaffold protein HPIP forms complexes with PBX proteins in hematopoietic cells, whereas in breast cancers cells, it form a signaling complex involving microtubules, ERα, Src and p85 subunit of PI3 kinase (Abramovich *et al*., 2000, Manavathi *et al*., 2006). As HPIP contains two coiled coil domains, which are known to involve in protein-protein interactions, we sought to identify HPIP interacting proteins using affinity approach, thus it provides further insights about HPIP functions with respect to its role in cell migration and invasion. To accomplish this, we carried out GST pull down assay using CC1, CC2 domains of HPIP. Purified GST-CC1 and GST-CC2 protein beads were incubated with MDA-MB231 cell lysate, after thorough washing to eliminate the non-specific proteins, the eluted proteins were separated on SDS PAGE. The prominent proteins resolved on the gel were sliced off and analyzed by MALDI analysis (Figure 32A). The details of these four proteins are listed below (Figure 32B-D).

RUFY3: It is a RUN domain containing protein and has been implicated in the formation of a single axon by developing neurons through inhibition of PI3K (Mori *et al*., 2007). Further it has been shown to interact with actin bundling protein Fascin to control the growth of axons, implying an essential role for RUFY3 in neuronal development (Wei *et al*., 2014). Interestingly, RUFY3 is also shown to interact with Rab5, an endosomal protein (Yoshida *et al*., 2010). However, the functional interaction between Rab5 and RUFY3 are largely unknown. Recent studies revealed that RUFY3 regulates gastric cancer cell migration and invasion through p21 activated kinase (PAK1)-dependent manner by promoting cell protrusions at cell periphery (Wang *et al*., 2015).

A



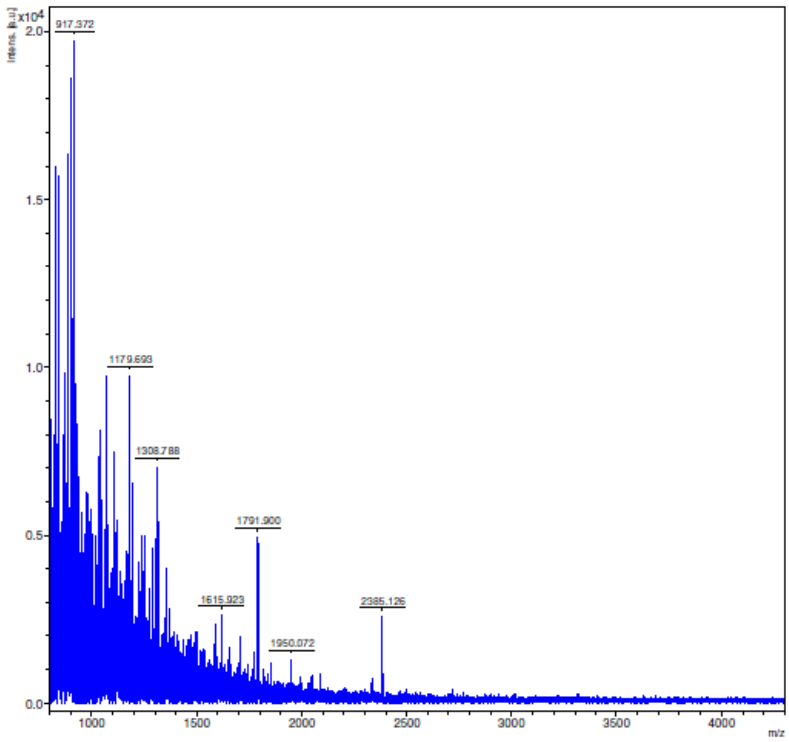
B

S.No	RUFY3 peptide sequences	HKR1 peptide sequences
1	VSMDGEWLCLR	LIAQLERGE APWR
2	HGLKAKK TFLGQNK	QQQDPFCFSG K
3	LA LMQKKLSEYMKALINKK	THSGGKPYVCRECGRGFTWK
4	IITLQ EEMER	THSG EKPYICRECE
5	QEMELAMK	SGEKPFVCAECGRGFNDKST
6	DVCEKQDA LVSLR	AHAGGKPHVCR

C

S.No	LL5β (PHLB2) peptide sequences	CCDC105 peptide sequences
1	SPSPLGTSVR	SQDTRVGAPA WREAAQAMAR
2	IQG SKQFSYDGTDK	CGQEAVTMWQPK
3	MSIQDSLALQ PKLTR	AAYIQPWRFR
4	ATENQLTPL SLPPRNSLGNSK	GKMKPPAWYARLPLPLHRK
5	QASGTPQPALR	ALQTTEVVHAHARGARLTAARLGR
6	SSISSIS GRDDLMDYHR	SEKVPDK
7	LREQEMER	TLASCR
8	ETILSLCAEY TKPDSRLSTG TTVEDVQ	ERL NMTLGLMRGT ILRCTK
9	TPEGISE EQRSQELAAM EETRIVILNN LEELK	NLSWGLNCKNIGHEVDGNVVR
10	LQELYSEQK TQLDNCPESM R	SKSSADP

D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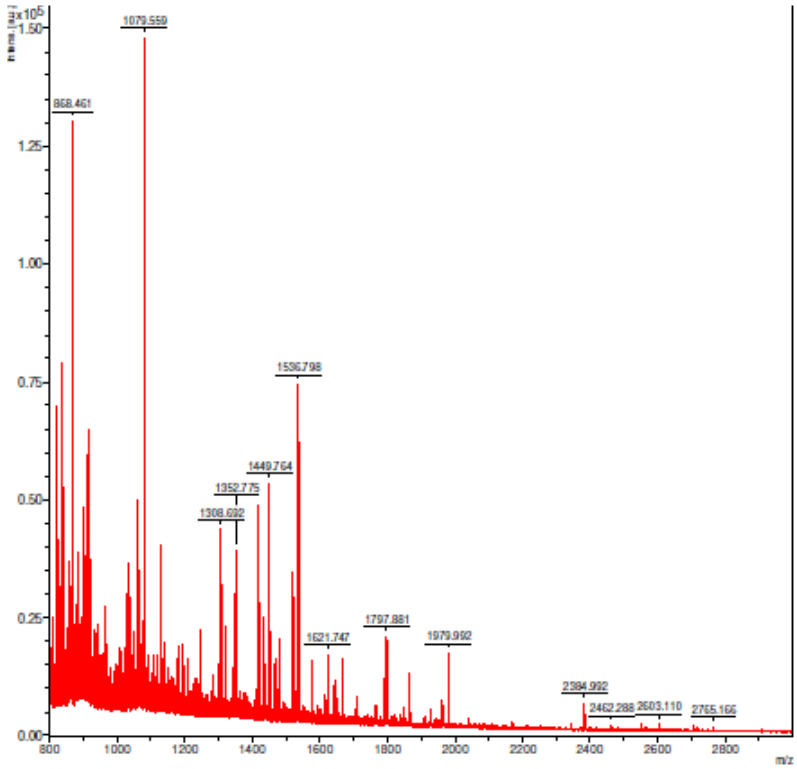
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1797.881
1979.992
2384.992
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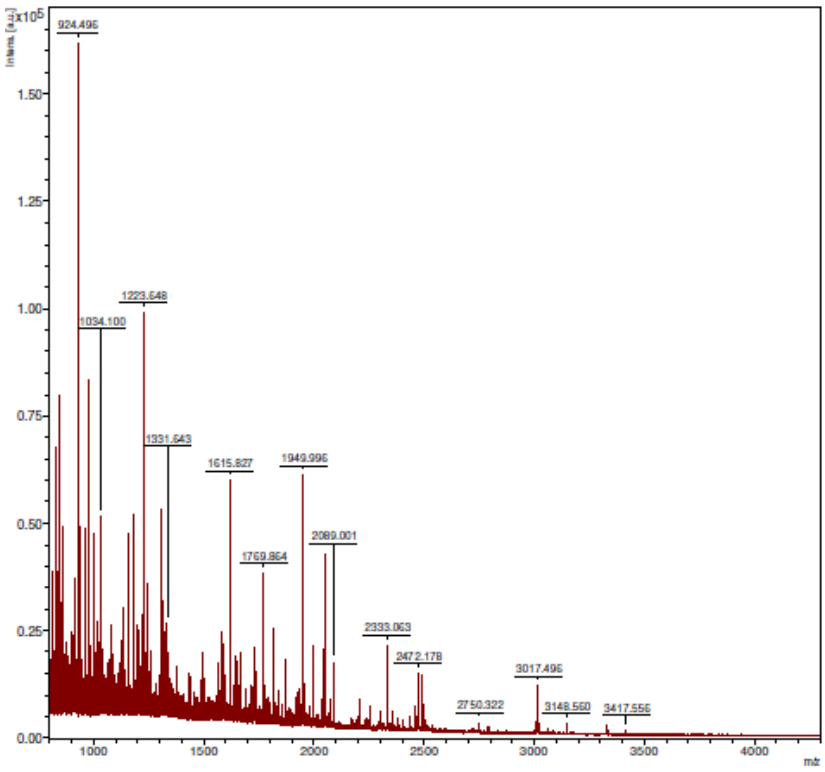
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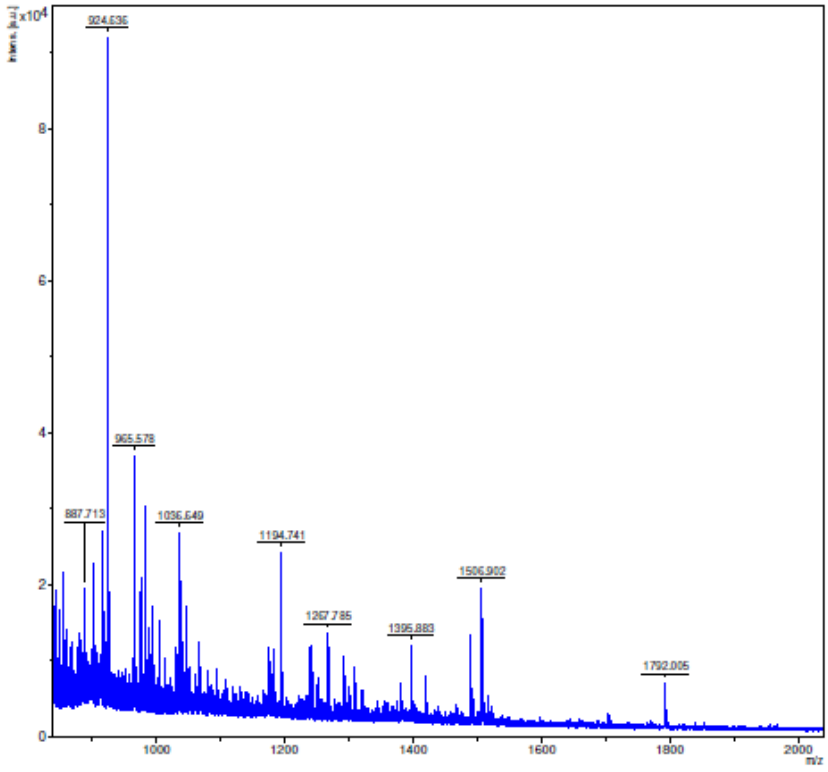
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1267.785
1395.883
1792.005
1000
1200
1400
1600
1800
2000
m/z

Figure 32. Identification of HPIP interacting proteins. (A) GST pull down assay was performed to identify HPIP interacting proteins using MDA-MB231 cell lysate. Equal amounts of GST–CC1, GST–CC2 and GST bound beads were incubated with 2 mg of total protein extract. After incubation, beads were recovered by a brief centrifugation and washed thrice with washing buffer and loaded onto gradient SDS PAGE. Unique protein bands were excised from gel and subjected to MALDI-TOF analysis. (B-C) List of four novel interacting partners of HPIP and their peptide sequences. (D) MALDI TOF/TOF spectrum. Peptide Mass Fingerprints (PMF) of RUFY3, HKR1, LL5β and CCDC105, respectively (from top to bottom).

CCDC105: It is also a coiled coil domain containing protein. The functional significance of CCDC105 is not much explored. However, it is proposed to have a role in microtubule cytoskeletal organization and biogenesis.

Krueppel-related zinc finger protein 1 (HKR1): Krueppel-related zinc finger protein 1 is a transcriptional factor known to overexpress in lung cancer (Oguri *et al*., 1998). It was located at the gene position chromosome 19q, and this region was shown to highly amplify in advanced ovarian cancer as well as in cervical cancer (Arnold *et al*., 1996).

LL5β (PHLB2): PHLB2, also known as LL5β, is a cytoplasmic protein with PtdIns (3,4,5)P_3 binding activity. Furthermore, it is known to interact with cytoskeleton adaptor protein γ-filamin as well as CLASPs (microtubule associated protein) (Dowler *et al*., 2000; Paranavitane *et al*., 2003), implying that it has role in microtubule functions. Accordingly, a recent study showed that it involves in regulation of the density of growing microtubules at the basal cortex of the cell (Hotta *et al*., 2010). In another study, LL5β was shown to localize to cell adhesion sites and interacts with laminin receptor, and affects the localization of integrin α3 to basal cell cortex (Hotta *et al*., 2010).

3.4.3 HPIP interacts with early endosome marker Rab5

As both HPIP and RUFY3 are known to modulate PI3 kinase/AKT pathway (Yoshida *et al*., 2010; Manavathi *et al*., 2006) and are known to involve in cell migration, we rationalized that HPIP and RUFY3 interactions are important for cell migration. To address this, we first subcloned RUFY3 from pBSKII-RUFY3 into pDsRed vector. Colocalization studies were carried out for DsRed-RUFY3 and endogenous HPIP in MDA-MB231 cells using confocal microscopy. As shown in Figure 33, RUFY3 and HPIP readily colocalized with each other in speckle-like structures (puncta) in MDA-MB231 cells.

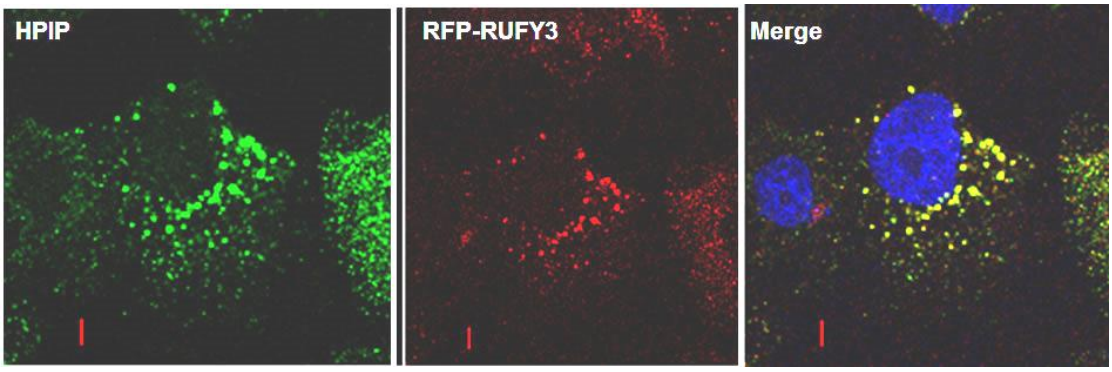

Figure 33: Colocalization of HPIP with RUFY3 in MDA-MB231 cells. Cells were transfected with DsRed-RUFY3 and 48 hours post transfection, cells were subjected to immunofluorescence analysis. Localization of DAPI (blue), HPIP (green), pDsRed-RUFY3 (red) was observed by confocal microscopy. Representative 40x magnified immunofluorescent images are shown.

The unique speckle like staining of HPIP and RUFY3 in MDA-MB231 cells, puzzled us to know their exact cellular localization. Based on the following facts that; 1) RUFY3 interacts with Rab5, an endosomal protein, 2) HPIP being a microtubule binding protein 3) and the reliance of endosomal transport on microtubules, we presumed that the staining pattern of HPIP may correspond to endosomes. To further validate the endosomal localization of HPIP, we performed

confocal microscopy using early endosome markers such as EEA1, Rab5 and late endosomal marker Rab7. MDA-MB231 cells were transfected with GFP-Rab5, GFP-Rab7 or GFP-EEA1 and 36 hrs of post transfection, cells were fixed and probed with anti-HPIP antibody. As shown in Figure 34, HPIP was readily colocalized with Rab5 but not with either EEA1 or Rab7 in MDA-MB231 cells. These studies indicated the endosomal colocalization of HPIP with either RUFY3 or Rab5 in MDA-MB231 cells.

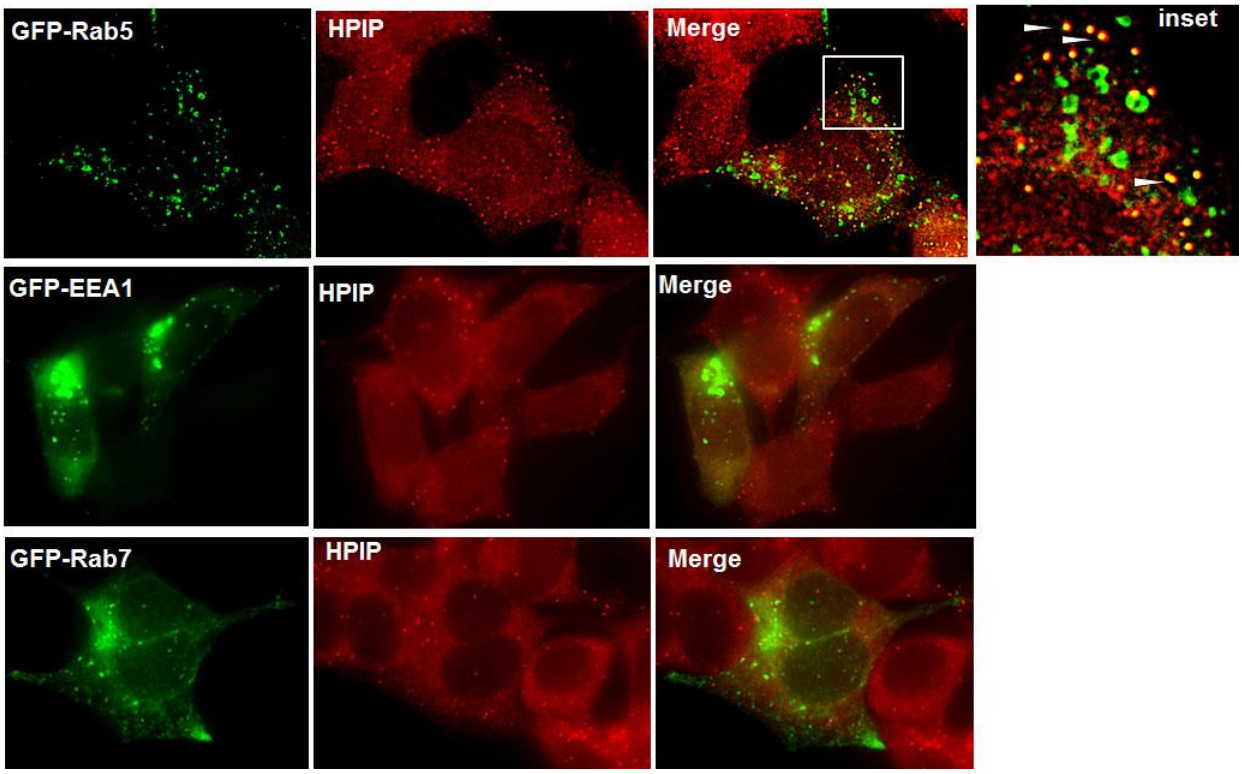

Figure 34. HPIP interacts with early endosomal marker Rab5: MDA-MB231 cells were transfected with GFP-Rab5, GFP-Rab7 or GFP-EEA1 and 36 hours post transfection, cells were fixed and subjected to immunofluorescence analysis. Localization of HPIP (red), GFP-Rab5, GFP-Rab7 and GFP-EEA1 (green), DAPI (blue), was observed under fluorescence microscope. Representative 60x magnified immunofluorescent images are shown. Arrow heads denotes colocalization sites (yellow) of Rab5 and HPIP.

Previous studies revealed that ectopic expression of Rab5 (Q79L), a GTPase-defective mutant form of Rab5 (active Rab5), enforces the formation of enlarged early endosomes which were due to the defective budding of endosomal vesicles (Stenmark *et al*., 1994). Furthermore, the GTP-bound form of Rab5, localized on the cytoplasmic face of early endosomes, is known to promote endosomal vesicular trafficking through interaction with specific effector molecules (Christoforidis *et al*., 1999). To further identify the interaction of HPIP with Rab5 is dependent on GTP, we performed immunofluorescence studies using Rab5-Q79L (Rab5-GTP) and Rab5-S34N (Rab5-GDP) mutants. Immunofluorescence studies demonstrated that HPIP colocalized with Rab5-GTP i.e Rab5-Q79L in large vesicle structures but not with Rab5-GDP in MDA-MB231 cells, indicating that the interaction between HPIP and Rab5 may be altered by the nucleotide-bound state of Rab5 (Figure 35).

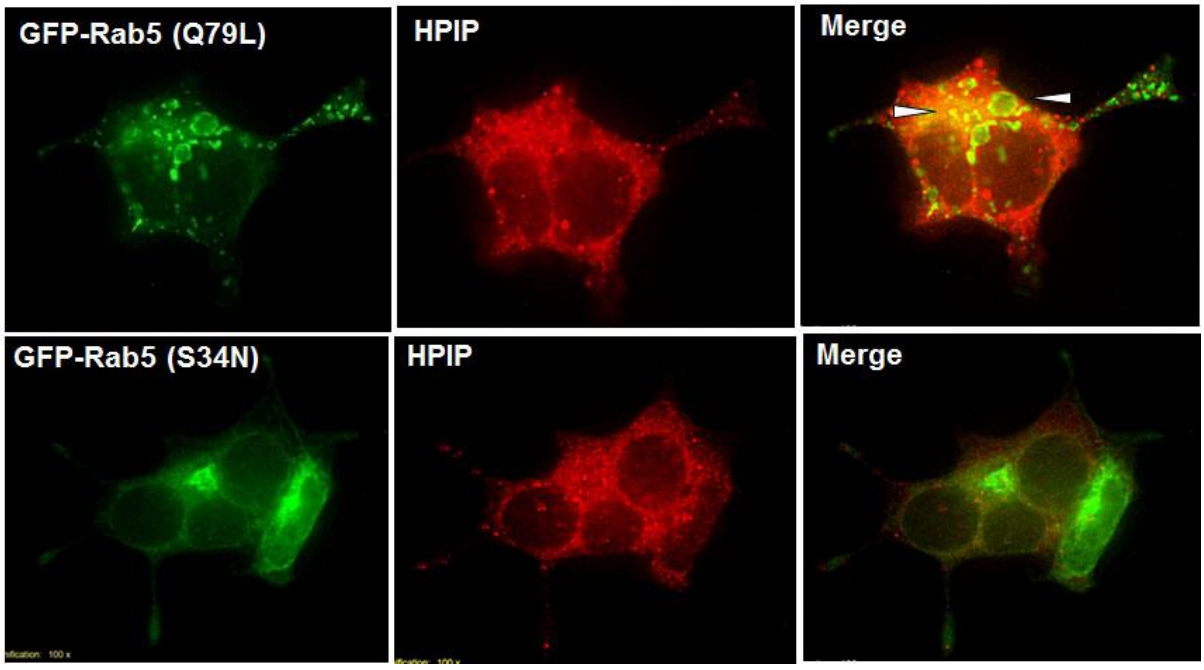

Figure 35. HPIP colocalizes with Rab5 active mutant (Rab5-Q79L) but not with Rab5-GDP. MDA-MB231 cells were transfected with either GFP-Rab5-Q79L or Rab5-S34N. Cells were fixed and incubated with HPIP

antibody and images were obtained by fluorescence microscope. Representative 60x magnified immunofluorescent images are shown.

Previous studies revealed that RUFY3 and Rab5 interact with each other (Yosidha *et al*., 2010) and results from our study indicate that HPIP interacts with both Rab5 and RUFY3, suggesting the formation of a **H**PIP-**R**UFY3-**R**ab5 (HRR) protein complex in migrating cells. To test this hypothesis, first we performed an *in vitro* GST pull down assay by incubating recombinant GST-**H**PIP, His-**R**UFY3 and His-**R**ab5. As shown in figure 36A, these proteins interact with each other and form a complex which we named as 'HRR complex'. To further validate this *in vivo*, MDA-MB231 cells were transfected with GFP-RUFY3 and 48 hrs post transfection, cell lysates were subjected to co-immunoprecipitation (CoIP) analysis using GFP antibody. Consistent with our notion, Rab5, HPIP and RUFY3 were coprecipitated with GFP-RUFY3 in MDA-MB231 cells but not with GFP (Figure 36B), indicating that these three proteins form a complex both *in vitro* and *in vivo.* The formation of this complex was previously unknown and HRR complex might be crucial for HPIP-mediated cellular functions and cell migration.

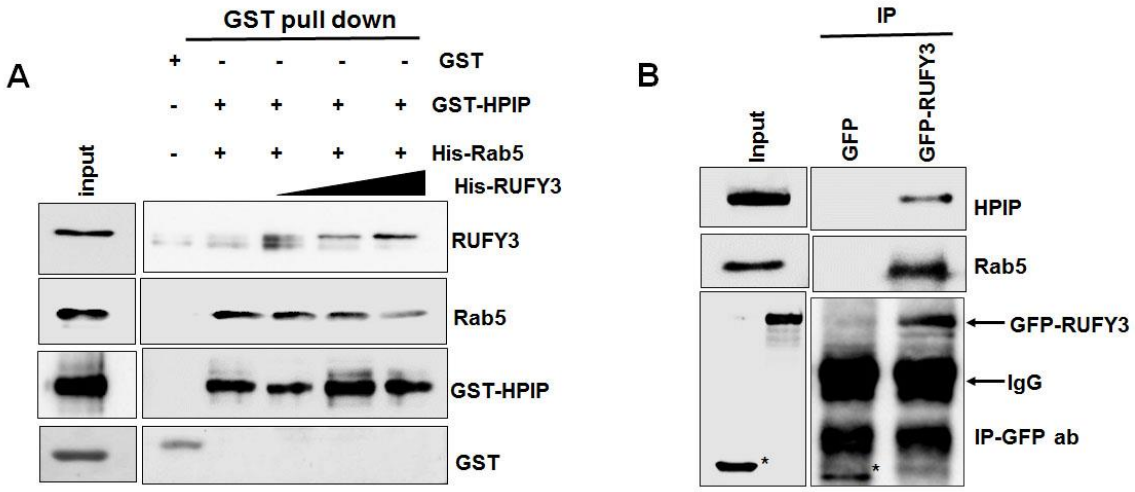

Figure 36: Interaction of HPIP, Rab5 and RUFY3 both *in vitro* and *in vivo*. **(**A**)** GST-pull down assay was performed by incubating GST-HPIP (bound to glutathione sepharose beads) and increasing concentration of His-Rab5 and His-RUFY3 (purified and eluted). The HPIP/Rab5/RUFY3 complexes were pulled down by glutathione sepharose beads and subjected to Western blotting using indicated antibodies. (B) Coimmunoprecipitation (Co-IP) experiments to assess the interaction between HPIP/Rab5/RUFY3 complexes *in vivo*. pEGFP-RUFY3 transfected into MDA-MB231 cells were coimmunoprecipitated with GFP antibody and blotted as indicated. Input lysates (10%) were loaded to verify the expression of indicated proteins, (*) GFP.

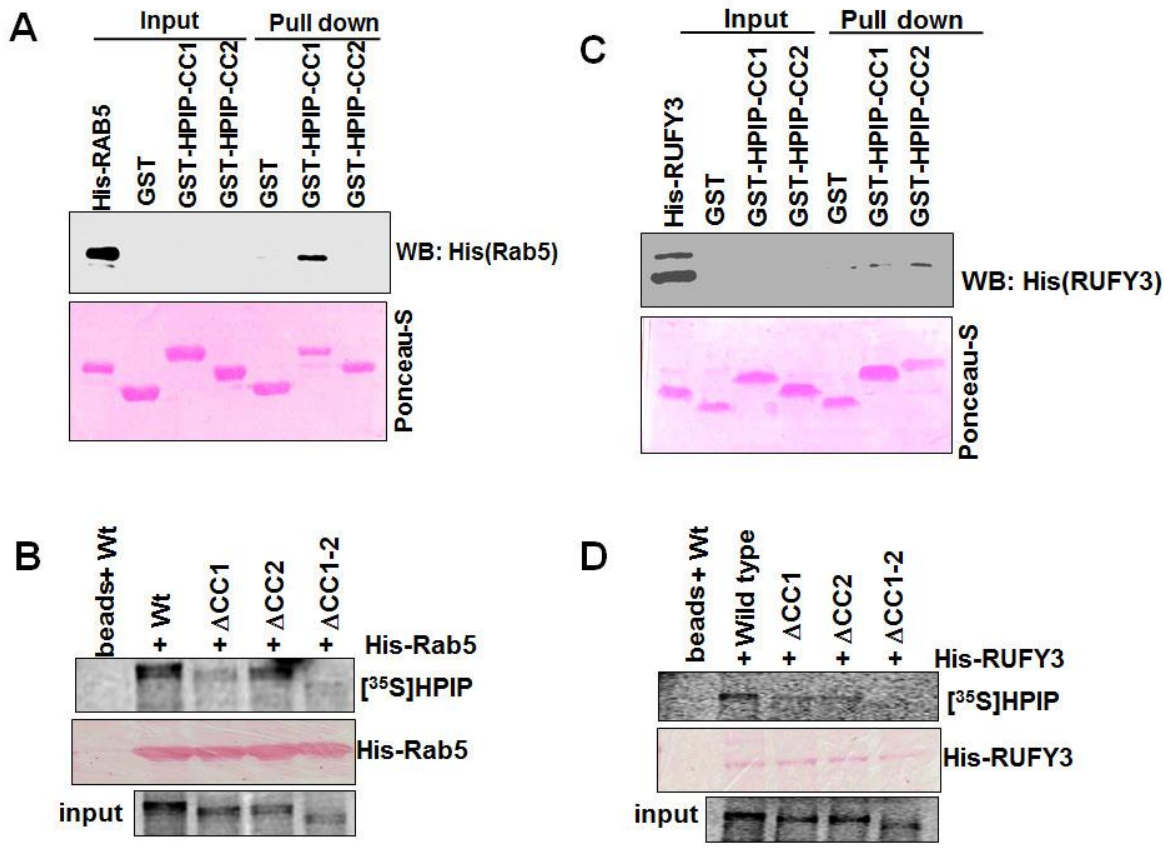

Figure 37. Requirement of coiled coil domains for HPIP interaction with Rab5 and RUFY3. (A, B) Dynamic exchange assay as described in the Methods section. Either recombinant His-Rab5 or His-RUFY3 and GST-HPIPCC1/GST-HPIP-CC2 bound to beads were co-incubated at the room temperature and coprecipitated using glutathione sepharose beads. The presence of interaction was checked by Western blotting using anti-His antibody.

(C, D) Either recombinant His-Rab5 or His-RUFY3 was incubated with 10 µl of *in vitro* translated [S^{35}]HPIP proteins (wt and mutants) at room temperature and coprecipitated using His-Talon beads. The presence of interaction was checked by SDS-PAGE followed by exposing the membrane to phosphoimager cassette and autoradiography.

3.4.4 Rab5 and RUFY3 interact with HPIP through coiled coil domains:

After establishing the interaction of HPIP with Rab5 and RUFY3, we next proposed to determine the association of Rab5 and RUFY3 with coiled coil domains of HPIP using *in vitro* interaction assays. For *in vitro* interaction assays, bacterially purified GST, GST-CC1 or GST-CC2 were incubated with His-Rab5 at room temperature for 20 min and the unbound proteins were washed with NP40 lysis buffer, and then probed with anti-His antibody. We found that CC1 domain, but not CC2 domain, of HPIP was associated with Rab5 (Figure 37A). Further this observation was confirmed by *in vitro* interaction assay using [S^{35}]HPIP coiled coil deletion mutants by pull-down assay. Consistent with this observation, Rab5 was able to interact with wild type [S^{35}]HPIP and [S^{35}]HPIPΔCC2 but not with either [S^{35}]HPIPΔCC1 or [S^{35}]HPIPΔCC1-2 (double mutant lacking both coiled coil domains), indicating that Rab5 specifically interacts with CC1 domain of HPIP (Figure 37B). Similarly *in vitro* studies to determine the involvement of CC domains of HPIP in the interaction of RUFY3 were performed (Figure 37C-D). Unlike to Rab5, RUFY3 interacted with both CC1 and CC2 of HPIP as neither [S^{35}]HPIPΔCC1 or [S^{35}]HPIPΔCC2 interacted with RUFY3 (Figure 37C-D), indicating that Rab5 and RUFY3 explores distinct CC domains to interact with HPIP.

3.4.5 Rab5-mediated cell migration requires HPIP:

After establishing the physical interaction between HPIP, RUFY3 and Rab5 that forms HRR complex, we analysed the role of HRR complex in cell migration. Rab5 promotes cell migration

and invasion mainly through integrin internalization and recycling (Pellinen *et al*., 2006; Palamidessi *et al*., 2008; Torres *et al*., 2008; Torres *et al*., 2010; Diaz *et al*., 2014). Furthermore, recent studies revealed that FAK activation is also required for Rab5-mediated cell migration and invasion (Mendoza *et al*., 2013). Our previous results showed that HPIP activates FAK and HPIP-dependent cell migration requires FAK (Bugide *et al*., 2014), implying that both Rab5 and HPIP converge onto FAK pathway in the regulation of cell migration. Therefore, we next analysed the effect HPIP expression on Rab5-mediated cell migration. Consistent with previous results, Rab5 overexpression stimulated the migration of MDA-MB231 cells (Mendoza *et al*., 2013). Conversely, HPIP knockdown significantly decreased Rab5-meadiated cell migration (Figure 38). This result demonstrates that HPIP is required for Rab5-dependent cell migration.

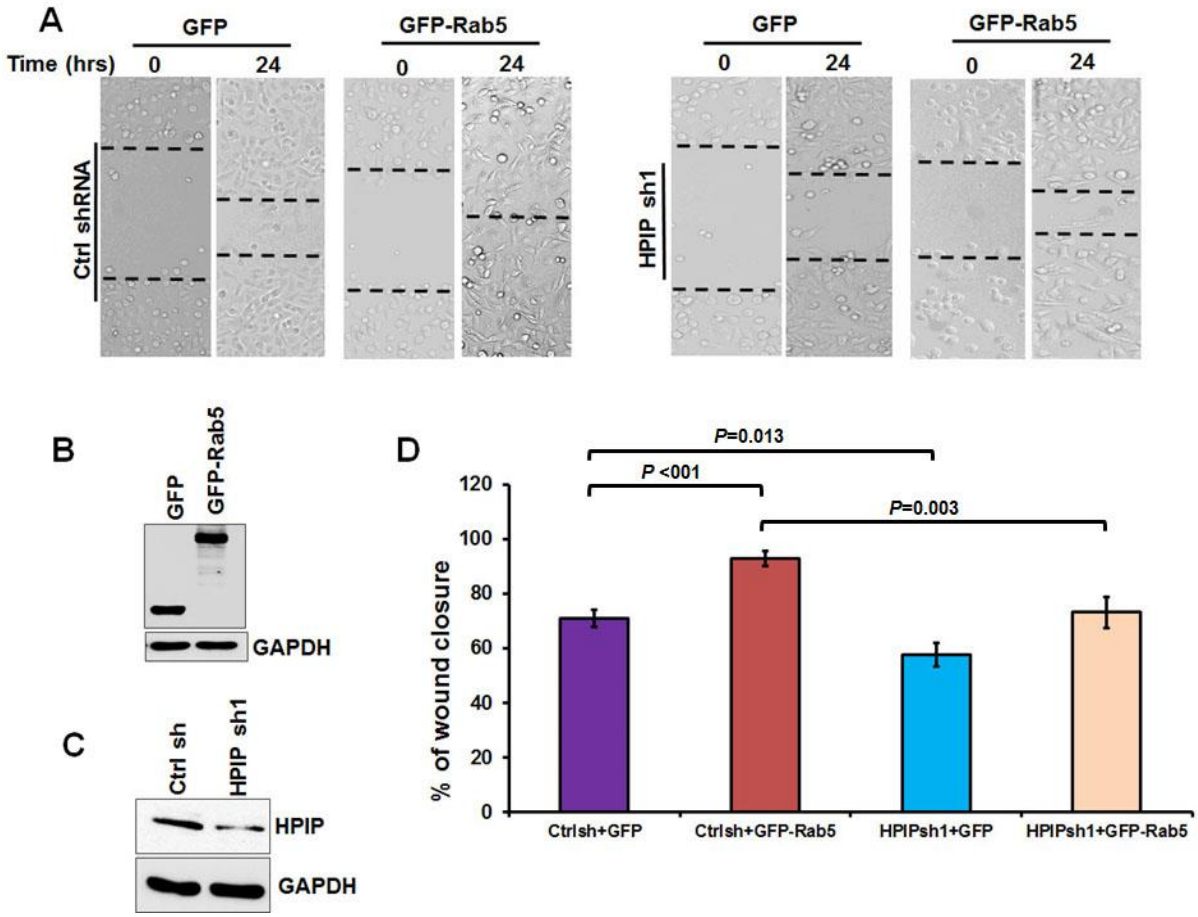

Figure 38. Effect of HPIP knockdown on Rab5-mediated cell migration. (A) Representative bright field microscopy images (10x magnification) of MDA-MB231 cells expressing ctrl sh+pEGFP, HPIP sh1+ pEGFP, ctrl sh+pEGFP-Rab5 or HPIP sh1+ pEGFP-Rab5 after creating wound and at later time points. (B-C) Western blot analysis shows effective knockdown of HPIP in MDA-MB231 cells. Similarly Western blot analysis shows over expression of GFP-Rab5 in MDA-MB231 cells. (D) Quantification of the kinetics of wound closure assay showing the effect of either HPIP knockdown or Rab5 overexpression on migration of confluent monolayers of MDA-MB231 cells.

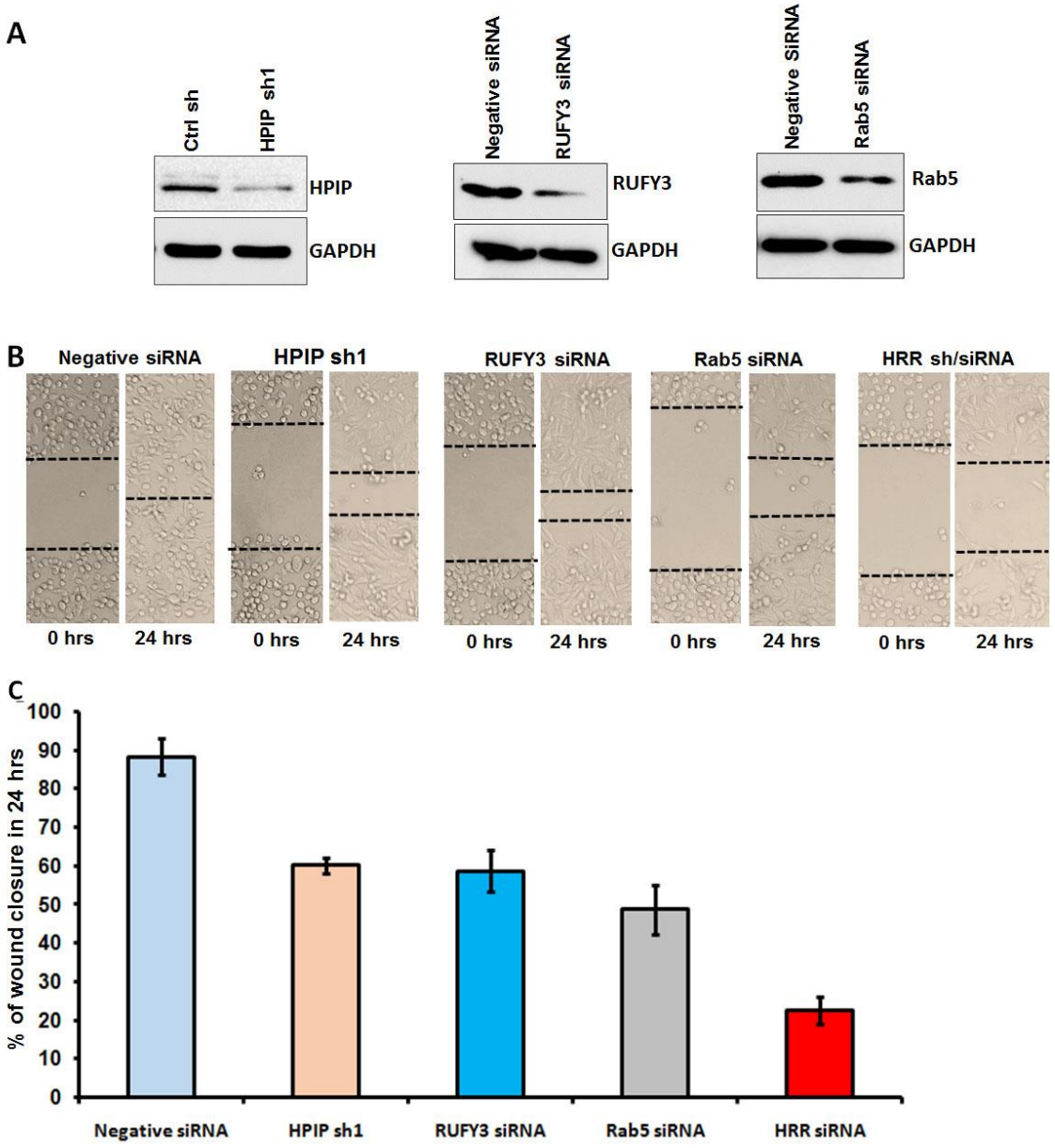

Figure 39. HRR complex regulates cancer cell migration. (A) Western blot analysis showing knockdown of either HPIP, RUFY3 or RAB5 using gene-specific siRNAs in MDA-MB231 cells. (B) Representative bright field microscopy images (10x magnification) of MDA-MB231 cells expressing negative siRNA, HPIPsh1, RUFY3siRNA or Rab5siRNA alone or combined knockdown of RUFY3, HPIP and Rab5 after creating wound and at later time points. (C) Quantification of the kinetics of wound closure assay showing the effect of HPIP, Rab5 or RUFY3 knockdown or combined knockdown of HPIP, Rab5 and RUFY3 on migration of confluent monolayers of MDA-MB231 cells. P=0.001, samples between negative siRNA and HPIP sh1; samples between negative siRNA and RUFY3 siRNA, P=0.002; samples between negative siRNA and Rab5 siRNA, P=0.001; samples between negative siRNA and HRR siRNA (HPIP sh1, RUFY3 siRNA and Rab5 siRNA), P<0.001; samples between HRR siRNA and HPIP sh1, P<0.001; samples between HRR siRNA and RUFY3 siRNA, P=0.001; samples between HRR siRNA and RUFY3 siRNA, P=0.004.

As HPIP forms HRR complex by associating with Rab5 and RUFY3, we next examined whether HRR complex regulates cell migration. As anticipated, a significant decrease in MDA-MB231 cell migration was observed upon knockdown of RUFY3, HPIP and Rab5 as compared to either ctrl-siRNA or individual siRNA-treated MDA-MB231 cells (Figure 39), indicating that HRR complex is crucial for cell migration.

3.4.6 HPIP promotes Rab5 activation in breast cancer cells:

After establishing the role of HRR complex in cell migration, we next investigated the mechanism of cell migration mediated by HRR complex. First we analyzed whether Rab5 and HPIP colocalizes to endosomes during microtubule regrowth as this was the case for proteins involved in focal adhesion-induced disassembly followed by integrin internalization. To test this, MDA-MB231 cells were transfected with GFP or GFP-Rab5 and then, focal adhesion disassembly was stimulated by microtubule regrowth using nocadazole washout. Before MT regrowth (Nocadazole 0 min), only a small subset of the Rab5 containing endosomes were

colocalized with HPIP. However, after microtubule regrowth (Nocadazole washout 60 min) and focal adhesion disassembly, large number of the Rab5-endosomes colocalized with HPIP. The extent of colocalization of HPIP with Rab5 increased more than threefold after microtubule regrowth (Figure 40), indicating that HPIP and Rab5 interaction was increased during integrin internalization and recycling.

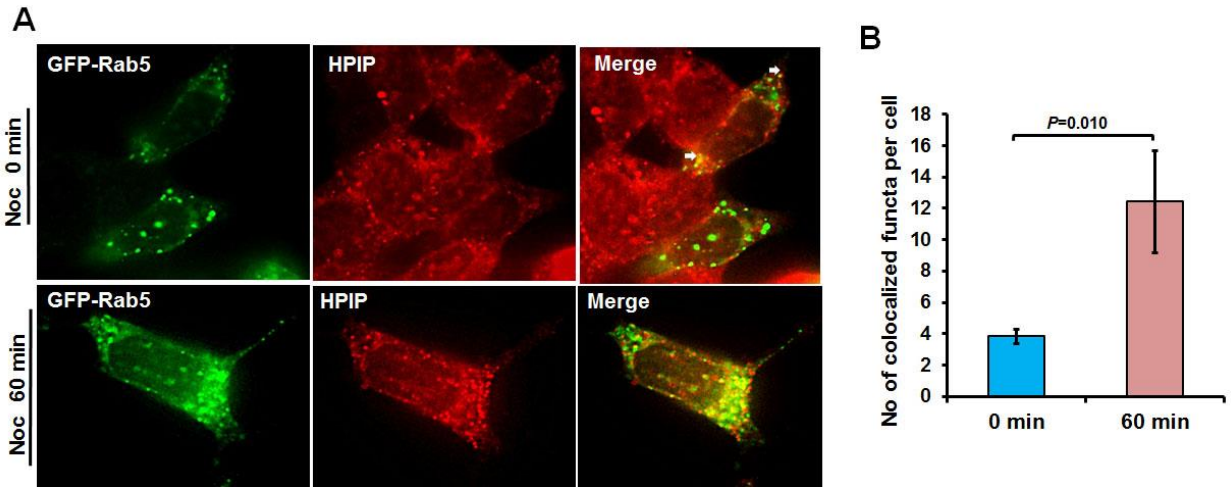

Figure 40. Rab5 and HPIP colocalization was increased during integrin endocytosis. (A) Immunofluorescence of HPIP in MDA-MB231 cells transiently transfected with GFP-RAB5 and treated with nocodazole (10 µM) for 4 hrs. Sixty minutes after treatment which establishes microtubule regrowth, cells were fixed and subjected to immunofluorescence analysis. Arrowheads indicate colocalization points of Rab5 and HPIP. (B) Quantification of the extent of colocalization of HPIP and Rab5 in endosomes at 0 and 60 min of microtubule regrowth. The graph represents analysis of colocalised puncta in 10-15 cells for each condition.

As Rab5 was reported to control cancer cell migration and invasion by activating FAK and modulating FA dynamics in a GTP dependent manner, we reasoned that HPIP might activate Rab5 to induce endocytosis and cell migration. Therefore, we next evaluated the effect of HPIP

knockdown on Rab5 activation in MDA-MB231 cells by measuring Rab5-GTP using Rab5 activation assay. As anticipated, over expression of HPIP in MDA-MB231 cells significantly increased Rab5–GTP when compared with vector transfected cells (Figure 41A). Further, knockdown of endogenous HPIP significantly decreased the Rab5-GTP loading as compared to Ctrl-shRNA transfected cells. Conversely, forced expression of HPIP in which HPIP had been knocked down by HPIPsh1 restored Rab5-GTP levels (Figure 41B), suggesting that HPIP promote Rab5 activation in breast cancer cells, which partly explains the role of HPIP in HRR-mediated cell migration.

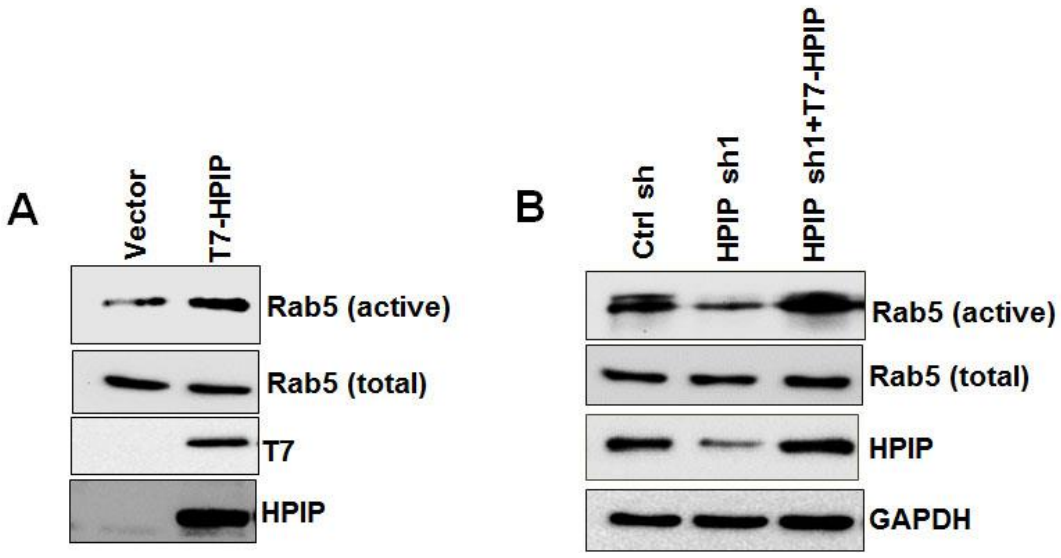

Figure 41. HPIP regulates Rab5 activation in cancer cells. (A) MDA-MB231 cells stably transfected with either ctrl shRNA or HPIP sh1 or HPIP sh1+T7-HPIP were (Bugide *et al*., 2014) utilized for Rab5 activation assay. Rab5-GTP loading assay were performed using Rab5 activation kit. Cell extracts were immunoprecipitated with Rab5 active antibody, which specifically binds to Rab5-GTP form, and subjected to Western blot analysis using indicated antibodies. (B) MDA-MB231 cells were transiently transfected with either pcDNA or pcDNA-HPIP plasmids. Post transfection, cell extracts were subjected to Rab5 activation assay and analysed by Westernblotting as indicated.

3.5 Discussion:

Increasing body of evidence suggest that HPIP regulates cell migration and invasion in variety of cancer cell lines (Manavathi *et al*., 2006; Xu *et al*., 2013; Van Vuurden *et al*., 2014; Feng *et al*., 2015). Our recent findings using breast cancer cell lines MDA-MB231 and MCF7 revealed that HPIP regulates these processes by modulating focal adhesion disassembly through FAK/MAPK/calpain2 pathway (Bugide *et al*., 2014). However the detailed mechanisms that underlie these processes remain elusive. Here, we report a novel function of HPIP in regulating Rab5 activity and thereby cell migration. *In vitro* interaction studies supported the notion that HPIP interacts with Rab5 and RUFY3 and forms a protein complex, which we named as 'HRR complex' (pronounced as HoRRor complex) in MDA-MB231 cells. Indeed, both HPIP and RUFY3 were found to colocalize with Rab5, an early endosome marker, within the endosomes. Further, our studies revealed that HPIP promotes Rab5 activation and is involved in Rab5-meadiated cell migration.

HPIP is a scaffolding protein and known to interact with FAK, calpain2, PI3K, Src, ERα and microtubules to activate cell survival and cell migration pathways (Manavathi *et al*., 2006; Bugide *et al*., 2014). In this study we sought to identify novel interacting partners thus it will provide new insights into HPIP-mediated cellular signaling. Through *in silico* analysis, we identified two highly conserved coiled coil domains in HPIP protein. Coiled coil domain is the versatile motif present in myriad number of proteins and is mainly involved in protein-protein interactions (Wolf *et al*., 1997; Newman *et al*., 2000). Using coiled coil domains of HPIP, we identified four novel interacting partners of HPIP such as RUFY3, PHLB2/LL5β, Krueppel-related zinc finger protein 1 and CCDC105. Of these, RUFY3 was recently shown to involve in gastric cancer cell migration and invasion by promoting the protrusions at cell periphery that are

necessary for cell motility (Wang *et al*., 2015). CCDC105 might involve in microtubule cytoskeletal organization and thus may have role in cell migration, but its functions are not well understood. Krueppel-related zinc finger protein 1 (KFP1) is over expressed in lung cancers. Gene locus on chromosome 19q, where this gene represents is amplified in ovarian and cervical cancers implying a role for KFP1 in oncogenesis (Oguri *et al*., 1998; Arnold *et al*., 1996). PHLB2/LL5β, another HPIP interacting protein identified, was recently shown to involve in regulation of microtubule growth at the basal cortex of the cell and interacts with cell adhesion molecules such as laminin receptor at cell adhesion sites (Hotta *et al*., 2010). Although all four interacting proteins of HPIP identified in this study have putative or demonstrated role in cell migration and cancer progression, we particularly focused on deciphering the functional significance of HPIP-RUFY3-Rab5 complex in regulation of cell migration due to the following reasons: 1) recent studies have implicated a role for RUFY3 in cell migration (Wang *et al*., 2015), 2) RUFY3 was previously shown to interact with Rab5, an early endosomal marker (Yoshida *et al*., 2010), 3) emerging studies showed a role for Rab5 in focal adhesion disassembly, cell migration and invasion (Torres *et al*., 2008; Torres *et al*., 2010; Diaz *et al*., 2014) similar to HPIP.

Our initial investigation finds that HPIP is distinctly localized to speckle like (puncta) structures which are similar to endosomes in OAW42 and MDA-MB231 cells (Figure 33). In support of this, immunofluorescence studies indeed showed localization of HPIP into endosomes similar to early endosomal marker Rab5. Further localization studies indicated that HPIP colocalizes with Rab5 but not with either EEA1 or Rab7, a late endosomal marker, indicating that HPIP specifically associates with Rab5. In particular, HPIP associated with either wild type Rab5 or constitutively active mutant Rab5 (Q79L), but not with constitutively inactive Rab5

(S34N) in MDA-MB231 cells, paralleling observations with the newly identified HPIP interacting protein RUFY3 which showed similar interactions with Rab5 (Yoshida *et al*., 2010). Residing on the cytoplasmic face of endosomes, active Rab5 (Rab5-GTP) associate with effector proteins to control the endosomal trafficking (Christoforidis *et al*., 1999). Preferential binding of HPIP with active Rab5 implies that HPIP could serves' as an effector protein. Microtubules are known to involve in endosomal trafficking by acting as a track upon which endosomes move as 'cargo' (Caswell *et al*., 2009). Our lab previously reported that HPIP is a microtubule interacting protein (Manavathi *et al*., 2006). As evident from our results shown in figure 35, HPIP associates with Rab-Q79L from outside of enlarged endosome, HPIP might link endosomal Rab5 and microtubule and acts as an anchor protein for endosomal transport. The colocalization studies clearly revealed that HPIP interacts with Rab5 but not EEA-1, indicating that HPIP partners with endosomal components towards the cytoplasmic face and help in anchoring the endosomes to microtubules required for cargo trafficking/sorting. Also, association of HPIP with early endosomal marker Rab5 but not with Rab7, a late endosomal marker involved in degradation of proteins, suggests that HPIP is part of early endosomal machinery.

The two coiled coil (CC) domains present in HPIP may impart a unique architecture to this protein that acts as recognition site for many interacting partners. It is clearly evident from our investigations that Rab5 interacts with CC1 domain of HPIP, while RUFY3 interacting with both CC1 and CC2 regions of HPIP. Further, both *in vitro* and *in vivo* binding studies demonstrated that HPIP forms a HRR complex involving Rab5 and RUFY3, indicating that CC domains are bridging this molecular association.

Next we focused analyzing the functional significance of HRR complex using MDA-MB231cells. The roles of Rab5, RUFY3 and HPIP in cell migration have already been reported

(Bugide *et al*., 2014; Torres *et al*., Wang *et al*., 2015), but the molecular interdependence in regulation of cell migration is largely unknown. RUN domain containing proteins like RUFY3 are known to interact with Rab family members to regulate membrane trafficking and cell migration (Yoshida et al., 2010; Kitagishi *et al*., 2013; Wang *et al*., 2015). Accumulating evidence indicate that Rab5 activation is crucial for tumor cell migration, integrin internalization and recycling (Pellinen *et al*., 2006; Palamidessi *et al*., 2008; Torres *et al*., 2010). Further studies indicated that FAK activation is required for Rab5-mediated cell migration and invasion (Mendoza *et al*., 2013). Similar to Rab5, our recent studies clearly shown that HPIP-mediated cell migration is FAK-dependent (Bugide *et al*., 2014) implying that Rab5 may require HPIP for FAK activation and cell migration. Accordingly, HPIP knockdown significantly inhibited Rab5-meadiated cell migration, suggesting that HPIP is partly involved in Rab5-dependent cell migration by promoting Rab5 and FAK activation. Furthermore, combined knockdown of HPIP, Rab5 and RUFY3 showed cumulative suppressive effect on MDA-MB231 cell migration as compared to knockdown of HPIP, RUFY3 or Rab5 alone. However, the effect of HPIP knockdown on downstream effectors of Rab5 such as TIAM1 and Rac activation or Rab5 interaction with focal adhesion proteins remains to be investigated and further studies are warranted.

Recent studies revealed that caspase-8 promotes Rab5-mediated internalization and recycling of β1 integrins by increasing Rab5 GTP loading through interacting with p85α (Torres *et al*., 2008; Torres *et al*., 2010). Supporting this, microtubule regrowth studies shown increased Rab5 and HPIP colocalisation during integrin internalization and recycling, suggesting a possible involvement of HPIP in integrin internalization and recycling. We presume that RUFY3 may

recruit HPIP to endosomes by interacting with it and forms HRR complex. Future studies are warranted to unravel the mechanism of HRR complex formation *in vivo*.

From this study, we conclude that HPIP is a new activator of Rab5, and HPIP, RUFY3 and Rab5 function collaboratively to promote cell migration. These results provide a new molecular mechanism by which HPIP promotes cancer cell migration.

Importance of the study and future perspectives:

Ever since the role of HPIP in tumor development is reported (Manavathi *et al*., 2006), over expression of HPIP in more than half a dozen different cancers has been documented. For instances, HPIP over expression is found in hepatocellular carcinoma (Xu *et al*., 2013), leiomyosarcoma (Silveira *et al*., 2013) glioma (van Vuuden *et al*., 2014), oral carcinoma (Okada et al., 2014), colorectal carcinoma (Feng et al., 2015) and gastric cancer (Feng *et al*., 2015). A recent study by Shostak *et al*., 2014 shown that HPIP is subjected to TBK1-mediated phosphorylation and followed by MDM2-depenendent ubiquitination and degradation, which restrains estrogen-mediated AKT activation that confers the resistance to tamoxifen in breast cancer cells (Shostak *et al*., 2014). Here we found that HPIP is over expressed in infiltrative ductal carcinoma (Bugide *et al*., 2014), and its expression was positively correlated with poor patient survival suggesting the consideration of HPIP as a potential prognostic marker for invasive/infiltrative ductal carcinoma (IDC).

We and others showed that HPIP regulates cell migration and invasion by activating various signaling cascades (Manavathi *et al*., 2006; Xu *et al*., 2013). However, the precise mechanism by which HPIP regulates the cell migration and invasion remains elusive. In our study, using breast cancer cell lines as model system, we discovered a regulatory mechanism by

which HPIP controls cancer cell migration. We identified that HPIP is involved in cell migration by virtue of its interaction with crucial components of focal adhesion as well as endosomal machinery. It physically interacts with FAK, a game changer of FA dynamics, and Rab5, the counterpart of FAK in respect to regulation of endosomes. Activation of FAK leads to faster turnover of focal adhesions, whereas activation of Rab5 is associated with quicker recycling of FA components and their availability at cell membrane. Displaying this dual effect, HPIP accelerates the adhesion-deadhesion cycle at leading edge, thereby directly controls the rate of cell mobility (Figure 42). HPIP interacts with and activates FAK in a migrating cell. Mechanistic insights into this interaction further revealed that HPIP induces focal adhesion disassembly by enhancing calpain2 activity through MAPK pathway. Activation of calpain2, a calcium-dependent protease, by HPIP leads to proteolysis of talin, a key component involved in maintenance of integrin that contacts with extracellular matrix, which results in focal adhesion disassembly and cell migration. Interestingly, activated calpain2 by HPIP in turn cleaves HPIP implying the tight regulation of HPIP-FAK-calpain2 signaling cascade during cell migration (Bugide *et al*., 2014; Bugide and Manavathi, 2015).

Role of endocytosis and small Rab GTPases in tumor metastasis is emerging as a key issue in cancer research. Recent evidence revealed that early endosomal markers such as Rab5 and Rab21 are elevated in different cancers and regulate cell migration by accelerating integrin internalization and recycling (Cheng *et al*., 2004; Pellinen *et al*., 2006; Stenmark *et al*., 2009; Subramani *et al*., 2010). Integrin endocytosis could be coupled to focal adhesion disassembly, in which microtubules are targeted to dynamin-dependent internalization sites (Burridge, 2005; Ezratty *et al*., 2005). Here, we discovered Rab5 and RUFY3 as novel interacting proteins of HPIP in regulation of endosome-mediated cell migration. Here we showed that HPIP forms a

complex involving RUFY3 and Rab5, which is critical for regulation of cancer cell migration. In addition, HPIP and Rab5 interaction was significantly increased during integrin internalization time, indicating that HPIP might involve in Rab5-mediated integrin internalization and recycling. The focal adhesion junction is a hub of diverse signaling pathways and scaffolding proteins such as HPIP link these diverse cellular pathways. The importance of HPIP as a signaling scaffold at focal adhesion points couldn't be denied owing to its rich amino acid diversity and existence of coiled coil domains which act as recognition sites for many proteins. Owing to this very nature, over expression of HPIP could have cascading effects on deregulated cellular motility and therefore tumor metastasis. The triumvirate of HPIP, Rab5 and RUFY3 (HRR complex) at the helm of migration signaling in a cell could be a key determining factor for activation of effector proteins that eventually results in cell mobility.

Further, tight regulation of focal adhesion dynamics by HPIP is clearly evident from the fact that HPIP itself is a calpain2 substrate and proteolysis of HPIP by calpain2 is associated with decreased FA dynamics, establishing a strong foundation that HPIP is an integral part of FA machinery. FAK is not only critical for FA disassembly but also for the proper FA contacts. The study involving the mapping of calpain2 cleavage sites in HPIP followed by investigations with calpain2 cleavage site defective mutant of HPIP may provide critical mechanistic insights into HPIP-mediated cell migration and invasion. Another intriguing finding from this study is identification of four interacting proteins of HPIP that are previously known to involve in cell migration, invasion, cytoskeletal organization and cancer progression. However the role of these proteins in such critical pathways is poorly understood. Hence, studying the functional interaction between HPIP and these proteins would reveal new mechanisms of cell migration and metastasis. RUFY3, one of the HPIP interacting protein, was shown to interact with Fascin, an

actin cytoskeletal remodeling protein that was shown to involve in FA dynamics (Wei *et al*., 2014). Further, RUFY3 was shown to promote lamellopodia like protrusions at cell periphery which promote cell migration (Wang *et al*., 2015). It might be interesting to study the role of HPIP in actin cytoskeleton regulation and stress fiber formation that are important for cell migration. HPIP activates Rab5, and Rab5 is known to be involved in Rac1, a key modulator of actin cytoskeleton activation, by regulating the Rac1-GEF TIAM1 (Lanzetti *et al*., 2004; Palamidessi *et al*., 2008; Torres *et al*., 2010). Thus studying the role of HPIP in Rac1 activation and actin cytoskeleton dynamics might provide additional mechanisms for HPIP-mediated cell migration.

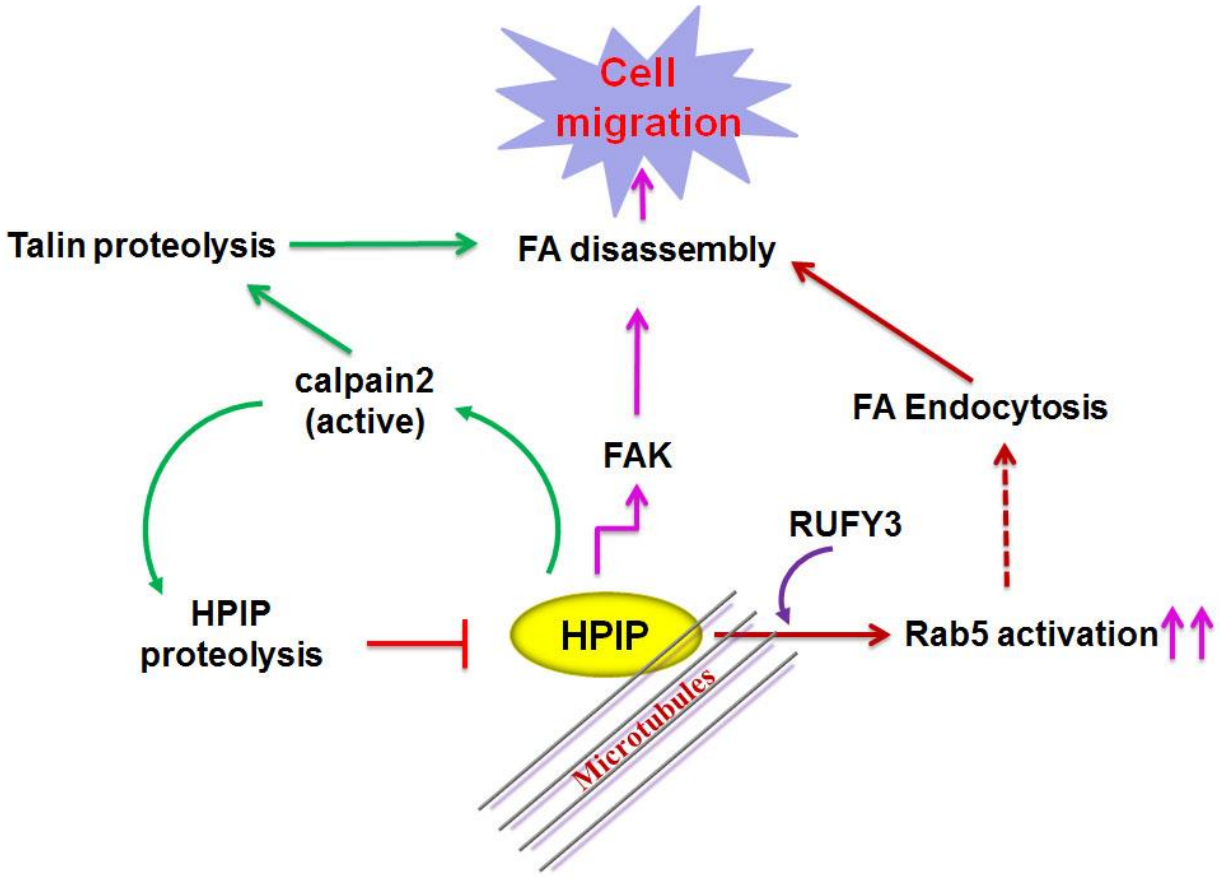

Figure 42: A proposed model for HPIP mechanism in cell migration: HPIP-mediated FAK and calpain2 activation leads to faster turnover of focal adhesions, whereas activation of Rab5 is associated with quicker recycling of FA components and their availability at cell membrane. Displaying this dual effect, HPIP might accelerates the adhesion-deadhesion cycle at leading edge, thereby directly controls the rate of cell mobility. Activated calpain2 in turn cleaves HPIP implying the tight regulation of HPIP-FAK-calpain2-Rab5 signaling cascade during cell migration. FA, focal adhesions; FAK, focal adhesion kinase.

To summarize, being over expressed in more than half a dozen popular cancers, complete functional evaluation of HPIP in the context of cell migration, invasion and metastasis is essential to consider HPIP as a druggable target in the treatment of this malicious disease of human.

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ORIGINAL ARTICLE

Hematopoietic PBX-interacting protein (HPIP) is over expressed in breast infiltrative ductal carcinoma and regulates cell adhesion and migration through modulation of focal adhesion dynamics

S Bugide[1], D David[2], A Nair[2], N Kannan[3], VSK Samanthapudi[1], J Prabhakar[4] and B Manavathi[1]

The scaffolding protein, hematopoietic PBX-interacting protein (HPIP/PBXIP1), regulates cell migration necessary for cancer cell dissemination. However, the mechanism that governs this process remains unknown. We show here that *HPIP* expression is associated with stages of breast cancer where cell dissemination results in poor patient outcome. Our investigation finds a novel association of HPIP with focal adhesion kinase (FAK) regulating FA dynamics. Interestingly, this interaction that led to activation of FAK protein was mediated by the C-terminal domain of HPIP and not the typical integrin-binding motif. Further, short hairpin RNA-mediated knockdown of FAK expression significantly reduced HPIP-induced cell migration indicating participation of FAK pathway. Live-cell time-lapse imaging and biochemical analysis further established the role of HPIP in microtubule-induced FA disassembly. We also found that HPIP-mediated MAPK activation led to phosphorylation and subsequent activation of calpain2, and the activated calpain2 in turn proteolyses FA protein, talin. Interestingly, HPIP is also proteolysed by calpain2 in breast cancer cells. The proteolysis of HPIP and talin by calpain2, and the activation of calapin2 by HPIP-mediated MAPK phosphorylation, is a novel regulatory axis to modulate the cell migration signal. Together, we have determined HPIP as a novel activator of FAK and a new substrate of calpain2. These molecular interactions between HPIP and FAK, and HPIP and calpain2 regulate cell adhesion and migration through modulation of FA dynamics.

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INTRODUCTION

Progression of epithelial cancer involves complex coordinated processes of highly regulated molecular events such as loss of cell adhesion, cell–cell and cell–extracellular matrix interactions resulting in cell migration and invasion.[1,2] These processes are to a major extent regulated by integrins and proteins associated with focal adhesion (FA) formation like FA kinase (FAK), paxillin, talin and others.[3] Cells move by applying traction forces against the substratum at focal contacts, besides these FAs also act as localized dynamic signaling centers that regulate cell migration. Such dynamic signaling centers involve FA turnover, leading edge protrusion, tail retraction and detachment.[4,5] Importantly, coordination between actin cytoskeleton and microtubules is essential for efficient cell adhesion and migration.[6,7]

FAK is an essential regulator of cell migration as cells deficient in FAK showed poor cell migration and reintroduction of FAK into such cells restored cell migration.[8,9] Overexpression and increased activity of FAK are found in several types of metastatic cancers.[10–13] On localization to FAs, FAK activates itself by autophosphorylation at tyrosine 397.[14,15] Phosphorylation at this site is important for controlling of FA turnover.[16] Recent report suggests that FAK phosphorylation at Y397 but not kinase domain is important for cell migration.[17] FAK appears to control FA turnover by modulating the activity of MAPK-calpain2 signaling pathway and is also a substrate of calpain2.[18]

Although actin cytoskeleton and FAK have major roles in FA formation and maturation, growing body of evidence suggests that highly polarized activity of cell migration is brought about by microtubules.[19] Cells treated with microtubule poison drugs such as nocadazole interfere with cell migration by affecting the FA disassembly and cell migration in a FAK-dependent manner.[20] Furthermore, microtubule motor protein, kinesin-1, has been implicated in regulating microtubule-induced FA disassembly.[21] However, to date, only few reports are available to suggest the role of microtubule-binding proteins in FA turnover. Here we suggest a role for hematopoietic PBX-interacting protein (HPIP), also known as pre B-cell leukemia homeobox-interacting protein (PBXIP1), a microtubule-binding protein, in FA turnover and cell migration.

HPIP was initially identified as a PBX1-interacting protein that regulates PBX1-mediated transcription functions and later its role in hematopoiesis has been determined.[22,23] We previously reported that HPIP interacts with microtubules and has a role in estrogen receptor signaling in cancer cells.[24] Recent studies revealed that HPIP is involved in proliferation, migration and anchorage-independent growth of breast cancer cells by

[1]Molecular and Cellular Oncology Laboratory, Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, India; [2]Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India; [3]Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, British Columbia, Canada and [4]Regional Cancer Centre, Thiruvananthapuram, India. Correspondence: Dr B Manavathi, Molecular and Cellular Oncology Laboratory, Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500046, Telangana, India.
E-mail: manavathibsl@uohyd.ernet.in
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Functional Regulation of Pre-B-cell Leukemia Homeobox Interacting Protein 1 (PBXIP1/HPIP) in Erythroid Differentiation*[S]

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Bramanandam Manavathi[‡1], Dennis Lo[§2], Suresh Bugide[‡2], Oindrilla Dey[‡], Suzan Imren[§], Mitchell J. Weiss[¶], and R. Keith Humphries[§3]

From the [‡]Molecular and Cellular Oncology Laboratory, Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad-500046, India, the [§]Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, British Columbia V5Z 1L3, Canada, the Department of Medicine, University of British Columbia, Vancouver, British Columbia V5Z 1L3, Canada, and the [¶]Division of Hematology, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104

Background: HPIP is a pre-B-cell leukemia homeobox 1 (PBX1) interacting protein with unknown function in hematopoiesis.
Results: The *HPIP* gene is a target of GATA1 and CTCF and regulates erythroid differentiation involving PI3K/AKT-dependent mechanisms.
Conclusion: *HPIP* is a novel downstream target of GATA1 and serves as an essential regulator of erythroid differentiation.
Significance: A new regulator of erythroid differentiation is discovered. This finding may help in better understanding erythropoiesis.

Pre-B-cell leukemia homeobox interacting protein 1 or human PBX1 interacting protein (PBXIP1/HPIP) is a co-repressor of pre-B-cell leukemia homeobox 1 (PBX1) and is also known to regulate estrogen receptor functions by associating with the microtubule network. Despite its initial discovery in the context of hematopoietic cells, little is yet known about the role of HPIP in hematopoiesis. Here, we show that lentivirus-mediated overexpression of HPIP in human CD34$^+$ cells enhances hematopoietic colony formation *in vitro*, whereas HPIP knockdown leads to a reduction in the number of such colonies. Interestingly, erythroid colony number was significantly higher in HPIP-overexpressing cells. In addition, forced expression of HPIP in K562 cells, a multipotent erythro-megakaryoblastic leukemia cell line, led to an induction of erythroid differentiation. HPIP overexpression in both CD34$^+$ and K562 cells was associated with increased activation of the PI3K/AKT pathway, and corresponding treatment with a PI3K-specific inhibitor, LY-294002, caused a reduction in clonogenic progenitor number in HPIP-expressing CD34$^+$ cells and decreased K562 cell differentiation. Combined, these findings point to an important role of the PI3K/AKT pathway in mediating HPIP-induced effects on the growth and differentiation of hematopoietic cells. Interestingly, *HPIP* gene expression was found to be induced in K562 cells in response to erythroid differentiation signals such as DMSO and erythropoietin. The erythroid lineage-specific transcription factor GATA1 binds to the *HPIP* promoter and activates *HPIP* gene transcription in a CCCTC-binding factor (CTCF)-dependent manner. Co-immunoprecipitation and co-localization experiments revealed the association of CTCF with GATA1 indicating the recruitment of CTCF/GATA1 transcription factor complex onto the *HPIP* promoter. Together, this study provides evidence that *HPIP* is a target of GATA1 and CTCF in erythroid cells and plays an important role in erythroid differentiation by modulating the PI3K/AKT pathway.

The human hematopoietic system is composed of a heterogeneous population of cells that range in function from mature cells with limited proliferative potential to pluripotent stem cells known as hematopoietic stem cells (HSC)[4] with extensive proliferation, differentiation, and self-renewal capacities (1, 2). This process is governed by the interplay of a number of transcription factors and various signaling pathways, which altogether facilitate proper hematopoietic development (3, 4). Emerging evidence indicates that human leukemias, lymphomas, and possibly myelodysplastic syndromes are initiated at the level of HSCs and/or early multipotent progenitors that have been transformed due to genetic/chromosomal aberrations or deregulation of gene expression (5). Of several regulators of HSC, PBX transcription factors play an important role in the establishment and maintenance of definitive hematopoiesis, and PBX overexpression has been linked to leukemia development (6). PBX proteins mainly act as cofactors for HOX pro-

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[1] To whom correspondence may be addressed: Dept of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad-500046, India. E-mail: manavathibsl@uohyd.ernet.in.
[2] Both authors contributed equally to this work.
[3] To whom correspondence may be addressed. E-mail: khumphri@bccrc.ca.

[4] The abbreviations used are: HSC, hematopoietic stem cell; PBX1, pre-B-cell leukemia homeobox 1; PBXIP1 or HPIP, pre-B-cell leukemia homeobox interacting protein 1; Epo, erythropoietin; q, quantitative; CFC, colony-forming cell assay; E/Meg, erythroid and megakaryocyte; 4-OHT, 4-hydroxytamoxifen; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CTCF, CCCTC-binding factor.

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RESEARCH HIGHLIGHT

Engagement, reception and breakup: Three steps of cell motility regulated by HPIP signaling

Suresh Bugide, Bramanandam Manavathi

Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad-500046, India

Correspondence: Bramanandam Manavathi
E-mail: manavathibsl@uohyd.ernet.in
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Tumor metastasis is the prime cause for increased morbidity and mortality for majority of the cancers. Invasive or infiltrative ductal carcinoma (IDC) is the predominant form of breast cancer, accounting approximately 80% of all breast cancers. The molecular portraits and the mechanism by which they promote IDC remain largely unknown. Hematopoietic PBX interacting protein (HPIP/PBXIP1), a microtubule binding protein, regulates cancer cell migration and invasion. However, functional mechanism underlying HPIP-mediated cell migration in cancer remains uncertain. Here, we describe our recent studies in which we identified the new mechanisms by which HPIP regulates cell migration in breast cancer cells. Our recent studies confirmed that *HPIP* expression is elevated in breast infiltrative ductal carcinoma and positively correlated with poor patient survival. We reported that HPIP directly interacts and activates FAK to promote the cell migration. Mechanistic studies further revealed that HPIP induces focal adhesion disassembly by enhancing calpain2 activity through MAPK pathway that led to talin proteolysis, focal adhesion turnover and cell migration. Interestingly, the activated calpain2 in turn cleaves HPIP suggesting the tight regulation of HPIP-FAK-calpain2 signaling cascade during cell migration.

Keywords: HPIP; FAK; calpain2; Invasive or infiltrative Ductal Carcinoma (IDC); cell migration

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Cell migration is a multistep and dynamic process involving focal adhesion turnover, leading edge protrusion, tail retraction, generation of tractional forces and detachment [1]. Focal adhesions, large macromolecular complexes that connects the cell physically with the extracellular matrix, play crucial role in cell migration by regulating multiple steps involving attachment of cell to ECM, tail end retraction, leading edge protrusion and detachment of cell at rear edge [2,3,4]. The rapid turnover rate and local rearrangements of focal adhesions often predict the speed of cell migration [5,6]. Diverse array of proteins with different activities such as kinases, proteases, phosphatases, dephosptases, adaptor proteins, microtubules, etc participates in the FA turnover and cell migration [5,7,8]. However, limited reports exist to support the role of microtubule-binding proteins in focal adhesion turnover. In our recent report, we found the regulatory role for hematopoietic PBX interacting protein (HPIP), a microtubule binding protein, in focal adhesion dynamics and cell migration [9].

Hematopoietic Pbx-interacting protein (HPIP) was first identified as a transcriptional repressor of Pbx proteins [10]. It is mostly localized in cytosol but traces amounts also found in nucleus. The transcriptional repression activity of HPIP is primarily attributed to the presence of a nuclear export signals and two functional nuclear localization signals [10]. Recent studies revealed that HPIP is involved in erythroid differentiation and display stem cell activity by modulating the PI3K/AKT/GSK3β signaling pathway [11].

Derailed Estrogen Signaling and Breast Cancer: An Authentic Couple

Bramanandam Manavathi, Oindrilla Dey, Vijay Narsihma Reddy Gajulapalli, Raghavendra Singh Bhatia, Suresh Bugide, and Rakesh Kumar

Molecular and Cellular Oncology Laboratory (B.M., O.D., V.N.R.G., R.S.B., S.B.), Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India; Cancer Research Program (R.K.), Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram 695014, India; and Department of Biochemistry and Molecular Biology (R.K.), The George Washington University, Washington, D.C. 20052

Estrogen or 17β-estradiol, a steroid hormone, plays a critical role in the development of mammary gland via acting through specific receptors. In particular, estrogen receptor-α (ERα) acts as a transcription factor and/or a signal transducer while participating in the development of mammary gland and breast cancer. Accumulating evidence suggests that the transcriptional activity of ERα is altered by the action of nuclear receptor coregulators and might be responsible, at least in part, for the development of breast cancer. In addition, this process is driven by various posttranslational modifications of ERα, implicating active participation of the upstream receptor modifying enzymes in breast cancer progression. Emerging studies suggest that the biological outcome of breast cancer cells is also influenced by the cross talk between microRNA and ERα signaling, as well as by breast cancer stem cells. Thus, multiple regulatory controls of ERα render mammary epithelium at risk for transformation upon deregulation of normal homeostasis. Given the importance that ERα signaling has in breast cancer development, here we will highlight how the activity of ERα is controlled by various regulators in a spatial and temporal manner, impacting the progression of the disease. We will also discuss the possible therapeutic value of ERα modulators as alternative drug targets to retard the progression of breast cancer. (***Endocrine Reviews*** **34: 1–32, 2013)**

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I. Introduction

Breast cancer is heterogeneous in nature that originates from the mammary epithelial cells. Despite advances made in the understanding of the molecular and cellular events that underlie the disease, it remains the leading cause of cancer deaths among females worldwide (1). A woman's risk of breast cancer is influenced by her reproductive history, *i.e.*, lifetime exposure to reproduc-

Abbreviations: AIB1, Amplified in breast cancer-1; AKT, serine/threonine protein kinase; ALDH, aldehyde dehydrogenase; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and rad3-related protein; BCAS3, breast carcinoma amplified sequence 3; BRCA1, breast cancer 1; BrCSC, breast cancer stem cell; BT-IC, breast tumor-initiating cell; CDK4, cyclin-dependent kinase; Ciz1, CDKN1A-interacting zinc finger protein 1; DACH1, dachshund homolog 1; DBC1, deleted in breast cancer 1; DNAPK, DNA-dependent protein kinase; E2, estrogen or 17β-estradiol; Efp, estrogen-responsive finger protein; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ER, estrogen receptor; ERE, estrogen response element; GPR30, G protein-coupled receptor 30; GREB1, growth regulation by estrogen in breast cancer 1; GSK3β, glycogen synthase kinase 3β; HAT, histone acetyl transferase; HDAC, histone deacetylase; HPIP, hematopoietic PBX-interacting protein 1; MaSC, mammary stem cell; miRNA, microRNA; MTA, metastasis-associated protein; MTA1s, MTA1 short form; NCOR1, nuclear receptor corepressor 1; NuRD, nucleosome remodeling and histone deacetylation complex; PAK1, serine/threonine p21-activated kinase; PELP1, proline, glutamic acid and leucine-rich protein; PHB, prohibitin; PI3K, phosphatidylinositol 3 kinase; PKA, protein kinase A; PR, progesterone receptor; PRMT1, protein arginine N-methyltransferase 1; REA, repressor of ER activity; SAFB, scaffold attachment factor B; SCID, severe combined immunodeficiency; SERM, selective ER modulator; SIRT1, sirtuin 1; S6K1, S6 kinase 1; SP, specificity protein; TFF1, trefoil factor 1; UTR, untranslated region.

Plagiarism report

HPIP Signaling in Cell Adhesion and Migration:Potential Role in Tumor Metastasis

by Bugide Suresh

HPIP Signaling in Cell Adhesion and Migration:Potential Role in Tumor Metastasis

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