Functional Analysis of Human Gene Hyaluronan Binding Protein Using Eukaryotic Expression System: *Schizosaccharomyces pombe*

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CERTIFICATE

The research work embodied in this thesis "***Functional Analysis of Human Gene Hyaluronan Binding Protein Using Eukaryotic Expression System: Schizosaccharomyces Pombe***" has been carried out in the SCHOOL OF ENVIRONMENTAL SCIENCES, Jawaharlal Nehru University, New Delhi-110067, INDIA. This investigation is original and has not been submitted in part or full for any degree or diploma for any University elsewhere.

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Once there was One. But the one felt alone.
So there was two.
A duality in perpetual union creating the cosmos out of stillness and motion.
Of this came we: unconscious and immindful yet a duality in our selves.
The motion of the cosmos took us in its flow from the moment of separation.
Thus started the journey to unity never to be measured. Just felt.
A journey of self-realization.
The flow propelled us ahead:
First the world of the body, then the world of the mind, then the world of spirit
and then the worlds of all.
From one to the other painfully, blissfully, tearfully and unconsciously
we went swimming in the waters of the cosmos.
Like an invisible flicker of fire,
consciousness burning brighter, illuminated further and further.
Somewhere one realized . . .. the flow of cosmos, the Divine dance of Shakti.
Where the power really existed.
And one submitted mind, heart and soul.
Surrendered the lotus of the heart to Her for he had nothing else to give.
Streams of tears to wash the ground before her feet.
Daring to pray that he may know that She sits on the Throne of His Heart eternally.
And never forget that he is but Her's.

This humble work is dedicated to Her and her most loved manifestations in my life – My Mother and My Father.

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Jaideep

ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometer
Å	Angstrom
AP	Alkaline phosphatase
APS	Ammonium persulfate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bp	basepair
BSA	Bovine serum albumin
C1q	Complement Factor 1 q
CD	Circular dichroism
cDNA	complementary Deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECL	Enhanced chemiluminiscence
EDC	N-ethyl-N'-(3-dimethyl aminopropyl) carbodimide
EDTA	Ethylene diamine tetra acetic acid
EGFP	Enhanced green fluorescent protein
GFP	Green fluorescence protein
EGTA	Ethylene glycol tetra acetate
RLC	Regulatory light chain
EMM	Edinburgh_Minimal Medium
GDB	Genome data bank
HA	Hyaluronan
Ig	Immunoglobulin
IgG	Immunoglobulin G
IP_3	Inositol 1, 4, 5-triphosphate
kb	kilobase
kDa	Kilodalton
LB	Luria-bertani medium

M	Molar
mg	Milligram
ml	Millilitre
mM	milimolar
mRNA	Messenger RNA
NBT	Nitro Blue Tetrazolium
ng	Nanogram
nm	Nanometre
nM	Nano molar
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
pI	Isoeletric point
PMSF	Phenyl methyl sulfonyl fluride
RNA	Ribonucleic acid
RNase A	Ribonuclease A
ROS	Reactive oxygen species
rpm	rotations per minute
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TEMED	N, N, N', N'-tetramethylene diamine
Tris	Tris-(hydroxymethyl) amino methane

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Introduction

1. INTRODUCTION

Ever since the dawn of civilization humans have tried to understand everything around him, including his own self. In this journey of self-assessment, he has had to study simpler organisms in order to understand the rules underlying the functioning of a living system. By studying the various living beings around him, he understood that there exists great diversity amongst them. One major distinguishing factor between was the unicellularity and multicellularity of the organisms. Till a long time after life began on this planet, all life forms were single-celled, then multicellularity developed for a yet unknown reason. However, multicellularity brought in its wake a host of problems and new ways to solve them. But the main difference this made was that it gave rise to a community life for cells. Like in a city, there are a lot of things other than human beings, similarly, in a multicellular organism, a lot exists outside the cells. Cells are in fact located in a matrix, which does lot more than just providing mere material support to the cells. This matrix is called the extracellular matrix or the ECM. ECM is like the supply, communication and accommodative infrastructure of a city with respect to the cells. It provides material support to individual cells and binds them as required in a three dimensional array. It is through the ECM that molecules mediating cell-cell communication pass. Thus, the molecules of ECM are extremely important, as they are the agents through which the cells keep in touch with their environment.

All cells are in fact, in contact with a network of ECM macromolecules. ECM is nothing but an intricate network of complex macromolecules, which again, are secreted by the cells. It is often the most crucial component of an organ (though, for a long time, it was thought to be an inert framework material). Since the ECM is involved in all the functions of the cell, the study of ECM assumes equal importance

in the study of cells. A variety of versatile proteins and polysaccharides constitute the ECM. These molecules are secreted locally and assembled into an interestingly organized meshwork according to the requirements of the organs. A variation in the relative amounts of the various macromolecules gives rise to amazingly diverse forms in different tissues, adapted for particular functional requirements. This diversity in the matrix forms ranges from the calcified rock hard matrix of the skeletal tissue to the transparent matrix of the tendons. Thus, it is expected that there is an enormous heterogeneity in the structure, function and composition of the ECM isolated from different sources. All these aspects of ECM depend on the tissue of origin, the state of development of an organism and even the precise condition of culture, in case of the ECM obtained from cells cultivated *in vitro*. Changes in the level of various components and composition of ECM, in general, also reflects certain disorders like tumor metastasis and inflammation. Most of the ECM contains a back bone of fibrillar proteins (e.g. elastin or collagen) into which a variety of glycoproteins, complex carbohydrates and proteoglycans are enmeshed, creating a bewildering variety of structurally and functionally unique network to suit a particular cellular milieu. Among these carbohydrates, a major ubiquitous component of the ECM is HYALURONIC ACID (HA), also referred to as Hyaluronan.

I.A HYALURONIC ACID (HA) OR HYALURONAN

Glycosaminoglycans are large carbohydrates that are composed of repeating disaccharide units and exist mainly in four forms : heparan sulphate and heparin, chondroitin sulphate and dermatan sulphate, keratan sulphate and hyaluronic acid. The first three are protein-bound glycosaminoglycans in their natural form and contain sulphate, while hyaluronic acid (HA) is usually found as a free glycosaminoglycan and lacks sulphate. All the GAGs, particularly the sulphated one, bear a

strong negative charge, which helps them to bind to a lot of substances including growth factors and cytokines.

HA is made up of repeating disaccharide unit containing ..D-glucuronic acid (β,1→3) and N-acetyl-D-glucosamine(β,1→4)... and is not sulphated. It was first described in vitreous humour of eyes by Karl Meyer in 1934 (Meyer and Palmer., 1934). Although, HA is ubiquitously present throughout the body, its classical courses apart from the vitreous humour are synovial fluid, umbilical cord, skin and roosters comb. It has also been detected from strains of bacteria like *Streptococci.*

Chemically, HA is a linear polymer and when extracted from different tissues, it shows variable molecular mass, the average molecular mass being several millions (Laurent, 1970). This molecule looks like a single chain in an electron microscope. The length of a chain of molecular weight 4 x 10^6 D is 10μM (Fessler *et al.,* 1966). The molecule is stabilized by hydrogen bonds parallel to the chain axis. The polymer, consequently, takes up a stiff helical configuration, which gives the molecule an overall expanded coil structure in solution. The radius of gyration is about 200 nm, i.e., the coil can be regarded as a highly hydrated sphere containing approximately 1000 fold more water than polymer (Laurent, 1970). The secondary structure of HA proposed by Scott leads to a clustering of hydrophobic groups and forming the basis of inter-chain interactions. (Scott., 1989)

Synthesis of HA is also unique when compared to other GAGs. While a majority of the GAGs are synthesised within the confined spaces of the Golgi compartment, HA is synthesised on the inner side of the plasma membrane by a single enzyme named HA synthase (HAS), using the sugar nucleotides UDP-glucuronic acid and UDP-N-acetyl glucosamine in the presence of Mg^{2+} (Markovich *et al.,* 1959; DeAngelis and Weigel, 1994). While HAS is located at the inner cell membrane, the

newly synthesised HA is extruded into the extracellular space (Prehm, 1984). This extrusion mechanism facilitates the formation of exceptionally large polymers. The rate of HA chain elongation has been estimated to be about 60-disaccharide units per minute in prokaryotic cells (DeAngelis and Weigel, 1994).

1.A.i BIOLOGICAL FUNCTIONS OF HA

HA being a major component of the ECM has a multi-faceted biological activity, which is now being seen not to be limited to the ECM. Earlier it was thought that HA mainly acted as a space-filler in tissues and helped maintain body shape. Later when research progressed, it was found that it interacts with other cells and proteins and modulated many cellular and extracellular activities. The molecular functions of HA broadly fall into three overlapping categories. Firstly, HA occupies an enormous hydrodynamic volume, thereby, influencing the hydration levels and other physical properties of tissues. Secondly, it interacts with other ECM macromolecules, being a major component of the ECM including proteoglycans, aggrecan, versican, etc, which are essential for structure and assembly of tissues. Finally, HA interacts specifically with many cell surface receptors (like CD44, RHAMM, HABP1, etc) and influences cell behaviour. Interestingly, recent evidence has shown that apart from its ECM related functions, HA also operates at the intracellular level. So it is quite obvious that the study of HA is an expanding field which has not yet divulged all its secrets.

1.A.i.a HA IN MORPHOGENESIS AND EMBRYOGENESIS

In the process of forming well defined tissues, cells have to divide, migrate and differentiate according to a very well defined path. This takes place within an extracellular matrix that is rich in hyaluronan and it has been seen that the level and the organization of hyaluronan dramatically changes numerous aspects of cellular behaviour. Striking examples of

dynamic events during which cells are surrounded by hyaluronan-rich matrices are mesenchymal cells invading the primary corneal stroma to form the mature cornea; neural crest cells traveling from the neural tube to form ganglia of the peripheral nervous system; sclerotomal cells approaching and surrounding the notochord to form vertebrae; cushion cells migrating from the endocardium towards the myocardium during formation of heart valves; neuronal and glial precursors moving and proliferating during brain development; mesenchymal cells dividing and migrating during embryonic limb development; salamander limb regeneration; tendon regeneration and fetal wound repair; and tumor cell growth and invasion. Cellular proliferation and migration in these systems usually lead to assembly of cells in appropriate numbers and positions prior to overt differentiation to tissues and organs. At this stage of morphogenesis, pericellular hyaluronan often decreases in concentration and rearranges in such a way as to allow or promote initiation of cell interactions essential for subsequent differentiation, for example during cellular condensation prior to formation of muscle and cartilage in the embryonic limb. One way in which hyaluronan facilitates cell migration is by creating hydrated pathways that allow cellular or fibrous barriers to be penetrated by cells. Formation of hydrated pericellular matrices may also facilitate cell rounding during mitosis. A subsequent decrease in hyaluronan concentration leads to decreased volume of intercellular matrix and closer apposition of cells prior to differentiation. In addition to these physicochemical effects, the studies noted above also led to the discovery of HA receptors on the surface of embryonic and tumor cells and to the enormous proliferation of modern studies that have demonstrated definitively that HA exerts a direct and profound effect on cell behavior. (Toole, 2002)

1.A.i.b CELL SURFACE RECEPTORS OF HA

The fact that HA influenced cellular behaviour so intimately led researchers to look for receptors of HA on the cell surface and two families of receptors were found and were named CD44 and the RHAMM. HA provides an appropriately hydrated, extracellular milieu that facilitates cellular invasion. *In vitro* studies strongly suggest that interactions of HA with RHAMM or CD44 are involved in cell movement and proliferation, which are critical events in morphogenesis (Toole, 1997).

CD44 is a widely distributed cell surface glycoprotein that is encoded by a single gene but expressed as numerous isoforms as a result of alternative splicing. The simplest and most widespread form is termed the standard isoform and is denoted as CD44s (often alternatively termed the hematopoietic isoform or CD44H). CD44s contains transmembrane, cytoplasmic, and extracellular regions that are common to all membrane-bound isoforms of CD44. The extracellular region of CD44s includes two major domains: (1) The amino-terminal domain is a "link module" similar to that found in many hyaluronan-binding proteins. This domain is the presumed binding site for hyaluronan, although binding is subject to numerous positive and negative influences from other regions of the molecule, *e.g.,* glycosylation, alternative splicing, dimerization, clustering in the plasma membrane, and integrity of the cytoplasmic domain (Kincade *et al.,* 1997) (2) The so-called membrane-proximal domain lies between the hyaluronan-binding and transmembrane domains. Various combinations of the products of approximately 10 variant exons can be spliced into a single position within the membrane-proximal domain to give rise to numerous variant isoforms of CD44 that exhibit different physiological properties and variable ability to bind hyaluronan. For example, HA-CD44 interactions have been shown to mediate endocytic removal of HA at critical stages of embryonic development.

Alternative splicing also generates several isoforms of RHAMM, including intracellular and cell surface isoforms. However, RHAMM does not contain a "link module" domain but does include two regions that contain a potential hyaluronan-binding "motif," namely, the linear sequence B-(X_7)-B, where B is a basic amino acid residue and X is any non-acidic amino acid. This sequence is present in most hyaluronan-binding proteins and, at least in part, accounts for their hyaluronan-binding capability. (Entwistle *et al.,* 1996.)

1A.i.c HA IN CANCER

The level of HA has been seen to be high in malignant tumors and these high levels of hyaluronan expression correlate with poor differentiation and decreased survival rates in some human carcinomas. This high level happens both by enhanced production of HA by the tumor cells themselves or by the sounding stromal cells under induction from the former. (Knudson *et al.,* 1989.) The pathways by which HA-receptor interactions influence tumor behavior is not exactly understood though perturbations of HA and its receptors have been seen to have a profound effect on the fate of tumors.(Sherman *et al.,* 1994.; Zeng *et al.,* 1998; Mohapatra *et al.,* 1996.; Yu *et al.,* 1997)

1.A.i.d HA IN OTHER DISEASES

In certain diseases such as rheumatoid arthritis (Laurent *et al.,* 1996), osteoarthritis, liver cirrhosis, scleroderma (Engstrom-Laurent *et al.,* 1985), Werner syndrome, renal failure (Laurent *et al.,* 1996) and psoriasis (Lundin *et al.,* 1985) hyaluronan level is seen to increase in serum. In Atherosclerosis and Restenosis, HA is seen to be a major component of plaques that form in veins and arteries. However, the mechanisms by which HA and its interacting proteins discharge these changes and their connection with the diseases in question are not yet known, but it has been seen that there is a direct link between these

diseases with HA. The accumulation of HA and water in knee of arthritic patients causes "morning stiffness" and similar accumulation in the lungs causes interstitial oedema and impairment of gas exchange (Engstrom-Laurent and Hallgren, 1987; Hallgren, *et al.,* 1989; Nettelbladt *et al.,* 1989; Vignola *et al.,* 1998).

1.A.i.e HA AS AN ANTI-OXIDANT

Extensive studies on patients suffering from Rheumatoid Arthritis (RA) and other inflammatory disorders have shown that HA functions as an anti-inflammatory substance by scavenging the Reactive Oxygen Species (ROS) (Brandt, 1970). An abundance of ROS in the synovial fluid of RA patients have been observed (Niwa *et al.,* 1983) and exogenous application of HA and its subcomponent, D-glucuronic acid can decrease levels of ROS. It has also been observed that HA degrades in the presence of ROS (McCord, 1974). Even though HA is degraded or depolymerised by ROS, it is considered as a potential scavenger for ROS and other free radicals. Since HA can efficiently scavenge hydroxyl radicals ($OH^{\bullet}$) (Myint *et al.,* 1987), protective effect of HA is due to its competition with the cells for $OH^{\bullet}$ hydroxylation (Presti and Scott, 1994). Apparently, the effect of HA is molecular weight and concentration dependent, suggesting that it is not solely related to its primary or secondary structures, but a higher order of organisation is involved. "The mesh-works" of HA formed by self-aggregation keep the ROS producing cells away from the target cells or tissues. This phenomenon may be important in the protection of cartilage by HA (Scott *et al.,* 1991).

Kvam *et al.* (1993) and Cortivo *et al.* (1996) have shown that chemical modification of HA protects it from oxidative damage and thereby improves its properties as an antioxidant. Cortivo *et al.* (1996) have developed a steroid derivative of HA by esterifying it with α-methyl-prednisolone which has much better scavenging properties and protects

cells and tissues from oxidative damage more efficiently than unmodified HA. Since esterified derivatives of HA are more resistant to damage by ROS, it will be interesting to exploit them in the treatment of inflammatory disorders of joints.

1.A.i.f INTRACELLULAR HA

Usually while commenting on the biological functions of HA, the action of HA in the ECM or on the cell surface was observed. However HA is not only present inside the cells but it also is involved in a variety of cellular processes. The presence of HA in the nucleus was reported as early as 1976 by Margolis *et al.* Recent data suggest that intracellular hyaluronan may be involved in growth regulation and mitosis. Hyaluronan is synthesized in large amounts by mitotic cells, (Brecht *et al.,* 1986.; Tammi *et al.,* 1991) where it forms distinct pericellular matrices that can be visualized using a particle exclusion assay (Evanko *et al.,* 1999). In the pericellular matrix, hyaluronan may promote membrane ruffling, focal adhesion turnover, and cell detachment and rounding, in part through the stearic exclusion properties of the hyaluronan-dependent matrix. This pericellular coat also includes hyaluronan-associated molecules such as the aggregating proteoglycans, aggrecan, versican, link protein, TSG-6 and inter-□-trypsin inhibitor, all of which contribute to the physico-chemical and biological properties of the hyaluronan-dependent matrix. Hyaluronan also regulates cell function through signaling, which is mediated by cell surface receptors like CD44 and RHAMM. (Evanko and Wight,2002)

The recognition that hyaluronan is present intracellularly at critical points during cell proliferation and migration suggests that it may have an intracellular mode of action in the regulation of these processes. One emerging possibility is that the processes of uptake, translocation, and possibly, degradation of hyaluronan may be integral to the signaling

and regulatory mechanisms associated with the intracellular hyaluronan-binding molecules (IHABPs), or hyaladherins. However, this field is in its infancy and most of the functional aspects of intracellular hyaluronan are still quite speculative.

1.A.i.g HA IN SPERM-EGG INTERACTION

In addition to playing an important role in embryonic development and cell differentiation, HA plays a critical role in cell-cell interaction. The best example of this interaction, which can be taken as model, is the sperm-oocyte interaction, where HA plays a vital role. Prior to ovulation, the cumulus cell-oocyte complex synthesises and organises an extensive extracellular matrix that is enriched in HA. In addition, HA is also found in follicular fluid as well as zona pellucida and perivitelline space of the oocyte (Epig, 1979; Grimek, 1984; Laurent *et al.,* 1992) Addition of HA to human sperm suspensions *in vitro* appears to improve motility and velocity (Huszar *et al.,* 1990). HA at a very low concentration induces acrosome reaction in rodent and bovine populations (Meizel and Turner, 1986; Ranganathan *et al.,* 1994). It also enhances the zona-induced acrosome reaction in *Cynomolgu macaque* sperm (Vandevoort *et al.,* 1997). The exact mechanism for this is not very clear. However, *in vitro* studies have suggested that HA interacts with PH 20 protein, a sperm HA binding protein, to induce this change in sperm function. The enhancing effect of HA on acrosome reaction involves increase in the basal levels of intracellular calcium and may also involve an increase in the rate of calcium metabolism at the initiation of acrosome reaction (Sabeur, 1998).

1.B.i PROTEINS INTERACTING WITH HA

HA discharges its diverse functions, both intracellular and extracellular, by means of an army of interacting proteins named Hyaladherins that exhibit a great diversity in terms of their tissue

expression, cellular localization, specificity, affinity and regulation (Toole., 1997). These proteins interact with HA by binding with it and there are two observed ways of this: one is by means of the Link Module, and secondly by means of the HA binding motif or the B-(X_7)-B motif. The Link Module is also referred to as the proteoglycan tandem repeat (PTR) and the proteins containing the link module have a common structural domain of about 100 amino acid residues (Day, 1999).). In the B- (X_7)-B motif, the B is either arginine or lysine and X_7 contains a stretch of 7 amino acids, none of whom are acidic residues and at least one is basic. This motif was defined by Yang *et al.* (1994). Some of these proteins have a series of cysteine residues whose relative positions are conserved and form disulfide bridges to establish loop structures in the protein (Goetinck *et al.,* 1987; Wolffe *et al.,* 1990). Functional relevance of these loop structures for HA- binding has been suggested by the finding that HA-binding is abolished under reducing conditions (Toole, 1990). Most of the matrix HA-binding proteins like cartilage link protein (Goetinck *et al.,* 1987), aggrecan (Fosang *et al.,* 1991), versican (Le Baron *et al.,* 1997), Brain enriched hyaluronan binding protein (BEHAB) (Jaworski *et al.,* 1994) and cell surface receptors CD44 (Aruffo *et al.,* 1990) and ICAM-1 (McCourt *et al.,* 1994)) are members of this group. The interaction between the proteins containing this motif and HA is ionic in nature (Yang *et al.,* 1994).

Sometimes, hyaladherins are also classified according to their site of occurrence like extracellular HABPs, cell surface HABPs and intra-cellular HABPs. It is believed that cell type specific functions of HA may be mediated through its interaction with HA-binding proteins. The interaction of HA with HA-binding proteins regulate many aspects of cell behaviour such as cell migration, cell-cell adhesion, cell differentiation - a phenomena, well documented in developing, regenerating, remodeling tissues and in tissues undergoing malignant tumor cell invasion

(Knudson *et al.,* 1993). The ability of HA and HA-binding proteins to influence cell physiology and behaviour may occur either due to alteration of the hydrodynamic and physical properties of the matrix itself or via direct interaction of HA with cell surface protein receptors. In support, several HA-binding proteins of different nature, isolated from various cell types have been reported.

Till date, both the extracellular or matrix HABPs and the cell surface HABPs have both been well characterised. However there is another group of HABPs, which are called intracellular HABPs whose physiological significance is not fully known, though their occurrence and localization have been well studied. This maybe because one does not know fully the intracellular functions of HA. The 34 kDa Hyaluronic Acid Binding Protein 1 (HABP1) is a member of this group.

1.B.i.a HABP1: PRELIMINARY STUDIES

The preliminary investigation demonstrated the isolation of a 34 kDa HA-binding protein from normal rat brain and liver by single HA-affinity chromatography (D'souza and Datta, 1985, 1986a). The HA-binding protein was shown to be heat susceptible and protease sensitive and was further characterized as a sialic acid containing glycoprotein. The binding studies revealed that this protein interacts specifically with HA among GAGs (D'souza and Datta, 1986b). Furthermore, the amino acid analysis of this HA-binding protein showed that it is rich in glycine and glutamic acid and was thus distinct from other well characterized matrix proteins which also bind to HA such as fibronectin, Link protein and gelatin-binding protein (D'souza and Datta, 1986a). Later, Gupta *et al.* (1991) also purified this protein (a homodimer of 34 kDa subunits) from rat kidney, showed its cell surface localization by immunocytochemical analysis, its glycoprotein nature by concanavalin-A binding and positive co-operative binding interaction

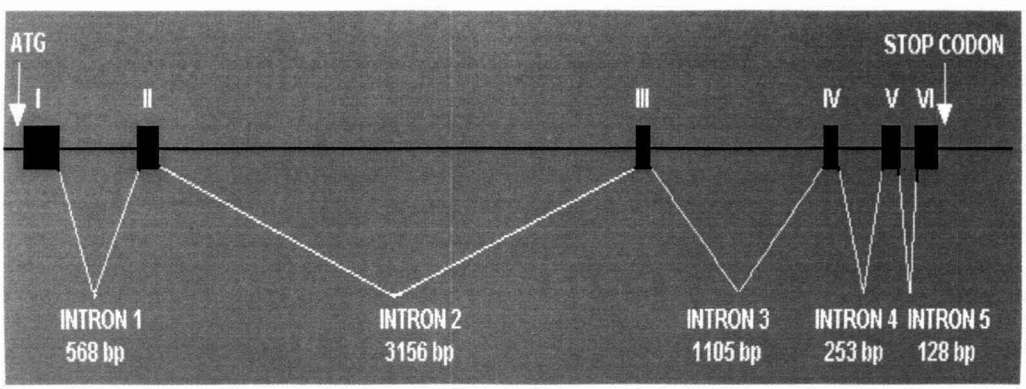
with other extracellular proteins like fibronectin, llaminin, implying ts role in structural organization. Using AK-5, a transplantable histiocytic macrophage cell line, the role of this protein in cell adhesion and tumor invasion as well as its secretory nature was demonstrated (Gupta and Datta, 1991). The specific binding of this protein with two fibrosarcoma membrane proteins of 37 kDa and 41 kDa was also reported by ^{125}I-HA-binding protein gel overlay experiment implying presence of the connecting proteins between the cell surface HA-binding protein and cytosol (Gupta *et al.,* 1993). This observation that HABP1 could be involved in solid tumour formation and cell adhesion along with its cell surface localization led Gupta *et al.* (1993) to test for its membrane localization. It was thus demonstrated that 34 kDa HABP1 and other HA binding proteins are present in plasma membrane fractions of rat brain, liver and ascitic fibrosarcoma cultures. Thereby, it was suggested that 34 kDa HABP could be one of the bonafide cell surface receptors for HA. Ranganathan and Datta (1995) analyzed the levels of 34 kDa HABP in cultures of neonatal cardiac myoblasts and fibroblasts and observed that its level was higher in fibroblasts as compared to myoblasts. They also confirmed its secretory nature and showed a gradual decline in its level during the progression of myoblast differentiation. The protein purified from both myoblasts and fibroblasts were identical in terms of molecular weight, immunological properties and secretory nature.

Since 34 kDa HABP is a phosphoprotein, studies were initiated to understand the interrelationship between phosphorylation of 34 kDa HABP and its interaction with HA. It was demonstrated that the phosphorylation of 34 kDa HABP is regulated by PMA, calyculin A and Ca^{2+} ionophore in various cell lines (Rao *et al.,* 1997). Using lymphocyte cultures, Rao *et al.* (1996) demonstrated that 34 kDa HABP is a specific receptor for HA and HA enhances its phosphorylation. The enhanced phosphorylation by HA and inhibition of cellular aggregation and IP_3

formation by anti-34 kDa HABP-antibodies revealed that this protein is one of the potential mediators in HA induced signal transduction. Involvement of 34 kDa HABP in HA induced signal transduction events was further demonstrated in other cell types (Rao *et al.,* 1997).

1.B.i.b MOLECULAR CLONING OF HABP1 AND ITS MULTIFUNCTIONAL NATURE

After having understood the role of this protein in various signal transduction pathways, it became imperative to uncover the molecular basis of its action and that necessitated pin pointing the gene of this protein and cloning it. This was done by screening of the human skin fibroblast cDNA expression library in λgt11 with polyclonal antibody raised against 34 kDa HABP and a partial cDNA (Genbank accession no. ASF275902) encoding 34 kDa HABP was isolated. The protein sequence predicted from this cDNA corresponded to an internal peptide sequence (83 residues) of 34 kDa HABP isolated from rat kidney (Deb and Datta, 1996). Subsequently, analysis of the cDNA showed its complete homology with the cDNA sequences of a human splicing factor associated protein, p32 (Honore *et al.,* 1993; Krainer *et al.,* 1991; Genbank accession no. L04636 and M69039, respectively) and the human receptor for the globular head of the complement factor 1q, gC1qR (Ghebrehiwet *et al.,* 1994; Genbank accession no. X75913). Furthermore, it had 92% homology with a Murine HIV type 1 Rev binding protein, YL2 (Luo *et al.,* 1994). The 34 kDa HABP has been given the accession ID: 9786126 and named HABP1 by Hugo Nomenclature Committee of GDB. Henceforth, in the following text 34 kDa HABP has been referred to as HABP1/p32/gC1qR. After the sequence homology of HABP1 with p32 was confirmed, the p32 cDNA was expressed in *E. coli,* functionally active recombinant HABP1/p32 was purified using HA-affinity column chromatography. It not only showed immunocross-reactivity with antibodies raised against rat kidney HABP1 but also showed a dose



Diag 1:Genomic organization of human *HABP1* gene

dependent binding to biotinylated HA. This protein shows a molecular weight of 34 kDa in spite of its formula weight being 23.8 kDa, as it is a highly acidic protein. The pI (4.2) of the recombinant protein was identical to that of the tissue purified 34 kDa HABP (Deb and Datta, 1996). Krainer *et al.* (1991) reported the sequence of p32 cDNA, which does not contain any conventional ATG (met) start codon but initiates with a CTG (Leu) codon. The matching of predicted amino acid sequence with the N-terminus of HeLa cell purified p32, confirmed the absence of ATG codon. Later, Honore *et al.* (1993) cloned and expressed the entire p32 cDNA, which extends beyond the 5' end reported by Krainer *et al.* (1991) and showed that ATG is indeed the start codon. However, the recombinant protein produced from cells infected with vaccinia virus harbouring full length p32 cDNA including the ATG start codon, had an N-terminal amino acid sequence as reported by Krainer *et al.* (1991). Thus, it implies that p32/HABP1 is synthesised as a proprotein of 282 amino acids which is subsequently processed by the removal of the initial 73 amino acids to form the mature protein of 209 amino acids.

1.B.i.c CHROMOSOMAL LOCALIZATION AND THE GENETIC STRUCTURE OF HABP1

The HABP1 gene was localized by STS marker in the human chromosome 17p12-p13 by fluorescence *in situ* hybridization (FISH) analysis. The chromosomal localization shows 99.5% similarity (from base 928 to base 1163) with STS WI-9242, an STS flanking marker of human chromosome 17 (Majumdar and Datta, 1998). An extensive analysis of the gene and the marker was done after this. After cloning and characterizing both the human gene and its murine homologue, it was found that they possess a similar exon/intron organization (Guo *et al.,* 1997; Lim *et al.,* 1998). The HABP1 gene is 6 kb long. It has 6 exons and 5 introns (Diag. 1). When the HABP1 cDNA was hybridized with the

digest of the full human genome, a simple pattern was observed indicating that there's a single copy of the gene.

When the 7.8 kb HABP1 gene containing 6 exons and 5 introns was cloned, the 1.3 kb DNA fragment at the 5' flanking region revealed the presence of multiple TATA, CCAAT and Sp1 binding sites (Tye *et al.,* 2001). Luciferase reporter assay in cell lines showed a ubiquitous expression of the promoter. Subsequent 5' deletions confirmed the presence of the promoter elements within 400 BP upstream of the translation start site. Since the removal of the 8 bp consensus TATATATA at --99/--406 and CCAAT at –410/-414 did not significantly affect the transcription efficiency of the promoter, a GC rich sequence downstream of the TATA box is possibly important for the gC1qR/HABP1 promoter activity. One of the seven GC rich sequences in this region bins specifically to PANC-1 nuclear extracts and gel shift assay established the binding of the transcription factor Sp1 to this GC rich region. Primer extension studies mapped three major transcription start regions, the farthest on being 496 bp upstream of the first ATG codon and this is in close proximity of a Sp1 binding site (Tye *et al.,* 2001).

1.B.i.d PSEUDOGENES OF HABP1

Pseudogenes are sequences of genomic DNA which are similar to normal genes but are non-functional and are considered to be defective copies or close relatives of genes (Proudfoot *et al.,* 1980). When the DNA sequencing of the smallest human autosome, the chromosome 21, was completed, the revealed sequence showed the presence of a lot of 'junk DNA' and pseudogenes formed a major part of this. Chromosome 21 has 127 known genes of which 59 are pseudogenes. These pseudogenes cant be express for the following reasons:

1) absence of intervening introns

2) presence of intermittent stop codons in the reading frame

3) insertions or deletions that have disrupted the ORF

The first pseudogene of HABP1 was reported to be localized in a gene poor region in chromosome 21 flanked by 3 pseudogenes on each side (Hattori *et al.,* 2000). When the sequencing of the entire human genome was done, it became possible to search for more such elements. When the genome was searched for sequences similar to HABP1 cDNA, it was seen that HABP1 pseudogenes were present in chromosomes 15,11 and 4. They were all of different lengths and bore varying degrees of similarity to the parental cDNA sequence. All these pseudogenes lack the 5' promoter sequence and possess multiple mutations. They also have premature stop codons in all three reading frames. All this confirms their identity as processed pseudogenes (Majumdar *et al.,* 2002).

1.B.i.e ROLE OF HABP1 IN REPRODUCTIVE SYSTEM

Since HA is one of the main carbohydrates involved in sperm-egg interaction, the role of HABP1 in the reproductive system was examined. Ranganathan *et al* (1994, 1995) reported its presence on spermatozoa and showed the differential localization of this protein on the sperm head, mid-piece and tail of different organisms. Later, it was seen that this protein is present in lower concentrations in teratozoospermic and asthenozoospermic patients in comparison to the normozoospermic spermatozoa, with enhanced phosphorylation in fertile spermatozoa as compared to the lower level of phosphorylation in infertile spermatozoa, thus assigning a marker status of the protein in assessing human fertility (Ranganathan, 1995). The declining pattern of HABP1 during the epididymal maturation was also observed. Pre-treatment of sperms with anti- HABP1-antibodies inhibited sperm-oolemmal adherence. Examination of the expression of HABP1 in adult rat testis during spermatogenesis revealed the presence of a pro-protein form of HABP1,

specifically in the testis (Bharadwaj *et al.,* 2002). Further, enhanced phosphorylation of this protein in motile sperms was also demonstrated. This report clearly demonstrated the involvement of HABP1 in sperm maturation, motility and fertilisation processes. Investigation of HA mediated signal transduction events in the spermatozoa showed that HA mediates sperm motility by enhancing the phosphorylation of HABP1. HABP1 is expressed in a stage specific manner in the testicular tubules and shows specific binding to ZP3, the sperm receptor in zona pellucida, which is involved in primary sperm-egg binding (Ghosh and Datta, 2003). All these amply demonstrate that HABP1 is an important protein in the whole process of fertilization. Along with this, it has also been observed that HABP1 level in sperm samples of infertile patients reveal a substantial decline, and a direct correlation could be drawn between sperm motility and HABP1 expression (Ghosh *et al.,* 2002). So, this protein could be used to serve as a prognostic marker for male infertility and can also be used in the analysis of testicular biopsies where spermatogenic arrest has occurred.

1.B.i.f HOMOLOGUES OF HABP1 BASED ON SEQUENCE HOMOLOGY SEARCH AND THEIR FUNCTIONS

From sequence homology searches, it was seen that the multifunctional HABP1 has complete sequence homology with the receptor of the globular head of C1q (gC1qR) and gets co-purified with alternate splicing factor SF2 associated protein p32 and also shows substantial homology (~92%) with a murine protein YL2 (Ghebrehiwet *et al.,* 1999; Deb *et al.,* 1996; Luo *et al.,* 1994 and Tenge *et al.,* 1996). So, it is apparent that different groups investigating very diverse areas have discovered this protein. Consequently, ligands interacting with this protein have been discovered from different backgrounds. In the following sections the different functions of HABP1 are given along with its interacting ligands.

1.B.i.f.1 gC1qR/HABP1INVOLVEMENT IN THE CONTACT PATHWAY OF SECONDARY HEMOSTASIS

This protein was isolated from Raji cells and was found to bind with the globular heads of C1q molecules at physiological ionic strength, and was seen to inhibit the complement mediated lysis of sheep erythrocytes by human serum. This was seen to be present on the surface of lymphocytes, polymorphonuclear leucocytes, neutrophils and eosinophils. Later, it was confirmed to be a membrane constituent of platelets and was suspected to have a role in platelet-C1q interactions (Ghebrehiwet *et al.,* 1994; Peerschke *et al.,* 1994). On phorbol myristate acetate (PMA) treatment of neutrophils, the expression of gC1qR was seen to be down regulated and its concentration in the cell media supernatant was seen to increase, suggesting receptor shedding or secretion. The interaction between C1q and gC1qR was also seen to be specific in nature as the latter did not bind to other molecules resembling C1q, like mannose binding protein, surfactant protein A and conglutinun (Eggleton *et al.,* 1995). Murine homologue of this protein was seen to express on the surface of murine mast cells. C1q mediated migration of murine mast cells were seen to be inhibited in the presence of a monoclonal antibody against gCq1R, suggesting the phenomenon to be modulated by surface receptors like gC1qR and cC1qR (which is the receptor of the collagen like stalk of C1q) (Ghebrehiwet *et al.,* 1995).

Subsequently, this protein was found to occur on the surface of HUVEC cells (human umbilical vein endothelial cell) and seen to interact with high molecular weight kininogen (HK) and factor XII in a zinc dependent manner. Antibodies against certain epitopes of gC1qR were seen to inhibit the binding of HK to HUVEC cells. Since C1q did not inhibit this interaction between HK and HUVEC, it follows that both HK and factor XII bind to the HUVEC cells by means of a 33 kDa cell surface protein. As it was observed that those antibodies which inhibit gC1qR-

C1q interactions do not inhibit binding of HK and factor XII to HUVEC cells. This means that gC1qR interacts with factor XII and HK by means of a motif different from the one required to interact with C1q. This is one of the first indications of gC1qR/HABP1 being a multifunctional protein (Joseph *et al.,* 1996). Later it was found that gC1qR binds to HK by means of its light chain (Joseph *et al.,* 1999). However the binding of gC1qR with kininogen has not been without controversy. Some research groups have shown that upon overexpression of gC1qR, it displays an intracellular localization, where it stays tightly associated to the mitochondria rather than being present on the surface. The overexpression of this protein also does not open up extra HK binding sites on the cell surface even though HK binds tightly to gC1qR in vitro (Dedio *et al.,* 1999).

Since it was seen that the endothelial cell surface proteins gC1qR and cytokeratin 1 are capable of binding to Factor XII and high molecular weight kininogen (HK) in a zinc dependent manner, it was investigated whether the signal cascade involved in the kinin forming pathway can be catalysed by gC1qR and cytokeratin 1. It was seen that incubation of Factor XII, pre-kallikrein and HK with gC1qR or cytokeratin 1 leads to a zinc dependent, Factor XII dependent conversion of pre-kallikrein to kallikrien. Normal plasma was seen to activate the kinin formation pathway when interacting with endothelial cells while plasma deficient in Factor XII, prekallikrein and HK does not. Further, this activation by normal plasma is inhibited by antibodies against gC1qR and cytokeratin 1. So, gC1qR and cytokeratin 1 form potential initiating surfaces for the activation of the kinin forming pathway on account of their expression on cell surfaces (Joseph *et al.,* 2001). However it was later seen that even in Bradykinin formation, zinc dependent binding of Factor XII and HK to gC1qR, cytokeratin 1 and urokinase plasminogen activator receptor is necessary. gC1qR, cytokeratin 1 and the urokinase plasminogen

activator (uPAR) receptor forms a complex within the cell membrane and the pathway of Bradykinin formation starts with the binding of factor XII to gC1qR of this complex (Kaplan *et al.,* 2002). It was later seen that a peptide of HK domain 5 known as HKH20 (amino acids 475-485) is particularly important for contact activation of the Bradykinin forming pathway along with prekallikrein, Factor XII, dextran sulphate, gC1qR and endothelial cells (Nakazawa *et al.,* 2002). Proteins of the plasma kinin-forming cascade can be activated by binding to aggregated A β protein of Alzheimer's disease. Here too, activation of the cascade using purified protein or upon addition of antibody to plasma require A-β and the reactions are zinc dependent (Joseph *et al.,* 1999).

1.B.i.f.2 INTERACTION WITH VITRONECTIN

Further investigations showed that this protein also binds to the heparin binding, multimeric form of vitronectin. The murine homologue of the human gC1qR has six exons interspersed with 5 introns and has a total length of 6 KB. The 70 amino acid long Exon 1 contains the putative signal peptide. Exons 2-5 code for extremely homophilic domains while exon 6 codes for a neutral domain. The amino acid sequence responsible for binding to vitronectin multimers is present in exon 2. The 1 kb upstream region before the first initiation codon of this murine gene was sequenced and this region was seen to contain four potential TATA boxes, seven CAAT boxes and six SP1 sites along with various putative transcription factor binding sites. This indicates that the promoter region is in close proximity to the promoter (Lim *et al.,* 1998). A truncated form of gC1qR was generated which did not have the first 21 amino acids. This mutant and the unchanged protein both could bind to C1q, but the mutant form did not bind to vitronectin implying that the N-terminal region of gC1qR is responsible for its binding to the multimeric form of vitronectin (Lim *et al.,* 1996).

1.B.i.f.3 ROLE OF gC1qR/HABP1 IN INFLAMMATION AND IMMUNE INJURY

Since endothelial cells express a variety of receptor systems involved in humoral response, it was tested whether the expression of gC1qR and cC1qR, the receptors for the globular 'heads' of C1q and the collagen like stalk of C1q are affected by vascular inflammatory reactions. It was found that the expression of both gC1qR and cC1qR in bone marrow vascular endothelial cells increase in response to inflammatory mediators, inteferon gamma, tumor necrosis factor X and a lipopolysaccharide (from *E. coli*) in a dose and time dependent manner as detected by enzyme linked immuno absorbant assay (ELISA). The expression of both the proteins increased substantially within 4-7 hours of stimulation and doubled after 22 hours of stimulation. Both 3H-thymidine incorporation studies and direct cell counts confirmed that this increase of the protein levels were not due to an increase in cell proliferation. Northern blot analysis revealed that rise of their mRNA levels preceded the rise in the protein levels. The increase of the mRNA levels could be stopped by actinomycin D and that of their protein levels could be blocked by cycloheximide, indicating that this increase in transcription and translational levels were induced in response to the given stimulation. Interestingly, when the bone marrow endothelial cells were stimulated by C1q, this up- regulation of cC1qR and gC1qR were not seen, instead an up-regulation of leucocyte adhesion molecule ICAM-1 was observed in the presence of aggregated C1q. Thus it could be concluded that there is a role of gC1qR and cC1qR in vascular inflammation and immune injury (Guo *et al.*, 1999).

gC1qR the cellular C1q binding protein interacts with a number of plasma proteins along with functions in coagulation of blood to kinin formation pathways. One plasma protein seen to interact directly with gC1qR is fibrinogen. When checked with ELISA, it was observed that

gC1qR binds to immobilized fibrinogen in a manner specific enough to be inhibited by excess soluble fibrinogen and polyclonal antibodies against gC1qR or fibrinogen. It is seen that gC1qR at a ratio of 2:1 (gC1qR: fibrinogen: 2:1) with fibrinogen, inhibits reptilase induced fibrin clotting. Repolymerization of thrombin monomers to form polymers was similarly abolished to such an extent that at equivalent concentrations, gC1qR seemed to be a more powerful inhibitor of thrombin polymerization than fibrinogen. When used together, the effect of fibrinogen and gC1qR seemed to be a more powerful inhibitor of thrombin polymerization than fibrinogen alone and their combined effect was seen to be additive. Binding assays of gC1qR with plasmin degraded fibrinogen showed that gC1qR binds to domain D of fibrinogen and the carboxy terminal segment of fibrin is important for this interaction. These observations suggest a role of fibrin formation, especially at sites of immune injury and inflammation. (Lu *et al.,* 1999)

1.B.i.f.4 INTERACTION OF gC1qR/HABP1 WITH PROTEIN A OF *Staphylococcus aureus*

gC1qR was found to interact with protein A of *Staphylococcus aureus*. The binding of *S.aureus* to platelets, an as yet incompletely understood phenomenon, is a major determinant of virulence in endocarditis. This makes the investigations into interactions between *S. aureus* and platelets is of great medical importance. Suspensions of fixed *S.aureus* cells or purified protein A bound to agarose beads picked up gC1qR from whole platelets. Biotinylated protein A was also seen to bind to fixed adherent platelets. This interaction was inhibited by unlabelled protein A, soluble recombinant gC1qR or F(ab')(2) fragments of antibodies against gC1qR. gC1qR preferentially recognized the protein A bearing Cowan 1 strain of *S. aureus* as compared to protein A deficient *S. aureus* Wood 46 strain. Binding of biotinylated gC1qR to immobilized protein A was seen to be specific by ELISA and this interaction was seen to be

inhibited by unlabelled gC1qR. Similar binding studies of protein A with a gC1qR mutant lacking residues 74 to 95 at the amino terminal shows that the protein A binding domain of gC1qR lies out side the amino terminal α helix, which bears the C1q binding site. All this data taken together implies that platelet gC1qR is a receptor for protein A of *S. aureus* which has a role in the adhesion of *S. aureus* to platelets. (Nguyen *et al.*, 2000)

1.B.i.f.5 INTERACTION OF gC1qR/HABP1 WITH INTERNALIN B OF *Listeria monocytogenes*

gC1qR is also involved in the invasion of *listeria monocytogenes* into mammalian cells by interacting with its protein internalin B (InlB). It had been observed that the entry of *L. monocytogenes* in some mammalian cell lines like epithelial cell line CACO-2 takes place by means of the bacterial protein Internalin A (Inl A) interacting with the mammalian protein E-cadherin, a protein involved in calcium dependent cell-cell adhesion. But in many other cell lines like *Vero, Hep 2* and *HeLa,* entry of *L. monocytogenes* seems to take place through Inl B which does not make use of E-cadherin. Inl B enters a mammalian cell by stimulating the tyrosine phosphorylation of adaptor protein Gab1, Cb1 and Shc and activation of phosphatidyl-inositol (PI) 3-kinase. It has been seen that Inl B binds to gC1qR *in vitro* and vice-versa. Soluble C1q and anti-gC1qR antibodies have also been seen to inhibit Inl B mediated entry of *L. monocytogenes* into mammalian cells. When GPC16, cells which are non-permissive to Inl B mediated entry, are transiently transfected with a plasmid bearing gC1qR, they become permissive to Inl B coated beads. Further, it was observed that membrane recruitment and activation of PI-3 kinase involves an Inl B-gC1qR interaction and gC1qR associates with Gab1 upon stimulation with Inl B in Vero cells. These observations conclude that gC1qR is a cellular receptor involved in

Inl B mediated invasion of *L. monocytogens* into mammalian cells (Braun *et al.*, 2000).

1.B.i.f.6 GUIDE RNA BINDING PROTEIN OF *Trypanosoma brucei*

RBP16 is a guide RNA (gRNA) binding protein which interacts with approximately 30% of total gRNAs in *Trypanosoma brucei*. To understand the functioning of RBP16, a search was made for its interacting proteins, by affinity chromatography and immunoprecipitation, which detected four proteins of 12,16,18 and 22 kDa respectively. This p22 protein has significant sequence homology with the SF2 associated p32 and *S. cerevisiae* Mam33p. Homomultimeric forms of this protein was observed upon glutaraldehyde cross linking of recombinant p22. Direct *in vitro* binding of RBP16 and p22 was also observed in ELISA. To check whether p22 has any effect on the RNA binding capacity of RBP16, recombinant p22 was titrated into UV crosslinking assays and it was seen that p22 enhances the gRNA binding capacity of RPB16, there by establishing its role as a regulatory molecule in *T. brucei* mitochondrial gene expression by modulating the RNA binding properties of RPB16 (Hayman *et al.*, 2001).

1.B.i.f.7 INTERACTION OF gC1qR/HABP1 WITH α_{1B} ADRENERGIC RECEPTOR

The α_{1B} adrenergic receptor is a G-protein coupled receptor, which interacts with the heterotrimeric G protein along with many other cytoplasmic proteins to discharge its role in the signal transduction pathways. A yeast two-hybrid screening of the cDNA library prepared from the rat liver was done to pick up proteins interacting with the adrenergic receptor using it as the bait. Its carboxyl-terminal cytoplasmic domain (173 amino acids, 344-516 residues) was seen to bind with gC1qR. Further analysis revealed that gC1qR co-immunoprecipitates with the α_{1B}-adrenergic receptor. Fluorescence confocal laser scanning

microscopy showed their co-localization in transfected COS-7 cells. However, the adrenergic receptors are seen to express only on the plasma membrane of the cells when expressed alone, while gC1qR was seen to be localized in the cytoplasm under similar circumstances. But in COS-7 cells, expressing both the adrenergic receptor and gC1qR, both co-localized in the intracellular region and a lower expression of the receptor was seen. This suggests that gC1qR has a role in the localization and level of expression of the α1B adrenergic receptors (Xu *et al.*, 1999).

1.B.i.f.8 INTERACTION OF gC1qR/HABP1 With GABA(A)

gC1qR was found to have co-immunpurified with gamma-aminobutyric acid type A (GABA(A)) from bovine brain. It was also co-immunoprecipitated with GABA (A) from solubilized rat brain membranes using an yeast two hybrid system. It was seen that gC1qR interacts with the beta subunits of GABA (A), but not the alpha 1 and gamma 2 subunits. N-terminal and C-terminal deleted mutants of the beta 2 subunit of GABA (A) were used to map the region responsible for binding and a stretch of 15 amino acids containing 7 positively charged residues were found (amino acids 399-415) as the interacting region with gC1qR. This region contains a serine at position 410, which is a kinase substrate whose phosphorylation status modulates the receptor activity. It has been speculated that the interaction between gC1qR and GABA (A) is tightly regulated and may be involved in receptor biosynthesis or modulation of the mature function (Schaerer *et al.*, 2001).

1.B.i.f.9 INTERACTION OF gC1qR/HABP1 WITH TYPE 1 MATRIX METALLOPROTEASE

Membrane type 1 matrix Metalloprotease (MT1-MMP), a key enzyme in cell locomotion is usually found on the leading edge of migrating cells. So it is quite possible that the C-terminal cytoplasmic tail of MT1-MMP interacts with intracellular regulatory protein which control

the movement of the protease across the cell. Its been observed that this tail indeed binds directly to gC1qR. Although the significance of this interaction is yet unclear, indications are that transient associations between these two proteins are involved in the mechanisms for regulating the presentation of the protease at the tumor cell surface (Rozanov *et al.*, 2002).

1.B.i.f.10 INTERACTION OF gC1qR/HABP1 WITH VIRAL PROTEINS

gC1qR/p32/HABP1 has been seen to interact with a large number of viral Uproteins, the undrlying significance of most of which is rather obscure. However, these interactions prove to be of great interest as they often form a pathway for viral invasion into the host cell and are thus potential drug targets.

1.B.i.f.10.I HEPATITIS C VIRUS

The Hepatitis C virus (HCV) core protein is the first protein expressed during the early phase of HCV infection. It suppresses host immune responses including anti-viral cytotoxic T-lymphocyte responses in the murine model. In order to understand the mode of immuno suppression by this protein, a search was made for host proteins capable of interacting with the HCV core protein by screening a human T cell enriched expression library using the HCV core protein as the bait. gC1qR, a ligand of the globular 'head' of C1q was picked up. C1q is involved in the early host defense system against infection and it suppresses T cell proliferation *in vitro*, quite like the HCV core protein. The HCV core protein induced suppression of T-cell proliferation is blocked upon addition of anti-gC1qR antibodies. Biochemical analysis showed that the HCV core protein binds to the amino acid residues 188-259 of gC1qR, a site distinct from that which binds to C1q (Kittlesen *et al.*, 2000). The HCV core protein affects the T-cell proliferation by inhibiting phosphorylation of extracellular signal regulated kinase (ERK)

and mitogen activated ERK kinase (MEK). This in turn inhibits the transcription of the genes IL-2 and IL-2Rα leading to the inhibition of IL-2 production and high affinity IL-2Rα expression. Anti-gC1qR antibody can reverse this HCV-core induced suppression of phosphorylation of ERK and MEK, revealing that the interaction between gC1qR and HCV core protein is somehow interfered by the ERK/mitogen activated MEK, protein kinase activation. This data implies that HCV-core protein induced suppression of T-cell activation by a complement dependent pathway may well play a critical role in the establishment HCV persistence during the acute phase of viral infection (Yao *et al.*, 2001).

1.B.i.f.10.II HUMAN IMMUNO-DEFICIENCY VIRUS TYPE 1 TAT

One of the most pathogenic viruses in today's world is HIV. Expression, function and mechanism of action of all the HIV proteins are of great interest to the scientific world. HIV-1 Tat is an 86 amino acid protein, which is essential for viral replication. It acts as a powerful transactivator of viral gene expression. So, to understand the pathway of its functioning, identification of its target protein in the host is of utmost importance. An interacting protein of Tat was picked up by affinity purification with the Tat peptide from human leucocyte cell line Molt3 lysate. On deletion analysis, it was seen that the C-terminal region of this protein, named TAP (Tat associated protein), is rich in acidic amino acids and leucine residues which acts as a strong transcriptional activator when bound through the GAL4 sites upstream of the core Long Terminal Repeat (LTR) promoter, as well as to flanking sequences, which mask the activation. Substitution of 2 leucines from within the core activation region results in the loss of TAP activation function. Further it is seen that promoter bound Tat recruits a TAP/VP16 fusion protein to the promoter and on transient expression of Tat, it co-localizes with TAP and can be co-immunoprecipitated with it. TAP also binds to the general transcription factor TFIIB quite strongly (kD = 2 to 5 x 10^{-7}M). A 17

amino acid sequence stretch in Tat has been observed to be its interacting site while binding to TAP. Single amino acid substitution in this region abolishes the binding of Tat with TAP. Interaction of TAP with TFIIB happens through a region located at the C-terminal of TFIIB, which is also required for binding of a strong acidic promoter VP16. Tat and TFIIB interact with TAP via it C-terminal region containing the TAP activation domain. From all these it can be concluded that TAP is a cellular coactivator that bridges the Tat tranactivator to the general transcription machinery via TFIIB. Sequence homology studies showed the identity of TAP with gC1qR/HABP1 (Yu *et al.,* 1995A; Yu *et al.,* 1995B).

1.B.i.f.10.III HUMAN IMMUNO-DEFICIENCY VIRUS TYPE 1 REV

Replication of HIV type 1 requires expression of another viral transactivator known as Rev, which binds to a highly structured RNA known as the rev response element (RRE), which is present in singly spliced and un-spliced genomic viral RNAs. Rev transports these transcripts from the host cell nucleus to the cytoplasm by a mechanism, which is not fully understood. In an attempt to understand the pathway and identify interacting proteins, a yeast two-hybrid screen was done and a murine protein YL2 was picked up which has 92% identity to SF2/p32. This protein interacts with the basic domain of Rev, which is essential in its *in vivo* functioning and for the inhibitory splicing activity of Rev *in vitro.* Even though, the expression of YL2 greatly increases the potential of Rev action, its been seen that anti sense YL2 transcripts blocks the effects of Rev in mammalian cells. YL2 was also seen to increase the effects of Rev on the Rev response element of hybrid Rev-Tat fusion protein and the coat protein of bacterio-phage MS2 on their respective RNAs (Luo *et al.,* 1994). It has been shown that the human homologue of YL2, p32 also binds to HIV Rev *in vitro* and the complex they form is resistant to high concentrations of salt or non-ionic detergents. Protein

foot printing assay suggests that Rev binds to the 196-208 amino acid region of p32. It was further seen that p32 acts as a bridge in the association between Rev and RRE. When added exogenously, p32 specifically relieves the inhibition of splicing *in vitro* exerted by the basic domain of Rev. Thus p32 acts as a link between Rev and the cellular splicing apparatus (Tange *et al.,* 1996). Thus it is quite clear that YL2 or SF2/p32 have a significant role in modulating the functions of HIV 1 Rev protein.

1.B.i.f.10.IV gC1qR/HABP1 INHIBITS THE ENTRY OF HIV TYPE 1 VIRUS IN A VARIETY OF MAMMALIAN CELL LINES

It was seen that gC1qR, the receptor for the globular heads of C1q has the ability to inhibit the growth of HIV Type 1 strains in many cell cultures like human T cell lines (MT-4 and H9) and human monocyte-derived macrophage cultures. Maximum inhibition was seen when the cells were preincubated with gC1qR and then removed before infecting them with the virus. Less inhibition was seen when the cells were infected with HIV 1 - gC1qR mixtures. The inhibition was much less when gC1qR was added a day or two after infection. In ELISA, gC1qR did not bind to immobilized gp120, an outer envelope protein of HIV 1, which is structurally and functionally similar to C1q, instead it bound strongly in a dose dependent manner to CD4. Thus, it could well be that gC1qR prevents viral entry into mammalian cells by blocking the interaction between CD4 and gp120. Since gC1qR is a human protein, the idea of using its ability of binding CD4 as a possible therapy is worth looking into (Szabo *et al.,* 2001).

1.B.i.f.10.V EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN 1

The Epstein Barr Virus Nuclear Antigen1 (EBNA-1) protein helps in maintaining the latent EBV infection by helping in replication and maintenance of the episomal form of the viral genome and also

transactivates the latency C and LMP-1 promoters. It is the only viral protein expressed in all EBV associated tumors. This protein lacks detectable enzymatic activity in the purified form, but is known to have transcriptional transactivating capability in vivo even though it lacks a specific transcriptional activation domain. Thus, the mechanism of EBNA-1 mediated transactivation is not understood and so a search was made for an interacting cellular protein, which together with EBNA 1 produces this transactivating ability. So, a yeast two-hybrid sytem screening of the human leucocyte cDNA library was done using EBNA-1 fused with Gal4 DNA binding domain as bait. This picked up a 32 kDa protein, which on subsequent sequencing revealed it to be the SF2 associated p32. In the yeast two hybrid system, two interactive N-terminal regions of EBNA 1 was mapped, both of which have arginine-glycine repeats and these regions interact with the C-terminal portion of p32. Expression of the full-length p32 translocates EBNA1 from the nucleus to the cytoplasm. Observations also indicate that p32 co-immunoprecipitates with EBNA-1. Deletions of p32 interacting regions in EBNA1 virus severely compromises the transactivating potential of EBNA1 (Wang *et al.,* 1997; Van Scoy *et al.,* 2000). Later, it was seen that p32 interacts with residues 1-102 and 325-357 of the EBNA 1 protein (Chen *et al.,* 1997). More interacting proteins of EBNA 1 have also been discovered and they are karyopherin α (Karyopherin α1, hSRP1 , NPI 1) and karyopherin α_a (hSRP1α, hRch1). It could well be that karyopherin α, the adaptor subunit of the nuclear localization signal receptor, mediates the nuclear transport of EBNA 1. Karyoherin α_2 is another NLS adaptor subunit and could also be involved in the nuclear transport of EBNA 1 (Ito *et al.,* 2000).

1.B.i.f.10.VI RUBELLA VIRUS CAPSID PROTEIN

gC1qR , one of the cellular defense molecules is seen to bind with the rubella virus capsid (RVCP) protein N-terminal 28 amino acid

domain. About 69 amino acid residues of gC1qR are responsible for this interaction. This interaction seems to elucidate a modus operandi where one of the viral proteins is recruited to interact with cellular proteins to exploit the loopholes in the cells defense and exploit them (Mohan *et al.*, 2002).

1.B.i.f.10.VII HERPES SIMPLEX VIRUS ORF-P

gC1qR is also seen to bind to ORF-P a Herpes simplex virus 1 though the physiological importance of this not yet known (Randall *et al.*, 1997a, b).

1.B.i.f.10.VIII ADENOVIRUS CORE PROTEIN V

The Adenovirus core protein V possibly has a role in delivering the viral genome to the nucleus of the host cell. A yeast two-hybrid system using the core protein as bait was used to screen a human cDNA library and the protein that got picked up was gC1qR. gC1qR was found to occur both in cytoplasm as well as in the nucleus in infected cells. Following the localization of protein V and gC1qR in the cell makes one speculate that p32 is a part of the machinery to deliver proteins into the nucleus which gets hijacked by the viral machinery to import its genome into the nucleus (Matthews and Russell *et al.*, 1998).

1.B.i.f.11 INTERACTION OF gC1qR/HABP1/p32 WITH CELLULAR PROTEINS

1.B.i.f.11.I SPLICING FACTOR (SF2)/ALTERNATE SPLICING FACTOR (ASF)

SF2, a protein factor essential for constitutive pre-mRNA splicing when purified from HeLa cell extracts was seen to co-purify with a 32 kDa protein which has complete sequence homology with HABP1. The function of this p32 protein was not clear, as it does not bind to RNA. Though its association with SF2 was quite clear, the physiological

significance of this association was not (Krainer *et al.*, 1991). Later it was seen that p32 interacts with both SF2 and another protein known as SRp30, both being members of the SR family of proteins. p32 inhibits the functioning of SF2 by preventing the latter from stably interacting with mRNA but does not block SRp30 function. p32 effects this inhibition by stopping SF2 from getting adequately phosphorylated. Thus SF2 associated p32 belongs to a group of proteins that affects mRNA splicing by sequestering an essential RNA splicing factor (Petersen-Mahrt *et al.*, 1999).

1.B.i.f.11.II PROTEIN KINASE C FAMILY

Protein Kinase C (PKC) proteins is a family of serine/threonine specific kinases having 11 identified members so far and are implicated in a wide range of biological functions including regulating changes in cell morphology, proliferation and differentiation. Of the three well defined major classes of PKC namely Ca^{2+} dependent classical PKC, Ca^{2+} independent novel PKC and Ca^{2+} and lipid independent a typical PKC, PKC μ falls in none of them and may form a new group on its own. PKC μ and its murine homologue PKD are distinguished by the presence of an amino terminal hydrophobic domain, an acidic domain, a pleckstrin homology domain within the regulatory region and lack of a characteristic pseudo-substrate site. In order to uncover new interacting proteins of PKC μ, its kinase domain and pleckstrin homology domain were used as bait in a yeast two-hybrid screening of a pACT λ bacteriophage library of human activated B-lymphocytes. The kinase domain picked up a protein, which showed a migration at 32 kD in SDS-PAGE and was detected by a monoclonal antibody against gC1qR a receptor for the globular 'heads' of C1q the complement pathway protein. It was further seen that PKC μ could be precipitated from whole cell extracts of PKC μ expressing SF158 cells by purified GST-p32 fusion protein, immobilized on glutathione sepharose beads. Reciprocal co-

immunoprecipitation was also seen to happen. In SKW 6.4 cells, p32 associated with PKC μ at the mitochondrial membranes, as is evident from the co-localization of p32 and PKC μ with cytochrome C. However, p32 seemed to be interacting with PKC μ in a compartment specific manner as co-immunoprecipitation of p32 occurs only from the particulate fraction but not from the soluble fraction of both 293T and SKW 6.4 cell lines. Inspite of p32 binding with PKC μ, it does not act as a substrate of the latter. In fact, the presence of p32 prevents phosphorylation of aldolase a well known substrate of PKC μ *in vitro* in a dose dependent manner possibly due to its binding to the kinase domain of PKC μ, thereby restricting access to aldolase by stearic hindrance. However the level of autophosphorylation of PKC μ remains unaffected by the presence of p32 (Storz *et al.*, 2000). Later, the interaction of p32 with other members of the PKC family was examined in rat hepatocytes. All PKC isoforms interacted with p32 *in vitro* but the dependence of these interactions on PKC activators differed for each one of them. PKC ζ and PKC α increase their binding when they were in the activated form. The bindings of PKC β, PKC ε and PKC θ are unchanged regardless of the presence of PKC activators. PKC μ binds better in the absence of PKC activators. It was also seen that as observed earlier, even though p32 is not a substrate of the PKCs, at times its presence enhances PKC activity for example the activity of PKC Δ doubles in its pressure. Upon phorbol ester treatment of rat hepatocyte (C9) cell line it was seen that p32 associated with PKC θ constitively and with PKC Δ upon activation. It was also seen that on phorbol ester treatment p32 translocated to the nucleus. Thus p32/gC1qR/HABP1 is seen to have a regulatory compartment specific role on the localization and function of the PKCs (Robles-Flores *et al.*, 2002).

1.B.i.f.11.III LAMIN B RECEPTOR

In higher eukaryotic cells, a fibrous lamina lines the inner nuclear membrane and remains tethered to it by means of several integral membrane proteins. One such protein is a 58 kDa polypeptide called the Lamin B receptor (LBR). This protein has a long N-terminal domain in the nucleus along with eight predicted membrane spanning segments. It forms a multimeric complex during interphase, consisting of nuclear Lamin A and B, a specific p58 kinase and two other polypeptides of 18 kDa and 34 kDa respectively. This multimeric complex was purified from turkey erythrocyte ghosts by immuno-affinity column using the anti p58 (Lamin B receptor) antibodies. Then p34 was separated from this complex by salt dependent dissociation from the complex. Micro sequencing of this protein revealed its sequence homology with the SF2 associated p32. The N-terminal region of the p58 lamin B receptor shares some similarity with that of SF2, in that both are highly charged and contain numerous basic amino acids. It was also seen that an arginine/serine (RS) rich region is present in the N-terminal domain of p58 and this motif is present in the C-terminal region of SF2. Usually an RS domain is found in proteins involved in splicing, so its presence in the Lamin B receptor, though unexpected, possibly means a wider function for the domain. So keeping in mind that p34 binds to SF2 and LBR and that its an acidic protein, one could speculate whether its a RS domain binding protein or not (Simos *et al.,* 1994). Indeed, further work proved that p34 binds to the N-terminal region of LBR and that a mutant LBR lacking the RS domain does not bind to HABP1/p32/gC1qR. Moreover, phosphorylation of the LBR by the RS kinase completely abolishes the binding of p34 with LBR. All this indicates the role of HABP1/gC1qR/SF2 associated p32 in the functioning of LBR complex and may help its interaction with the transcription machinery of the cell (Nikolakaki *et al.,* 1996).

1.B.i.f.11.IV ROLE OF YEAST HOMOLOGUES OF HABP1

A homologue of HABP1 has been discovered in bakers yeast *Saccharomyces cerevisiae* and named p32/Mam33p which has been seen to localize exclusively in the yeast mitochondrial matrix and thought to be involved in mitochondrial oxidative phosphorylation. Disruption of this gene in *S. cerevisiae* causes growth inhibition only in glycerol medium but not in glucose medium. The sequence of the mature cDNA of HABP1 show 53% similarity and 26% identity to this yeast protein applying the GCG program BESTFIT (Muta *et al.*, 1997, Seytter *et al.*, 1997).

1.B.i.f.11.V MYOSIN BINDING ROLE OF HABP1 HOMOLOGUE p38

Myosine forms thick filaments along with myosin binding proteins in smooth muscle cells. One major myosin binding protein that stabilizes these filaments is telokin. However in cells devoid of telokin, the formation of these filaments is observed which led to a search for another myosin binding protein and this protein was ultimately purified from the chicken gizzard. It turned out to be a 38 kDa polypeptide with sequence homology to human SF2 associated p32. Though this p38 was found equally to dephosphorylate and phosphorylate myosin, it helped only dephosphorylated myosin to form filaments and had no similar effect on phosphorylated myosin. It bound to myosin with both C and N-termini with the 20 terminal residues of the C-terminus and 28 residues of the N-terminus being particularly important for binding. Immunoblotting with anti-p38 antibodies showed the expression of this protein in various smooth muscle cells. Immunofluorescence studies show co-localization of p38 with myosin and other cytoskeletal elements in cultured smooth muscle cells (Okagaki *et al.*, 2000).

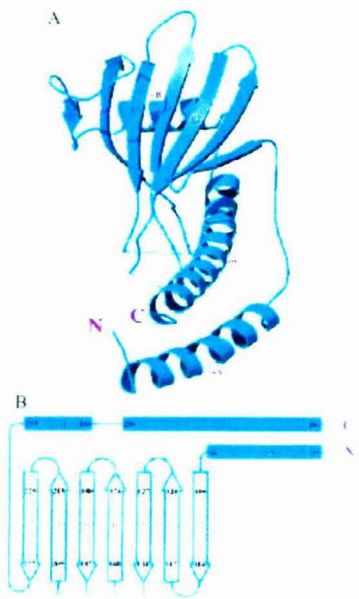
1.B.i.g MULTI-COMPARTMENTAL LOCALIZATION OF HABP1

HABP1 is a protein, which interacts with a wide assay of biomolecules from a complex polysaccharide to viral proteins to proteins of the complement pathway. This quite clearly points out to the fact that it shall be localized to a wide variety of cellular compartments, in a wide variety of cells. But this is not obvious from the structure of the protein. HABP1, as already mentioned, is synthesized as a pro-protein of 282 amino acids and the first 73 amino acids get chopped off proteolytically as a part of the post-translational modification. The amino acid stretch which gets cleaved off are quite basic in nature and contains 11 arginine residues making this sequence very similar to the nuclear localization signal and is considered to be so (Dedio *et al.,* 1999).

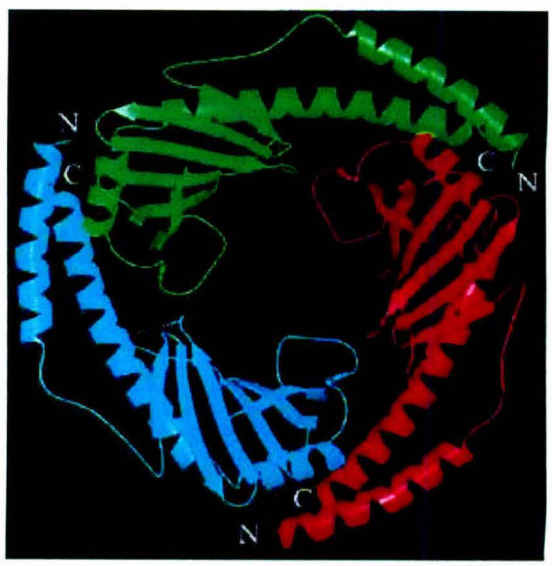
Further immunofluorescence and subcellular fractionation studies have shown the occurrence of the yeast homologue in the mitochondrial matrix (Muta *et al.,* 1997; Seytter *et al.,* 1997). However, this protein is a receptor of the 'globular' heads of HABP1, its presence is expected on the cell surface (Ghebrehiwet *et al.,* 1994). This has been shown by both by immunofluorescence and confocal microscopy on different cell lines including COS-1 (Van-Leeuwen *et al.,* 2001) and blood platelets (Peerschke *et al.,* 2003). Its localization in the nucleus has been shown by confocal microscopy and fluorescence microscopy in COS-1 and A459 cell lines (Brokstad *et al.,* 2001). These reports of varied and sometimes bewildering localization of HABP1 in different types of cells and tissues just go on to show the different facets of HABP1 activity and until all the biological functions of HABP1 are known the rationale behind the multiple localization of this protein can never be fully understood and appreciated.

1.B.i.h HABP1: STRUCTURAL ASPECTS

HABP1 has been crystallised and the crystal structure has been solved (Jiang *et al.,* 1999). It is a trimer, where three HABP1/p32 molecules form a doughnut shaped quaternary structure, with a sizeable central channel and an unusual asymmetric charge distribution on the surface. All the three monomers have very similar conformation. The monomeric HABP1/p32 does not have any distinct domains. Each monomer consists of seven consecutive β-strands, designated β1 through β7, which form a highly twisted anti-parallel β-sheets, with β1 nearly perpendicular to β7 (Diag. 2). The β-strands are flanked by one N-terminal (αA) and two C-terminal (αB and αC) α-helices. All the three helices are located on the same side of the β-sheet. Helix αB lies parallel to the β-sheet, with the helix axis perpendicular to the direction of individual strands. The helix αB and the N-terminal portion (4 turns) of helix αC make extensive hydrophobic contacts with the β-sheet, which appear to be essential for the stability of the structure. The N-terminal helix αA does not contact the β-sheet within the monomer, but forms an anti-parallel coiled coil with the C-terminal portion (5 turns) of αC. This coiled coil region is important for protein-protein interactions required for homo-oligomerization. Despite its structural simplicity, HABP1/p32 does not belong to any known protein fold. The channel of the doughnut shaped trimer has a diameter of about 20 Å, but the loops connecting β6 and β7 partially cover the channel, reducing the effective diameter of the channel opening to about 10 Å. The channel wall is formed by the β-sheets from all the three subunits. Due to the high degree of twisting, β-strands from adjacent monomers do not form contiguous β-sheets. Instead, the residues located at the tip of β1-turn-β2 and the loop connecting the β3 and β4 interact with β7 of the adjacent molecule. In particular, the main chain carbonyls of Asn-114 and amide group of Thr-



Diag. 2: The structure of a monomeric unit of HABP1. The N-terminal α helix followed by the 7 β strands forming the anti-parallel sheet. The structure is given schematically in panel B and the three dimensional conformation is given in panel A.



Diag. 3: The shape of HABP1 trimer is like that of a doughnut, with the central channel and the three monomers indicated by different colours.

116 form intermolecular hydrogen bonds to Oγ and the carbonyl group of Thr-222, respectively. The side chain atoms of Asn-134 and Asn-136 are involved in several intermolecular hydrogen bonds with main atoms of Asp-221, Asn-223 and Thr-225. The coiled coil region αA and αC forms extensive intermolecular contacts: αA packs with the anti-parallel αB of an adjacent monomer and the C-terminal region of αC packs against the back of the β-sheet. Most of these interactions are hydrophobic in nature, except for the intermolecular electrostatic pairing of Arg-246 and Asp-79. Asp-79 is not a conserved residue, but Arg-246 is invariant and is located in a region surrounded by several invariant residues. In summary, the overall architecture of the trimer can be visualised as if β-sheets form a hyperboloid shaped spool with α-helices wrapping around it (Diag. 3).

After the solution of the crystal structure it was also shown by size-exclusion chromatography under various conditions and glutaraldehyde cross-linking, HABP1 exists as a non-covalently associated trimer in equilibrium with a small fraction of a covalently linked dimer of trimers, i.e. a hexamer. The formation of a covalently linked hexamer of HABP1 through Cys-186 as a dimer of trimers is achieved by thiol group oxidation, which can be blocked by modification of Cys-186. The gradual structural transition caused by cysteine-mediated disulfide linkage is evident as the fluorescence intensity increases with increasing Hg^{2+} concentration until all the HABP1 trimer is converted into hexamer. In order to understand the functional implication of these transitions, we examined the affinity of the hexamer for different ligands. The hexamer shows enhanced affinity for hyaluronan, gC1q, and mannosylated BSA as compared to the trimeric form. Our data, analyzed with reference to the HABP1/p32 crystal structure, suggest that the oligomerization state and the compactness of its structure are factors that regulate its function (Jha *et al.*, 2002).

When the structural changes in HABP1 were investigated under a wide range of ionic environments it was seen that HABP1 exhibits structural plasticity, which is influenced by the ionic environment, under *in vitro* conditions near physiological pH. At low ionic strength HABP1/p32/gC1qR exists in a highly expanded loosely held trimeric structure, similar to that of the molten globule like state whereas presence of salt stabilizes the trimeric structure in a more compact fashion. It is likely that the combination of the high net charge asymmetrically distributed along the faces of the molecule and relatively low intrinsic hydrophobicity of HABP1/p32/gC1qR result in its expanded structure at neutral pH. Thus, the addition of counter ions in the molecular environment minimizes the intra-molecular electrostatic repulsion in HABP1/p32/gC1qR leading to its stable and compact conformations, which is reflected in its differential affinity towards ligands. While the affinity of HABP1 towards HA is enhanced on increasing the ionic strength, no significant effect was observed with the two other ligands, C1q and mannosylated albumin. Thus, while HA interacts only with compact HABP1, C1q and mannosylated albumin can bind to loosely held oligomeric HABP1 as well (Jha *et al.,* 2003).

Further structural analysis of the crystal structure of HABP1/p32 indicate the N- terminal sequence is similar to the WD 40 family of regulatory proteins. The WD 40 family of proteins which constitute a class of regulatory protein which has a signature sequence of G-H and W-D/E dipeptide usually separated by 25-27 amino acids. Such sequences predominantly form a α helix and biochemical evidence suggests that this signature motif/fold are critical for protein-protein interactions. RACK1, RACK2, Calreticulin and a number of PKC regulatory proteins are prominentt members of tis family (Iwasaki *et al.,* 1995; Mochly-Rosen *et al.,* 1991a; Mochly-Rosen *et al.,* 1991b).HABP1 has this WE signature motif at its N-terminal segment through which

HABP1 has been reported to interact with other proteins like gC1q, GABA(A) receptor, the β subunit light chain of unphosphorylated myosin thus showing its involvement in protein-protein interactions. This also implies that the HABP1 functions in a compartment specific manner rather like a molecular chaperon. These observations imply that there is great flexibility in the structural conformations of HABP1 and sets the foundation for the multifunctional role of HABP1.

Aim and Plan of Work

2.A Aim and Plan of Work

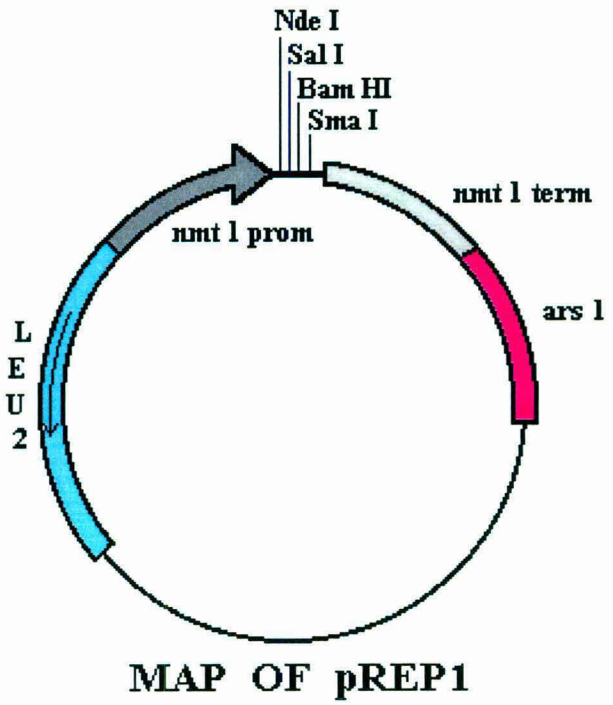
Any living system works like a self-replicating machine. The underlying processes for carrying out the basic activities for its continuing existence consists of, on the whole, a complicated network of the various biological macromolecules. One major class of which is proteins. When one takes the overall view of the system, one would observe that the whole system is being run and controlled by proteins through interactions amongst themselves and with nucleic acids, carbohydrates, lipids and some signalling molecules. Now to understand how this complicated machine works, one has to understand the functioning of the proteins and the interactions they have with the other molecules. This as one may have already surmised is not an easy task. In fact just trying to understand what one particular protein does may be favorably compared with trying to understand what a particular ant is doing in an extremely busy and crowded anthill. One way to solve this problem would be to take that ant out and put it in a similar setting which is smaller, simpler and not-so-busy and where the other ants are of a different color so that the ant of our interest can be easily tracked. Applying this idea to our problem of understanding the functioning of a protein would mean expressing the protein in a simpler system where it or a homologue was originally not present and observing the changes that take place. These changes would be due to the introduction of this protein and so if it could be understood what these changes are and how they are being brought about. That would entail analyzing the phenotypic changes being produced and identifying the other proteins, which are interacting, with the newly introduced protein. Once this is accomplished then we shall have a good idea about what the protein does in its native system.

Hyaluronic acid binding protein 1 (HABP1) is a molecule which interacts with many other molecules and is seen to localize in many cellular and extracellular compartments and is speculated to essay many different roles in so many different cellular processes that its truly bewildering. But leaving aside its function both in sperm-zona interaction and sperm cell maturation, the physiological significance of all the other processes are not very clear. Just to illustrate, it was observed that when the Alternate splicing factor (ASF/SF2) is purified from HeLa cellular extracts, HABP1 gets copurified with it. The physiological significance of why this happens is unknown and unexpected since HABP1 did not seem to have any role in splicing or transcription. Later *in-vitro* studies showed that it interacts with other members of SR family of proteins to which ASF/SF2 belongs. But whether this interaction is charge or structure dependent or has any physiological significance is still unknown (Krainer *et al.*, 1991; Petersen-Mahrt *et al.*, 1999). Hence it's of great interest to uncover the physiological role/s of this protein. To do this the simple eukaryotic expression system *Schizosaccharomyces pombe* was considered. The logic of using this system has been explained in the last paragraph.

Even though it may sound preposterous that a simple unicellular eukaryotic system was thought of, to study the function of a human protein, a closer look at the system would prove its appropriateness for this task. Schizosaccharomyces pombe is very similar to higher eukaryotes and mammals especially in terms of its cellular behaviour and genetic structure though much simpler. Some mammalian genes were isolated using *S. pombe* by complementation of the mutant homologue, for example the central cell cycle regulator cdc 2. The asexual reproduction of *S. pombe* is by means of septation and medial fission, a process much more common amongst higher eukaryotes then than lower eukaryotes. The mitochondrial genome of *S. pombe* very

similar to that of mammals and bears significant similarities to it. Quite a few mammalian promoters are seen to be fully functional in fission yeast in a manner identical to that in mammals. The transcription machinery and RNA splicing mechanism in fission yeast bears great similarity to higher eukaryotes. It has also been observed that the signal transduction pathways in fission resemble the mammalian G-protein-coupled signalling pathways.

Post-translational modification of mammalian protein produced heterologously in the fission yeast is indistinguishable from the protein in the native source. In fact functionally too the proteins are identical for example the human β 2-adrenergic retains its original pharmacological properties when expressed in fission yeast (Ficca *et al.,* 1995). In fact not just functioning of a single protein but interactions between multiple proteins have been studied in fission yeast. Interactions between PLC γ_2 (Phospholipase C γ_2) with its receptors PDGFβ or and the proto-oncogene product of c-src has been studied in fission yeast. Its been observed that when PDGFβ or c-src along with PLC γ_2 are co-expressed in *S. pombe,* then PLC γ_2 is activated by tyrosine phosphorylation by both the receptors and this triggers its enzymatic activity. Then PLC γ_2 is seen to hydrolyses the yeast membrane phosphoinositides (Arkinstall *et al.,* 1995). Inhibition of p110 α, the catalytic sub-unit of phosphatidylinositol 3-kinase by the p85 α , the regulatory sub-unit of phosphatidylinositol 3-kinase due to the binding of the p 110/p 85 complex with v-Ras a constitutively activated mutant of Ras has been seen to happen in *S. pombe* (Kodaki *et al.,* 1994) . Further, negative regulation of protein kinase c-Src by Csk a kinase, which phosphorylates tyrosine 527 of c-Src, has been observed to take place in *S. pombe* cells (Superti-Furga *et al.,* 1993). All these demonstrate the fact that fission yeast has been used to study both the function and protein-protein interactions with success



Diag.1: The map of the shuttle vector for *S.pombe* pRep1

and hence is a suitable system for studying the functional aspects of HABP1.

The Aim of this work is to uncover the biological function of the protein using the simple eukaryotic expression system Schizosaccharomyces pombe. To do this use has been made of the fission yeast shuttle vector pRep1 whose map is provided on the opposite page (Diag. 1).The cDNA of the mature HABP1 was subcloned between the Nde 1 and BamH1 sites of the MCS (multiple cloning site) of pRep1 (Diag. 1) under the strong promoter nmt1 (**n**o **m**essage in **t**hiamine). A gene under this promoter gets expressed at full strength in the absence of thiamine in the medium but in the presence of thiamine (10μM) the expression is completely repressed (Maundrell., 1990; Maundrell., 1993). The plasmid generated by subcloning the HABP1 cDNA in pRep1 was named pRHP1. *S. pombe* cells were transformed with this plasmid and then the following **Plan of Action** was adopted to fulfill the Aim of understanding the biological functioning of HABP1:

1. **Cell Growth Assay would be done of the transformed *S. pombe* cells to check their growth while producing HABP1.**

2. **The expression of the protein would be checked to ensure that the difference if any seen in the growth correlates with the protein expression.**

3. **A mutant of the cDNA clone HABP1 would be generated which bears a STOP codon after 6 amino acids from the START to stop the translation of the gene. This would be used to transform *S. pombe* cells to see the effect of just the transcript of HABP1 on the growth of the *S. pombe* cells without the protein.**

4. **Morphology of the *S. pombe* cells would be studied by means of different fluorescent dyes to check the prevailing intracellular condition on expression of HABP1 at different time points.**

5. **The localization of HABP1 would be studied at different time points by indirect immuno-fluorescence.**

6. **FACS Analysis of cells producing HABP1 would be done to check if the expression of the protein causes any cell cycle arrest.**

7. **Live cell staining would be done to ensure that the observed morphological changes are not artifacts.**

8. **Ultra-microscopy of the cells producing HABP1 would be done to study the fine structure of their morphology.**

9. **N and C terminal deletant mutants of HABP1 would be subcloned in the pRep1 shuttle vector and *S. pombe* cells would be transformed with them.**

10. **The expression of these deletant mutants of HABP1 would be checked.**

11. **The morphology of these cells expressing the deletant clones would be checked by the same way as the morphology of the cells expressing HABP1 was checked.**

12. **Attempts would be made to check for interacting proteins of HABP1 first by Far Western with biotinylated rHABP1 and then the interaction of HABP1 with CDC 25 and CDC 2 two of the main cell-cycle regulatory proteins shall be checked.**

Most of the work shall be done in the *Schizosaccharomyces pombe* strain BJ 7468 which is isogenic to 972 h^- strain. For the live cell staining the strain MBY 624 would be used which is isogenic to 972 h^- except for the fact that the regulatory light chain of its myosin is tagged with Green Fluorescent Protein (GFP). For studying the interactions of HABP1 with CDC 25 and CDC 2 the strain JK 2423 would be used which is isogenic to 972 h^- except for a 12 "myc" tag at the C-terminal end of CDC 25.

Material and Methods

3. MATERIALS AND METHODS

3.A MATERIALS

Chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO, USA) unless otherwise mentioned. Restriction enzymes and other molecular biology reagents were purchased from New England Biolabs (NEB, Beverly, MA, USA). Plasmid DNA has sometimes been purified with the help of Wizard Mini-Prep Kit from Promega Corporation.

Chemicals to prepare medium for *S. pombe* cells have been purchased from Invitrogen, Sigma and Merck. Site directed Mutagenesis (SDM) was performed using the of Quikchange™ kit from Stratagene Inc. La Jolla, CA 92037. Primers from SDM were purchased from GENSET, Genomic Research Center, Evry Cedex, France.

Rhodamine-phalloidin (Molecular Probes) was a kind gift from Dr. Suman Dhar, SCMM, JNU. Propidium Iodide was a kind gift from Dr. Chandrima Saha, NII, New Delhi. DAPI was a kind gift from Dr. Chetan Chitnis, ICGEB, New Delhi. Aniline Blue was purchased from Merck, India.

PVDF and nitrocellulose membranes were procured from Amersham (UK).Goat anti-rabbit and anti-mouse alkaline phosphatase conjugated antibodies were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-myc antibody, anti-PSTAIRE antibody, FITC conjugated anti-rabbit and anti-mouse antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA Monoclonal antibodies against p32 were a kind gift from Dr. A. R. Krainer, Cold Spring Harbour Laboratory, Cold Spring Harbour, USA.

α-[^{32}P]-dATP were obtained from Board of Radiation and Isotope Technology (BRIT), Bhabha Atomic Research Centre (BARC), India.

EZ-Link™ Sulfo-NHS-LC-Biotin used for biotinylating HABP1was purchased from Pierce (Rockford, IL, USA).

Water used for preparing media and reagents was either autoclaved triple distilled (distilled in our laboratory) or autoclaved Milli Q (obtained from water purification system, Millipore, MA, USA).

Of the *Schizosaccharomyces pombe* Strains used in this study, BJ 7468 was a kind gift from Prof Asis Datta ,Director, NCPGR, New Delhi; MBY 624 was a kind gift from Prof. Mohan Balasubramanian, Temasek Life Sciences Laboratory, Singapore and JK2423 was a kind gift from Prof Paul Russell,The Scripps Research Institute, La Jolla CA 92037 - USA.

3.A.i COMPOSITION OF MEDIUMS USED IN *S. pombe* CULTURE

a) Edinburgh_Minimal Medium (EMM) leu⁻:

Potassium hydrogen phthalate (3 g/litre), 2% (w/v) glucose, ammonium chloride (5 g/litre), disodium hydrogen phosphate (2.2 g/litre), 20 ml/litre salts (50X stock), 1 ml/litre vitamins (1000X stock), 0.1 ml/litre minerals (10000X stock) and supplements (50–250 mg/litre adenine and uracil). Solution to be autoclaved before use.

Salts stock (50X):

52.5 g/litre $MgCl_2.6H_2O$, 0.735 mg/litre $CaCl_2.2H_2O$, 50 g/litre KCl and 2 g/litre Na_2SO_4. Solution was to be filter sterilized before use.

Vitamins stock (1000X):

1 g/litre pantothenic acid, 10 g/litre nicotinic acid, 10 g/litre *myo*-inositol and 10mg/litre biotin. Solution was to be filter sterilized before use.

Minerals stock (10000X):

5 g/litre boric acid, 4 g/litre $MnSO_4$, 4 g/litre $ZnSO_4.7H_2O$, 2 g/litre $FeCl_2.6H_2O$, 0.4 g/litre molybdic acid, 1 g/litre KI, 0.4 g/litre $CuSO_4.5H_2O$ and 10 g/litre citric acid. Solution was to be filter sterilized before use.

b) Yeast Extract + Supplements (YES):

0.5% yeast extract, 3% (w/v) glucose, 50–250 mg/ml leucine, lysine, histidine, adenine and uracil. Solution was to be autoclaved before use.

c) Stop Buffer:

150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1mM NaN_3.

The solution to be used ice-cold.

d) Breaking Buffer:

150 mM NaCl, 50 mM PBS, 50 mM NaF, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM PMSF (Phenyl Methyl Sulphonyl Fluoride), 10% glycerol, 5 µg/ml each of pepstatin, aprotinin and leupeptin.

3.B. METHODS

3.B.i PROCESSING OF DIALYSIS TUBING

Convenient length of dialysis tubing were boiled in 1 mM EDTA, pH 8.0 for 10 min and thoroughly washed in distilled water and stored at 4°C for subsequent use.

3.B.ii PREPARATION OF HA-SEPHAROSE-4B AFFINITY MATRIX

Hyaluronic acid (HA) from human umbilical cord (grade 1) was coupled to EAH-Sepharose-4B beads by acid catalysed condensation reaction using N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) as the coupling reagent. Here, EDC acts as a homo-bifunctional reactant, which conjugates the carboxy group of the ligand (HA), to amino group of the gel beads. Reaction was performed in distilled water adjusted to pH 4.5-6.0 for 12 h. For 20 ml of EAH-Sepharose-4B (7-10 μmole-NH_2/ml), 310 mg of EDC and 50 mg of HA were added and the pH was checked intermittently and kept between 4.5-6.0. The slurry was washed thoroughly with water and then alternatively with 0.2 M Glycine-HCl, pH 2.2 and 0.1 N $NaHCO_3$ containing 0.5 M NaCl. The activated Sepharose was blocked by 1 M acetic acid, pH 4.0 for 4 h at 4°C under constant stirring condition and again washed as before. The amount of HA bound per ml of gel was estimated by carbazole test (Bitter and Muir, 1962) using glucuronic acid as standard. Column was packed with HA-Sepharose-4B matrix and equilibrated with 0.01 M phosphate buffered saline (PBS), pH 7.2.

3.B.iii PURIFICATION OF HYALURONAN-BINDING PROTEIN (HABP1)

3.B.iii.a INDUCTION AND EXPRESSION OF HYALURONAN-BINDING PROTEIN 1 (HABP1) IN *E. coli*

Sequence analysis of hyaluronan-binding protein (HABP1) was reported earlier and found to be identical to that of p32, a protein co-purified with the splicing factor SF2. p32 cDNA clone (in the bacterial expression vector pT7A.A32) was a kind gift from Dr. Adrian Krainer, CSHL, USA. pT7A. A32 plasmid construct harbouring the 209 amino acids long mature p32 protein (Krainer *et al.,* 1991; Honore *et al.,* 1993) under the control of T7 promoter was expressed in BL21 (DE3) cells according to Krainer et al (1991). Pre-innoculum of the BL21 (DE3) cells transformed with pT7A.A32 was grown overnight at 37°C in LB medium containing 50 µg/ml ampicillin, 0.2% glucose. One percent of the pre-innoculum was transferred to fresh LB medium containing 50 µg/ml ampicillin, 0.2% glucose and grown until OD_{600} reached 1.0. Then, 0.4 mM IPTG was added to the culture and it was grown at 37°C for the next 3 h. The cells were pelleted by centrifugation at 5000 x g for 30 min at 4°C and either stored at –70°C or immediately processed for protein purification.

3.B.iii.b PURIFICATION OF RECOMBINANT HABP1 USING HA-SEPHAROSE AFFINITY

Purification of HABP1 was carried out according to the procedure described by Deb and Datta, 1996. Bacterial pellet obtained from 50 ml culture was washed with PBS and suspended in 15 ml PBS. Triton X-100 (0.01%, v/v) was added and left at room temperature for 45 mins. Complete lysis was achieved by sonication (10-15 bursts each of 30 sec) on ice. Cellular debris were pelleted down and the supernatant was dialysed against PBS and the volume made upto 50 ml with PBS and loaded onto the HA-Sepharose-4B column. After washing with 20 bed-volumes of PBS (pH 7.2) containing 0.15 M NaCl and then, with 20 bed-

volumes of 0.01 M phosphate buffer (pH 7.2) containing 0.5 M NaCl; the column was eluted with 0.2 M Glycine-HCl (pH 2.2) at a flow rate of 20 ml/h and the eluent was collected as 2 ml fractions. The protein-containing fractions were identified by measuring their absorbance at 280 nm. The peak fractions were pooled and concentrated. Whole purification process was carried out essentially at 4°C. Functional activity of purified rHABP1/p32 was confirmed by its binding to biotinylated HA *in vitro*.

3.B.iv BIOTINYLATION OF HABP1

For biotinylation of rHABP1, it was incubated with N-hydroxy succinimide biotin (Pierce) in dimethyl sulfoxide followed by incubation with ammonium chloride and then dialyzed against PBS (pH7.2) and stored for further analysis with 20% glycerol at -20°C (Yang *et al.*, 1994).

3.B.v GROWTH, MAINTAINENCE AND MANIPULATION OF *S. pombe* CELLS

Wherever the reference has not been mentioned in this section, the protocol has been adapted from Moreno et al. (1991).

3.B.v.a BASIC GROWING PROCEDURE OF *S. pombe* CELLS

Transformed *S. pombe* cells were maintained on EMM leu$^-$ plates and grown when needed in EMM leu$^-$ Liquid Medium (composition given earlier) at 29° C with constant shaking at 200 rpm in shake flasks. From the plates, a 5 ml EMM leu$^-$ primary culture was grown for 72 hours and then a suitable volume of secondary culture was inoculated at a starting OD_{595nm} of 0.1. The OD_{595nm} of the secondary culture was measured at given time intervals and the cell number was calculated from the OD_{595nm} for experiments where cells had to be taken out from the culture and processed further. Wherever mentioned, thiamine was added to the medium while growing the cells.

3.B.v.b CELL GROWTH ASSAY FOR *S. pombe* CELLS

Cells were grown as given above and their OD_{595nm} checked and plotted against time in hours.

3.B.v.c TRANSFORMATION OF *S. pombe* CELLS WITH PLASMID DNA

i. Preparation of competent *S. pombe* cells:

This protocol is an adaptation from Sherman *et al* (1986) and Ito *et al* (1983). *S. pombe* cells are streaked on a YES (0.5% yeast extract; 3% glucose; 50 mg per litre of leucine, adenine and uracil) agar (2%) plate and grown at 29°C for 2-3 days. When individual colonies appeared, a single colony was picked up and was used to inoculate 10 ml YES medium and was grown again at 29°C overnight with proper shaking. Five ml of this primary culture was used to inoculate 50 ml of YES medium which was grown at 30°C until the optical density of the culture reaches 0.55 to 0.65. The cells were then pelleted at 7000-8000 rpm for five minutes and washed carefully with sterile MQ water, repelleted and finally dissolved in $1/100^{th}$ original culture volume with lithium acetate buffer (pH 4.9) (lithium acetate 11.2 gm/litre and pH adjusted with glacial acetic acid). The cells were once again washed again with lithium acetate buffer and finally resuspended in 0.5 ml lithium acetate buffer and stored in the refrigerator, until further use.

ii. Transformation of competent *S. pombe* cells with Plasmid DNA:

Transformation of *S. pombe* cells were carried out according to the protocol of Sherman *et al* (1986). 100 µl of the *S. pombe* cell suspension in the lithium acetate buffer was taken and 4 µl of Salmon Sperm DNA (10 µg/µl) and 1-2 µg of the recombinant pRep1 plasmid DNA was added to it. This mixture was incubated at room temperature for ten minutes. Following the incubation, 0.5 ml of PEG 3350 in lithium acetate buffer

(pH 4.9) was added to the mixture. The contents were incubated at 29°C for 45 minutes with tapping from time to time. The tube was then kept at 46°C for 25 minutes. After that the tube was allowed to cool down to room temperature. The tube was centrifuged at 4500 rpm for 5 minutes to pellet down the cells. The supernatant was discarded and the pellet was dissolved in 300 µl of MQ water. This cell solution was then plated on EMM-Agarose (Leu $^-$) plate for selection along with EMM-Agarose (Ade $^-$) and EMM-Agarose (Ura $^-$) plates (as negative controls) to test for transformed colonies. Here it must be pointed out that the strains of *S. pombe* used in this study are cells that do not grow in the absence of adenine or uracil or leucine. But when they are transformed with pRep1 or any of the other clones, they can grow in leucine free medium as the transforming plasmid has the gene Leu2 which enables the cells to grow in leucine free medium and this is used for selection.

3.B.v.d FIXING *S. pombe* CELLS WITH ETHANOL

From an exponentially growing culture about 10^7 are pelleted down at 2000 rpm for 5 minutes. The supernatant was discarded. The cells were then resuspended in 1 ml distilled water, vortexed well and then centrifuged for 15 sec to spin them down and resuspended in 1-ml cold 70% ethanol. The microcentrifuge tube was vortexed briefly and the cells were stored at 4°C till further use.

3.B.v.e FIXING *S. pombe* CELLS WITH PARAFORMALDEHYDE

First, 17.5% of paraformaldehyde is prepared as follows: 8.75 grams of paraformaldehyde is weighed out and added to 50 ml PBS, 1 ml of 1M NaOH is added to it and the mixture is incubated at 65° for 20 minutes. The solution is shaken for solubilization and centrifuged for 5 minutes at room temperature at 3000 rpm to remove the polymers. The clear supernatant is taken and used to fix cells.

The OD of the growing cells is checked at 595 nm and approximately 10^8 cells are taken and paraformaldehyde is added to it such that its volume is one fourth the volume of the cell culture taken. The mixture is then rotated slowly on a wheel for 30 minutes for through mixing. Following this, the cells are centrifuged at 3000 rpm for 5 minutes at room temperature and washed three times with PBS. These fixed cells are stored in PBS having 1mM sodium azide at 4° C until further use.

3.B.v.f PROPIDIUM IODIDE/ CALCOFLUOR STAINING OF *S. pombe* CELLS

Three hundred microlitres of ethanol fixed cells are taken (containing around 2 - 3 x 10^6 cells, after washing), centrifuged to remove the ethanol and the cells are rehydrated by resuspending in 1 ml of 50 mM sodium citrate. The cells are centrifuged briefly at 14000 rpm for one minute at room temperature) and the supernatant was discarded. The pellet was then resuspended in 0.5 ml 50 mM sodium-citrate containing 0.1 mg / ml RNase A incubated at 37°C for at least 2 hr. To this suspension of cells, 0.5 ml 50 mM sodium citrate containing 4 µg / ml propidium iodide (PI), was added bringing the final concentration of PI to 2 µg / ml (PI can be added together with the RNase if desired). Next the suspension was centrifuged at full speed for 4 minutes and most of the supernatant is discarded leaving around 50 µl of the liquid. From this suspension about 4 µl of was put on the slide and the slide was heat fixed at 70° for two minutes. Then the slide was cooled to room temperature and 1 µl of Calcofluor working stock(Calcofluor 50 µgm/µl in 1mg.ml PNPP and 50% glycerol) was added to the slide and then the cover slip was sealed with nail varnish and observed under suitable excitation in a Zeiss Axipscope II Fluorescence Microscope.

3.B.v.g STAINING OF ACTIN POLYMERS IN *S. pombe* BY RHODAMINE CONJUGATED TO PHALLOIDIN

The protocol for staining actin polymers was done according to that given by Balasubramanian *et al.*, 1997. Twenty milliliters of growing cells were taken at various time points and 5 ml of 17.5% formaldehyde solution (prepared as mentioned earlier) was added to it and incubated at the same temperature at which the cells were growing for 30 minutes. The cells were then centrifuged at 2000 rpm for five minutes and resuspended in 1 ml PBS in a microcentrifuge tube and centrifuged at 2000 rpm. This step was repeated three more times. The cells were then suspended in PBS containing 1% NP-40 and mixed gently for 1 minute. The cells were again centrifuged for five minutes at 2000 rpm at room temperature and washed with PBS as done earlier and resuspended in 1 ml of PBS. From this suspension, 270 µl were taken and 30 µl of Rhodamine-conjugated phalloidin stock (0.1 mg/ml in PBS) was added. Then the microcentrifuge tube was wrapped in aluminium foil and incubated at room temperature on a rotator for 30 minutes. Then the cells are centrifuged at 2000 rpm at room temperature and washed three times with 1 ml PBS as done earlier and finally resuspended in 50 µl of PBS. Four microlitres of this suspension was put on the slide and heat fixed at 70 ° C for five minutes. After the slide cooled to room temperature 1 µl of DAPI and 1 µl of Calcofluor are put on the cells and then the cover slip was sealed with nail varnish and observed under suitable excitation in a Zeiss Axioscope II Fluorescence Microscope.

3.B.v.h DAPI STAINING OF LIVE *S. pombe* MBY 624 CELLS

Transformed *S. pombe* cells belonging to the strain MBY 624 cells were grown in EMM leu- medium and their cell growth rates were measured spectrophotometrically at 595 nm. Accordingly approximately 10^7 cells were taken out and washed in sterile distilled water and resusrended in 50 mM sodium citrate Buffer. Four microlitres of this

suspension was heat fixed at 70°on a glass slide and cooled to room temperature and 1 µl of DAPI (4',6-diamidino-2-phenylindole. 2HCl) working solution (DAPI 50 µlgm/ml, PNPP 1mg/ml, 50% glycerol) was added onto it. Then a cover slip was put on it, sealed with nail varnish and observed under a fluorescent microscope (Zeiss Axioscope II).

3.B.v.i ANILINE BLUE/DAPI STAINING OF LIVE *S. pombe* MBY 624 CELLS

Cells of the *S. pombe* MBY 624 strain was grown and washed as stated earlier and resuspended in 59 mM Sodium citrate Buffer containing 0.5 mg/ml of Aniline Blue and then 4 µl of this solution was heat fixed onto a glass slide. Mounting was then done with DAPI working solution was described earlier and observed under a fluorescent microscope under suitable excitation.

3.B.v.j INDIRECT IMMUNO-FLUORESCENCE OF *S. pombe* CELLS

The protocol followed here was developed by Moreno et al 1991. Cells are taken at various time points and fixed with formaldehyde as mentioned earlier. Following fixation, the cells they were suspended in PBS containing 1mM sodium azide. From this suspension the cells were centrifuged at 2000 rpm for 5 mins at room temperature and resuspended in PBS containing 1.2 M Sorbitol. To this Novozym and Zymolase 20T were added to a final concentration of 0.5mg/ml each. This suspension was then incubated at room temperature for 20 minutes, until most of the cell walls of the *S. pombe* cells have been digested. This was checked by mixing 10 µl of 10% SDS and checking under a phase contrast microscope. Cells, whose cell wall has been completely digested, lose their refringence. After this the tube was filled immediately with PBS containing 1% Triton X-100 and the cells were again centrifuged at 2000 rpm at room temperature. The supernatant was then discarded and the cells resuspended in PBS and washed thrice.

After this the cells were resuspended in PBS containing 100mM lysine-HCl and 1% fatty acid free BSA (PBAL) and incubated on a rotator for 30 minutes at room temperature. Then the cells were pelletted at 2000 rpm at room temperature and resuspended in 100 µl of primary anti-body solution (dilution 1:100) and incubated on a rotator overnight at room temperature. The cells were again pelletted down at 2000 rpm at room temperature and washed thrice with PBAL. These washed cells were left on the rotator for 20 minutes. Then the cells were resuspended in 100 µl of secondary antibody (dilution 1:100) and kept on the rotator for 2 hrs at room temperature. Following this the cells were pelletted down at 2000 rpm at room temperature and washed thrice with PBAL as above. The cells were then pelletted and resuspended in 50 µl of PBAL. 4 µl of this suspension was taken on the slide and heat fixed at 70 ° C for five minutes and 1 µl of anti-fade (1 mg/ml p-phenylenediamine in 50% glycerol) was put on the dried cells after cooling the slide to room temperature and then the cover slip was sealed with nail varnish and observed under suitable excitation in a Zeiss Fluorescence Microscope Axioscope II.

3.B.v.k STAINING OF CELL SEPTUM AND NUCLEUS OF *S. pombe* BY ANILINE BLUE/PROPIDIUM IODIDE

Three hundred microlitres of ethanol fixed cells are taken (containing around 2 - 3 x 10^6 cells, after washing), centrifuged to remove the ethanol and the cells are rehydrated by resuspending in 1 ml of 50 mM sodium citrate. The cells are centrifuged briefly at 14000 rpm for one minute at room temperature) and the supernatant was discarded. The pellet was then resuspended in 0.5 ml 50 mM sodium-citrate containing 0.1 mg / ml RNase A incubated at 37°C for at least 2 hr. To this suspension of cells, 0.5 ml 50 mM sodium citrate containing 4 µg / ml propidium iodide (PI), was added bringing the final concentration of PI to 2 µg / ml (PI can be added together with the RNase if desired). Next

the suspension was centrifuged at full speed for 4 minutes and the supernatant was discarded leaving 10 µl .To this aniline blue solution (0.5 mg/ml made in 50 mM Sodium Citrate) was added and the microcentrifuge tube was vortexed briefly and incubated at 4° C for one hour in the dark. Then the cells were again vortexed briefly and 4 µl of the cell suspension was put on a slide, heat fixed at 70° C for five minutes and a cover slip was sealed with nail varnish over it and observed under suitable excitation in a Zeiss Axioscope II Fluorescence Microscope (Kippert F and Lloyd D, 1995)

3.B.v.l PROCESSING OF *S. pombe* CELLS FOR FACS

Three hundred microlitres of ethanol fixed cells are taken (containing around 2 - 3 x 10^6 cells, after washing), centrifuged to remove the ethanol and the cells are rehydrated by resuspending in 1 ml of 50 mM sodium citrate. The cells are centrifuged briefly at 14000 rpm for one minute at room temperature) and the supernatant was discarded. The pellet was then resuspended in 0.5 ml 50 mM sodium-citrate containing 0.1 mg / ml RNase A incubated at 37°C for at least 2 hr. To this suspension of cells, 0.5 ml 50 mM sodium citrate containing 4 µg / ml propidium iodide (PI), was added bringing the final concentration of PI to 2 µg / ml (PI can be added together with the RNase if desired). Then the solution was briefly sonicated and then their DNA content was checked at the FACS machine.

3.B.v.m PROCESSING OF *S. pombe* CELLS FOR ELECTRON MICROSCOPY

The OD 595 of growing fission yeast cells were checked and 10^8 cells were taken and fixed in modified Karnovsky's fluid buffered with 0.1M Sodium Phosphate (pH 7.4). Fixation was done for 10-18 hours at 4° C after which they were washed with fresh phosphate buffer and post fixed for two hours in 1% Osmium Tetraoxide in phosphate buffer at 4°

C. Then after several washes with phosphate buffer, the cells were dehydrated in graded acetone solutions and embedded in CY 212 Araldite Resin.Ultrathin Sections of 60-80 nm thickness were generated using ultracut E Ultramicrotome and the sections were stained with alchoholic uranyl acetate and lead citrate for appropriate time intervals and then the grids were examined in a transmission electron microscope (MORGAGNI 268 Model from Phillips) operated at 80 kv.

3.B.vi ANALYSIS OF PROTEINS

3.B.vi.a ESTIMATION OF PROTEINS

Estimation of proteins was done with the help of Bradford's Reagent from Biorad following the manufacturer's instructions (Bradford 1976).

3.B.vi.b EXTRACTION OF PROTEINS FROM *S. pombe* CELLS

Cells were centrifuged in SM 24 tubes and washed once in stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA and 1mM NaN_3) and stored in –80 °C till further use. When needed, the pellets were thawed on ice and a suitable amount of breaking buffer [150 mM NaCl, 50 mM Phosphate, 50 mM NaF, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM PMSF (Phenyl Methyl Sulphonyl Fluoride), 10% glycerol, 5 µg/ml each of pepstatin, aprotinin and leupeptin] was added (100 µl of breaking buffer for every 10^8 cells) and 1.5 ml of 50 micron glass beads were added and vigorously vortexed 10 times for one minute each. Between two bursts, a gap of one minute was given to avoid over heating.

3.B.vi.c SDS-PAGE OF PROTEINS EXTRACTED FROM *S. pombe* CELLS

Polyacrylamide gel electrophoresis under denaturing condition (in the presence of 0.1% SDS) was performed according to the method of

Laemmli (1970). The proteins were stacked at pH 6.8 in a stacking gel containing 3.5% acrylamide, 0.106% N, N'-methylene bisacrylamide, 0.125 M Tris-HCl, pH 6.8, 0.01% TEMED and 0.1% ammonium persulfate. The running (separating) gel was made of 12.5% acrylamide, 0.33% N, N'-methylene bisacrylamide, 0.375 M Tris-HCl, pH 8.8, 0.01% TEMED and 0.1% ammonium persulfate. The protein samples were electrophoresed in running buffer consisting of 0.025 M Tris-base, 0.192 M glycine, pH 8.3 and 0.1% SDS. The protein samples were prepared in sample buffer containing 0.0625 M Tris-HCl, pH 6.8; 2% SDS, 10% glycerol, and with or without 5% ß-mercaptoethanol (Laemmli, 1970) and immersed in a boiling water bath for 5 min. For lysates prepared by the direct boiling method (in 0.5% SDS-PBS or 1X Laemmli buffer), the samples were loaded as such onto the gel. Standard molecular weight marker (Pharmacia Biotech Inc., Uppsala, Sweden) was also electrophoresed alongside to calculate the subunit molecular size of the proteins.

3.B.vi.d VISUALISATION OF PROTEINS ON SDS-PAGE:

a) Coomassie Brilliant Blue staining:

SDS-Polyacrylamide gels containing more than 2 µg protein concentration were visualized by standard Coomassie Brilliant Blue (CBB) staining (0.1% (w/v) CBB dissolved in 25% (v/v) methanol and 10% (v/v) acetic acid in water), followed by de-staining in 25% (v/v) methanol and 10% (v/v) acetic acid in water.

b) Silver staining of proteins on SDS- Polyacrylamide gels:

SDS-Polyacrylamide gels containing very little amount of protein samples were detected by silver stain using the protocol described by Merril *et al.,* 1984. Briefly, following electrophoresis, the gel was fixed for 2 x 30 min or overnight in ethanol (40%) and acetic acid (10%). After

fixation, the gel was washed 2 x 10 min in Milli-Q water and then sensitised in sodium thiosulphate pentahydrate solution (0.3 g/l) for 1 min. A brief wash (2 x 1 min) in Milli Q water was given after sensitisation, followed by a 30 min incubation in silver nitrate solution (2 g/l silver nitrate and 250 µl/l 37% formaldehyde). After staining, the gel was rinsed for about 10-20 sec in Milli-Q water and then the bands were developed with 30 g/l sodium carbonate, 250 µl/l 37% formaldehyde and 10 mg/l sodium thiosulphate pentahydrate till the desired intensity was reached. The gel was then rinsed several times with Milli Q water and then photographed. Caution was taken not to touch the gel with bare hand while processing.

3.B.vi.e IMMUNO-BLOT ANALYSIS

Proteins were separated on 12.5% SDS-PAGE under reducing conditions and electroblotted onto a Hybond-C™ nitrocellulose membrane or PVDF (Amersham, UK) in a buffer containing 0.025 M Tris, 0.192 M glycine, 0.037% SDS and 20% methanol, pH 8.3 following the procedure described by Towbin et al (1979). When PVDF membrane was used then the membrane was equilibrated once with methanol before equilibrating it in the transfer buffer. After blocking in 3% (w/v) BSA in phosphate buffered saline with detergent (PBST – 10mM phosphate, pH 7.2; 0.15 M NaCl; 0.05 % Tween-20) at room temperature, the blots were probed with primary antibody for 1 h at room temperature. All dilutions were made in 1.5 % (w/v) BSA-PBST. Membranes were then washed 5 x 5 min in PBST and further incubated with secondary antibody (goat-anti-rabbit IgG conjugated to alkaline phosphatase (AP) or goat-anti-mouse IgG conjugated to AP; 1:10,000 in 1.5% BSA-PBST) for 1 h at room temperature. The bound antibody was detected with the NitroBlue Tetrazolium (NBT) / 5-Bromo-4-chloro-3-indolyl Phosphate (BCIP) colour system. Ten microliters of NBT (30 mg/ml) and 10 µl of BCIP (15 mg/ml)

in 10 ml of AP buffer was normally used for the same. The reaction was continued until the bands developed to desired intensity.

3.B.vi.f FAR-WESTERN BLOTTING

The process for Far-Western was exactly the same as in immuno-blotting as given above but instead of the primary anti-body, biotinylated HABP1 was used as the primary probe with was detected by the secondary anti-body extravidin labeled AP from Santa Cruz (Macgregor *et al.*, 1990).

3.B.vi.g CO-IMMUNO-PRECIPITATION

First *S. pombe* cells were lysed as given above the cell debris removed by centrifugation and the total protein content estimated. Samples containing 1mg/ml was prepared from it and to this suitable dilution of the first antibody was added and incubated with gentle shaking on ice for 2 h, following which 100 µl protein A sepharose beads (Pharmacia) from a stock of 40 mg/ml was added. This was also incubated with shaking on ice for 2 h. The beads were then pelletted and washed thoroughly by centrifugation at 3000 rpm for 10 min at 4°C. The beads were then washed for at least six times with PBS. The washed beads were boiled in a suitable volume of Laemmli's buffer for 15 min. Finally the boiled samples were centrifuged at 12000 rpm for 10 min and resolved on 12.5% SDS-PAGE. Then the proteins were electro blotted onto a PVDF membrane and probed with the primary antibody of the other protein as processed as in immuno-blotting.

3.B.vii DNA PREPARATION AND ANALYSIS

3.B.vii.a MINISCALE DNA PREPARATION BY MODIFIED ALKALINE LYSIS METHOD

Rapid mini scale DNA purification from *E. coli* was done by the method described by Ahn et al (2000). *E. coli* cells were grown in 2 ml of Luria Broth (LB) with appropriate antibiotic and the cells were harvested by centrifugation at 11000 X g for 1 min. Cells were then resuspended in 100 µl resuspension buffer containing 50 mM Tris, pH 8.0; 10 mM EDTA and 20 µg RNase A and then 100 µl lysis buffer (200 mM NaOH; 1% SDS) was added and mixed thoroughly. The lysed suspension was then neutralised with 120 µl neutralising buffer (3 M Potassium acetate, pH 5.5). It was mixed properly and incubated at room temperature for 3 min. The bacterial debris were removed by centrifugation at 11000 X g for 1 min. The supernatant was then added to 200 µl of isopropanol and incubated for 1 min at room temperature to precipitate DNA. The DNA pellet was collected by centrifugation at 11000 X g for 30 sec and washed with 500 µl of 70% (v/v) ethanol. Finally, the pellet was air dried and resuspended in 100 µl of sterile water or TE (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA, pH 8.0).

3.B.vii.b MIDI SCALE DNA PREPARATION BY ALKALINE LYSIS METHOD

Medium scale DNA purification from *E. coli* was done by the method described by Birnboim and Doly (1979). *E. coli* cells were grown overnight in 50 ml LB medium containing ampicillin (50 µg/ml) or kanamycin (30 µg/ml) and collected by centrifugation (5000 X g at 4°C for 15 min). The cell pellet was resuspended in 1.5 ml of TES Buffer (50 mM Tris, pH 8.0; 10 mM EDTA) containing 2 mg/ml lysozyme, and kept on ice for 10 min. The lysed cells were subjected to 3 ml of denaturing solution (1% SDS and 0.2 N NaOH) on ice for 10 min. The chromosomal DNA-Protein complex was selectively removed by incubating with 1.6 ml

of 3 M sodium acetate (pH 4.6) for 20 min on ice followed by centrifugation at 12000 X g for 20 min. The RNA was digested by treating with 5 µl of RNase A for 45 min at 37°C. The crude preparation was further purified by extracting twice with an equal volume of phenol/chloroform/ isoamyl alcohol [25:24:1 (v/v/v)] and once with equal volume of chloroform/ isoamyl alcohol [24:1 (v/v)]. The crude plasmid DNA was precipitated by the addition of three volumes of pre-chilled ethanol, and the precipitate was collected by centrifugation at 12000 X g for 30 min at 4°C. The pellet was resuspended in 0.4 ml of nuclease-free water and 0.12 ml of 4 M NaCl. DNA was precipitated with 0.5 ml of 13% polyethylene glycol (average Mol. Wt. 8000), and collected by centrifugation at 12000 X g for 10 min at room temperature. The pellet was washed with 70% (v/v) ethanol, and dissolved in 50 µl of TE (10 mM Tris, pH 8.0; 1 mM EDTA).

3.B.vii.c AGAROSE GEL ELECTROPHORESIS FOR DNA

Agarose gel electrophoresis was performed as described by Sambrook et al. (1989). For DNA samples, the required amount (0.8%) of agarose was melted by heating in 1 X TAE buffer, cooled to 55°C and ethidium bromide (0.5 µg/ml) was added prior to casting of the gel on the gel tray. One-sixth volume of DNA gel loading buffer was mixed with samples and loaded in the wells. The electrophoresis was performed at 5 V/cm in 1 X TAE buffer and the DNA fragments were visualised on an UV transilluminator at 302 nm.

3.B.vii.d SITE-DIRECTED MUTAGENESIS

In order to introduce a STOP codon after the first six amino acids of the mature HABP1 the process of Site Directed Mutagenesis was adopted using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene Inc.). The amino acid sequence of the mature HABP1 was changed from

^{1}L H T D G D K A F V ^{10} to ^{1}L H T D G D [STOP] A F V ^{10}

For this, two primers were used; namely

HMF1: 5 / cacaccgacggagacTaagcttttgttgatttc 3 /

HMR1: 5 / gtgtggctgcctctgAttcgtaaacaactaaag 3 /

This kit was based on polymerase chair reaction by Pfu Turbo™ DNA polymerase 50 ng of pRHP1 plasmid (used as template), 5 µl 10 X reaction buffer, 125 ng of each primer, 1 µl dNTP mix were added in a final volume of 50 µl reaction and 1 µl of *Pfu Turbo* DNA polymerase (2.5 U/µl) was added finally to the reaction mix. The reaction mix was overlaid with 30 µl of mineral oil. PCR cycle comprised of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min and extension at 68°C for 20 min. This was repeated from the denaturation step for 12 cycles. After the completion of these cycles the reaction mixture was cooled to 4° C till further use. 1 µl of *DpnI* (10 U/µl) was added directly to the amplification reaction and thoroughly mixed by pipetting the solutions up and down and incubated at 37°C for 1 h to digest the parental supercoiled dsDNA. 10 µl of this reaction mix was added in 100 µl E.coli XL-1 Blue MRF' competent cells and incubated on ice for 45 min. Heat pulse of 45 sec at 42°C was given to the transformation mix and added to 900 µl of LB. It was then allowed to grow for 1 h at 37°C and the entire volume of transformation reaction on LB-agar plate containing ampicillin (100 µg/ml). DNA was isolated from different colonies and named pRHPΔ1. The mutated plasmid was purified using Wizard Mini-prep DNA Purification System.

3.B.viii RNA ISOLATION AND ANALYSIS FROM *S. pombe* CELLS

Glassware used for RNA isolation and analysis were baked at 200°C for 16-18 h. Plastic ware were treated with 3.0% (w/v) H_2O_2 for 2

h and later autoclaved at 15 lb/sq. in for 30 min. All the solutions (except Tris buffer) were prepared in DEPC water. DEPC was dissolved (0.1%, v/v) in Milli-Q water with vigorous stirring for 2 h followed by an overnight incubation at 37°C and then autoclaving.

3.B.viii.a RNA ISOLATION

Fission Yeast cells ($1X10^7$) were taken from exponentially growing culture (OD_{595}) and washed twice with sterile PBS and total RNA was also isolated using a commercial reagent TRIZOL® (Life Technologies, UK) according to the manufacturer's protocol. Quality of RNA was checked by agarose gel electrophoresis. Confirmation of quality and quantitation was done by recording the absorbance at 230, 260 and 280 nm.

3.B.viii.b FRACTIONATION OF RNA BY AGAROSE GEL ELECTROPHORESIS

The agarose gels were prepared in formaldehyde gel running buffer and electrophoresed without sample for 5 min at 5 V/cm. ~20 µg of RNA was denatured (V_f-15 µl) in the presence of 50% (v/v) deionized formamide (pH 4.0), 2.2 M formaldehyde and 1 x formaldehyde running buffer at 65°C for 15 min, chilled on ice and 5 µl of DEPC treated formaldehyde gel loading buffer was added. The RNA samples were loaded onto the gel and electrophoresed in 1x formaldehyde gel running buffer at 3 V/cm for 3 h. The gel while setting itself contained ethidium bromide (0.4 µg/ml) (Sambrook *et al.*, 1989).

3.B.viii.c NORTHERN BLOT ANALYSIS

The formaldehyde gel was rinsed with several changes of DEPC H_2O to remove formaldehye. RNA was then transferred from the gel to the nylon membrane essentially as described for Southern blotting. The blots were UV-cross linked after the transfer.

3.B.viii.d HYBRIDIZATION OF RADIOLABELLED PROBES TO IMMOBILIZED NUCLEIC ACIDS

Preparation of radiolabelled DNA by random priming method developed by Feinberg and Vogelstein, 1983.About 50-100 ng of HABP1 cDNA was denatured by heating in boiling water bath for 10 min and immediately chilled on ice. To the tube containing denatured DNA, 2 µl each of 0.5 mM dGTP, dCTP and dTTP, 5 µl of hexanucleotide labelling mixture (containing random hexamers and reaction buffer at 10 x concentration (2M HEPES, pH 6.6, 2 mM Tris-Cl (pH 7.0), 0.1 mM EDTA and 4 mg/ml (BSA), 30-50 µCi α-[^{32}P] -dATP (3000 Ci/mMol, 10 µCi/µl)] and nuclease free water to make up the volume to 50 µl. The reaction was initiated by adding 5 units of Klenow enzyme. Incubation was carried out for 2-3 h at 37°C before stopping by addition of EDTA (to a final concentration of 20 mM). The unincorporated dNTPs were removed by ethanol precipitation in the presence of 2.5 M ammonium acetate and absolute alcohol.

For the hybridization of radiolabelled probe to RNA immobilized on Hybond-N^{TM} nylon membrane, the membrane was first incubated in pre-hybridization solution containing 100 µg/ml denatured Salmon sperm DNA at 42°C in hybridization bottles. After 2-3 h heat denatured radiolabelled probe (2-5 x 10^5 cpm/ml) was added to the prehybridization mix. The hybridization was carried out at 42°C for 16-18 h.

Removal of bound probe from nylon membrane:

The probes were removed by washing the membranes sequentially to remove the non-specifically bound probe using the following protocol: twice with 2 x SSC containing 0.1% SDS at room temperature for 5 min each and twice with 0.5 x SSC containing 0.1% SDS at 65°C for 15 min each and finally in a solution containing 0.1x SSC, 0.1% SDS at 15 min

at 65°C.

3.B.viii.e AUTORADIOGRAPHY

After hybridization and washing, the blots were wrapped in a thin plastic film and exposed with intensifying screens in cassettes at -70°C for an appropriate amount of time.

Chapter I:
Expression of HABP1 in Schizosaccharomyces pombe Alters its Morphology and perturbs its Growth

4.A Introduction

It has already been discussed under 'INTRODUCTION' that HABP1 interacts with a number of proteins and other non-protein molecules. However its presence in the different subcellular and extracellular compartments are not yet well explained as the pathways in which this protein functions and their physiological significance are not well mapped. Since this protein is well conserved from the budding yeast to humans, though with exceptions, it ought to be active in major physiological pathways like other such evolutionarily conserved proteins. But till date nothing like this has come to light. This prompted us to use a novel approach to investigate the functional face of this protein.

To reveal an unknown biological function/s of a protein two approaches can be taken namely i) the gene of the protein in question could be disrupted or the production of the protein stopped otherwise in a system where the protein is naturally present and then the effect of its absence can be studied; ii) the gene of the protein in question can be introduced in a system where it is not present naturally and the consequences studied. Though either of these approaches could be adopted we have taken the latter approach. For the latter approach, the choice of system where the gene of interest is to be introduced has to be chosen with care since here the focus is not on whether the protein gets expressed but whether it retains its native function.

To examine the functional profile of HABP1, the expression system of Fission yeast *Schizosaccharomyces pombe* was chosen for two reasons: first,its similarity with other higher eukaryotes and secondly because it does not possess a homologue of the HABP1 gene even though it was observed that the budding yeast *Saccharomyces cerevisiae* has one (Seytter *et al.*, 1998; Muta *et al.*, 1997). Though *Schizosaccharomyces pombe* is a unicellular, simple eukaryote and a member of the phylum

ascomycete fungi it has been found to bear remarkable similarity to higher eukaryotes and in some cases to humans. Some of the major similarities are given below.

Some mammalian genes have been isolated using *S. pombe,* by complementation of the mutant homologue. The functional substitution of the human homologue of the cell-cycle regulator *cdc2* for the *S. pombe cdc2* gene (which is homologous to the *S. cerevisiae CDC28* gene) is possible (Lee *et al.*, 1987). The similarity of the human cdc2 gene and that of *S. pombe* has been confirmed at the protein level. The equatorial process of cell division characterized by septation and medial fission is more common among eukaryotes than budding yeast. Further, comparison of 18 s RNA amongst the different species of Ascomycetes implies that S. pombe diverged early from them (Kurtzman *et al.*, 1994). The mitochondrial DNA of fission yeast is the smallest amongst the other yeasts and is very similar to that of mammalian cells (Sankoff *et al.*, 1992).

Some mammalian promoters are also known to work in S. pombe (Toyama *et al.*, 1990). When the human chorionic gonadotropin and the human cytomegalovirus promoters are used in *S. pombe* to produce a heterologous protein, it was seen that the transcripts initiate at the same position as in mammalian cells with the same promoter (Jones *et al.*, 1988). The transcription-initiation mechanism of *S. pombe* is more similar to higher eukaryotes than that of *S. cerevisiae* (Russell, 1983; Russell, 1985; Fischli *et al.*, 1996). The *S. pombe* TATA element is located 25–30 base pairs upstream to the start of transcription, exactly as in mammalian cells (Benoist *et al.*, 1980). Promoters from *S. cerevisiae* genes generally function poorly in *S. pombe*, resulting in inefficient and aberrant initiation of transcription even though some *S.cerevisiae* promoters have been used in *S. pombe* successfully. (Belsham *et al.*, 1986; Broker *et al.*, 1987).

When higher eukaryotic genes containing introns are introduced into *S. cerevisiae*, they are not expressed, whereas the same genes have been successfully expressed in *S. pombe*. The splicing system in *S. pombe* removes one intron of the SV40 gene encoding the small T antigen, although the efficiency is low (Kaufer *et al.*, 1985). *S. cerevisiae* genes rarely possess introns, while in *S. pombe* several introns per gene are usually distributed throughout the coding region (Woolford, 1989); (Prabhala *et al.*, 1992). Indeed, in the genes for β-tublin (Hiraoka *et al.*, 1984), ras homologue (Fukui *et al.*, 1985), calmodulin (Takeda *et al.*, 1987) and cell-cycle regulator protein cdc2 (Hindley *et al.*, 1984), *S. pombe* has several introns, whereas *S. cerevisiae* has none (Davis *et al.*, 1986).

The RNA splicing mechanism in fission yeast is more similar to that of higher eukaryotes than *S. cerevisiae*. *S. cerevisiae* has consensus intron signal sequences that are very conservative (Guthrie *et al.*, 1986). In *S. pombe* the intron signal sequences are not so tightly conserved. CTNAC, located near the 3′ end of the intron, is a degenerate version of the TACTAAC sequence in *S. cerevisiae* (Hindley *et al.*, 1984) and is similar to the consensus found in higher eukaryotes (Mount, 1982). The components of the spliceosomes, which remove the introns in S. pombe, are comparable to those of mammalian species (Guthrie *et al.*, 1986). The small nuclear ribonucleoproteins U1 and U2 in *S. pombe* contain U1 and U2 small nuclear RNA, respectively, whose size and structure resemble those of human U1 and U2 small nuclear RNA rather than those of *S. cerevisiae* (Brennwald *et al.*, 1988; Porter *et al.*, 1990).

The signal-transduction system of *S. pombe* shows marked similarities to the mammalian G-protein-coupled system. Signals initiated by the mating factors of *S. pombe* are also transmitted through a G-protein to the effector(s) (Xu *et al.*, 1994). *S. pombe* utilizes a G-protein subunit to positively transmit the signal from the mating-factor

receptor to the effectors. This resembles the role of G α during signal transduction in mammalian cells, and makes *S. pombe* an appropriate model system for investigating mammalian receptor function (Obara *et al.*, 1991).

Mammalian endoplasmic-reticulum-retention signal KDEL can be recognized in *S. pombe* (Hildebrandt, 1997) In addition, the components of *S. pombe* cell walls differ from those of *S. cerevisiae*. Most species of yeast have a mixture of glucan and mannan in their cell wall, whereas the cell wall of *S. pombe* contains alkali-soluble α- (1-3)-linked glucan and galactomannan instead of mannan (Font de Mora *et al.*, 1990). This galactomannan is present in the outer layer of the cell wall and the layer adjacent to the plasma membrane (Horisberger *et al.*, 1985). Similar to mammalian cells the carbohydrate chains of fission yeast glycoproteins are composed of N-linked oligosaccharides with asparagine residues and O-linked species with serine/threonine residues. Here it maybe recalled that HABP1 also has three potential N-linked glycosylation sites and so its very likely to get correctly glycosylated when expressed in *S. pombe*.

Based on these facts it is obvious that in-spite of being a simple lower eukaryote, the fission yeast has unique characteristic which makes it very suitable for studying the functional aspect of mammalian proteins. Consequently many mammalian proteins when expressed in *S. pombe* retain their native functionality along with proper post-translational modifications, due to the great similarity of the fission yeast system with higher eukaryotes. All this makes it evident that Schizosaccharomyces pombe would be the right system to study the functional profile of HABP1. Here it also needs to be pointed out that that even though this protein is deceptively simple in structure, its behaviour points to a very complicated functional profile. It is highly probable that it discharges its varied functions depending upon the microenvironment in which it exists.

4.B RESULTS

4.B.i EXPRESSION OF HABP1 IN *Schizosaccharomyces pombe* CAUSES GROWTH INHIBITION

S. pombe strain BJ7468 (isogenic to 972 h^-) transformed with the plasmid pRHP1 (containing the cDNA of the mature HABP1 subcloned in the shuttle vector pRep1) were grown in EMM leu^- Medium, both in the presence [pRHP1(+T)] and absence [pRHP1] of thiamine. Since the HABP1 clone in pRep1 was under the promoter nmt1 (no message in thiamine) which is completely repressed in the presence of thiamine, there is no expression of HABP1 in pRHP1 transformed cells are grown in presence of thiamine. Fission yeast cells transformed with the shuttle vector pRep1 were also grown as a control set alongside in the same medium as described in Materials and Methods. The growth rate of the cultures was monitored spectrophotometrically at different time points as seen in Fig 1. From the results it is obvious that when pRHP1 transformed cells are grown in the absence of thiamine and HABP1 is supposed to express, the growth of fission yeast cells exhibit inhibition as compared to the vector transformed cells.

4.B.ii GROWTH INHIBITION OF *Schizosaccharomyces pombe* (BJ 7468) IS CAUSED BY TRANSLATION BUT NOT BY JUST TRANSCRIPTION OF THE HABP1 GENE

After observing the growth inhibition in *S. pombe* cells transformed with pRHP1, (grown without thiamine) the reason for this growth inhibition was investigated. It was hypothesized that the inhibition might be due to some abnormality of the transcription process or due to the action of the protein. To clarify and resolve this issue, it was decided to introduce a premature stop codon early in the cDNA clone of pRHP1. This was done by site directed mutagenesis (SDM). The stop codon was introduced after six amino acids from the start site using the Qukchange™ kit from Stratagene following the manufacturer's protocol

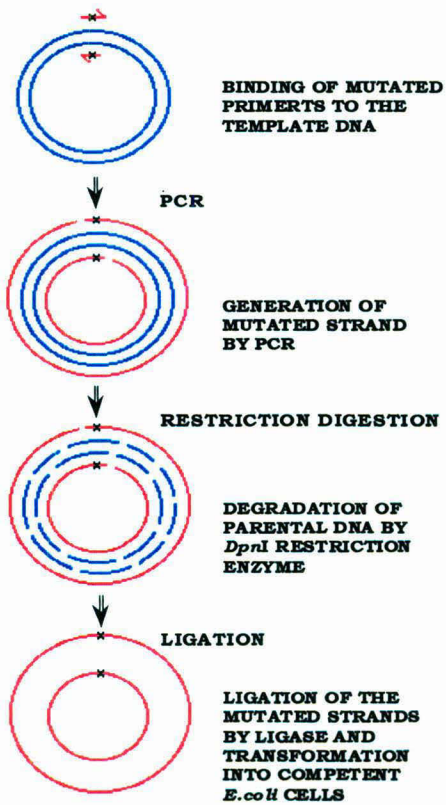


Diagram 1: Schematic diagram of the generation of the plasmid pPHPΔ1 in which the STOP codon has been introduced in the HABP1 gene by site directed generation (SDM).

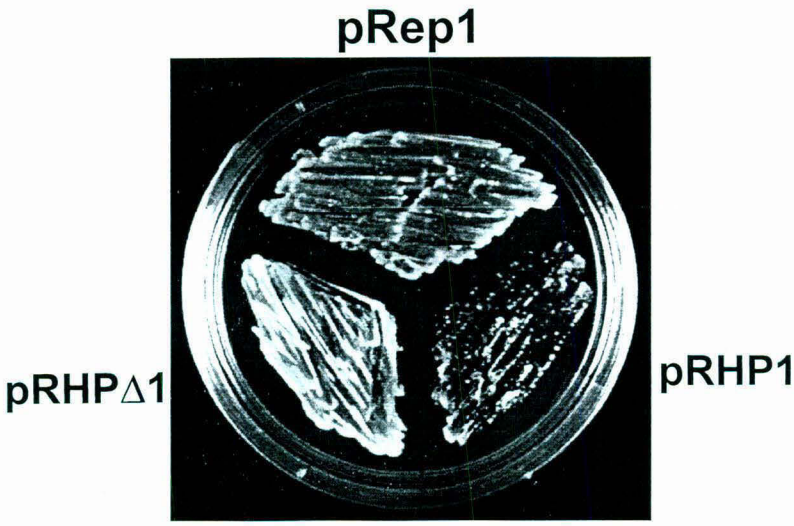


Fig2: Shows the Growth inhibition of HABP1 producing S.pombe cells on an EMM leu$^-$ Agar Plate.The pRHPΔ1 transformed cells show no growth inhibition

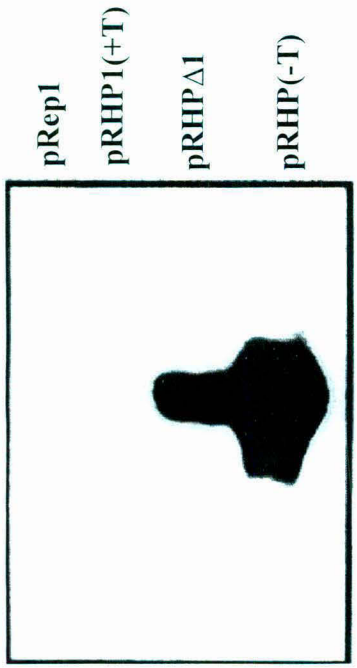


Fig 3A: Northern Blot of RNA extracted from S.pombe cells transformed with pRep1, pRHP1 (both in presence and absence of thiamine) and pRHPΔ1.

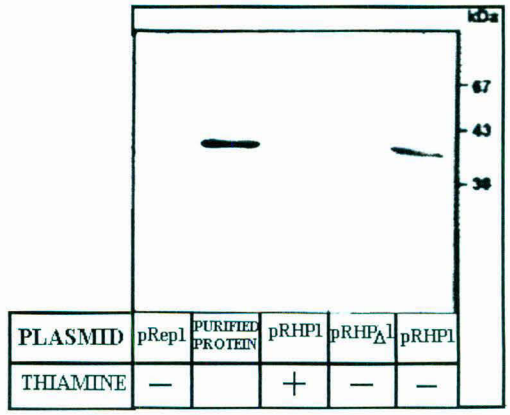


Fig 3B: Immuno-Blot of protein lysates from S.pombe cells transformed with pRep1,pRHP1(both in presence and absence of thiamine) and pRHPΔ1.Purified HABP1 has been used as a positive control

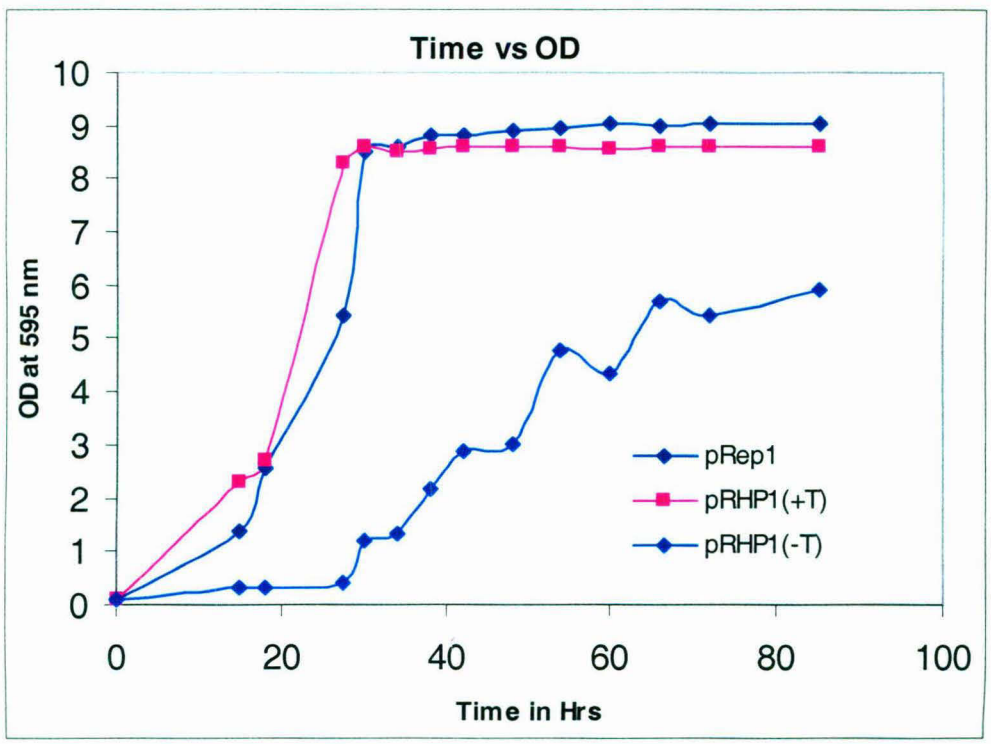


Fig 1:Cell Growth Curves of *S.pombe* cells transformed with the vector pRep1, pRHP1 (bearing the clone of HABP1) both in presence and the absence of thiamine as marked by +T and -T respectively. The pRHP1 transformed cells show growth inhibition when grown in the absence of thiamine.

as elaborated in Section 3.B.vii.d of Material and Methods. A PCR based mutation was done using two primers HMF1 and HMR1 as given below:

HMF1: cacaccgacggagacTaagcttttgttgatttc

HMR1: gtgtggctgcctctgAttcgaaaacaactaaag

The 19th base was changed from A to T creating a STOP codon. The mutant plasmid thus generated was named pRHPΔ1.A schematic diagram of the process is given in the opposite page (Diag. 1). When *S. pombe* cells were transformed with this plasmid and grown on EMM leu⁻ Agar plate, it was seen that the growth inhibition was no longer visible (Fig 2). Next the transcription of the mutant HABP1 gene was examined vis-a-vis the normal one. The total RNA from mid-log phase *S. pombe* cells transformed with pRHP1, pRep1 and pRHPΔ1 were extracted and resolved in a folmaldehyde gel. The resolved RNA was then transferred onto a nylon membrane and probed with HABP1 cDNA labelled with α p^{32}. The result shown in Fig 3a shows a single transcript being produced from cells transformed with pRHPΔ1 and pRHP1 grown without thiamine. But no transcript was seen in cells transformed with pRep1 and pRHP1 grown in presence of thiamine. This confirms the production of the HABP1 transcript in the mutant containing the STOP codon and the normal gene since the transcript was seen to hybridise with the HABP1 cDNA probe. Next the expression of proteins were checked in *S. pombe* cells transformed with pRHP1, pRep1 and pRHPΔ1. This was done by resolving the proteins from the lysates of these cells in a 12.5% SDS-Page, transferring them onto a nitrocellulose membrane and probing the membrane with monoclonal anti-HABP1 antibody. HABP1 was seen to be produced in pRHP1 transformed cells grown in the absence of thiamine (Fig 3b) but not in pRHPΔ1 transformed cells. Thus the introduction of the STOP codon stops the translation of the HABP1 gene while its transcription continues undisturbed. When the growth rates of these

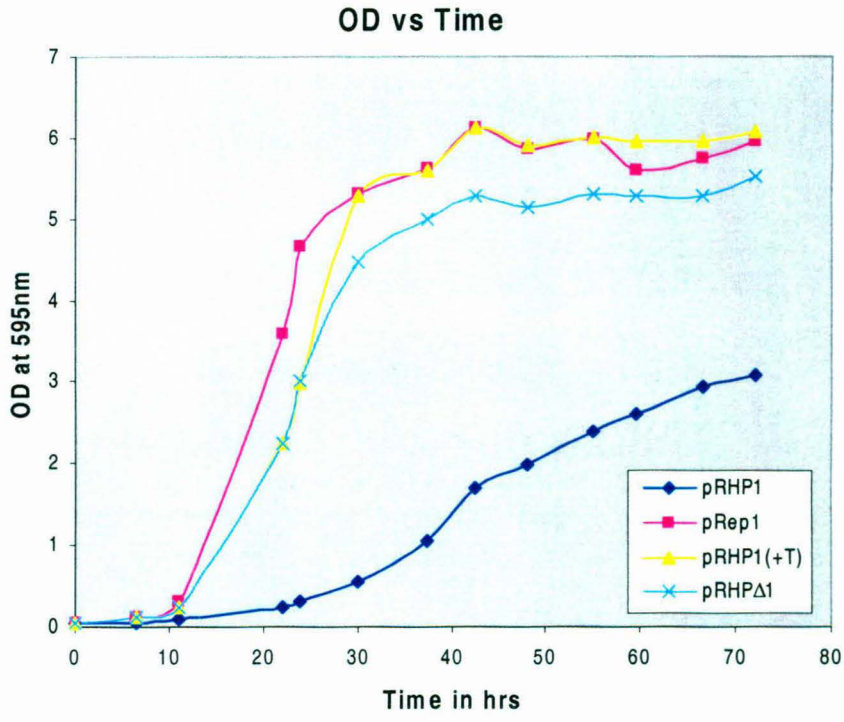


Fig 3c: The cell growth curves of pRep1 ,pRHP1 grown both in presence[pRHP1(+T)] and absence of thiamine [pRHP1] and pRHPΔ1 transformed *S.pombe* cells of the BJ 7468 strain. pRHPΔ1 transformed cells show no growth inhibition.

cells were measured it was seen that pRHPΔ1 transformed cells do not show any growth inhibition as is evident from Fig 3c. Thus the observed growth inhibition in *S. pombe* cells producing HABP1 gets abolished in the absence of the protein even in the presence of the RNA transcript of the gene indicating the HABP1 protein to be responsible for the observed growth inhibition.

4.B.iii MORPHOLOGY OF *S. pombe* (BJ 7468) EXPRESSING HABP1 SHOW CELL ELONGATION MULTI-NUCLEATION AND ABNORMAL CELL-SEPTUM FORMATION

4.B.iii.a PROPIDIUM IODIDE (PI) /CALCOFLUOR DOUBLE STAINING SHOW MULTINUCLEATION AND ABERRANT CELL SEPTUM FORMATION IN *S. pombe* (BJ 7468) CELLS ON HABP1 EXPRESSION

When it was established that the HABP1 protein was responsible for the growth inhibition of *S. pombe* cells transformed with pRHP1, the morphology of these cells were checked to see if the expression of HABP1 causes any visible phenotypic changes. For this purpose, cells expressing HABP1 were observed under the phase contrast light microscope to study morphological changes. It was apparent that the HABP1 expressing cells were much elongated and thicker than the cells transformed with just the vector (pRep1). Thus it was clear that expression of HABP1 led to a substantial change in the morphology of the *S. pombe* cells. To study the morphological change in details, first the nucleus and the cell wall were checked. This task was accomplished by a propidium iodide (PI) and Calcofluor Staining of the *S. pombe* (BJ 7468) cells transformed with pRHP1, pRep1, pRHPΔ1 at different time points. Propidium iodide stains the nucleus and Calcofluor stains the cell wall and the medial cell division septum. The results obtained are shown in the figure 4. It is quite evident that the pRHP1 transformed cells not only get elongated, but also exhibit other abnormalities in the form of multiple cell division septa and multinucleation throughout the observed period

Fig 4: Shows the Propidium Iodide/Calcofluor Staining of the nucleus and cell septum of pRHP1, pRep1 and pRHPΔ1 transformed *S. pombe* cells at different time points. The pRHP1 transformed cells shown in the panel to the right show elongation, multinucleation and abnormal cell septum formation all the time points as compared to the pRep1 and pRHPΔ1 transformed cells. pRHPΔ1 transformed cells show normal morphology just like the pRep1 transformed cells.

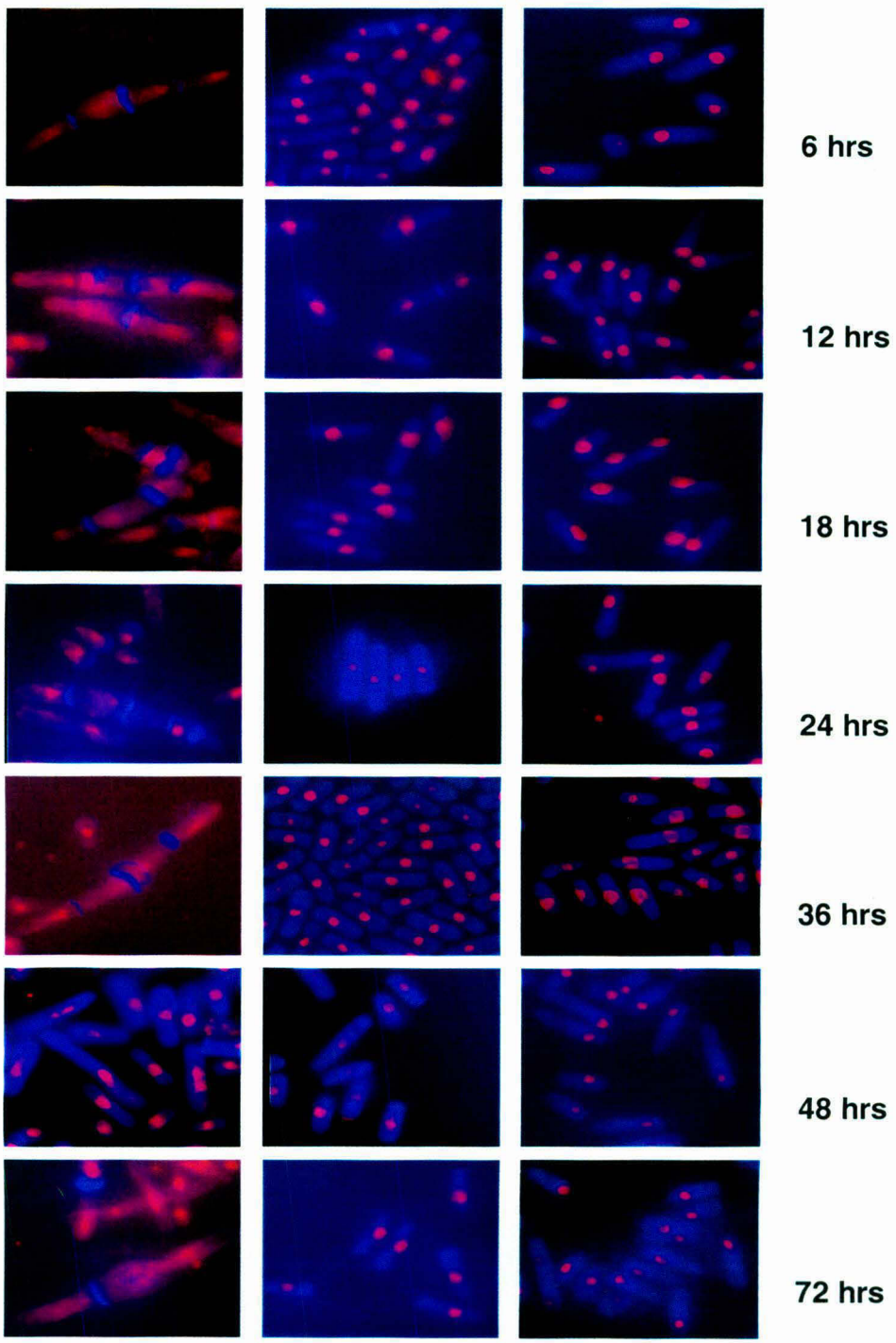

6 hrs
12 hrs
18 hrs
24 hrs
36 hrs
48 hrs
72 hrs
pRHP1
pREP1
pRHPΔ1

Fig 4

Fig 5: Staining of Actin polymers in pRHP1, pRep1 and pRHPΔ1 transformed *S. pombe* cells at different time points. Actin polymers have been stained with Rhodamine-Phalloidin, the nuclei have been stained with DAPI and the cell wall and cell septum have been stained with Calcofluor. No difference is observed either in localization and level of expression of Actin polymers on expression of HABP1 compared to the vector transformed cells. However elongation, multinucleation and abnormal cell septum are observed in pRHP1 transformed cells.

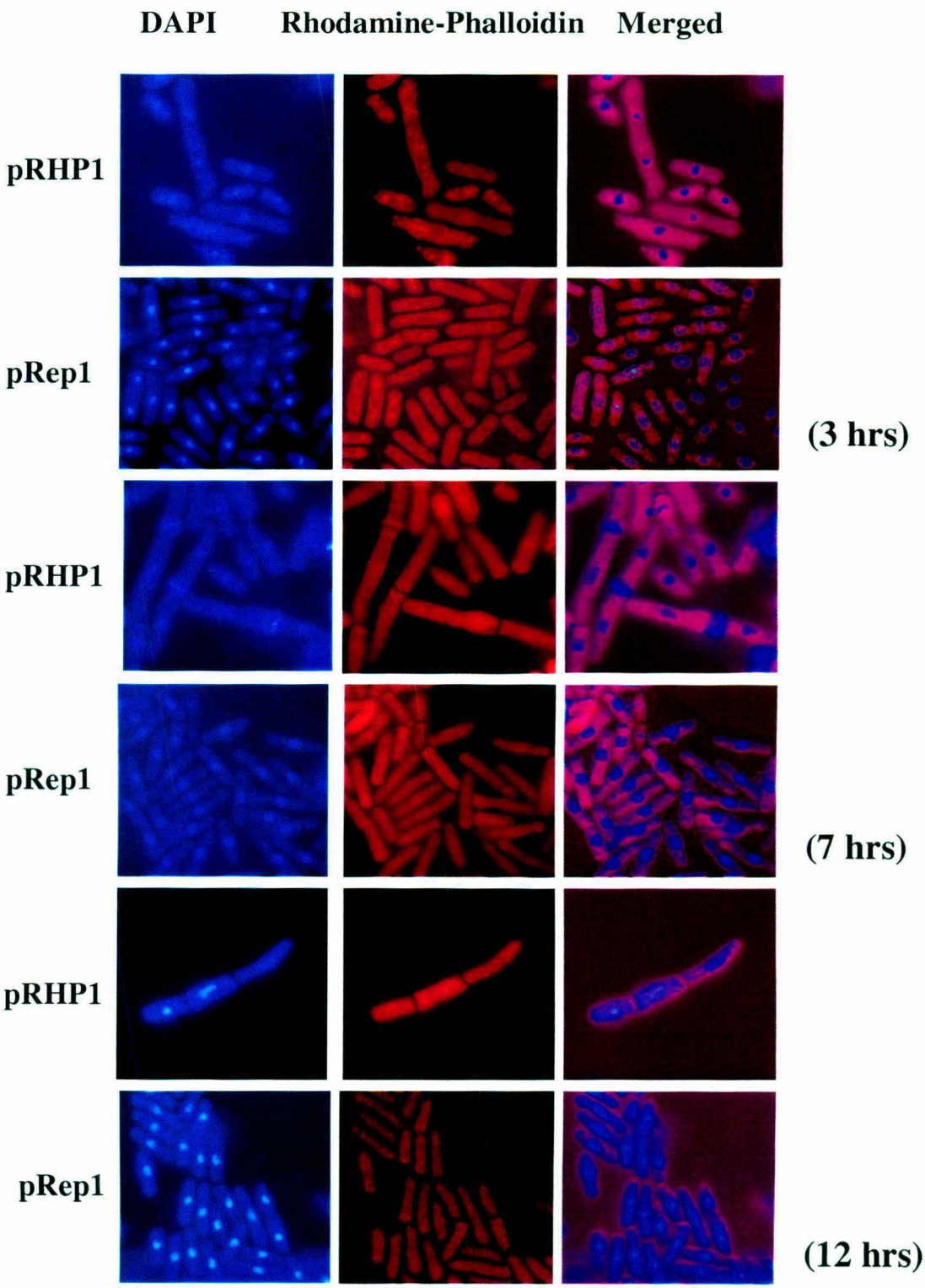

DAPI
Rhodamine-Phalloidin
Merged
pRHP1
pRep1
(3 hrs)
pRHP1
pRep1
(7 hrs)
pRHP1
pRep1
(12 hrs)

Fig 5

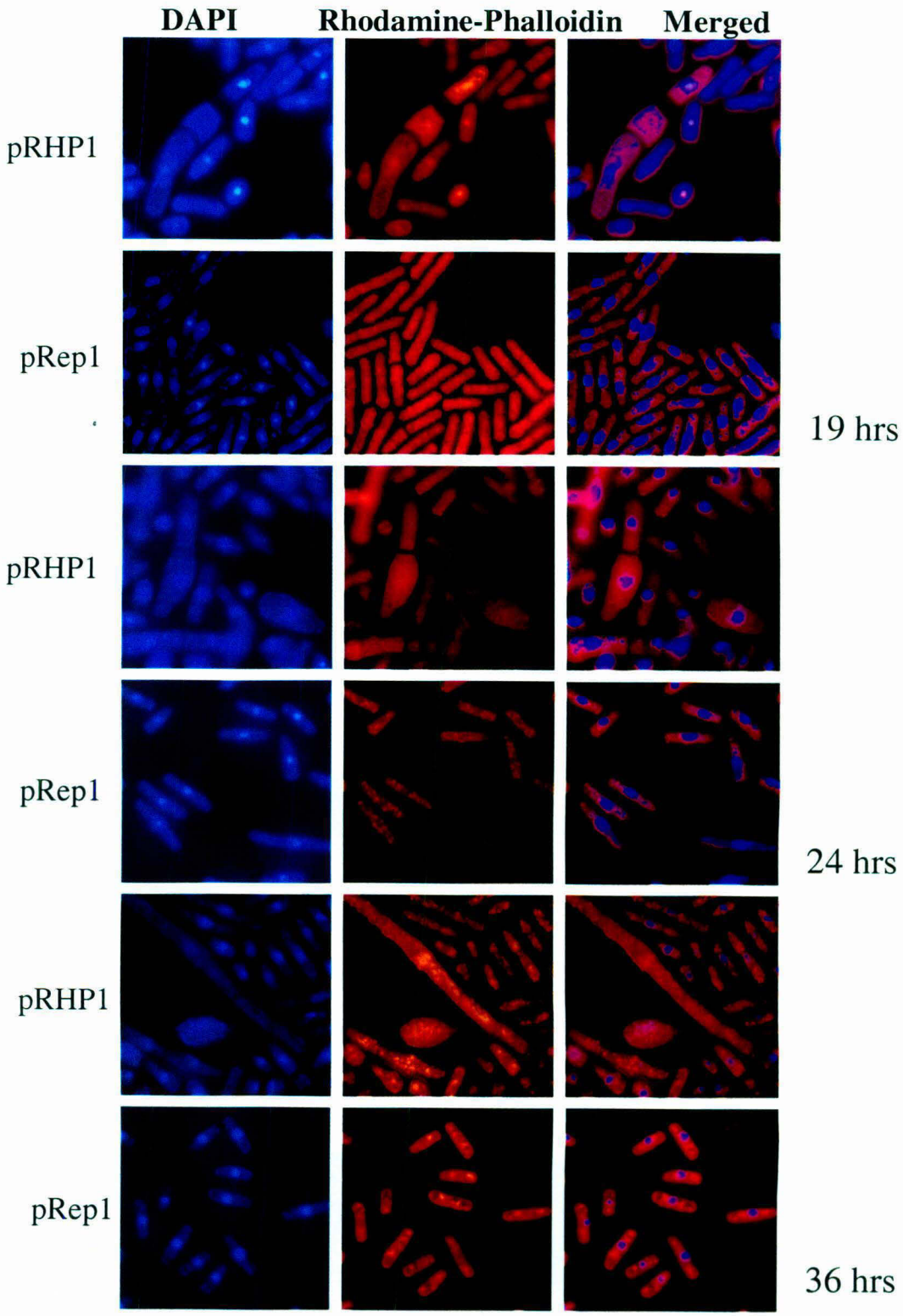

DAPI
Rhodamine-Phalloidin
Merged
pRHP1
pRep1
19 hrs
pRHP1
pRep1
24 hrs
pRHP1
pRep1
36 hrs

Fig 5
(Contd.)

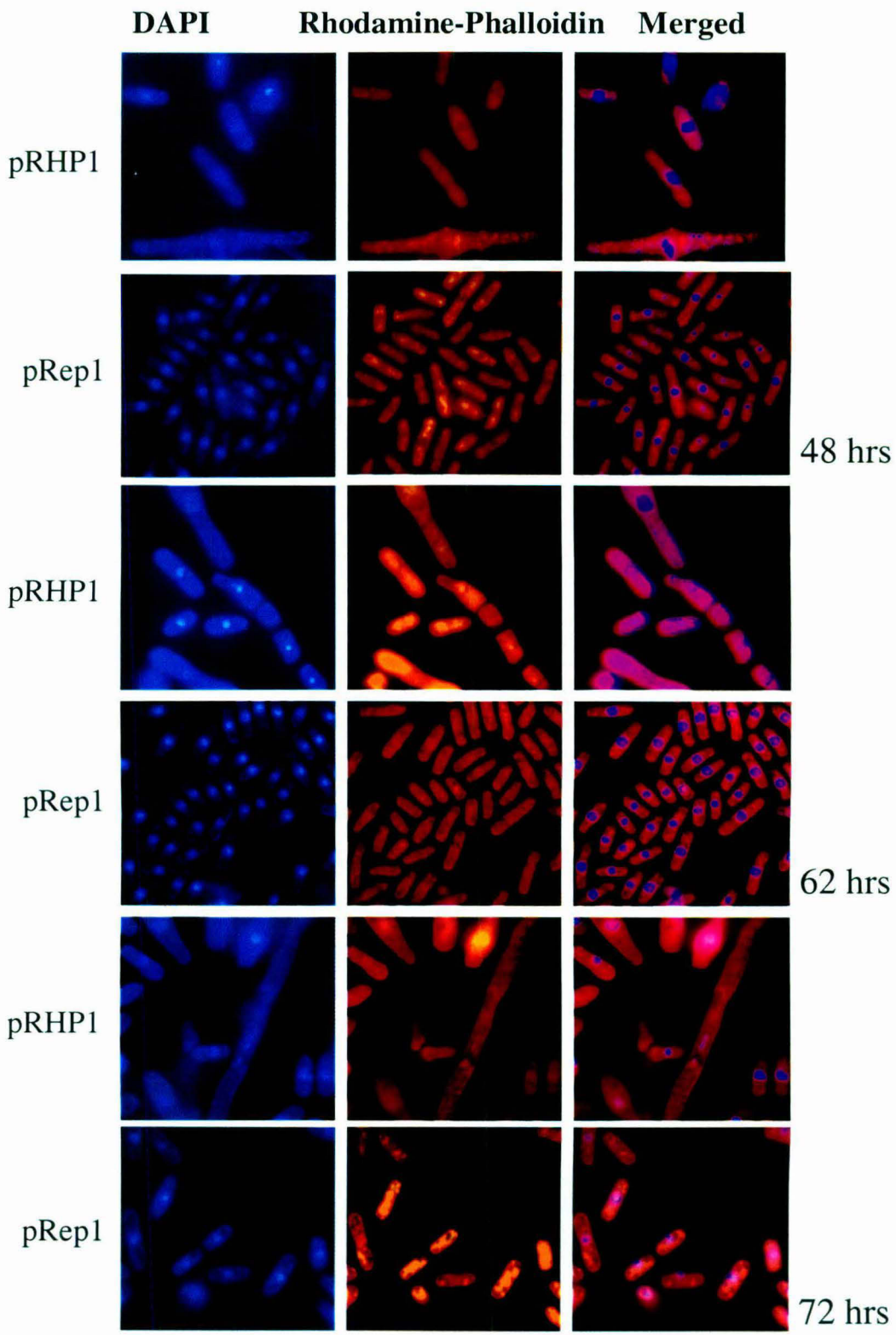

DAPI
Rhodamine-Phalloidin
Merged
pRHP1
pRep1
48 hrs
pRHP1
pRep1
62 hrs
pRHP1
pRep1
72 hrs

Fig 5 (Contd.)

Fig 6: Localization of HABP1 in pRHP1 transformed cells at different time points by indirect immuno-fluorescence. HABP1 is seen to translocate from the cytoplasm to the nucleus when the cells shift from the lag phase to the log phase. The occurrence of HABP1 in the nucleus is clearly visible at the 24 and 48 hours time points.

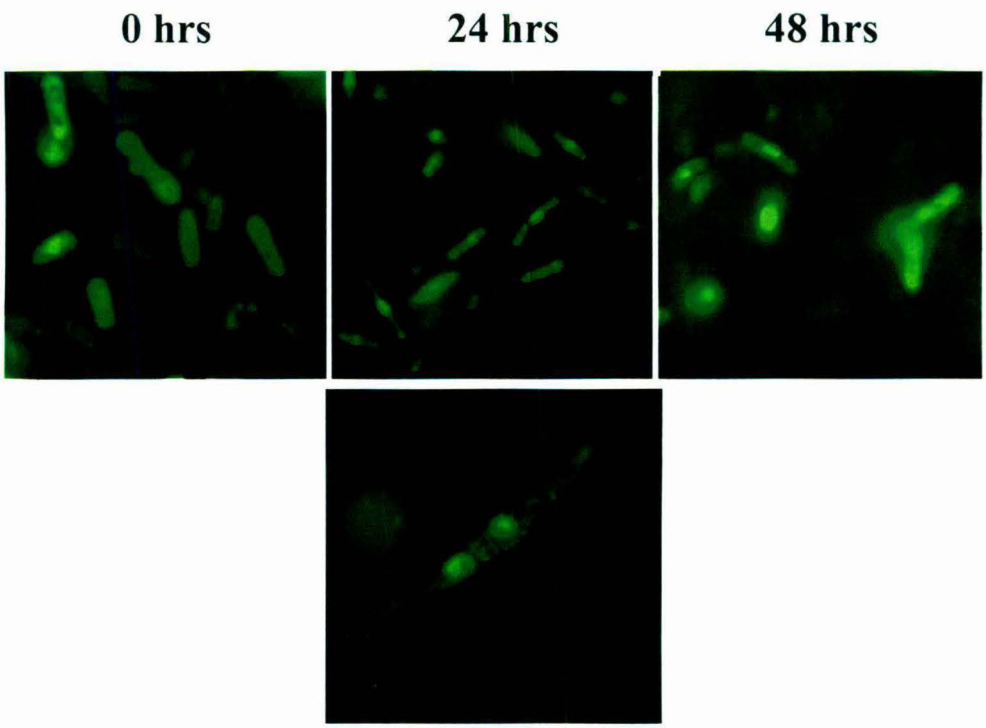


Fig 6.

from 6 to 72 hours. However the pRHPΔ1 transformed cells displayed a normal morphology just like pRep1 transformed cells. Here, it must be stated that the formation of multiple septa and multinucleation are indicative of an abnormality in cell division/cytokinesis. So this observation suggests that the expression of HABP1 causes some problem in the smooth functioning of the cell-growth processes specially in the cell division/cytokinesis phase.

4.B.iii.b RHODAMINE-PHALLOIDIN/DAPI/CALCOFLUOR STAIN OF ACTIN POLYMERS SHOWS NO CHANGE IN ACTIN POLYMERIZATION ON HABP1 EXPRESSION

Since septum formation, a major step in cytokinesis, gets disrupted here, it was thought that the level of expression and distribution of actin polymers, intimately connected to this phenomenon, should be checked. This was done by staining the actin polymers in the cells with Rhodamine conjugated to phalloidin. Transformed *S. pombe* (BJ 7468) cells were grown in EMM *leu*- medium, samples taken at different time points, fixed with para-formaldehyde and stained with Rhodamine-Phalloidin, DAPI and Calcofluor following the procedure described in Materials and Methods. The results are shown in Fig 5. No changes were observed in the localization and level of expression of actin polymers in *S. pombe* cells on expression of HABP1 indicating that the observed abnormalities do not involve actin polymers.

4.B.iii.c HABP1 LOCALIZES IN THE NUCLEUS OF THE ACTIVELY GROWING CELLS

Indirect immuno-fluorescence study of the cells transformed with pRep1 and pRHP1 was done at the time intervals of 0 h, 24 h and 48 h respectively, following the procedure described in Materials and Methods. The cells were fixed with para-formaldehyde and probed with monoclonal antibody against HABP1 which was detected FITC labeled secondary antibody. The pRep1 transformed cells did not display any fluorescence.

Fig 7a: FACS analysis of pRHP1 and pRep1 transformed *S.pombe* cells at different time points. The shifting of a substantial fraction of the cells to higher DNA content with accumulation of HABP1 with time is clearly visible. This has also been observed microscopically.

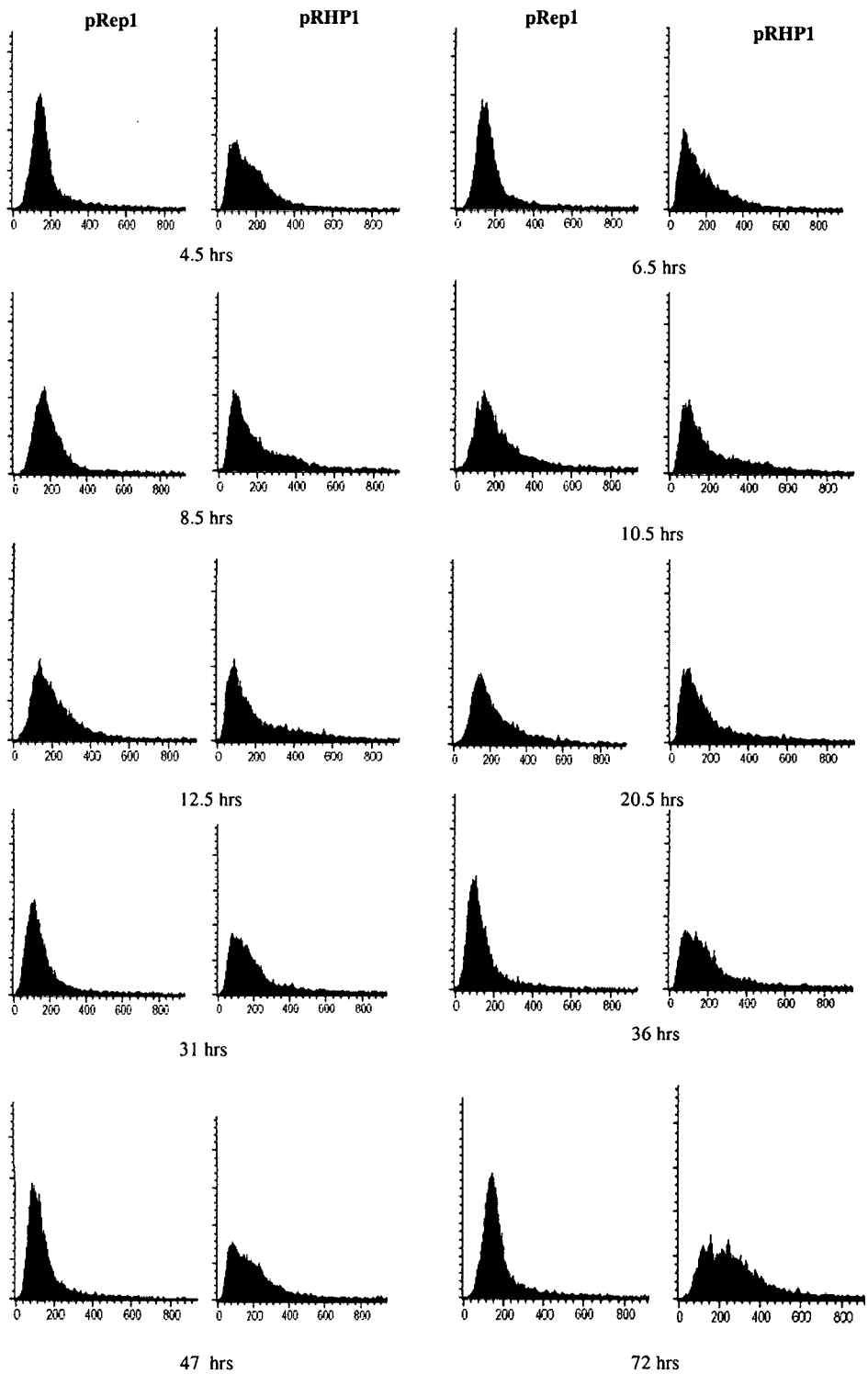

pRep1
pRHP1
pRep1
pRHP1
4.5 hrs
6.5 hrs
8.5 hrs
10.5 hrs
12.5 hrs
20.5 hrs
31 hrs
36 hrs
47 hrs
72 hrs

Fig 7a

Fig 7b: Forward and side scatter diagrams of pRHP1 and pRep1 transformed *S. pombe* cells at different time points from the FACS analysis. The X-Axis represents the length of the cells while the Y-axis represents the granularity of the cells. pRHP1 transformed cells are seen to be larger in size and have a higher granularity compared to the pRep1 transformed cells.

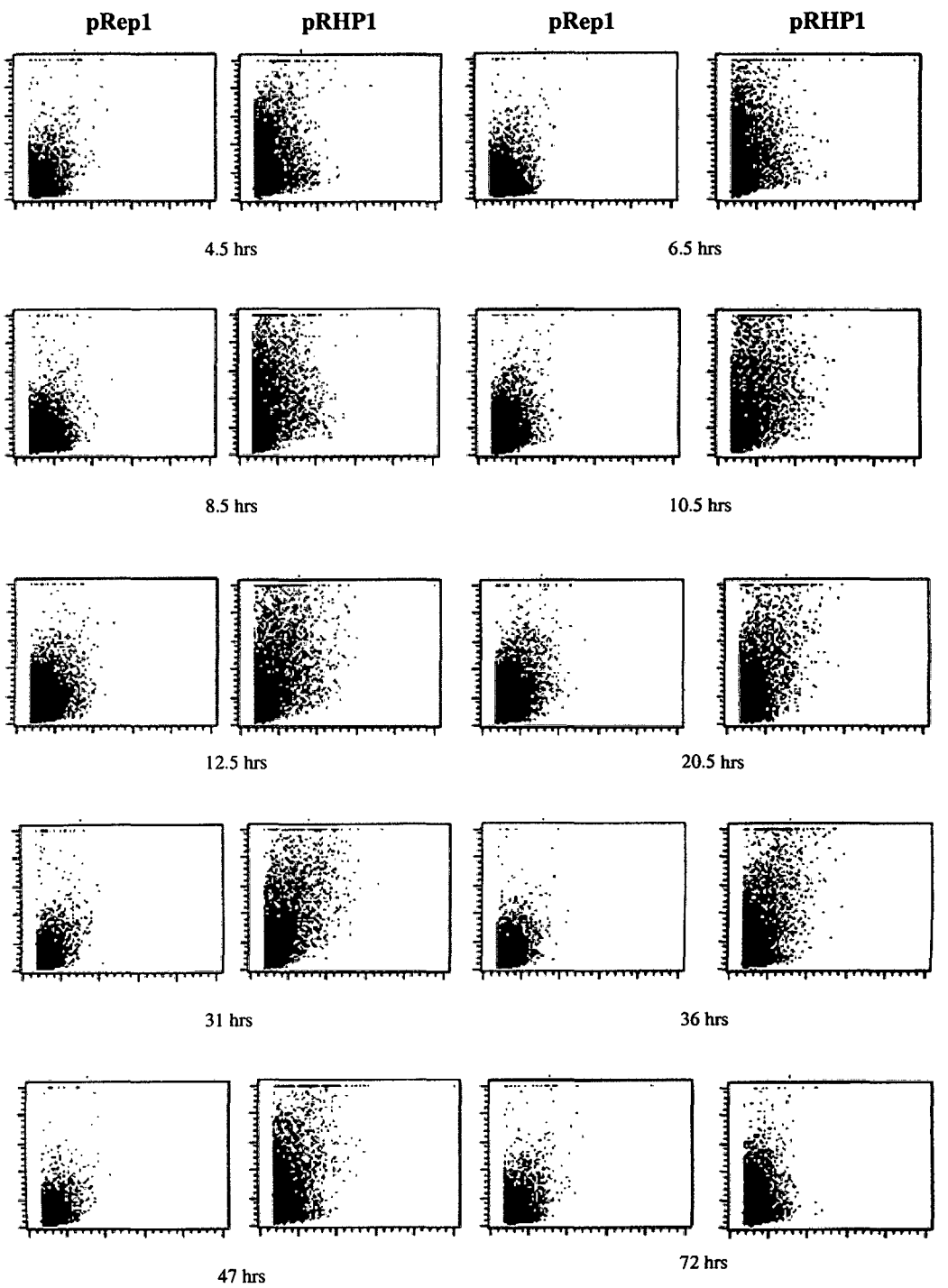
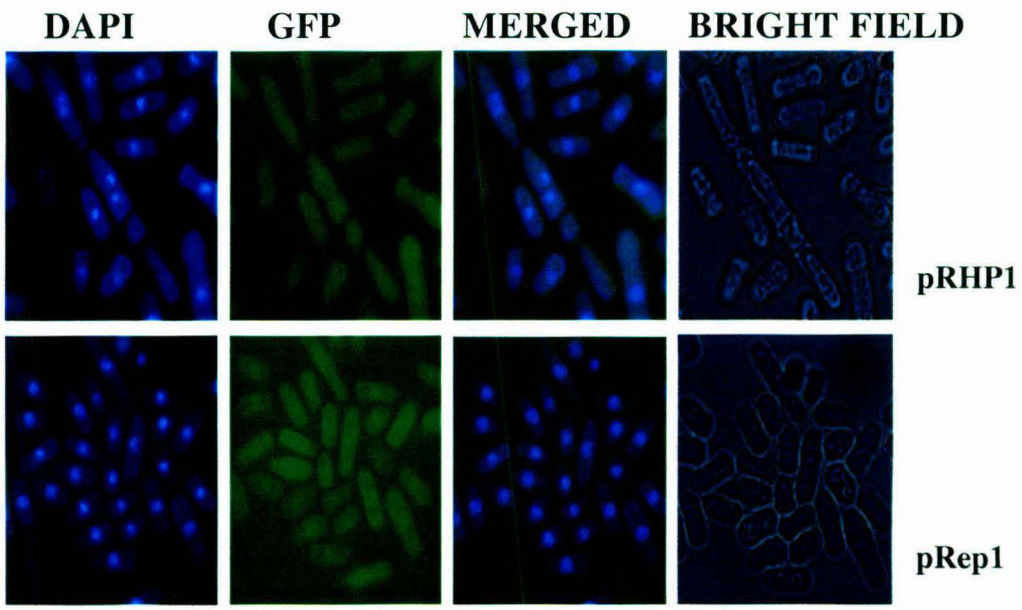
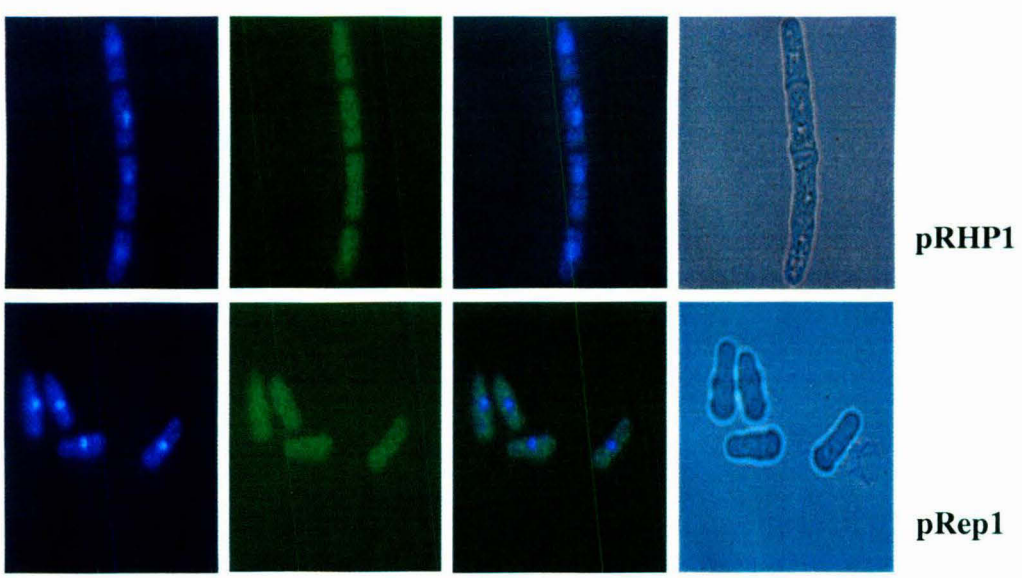

pRep1
pRHP1
pRep1
pRHP1
4.5 hrs
6.5 hrs
8.5 hrs
10.5 hrs
12.5 hrs
20.5 hrs
31 hrs
36 hrs
47 hrs
72 hrs

Fig 7b

Fig 8: Live cell staining of *S. pombe* MBY 624 strain transformed with pRHP1 and pRep1 at different time points. The regulatory light chain (rlc) of myosin has been tagged with GFP in this strain and the nucleus has been stained with DAPI. The pRHP1 transformed cells display elongation, multinucleation and abnormal cell septum formation. The cell septa in pRHP1 transformed cells do not show the green fluorescence indicating it's the primary septum. This indicates that this is a cell separation defect.

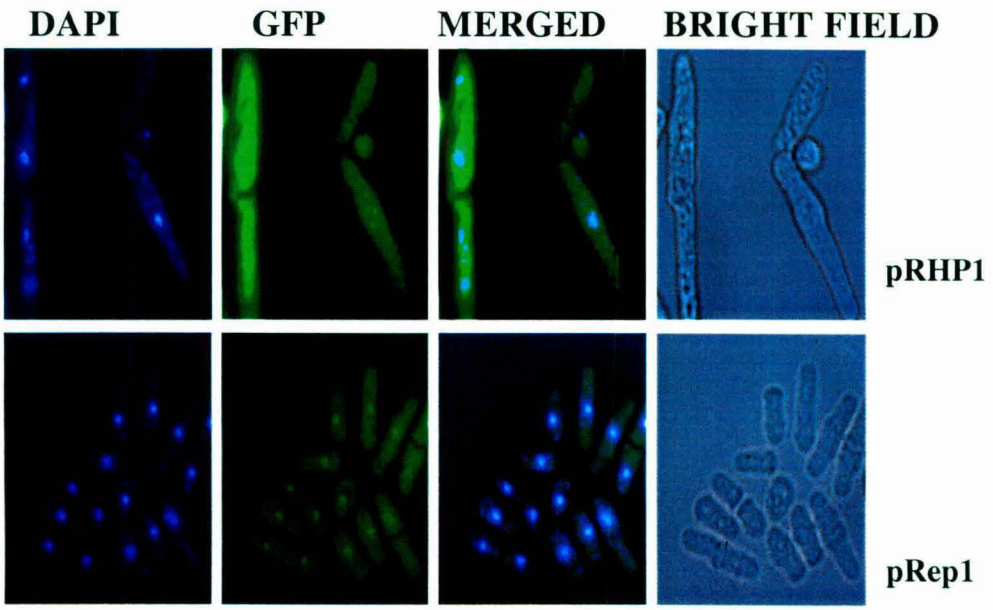


0 hrs

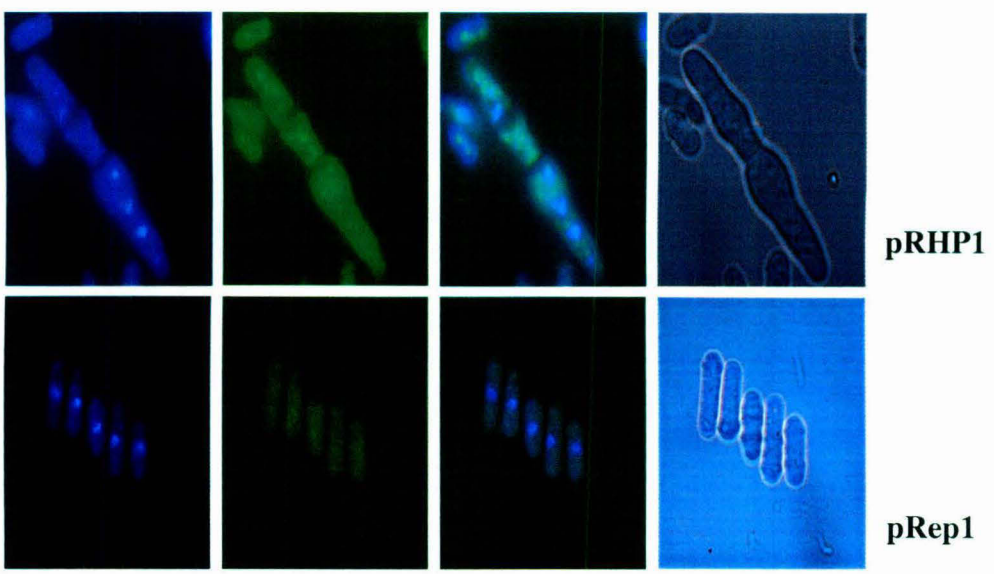


6 hrs

Fig 8

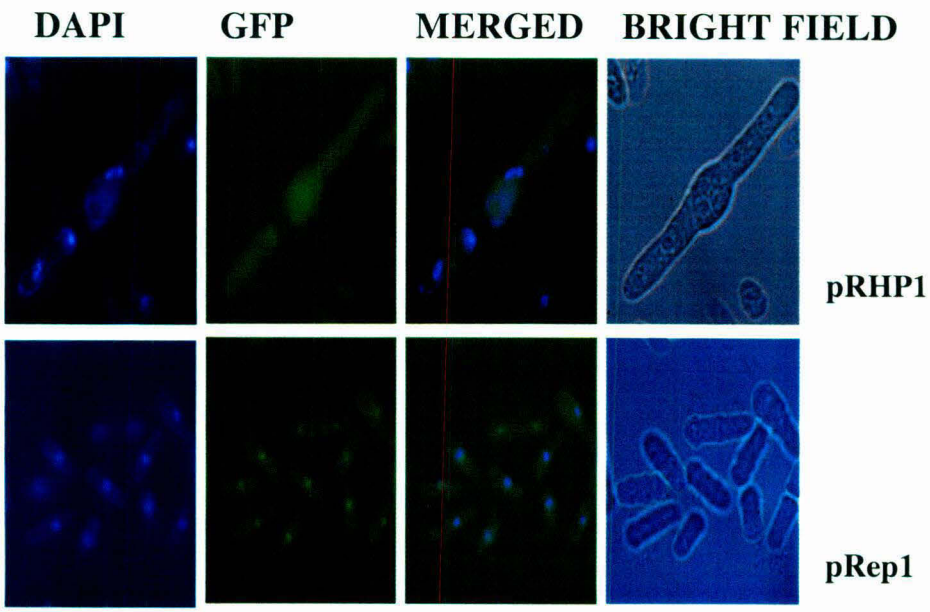


12 hrs



18hrs

Fig. 8(Contd.)


DAPI
GFP
MERGED
BRIGHT FIELD
pRHP1
pRep1
24 hrs

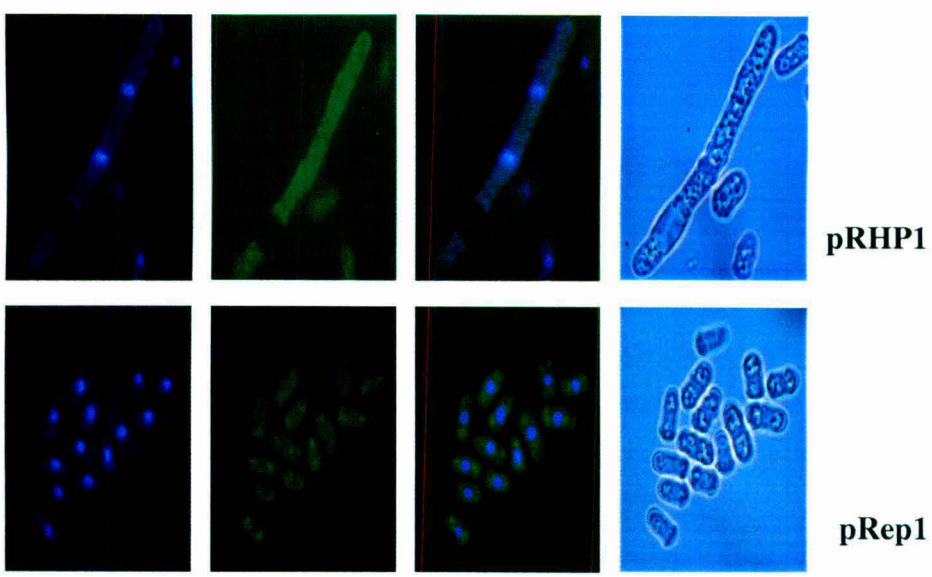
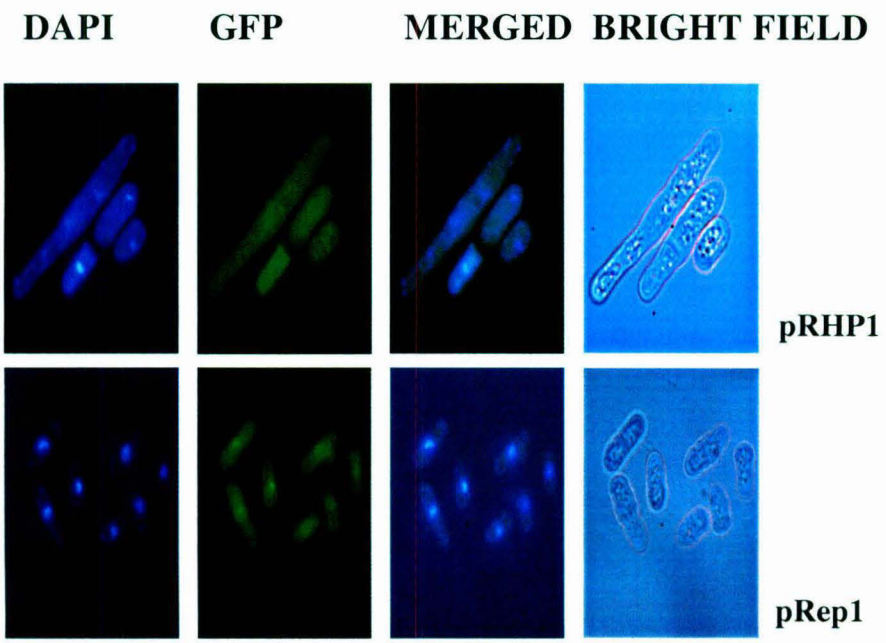
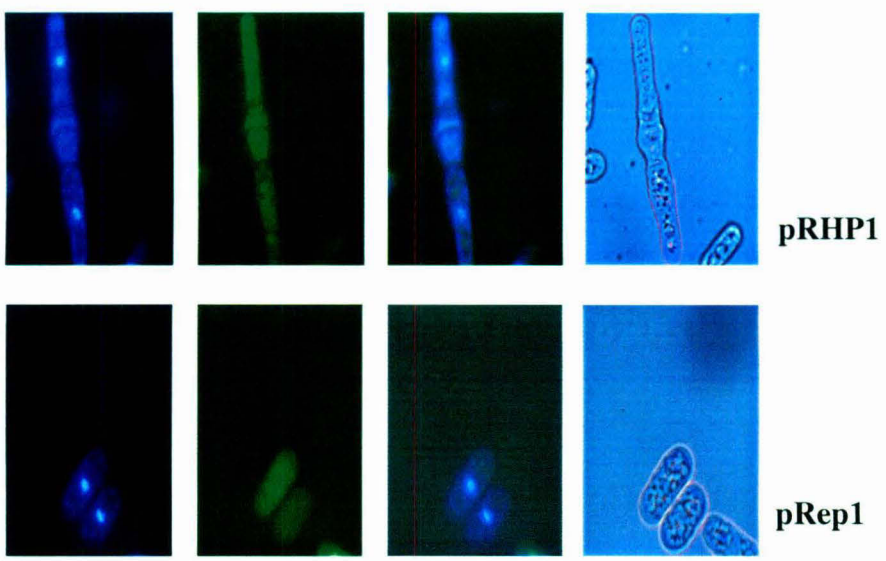

pRHP1
pRep1
36 hrs

Fig 8(Contd.)



47hrs



72 hrs

Fig 8(contd)

The images of pRHP1 transformed cells (Fig 6) clearly reveal that HABP1 translocates to the nucleus at 24 h, but at 0 h, it is seen to be more or less uniformly distributed throughout the cytoplasm of the cell. Though the exact reason for this is not clear, it may be assumed that upon expression, HABP1 interacts with *S. pombe* cellular protein/s, which modulates its nuclear translocation.

4.B.iv FACS ANALYSIS OF SCHIZOSACCHAROMYCES POMBE (BJ 7468) CELLS

S. pombe cells transformed with pRep1, pRHP1 and pRHPΔ1 were grown in EMM leu$^-$ Medium, samples taken at different time points and processed for FACS analysis as described in Materials and Methods. The results do not show any clear evidence of the expression of HABP 1 displaying a cell cycle arrest. This maybe due to the fact that the cells processed for FACS were unsynchronized. However it is observed that on expression of HABP1, the proportion of cells with higher DNA content increases (Fig 7a). This has also been observed microscopically. The forward and side scatter diagrams show that the HABP1 producing cells are larger and possesses a higher granularity (Fig. 7b) than normal cells.

4.B.v.a STAINING OF LIVE CELLS OF SCHIZOSACCHAROMYCES POMBE STRAIN MBY 624

It has often been observed that when cells are fixed either with ethanol or with formaldehyde, there is always a chance that the observed morphology might be partially artifactual in nature. So to overrule such possibilities, live cell staining was done. The strain of *S. pombe* used for this study was MBY 624. In this strain, the regulatory light chain (RLC) of myosin is tagged with GFP. So, the cell membrane and the medial actomyosin ring would be clearly visible when observed through the appropriate filter set for GFP. The cells transformed with pRHP1 and pRep1 were grown in EMM leu$^-$ as described in Materials and Methods, samples taken at different time points and processed accordingly. In this

Fig 9: Formation of abnormal cell septum in pRHP1 transformed *S. pombe* MBY 624 cells at different time points. Aniline Blue specifically stains the septum and DAPI has been used to stain the nucleus.

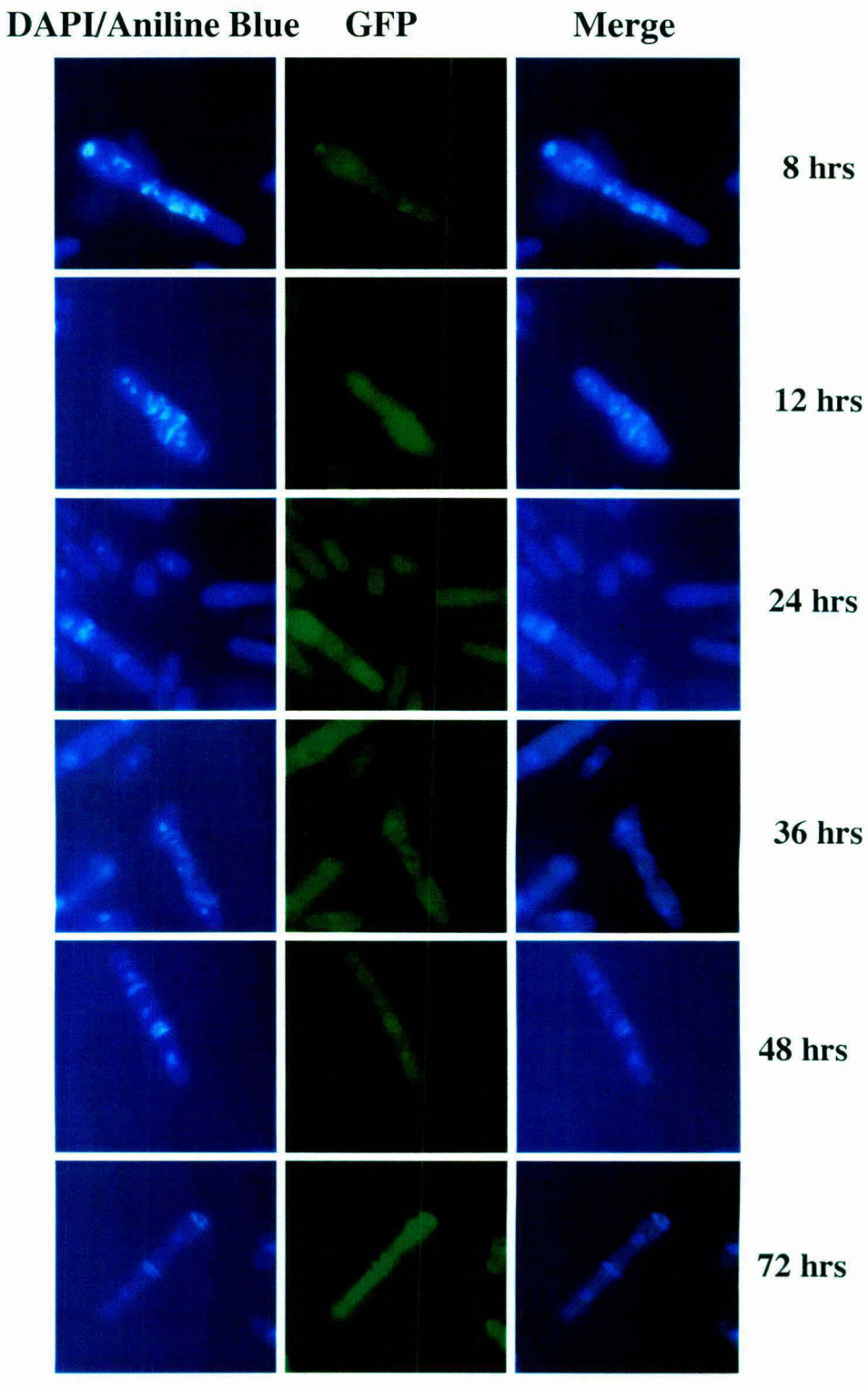

DAPI/Aniline Blue
GFP
Merge
8 hrs
12 hrs
24 hrs
36 hrs
48 hrs
72 hrs

Fig 9

study, the nucleus was stained with DAPI. As seen in figure 8, the observed morphological changes were as seen earlier. The fact that there is a cell separation defect seen is very clearly here. An interesting observation was that the green fluorescence of RLC-GFP was not visible in the abnormal septal region. This implies that this septal region consists of the primary septum, which has remained uncleaved, causing the cells to stay attached with each other.

4.B.v.b DAPI/ANILINE BLUE STAINING OF pRHP1 TRANSFORMED *S. pombe* MBY 624 CELLS SHOW ABNORMAL CELL SEPTUM FORMATION

pRHP1 transformed cells were grown and their cell growth rates measured. At different time points 10^7 cells were taken out and fixed with ethanol. Later, when processed, the cells are rehydrated and stained with DAPI and Aniline blue to visualize the nucleus and the cell septum. The results as seen in figure 9, demonstrate that the MBY 624 cells transformed with pRHP1 show the presence of multiple abnormal cell septum.

4.B.vi ULTRA-MICROSCOPY STUDY OF *S. pombe* MBY 624 CELLS SHOW MORPHOLOGICAL ABNORMALITIES ALONG WITH A CHANGE IN THE NUMBER AND LOCALIZATION OF VACUOLES

S. pombe cells transformed with pRHP1 and pRep1 plasmids were grown in EMM leu- Medium and an appropriate volume taken after 6 h, 12 h, 24 h 36 h and 48 h respectively and processed for ultramicroscopy as described in Materials and Methods. Then they were observed under an electron microscope. From the results obtained (Fig 10), it is very clear that the cells display severe abnormalities, as observed earlier. The formation of multiple septa is clearly seen in *S. pombe* cells producing HABP1 at all the time points. Compared to the cells transformed with the vector pRep1 they seem much longer and seem to have a larger number of vacuoles, whose distribution also seems to be different. This confirms

Fig 10: Shows the ultrastructural analysis of pRep1 and pRHP1 transformed *S. pombe* cells at different time points. In the panel to the right the observed morphological abnormalities of multiple cell septum increase in vacuoles and elongation in pRHP1 transformed *S. pombe* cells are clearly visible vis-à-vis the pRep1 transformed cells.

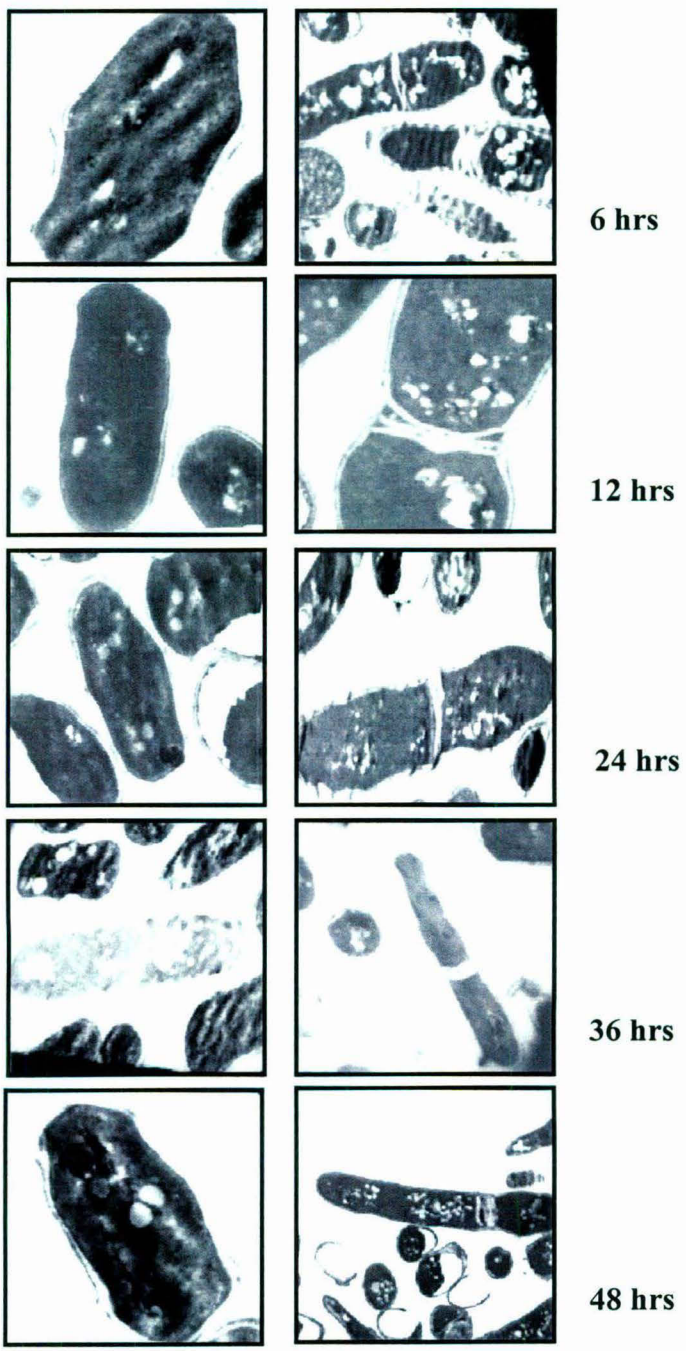


Fig 10

our earlier observations of the morphological abnormalities in pRHP1 transformed *S. pombe* cells in the fluorescence microscope.

4.C DISCUSSION

The expression of HABP1 in *S. pombe* cells of both the BJ7468 and MBY624 strains results in growth inhibition, elongation, multinucleation and multiple cell septa formation. These phenotypic changes were confirmed to be the consequence of HABP1 expression since just the absence of the protein abolished all of them even in the presence of the RNA transcript of the HABP1 gene. The appearance of the morphological abnormalities along with growth inhibition in *S. pombe* producing HABP1 is indicative of defective progression of its cell cycle and cytokinesis. The FACS Analysis too confirms the microscopically observed increase in DNA content of the fission yeast cells in the form of multi-nucleation. The rationale of *S. pombe* cells to show growth inhibition may be due to a variety of causes. It has been observed that the expression of pro-apoptotic proteins like Bax produced a significant inhibition on the cell growth of *S. pombe* and the cells were seen to die. This phenotype was rescued when the anti-apoptotic protein Bcl-XL was co-expressed in them (Jurgensmeier *et al.,* 1997). Interestingly it must be pointed out here that the expression of Bax increases with over-expression of HABP1 in mammalian fibroblast cell line F111 (Meenakshi *et al.,* 2003). The fission yeast cells exhibit growth inhibition when the tumor suppressor protein p53 was expressed in it (Bischoff *et al.,* 1992). p53 has also been reported to interact with CDC 25 and overexpression of CDC 25 in *S. pombe* was seen to over-come this inhibition (Rief *et al.,* 2000). These reports indicate that HABP1 may be interacting with proteins crucial for proper growth and cell cycle control of *S. pombe* cells.

S. pombe just like a typical eukaryote has a cell cycle with discrete G_1, S, G_2 and M phases. In minimal or complex medium its doubling time varies from 2 to 4 hours with the G_2 phase occupying more than 70% of this time. Under normal growth conditions, *S. pombe* cells are

haploid and they reproduce asexually by septation and medial fission. A newly divided cell in the G_1 phase actively synthesizes proteins it would require later for cell cycle progression. In most eukaryotic cells including the fission yeast, co-ordination of the different phases and events of the cell cycle occurs by means of 'checkpoints'. These can be called events where the progress of the cell is momentarily suspended while certain 'monitoring devices' within the cell examine whether the current conditions are suitable for proceeding to the next stage. Only when the conditions under scrutiny are deemed to be satisfactory, a green signal is given and the cell proceeds to the next stage. The cell mainly passes through 3 such major checkpoints throughout one complete cycle. The first checkpoint is the G1 checkpoint. Here the cell monitors the external environment (sensing the nutritional conditions, growth factor, pheromones etc) along with its own size before committing itself to a round of DNA synthesis. This checkpoint is also known as 'START'. After the G1 →S boundary approaches the S phase the DNA replication begins to take place. After the S phase is over, the cells have completed one round of DNA replication and they enter into G2 phase where the cells grow in size and prepare for the mitotic phase (M phase). Here the cell division septum forms and a fission yeast cell divides into two cells (Lodish *et al.,* 2000).

In *S. pombe* cells expressing HABP1 there seems to be no problems upto S phase as DNA replication seems to be happening normally though there are a few cells with a larger nuclei but these are too few in number. At the end of the G2 phase is the G2 – checkpoint. Here the cell monitors two things, namely completion of DNA replication and the cell reaching a necessary critical size. The cell does this by controlling the activation of cdc2 in MPF (Mitosis/Maturation Promoting Factor) which in *S. pombe* consists mainly of the cdc2-cdc13 heterodimer (Lodish *et al.,* 2000).

The entry of cells into the mitotic phase is controlled MPF. Identification of the genes whose products constituted the MPF, came generally from the analysis of temperature sensitive mutants of *S. pombe* cells. The morphology of these temperature sensitive mutants when grown in non-permissive temperature divided them into two classes – one formed extremely long cells which continued to grow in length but failed to divide and were called cdc mutants, while the other, known as wee mutants gave rise to smaller-than-normal cells and these were defective in proteins which stopped a cell from premature division (Lodish *et al.*, 2000).

As the genes responsible for these aberrant morphologies were identified, it was seen that the absence of cdc2 prevents the cell from entering into mitosis while an excess of it brings it on prematurely. These findings identified cdc2 as a key regulator of the M phase. It was seen that cdc2 was a 34 kDa protein having functional homologues in higher eukaryotes including humans. In fact the human homologue of cdc2 has 63% identity with the fission yeast protein. This along with the fact that the human homology of cdc2 can rescue the cdc phenotype in fission yeast showed that this gene is evolutionarily conserved and functionally similar. This further implies that the mechanism of controlling the onset of mitosis is generally the alike across the species (Giga-Hama and Kumagai, 1999).

After cdc2 was discovered and its function accepted, a new gene cdc13 was discovered and sequenced bringing forth two facts. Firstly it had substantial homology with sea urchin and *Xenopus laevis* cyclin B. Secondly it was also observed that cdc2 and cdc13 were the two components of the MPF. (Lodish *et al.*, 2000)

The inability of a *S. pombe* cell to enter mitosis is not only due to the absence of cdc2. There are other genes whose absence also caused

the same effect and influenced the activity of the cdc2-cdc13 heterodimer. Cdc25 is one gene whose absence prevents a S. pombe cell from entering mitosis. Incidentally there is another gene Wee1 whose absence is seen to cause the opposite effect that is a premature entry of the cell into the mitotic phase. Further investigations brought forward a clearer picture. It was seen that the protein kinase cdc2 was active as a kinase only when bound to the cyclin cdc13. Phosphorylation of threonine 161 activates the MPF while that of tyrosine 15 inactivates it. These regulatory phosphorylations events of cdc2 occur only when it is bound to cdc13. Wee1 is the protein kinase that phosphorylates the inhibitory tyrosine 15 residue while another protein kinase known as cdc2-activating kinase (CAK) phosphorylates the activating threonine 161 residue. However, even after these events have taken place the MPF is not activated. Finally cdc25 that has phosphatase activity removes the phosphorylation from the tyrosine 15 residue and this activates the MPF (Lodish *et al.*, 2000).

So this overview of the cell cycle regulation of *S. pombe* helps us in understanding the perspective of the aberrant morphology exhibited by the *S. pombe* cells expressing HABP1.These HABP1 expressing *S. pombe* cells show elongation and multiple nucleation. This could indicate either malfunctioning of the cdc2-cdc13 heterodimer, specially the cdc 2 part since cdc 2 $^-$ cells showed abnormal elongation and this phenotype came to be called the cdc phenotype due to this reason. Now the activation of this heterodimer is controlled in part by the CDC 25, a phosphatase as already mentioned earlier. In the absence of CDC 25 too the cells are observed to display the cdc phenotype and they also exhibited a cell cycle arrest at the G_2/ M boundary.(Daga and Jimenez., 1999). The observed phenotype of elongated cells with multiple nuclei thus could suggest a possible interaction of HABP1 either with CDC 2 or CDC 25 or both of them. However some cells seem to be crossing this boundary by some

alternative pathways and going into the M phase since they seem to undergo multiple rounds of cytokinesis as evident from the multiple septa.

Apart from growth inhibition, cells expressing HABP1 exhibits two types of abnormal septa. In one the positioning of the septa is abnormal, there being multiple septa one beside the other, and the other seems to be a cell separation defect where the primary septum has not dissolved after the end of mitosis. Abnormal septa stacked beside one another is observed seen in fig 9 and in the EM study (Fig. 10) of the cells producing HABP1. The occurrence of cell separation defects is also clearly seen in the live cell staining of MBY 624 strain of *S. pombe*. To understand the possible cause of this phenomenon, the detailed process of events in cytokinesis need to be considered. It is known that the actomyosin ring formation requires many proteins including products of $Cdc3^+$, $Cdc4^+$, $Cdc8^+$, $rng2^+$, $rng3^+$ and $myo2^+$ genes. F-actin patches are recruited to the medial ring where the actomyosin contractile ring will be formed. Coordination of contraction of this ring and the nuclear cycle requires interaction amongst a large number of proteins that are collectively "Septation Initiation Network" or SIN in short. The proteins involved in SIN are also involved in the formation of the primary septum as the actomyosin ring contracts. Genetic studies indicate that the activation of the SIN pathway might regulate cps1p, a β-1,3-glucan synthase subunit essential for the assembly of the division septa (Le Goff *et al.*, 1999; Liu *et al.*, 2000). But since no incomplete or arrested actomyosin rings were observed, the proteins of the pathways involved in the formation of the actomyosin ring seems to be functioning normally. Our observation of abnormal septa being stacked one beside another may be attributed to the proteins responsible for proper positioning of the actomyosin ring that determines the position of the septa and its normal functioning. Recent reports indicate that the products of the

mid1$^+$, plo1$^+$ and pom1$^+$ genes are needed for accurate positioning of the actomyosin ring. So the functioning of these genes needs to be checked to pinpoint the protein responsible for the faulty positioning of the septa in this case (Martin-Cuadrado *et al.,* 2003). However more investigations are required to elucidate which of these proteins interact with HABP1 to produce this abnormality.

The other abnormality observed is that the cells are not separating into two daughter cells after the end of cytokinesis as especially seen in the live sell staining. This is a cell separation defect. Cell separation entails the dissolution of the primary septum, which happens by autolytic degradation after the completion of mitosis and is accompanied by local erosion of the adjacent regions of the cell wall. Though much is known about the mechanisms of actomyosin ring assembly, constriction and formation of the division septa, very little is known about how the cleavage of the cell wall and primary septum is achieved. Some proteins implicated in having a role in cell-separation have been discussed below but their involvement in causing this problem in *S. pombe* cells needs to be pinpointed.

Since not much is known about proteins involved in the actual separation of *S. pombe* cells after cytokinesis, mutants showing partial or complete defects in cell separation have been isolated and classified in 16 different groups, namely sep1$^+$ to sep16$^+$ (Grallert *et al.,* 1999; Sipiczki *et al.,* 1993). Sep1$^+$ encodes a transcription factor highly homologous to the HNF-3/forkhead family present in higher eukaryotic cells and also in other microorganisms (Ribar *et al.,* 1997). There have been other proteins (Fkh1p and Fkh2p) in *S. cerevisiae* too which have been implicated in cell separation (Hollenhorst *et al.,* 2000). sep15$^+$ has recently been cloned and characterized and found to encode for an essential protein showing a high degree of similarity to Med8p, one of the subunits of the mediator complex of *S. cerevisiae* RNA polymerase II (Zilahi *et al.,* 2000).

A recent work (Wang *et al.,* 2000) shows the involvement of the exocyst complex in cell separation. It consists of an octameric protein complex present in many organisms and is involved in tethering vesicles to specific sites on the plasma membrane. Since mutations in the various subunits in the exocysts show separation defect, it has been suggested that this complex may be involved in the delivery of hydrolytic enzymes responsible for cell cleavage. Supporting evidence comes from another group showing that cell separation of *S. pombe* is an enzymatic process involving hydrolysis of certain cell wall components. They have further shown that the product of the gene eng1$^+$, having endo-β-1,3 glucanase activity localizes transiently at the septal region in the form of a ring - implying its involvement in cell separation. It seems that the eng1p glucanase is required to degrade the β-1,3-glucan, a major component of the primary septum (Martin-Cuadrado *et al.,* 2003).

Another group of proteins clearly required for daughter cell separation in *S. pombe* are the septins as deletion of septin genes generates a non-lethal chained cell phenomena (Tasto *et al.,* 2003). Septins are a group of conserved GTPases originally discovered in budding yeast and later found in many other organisms. In budding yeast they are multifunctional proteins reported to provide boundaries to restrict movement of certain chemicals and also act as a scaffold helping in proper localization of many factors involved in polarity and cell division. Deletion of septins leads to defects in cytokinesis in many cell types. Another factor important for septin organization is the conserved protein anillin, which has been seen to concentrate in the cleavage furrow of dividing cells where it links the actin and septin cytoskeletons. Disruption of anillin also leads to defects in cytokinesis. Only one anillin homologue was observed in *S. pombe* named mid1p. But there was no previous evidence to show that this protein has any role in interacting with the septin cytoskeleton. Loss of Mid1p, function resulted in

misplaced actomyosin ring and septa while deletion of a septin gives rise to a cell separation defect, implying that Mid1p and septin appears to be in involved in the different stages of cell division. Examination of the recently completed *S. pombe* genome led to the discovery of a new ORF bearing significant homology with human anillin and the mid1 gene and was named mid2. This protein was seen to have 24.3% over all similarity to Mid1p and 19.3% overall similarity to human anillin. Deletion of this gene causes a distinct cell separation defect. A double deletion strain where both mid 1 and mid 2 are disrupted shows misplaced cell septum along with the cell separation defects. Mid 2p was seen to localize as a ring on both sides of the septum as it formed. It was observed to be involved in the establishment of the septin ring and also that destruction of this protein may somehow be involved with its disassembly. This disassembly of the septin ring seems to be necessary for the smooth progress of the cell cycle.

So far we have discussed a group of proteins which have recently been seen to have an effect on cell separation in *S. pombe* at the end of mitosis. Our hypothesis is that the observed cell separation defect and abnormal septum formation, is due to the interaction of HABP1 with multiple proteins in *S. pombe*. Earlier when the alternate splicing factor (ASF/SF2) was purified from the HeLa (a human cervical cancer cell line) cell lysates, its was seen that p32/HABP1 gets co-purified with it (Krainer *et al.,* 1991). However the physiological significance of this was discovered much later when it was found that p32/HABP1 belongs to a group of proteins that affect mRNA splicing by sequestering the essential RNA splicing factor (ASF/SF2) (Petersen-Mahrt *et al.,* 1999). Now the activity of ASF/SF2 depends greatly on its phosphorylation status, which is modulated by the phosphorylation status of CLK/STY, a member of a family of dual specific serine/threonine kinases, of which it is a substrate (Prasad *et al.,* 1999, 2003). However there is another protein SRPK1

(human SR protein specific kinase 1) which also interacts with ASF/SF2 in a manner very similar to that with CLK/STY, phosphorylation being the main area of interaction (Colwill *et al.,* 1996). The interactions between CLK/STY and SRPK1 with ASF/SF2 seem competitive in nature. Now the *S. pombe* homologue of SRPK1 is a gene known as dsk1$^+$ and a *S. pombe* homologue of CLK/STY has been recently identified, known as kic1$^+$ (Tang *et al.,* 2000, 2003). So these families of proteins are conserved across species from fission yeast to humans and so the possibility of a functional interaction between a protein of this family and a human protein cannot be ruled out. Its been further observed that kic1$^+$ and dsk1$^+$ interact with each other and regulates the cell surface and septum formation as well as a late step in cytokinesis in *S. pombe.* So it could well be speculated that when HABP1 is expressed in *S. pombe,* it interacts with either or both of these proteins to produce the abnormal cell septum formation.

The ultramicroscopy of pRHP1 and pRep1 transformed cells show the presence of a larger number of vacuoles in cells producing HABP1 with a different distribution pattern as compared to normal cells. Vacuoles occur naturally in *S. pombe* and are used as a storehouse of necessary biomolecules and also to maintain homeostasis. This feature was not observed when cells were examined under a fluorescence microscope. In this connection it must be mentioned that over production of HABP1 in mammalian cells has also shown to increase in vacuolation (Meenakshi *et al.,* 2003). In mammalian cells this has been hypothesized to be an indication onset of apoptotsis but its significance in yeast cell fission is not yet clear.

4.D SUMMARY

To summarize it can be concluded that:

1) **Translation of the HABP 1 gene in *S. pombe* cells causes growth inhibition and other morphological abnormalities while just its transcription does not display any of these abnormalities.**

2) **Expression of HABP1 in *S. pombe* causes growth inhibition, elongation, multinucleation and formation of multiple abnormal septa.**

3) **All the growth and morphological abnormalities observed in *S. pombe* cells on expression of HABP1 is independent of whether live cells or fixed cells were observed indicating that all the abnormalities observed were not artefactual in nature.**

4) **HABP1 localizes to the nucleus in log phase of cell growth and displays a cytosolic distribution in the lag phase.**

5) **No change in the level of expression and localization of actin polymers was detected on expression of HABP1 in *S. pombe* cells.**

6) **Ultrastructural analysis of *S. pombe* cells confirms the earlier observed morphological changes and additionally shows increase and difference in distribution of vacuoles in *S. pombe* cells expressing HABP1.**

Chapter II: Evidence for HABP1 Interacting with Cell Cycle Regulatory Protein

5.A INTRODUCTION

Understanding the behaviour of a protein in a cell is often very difficult and usually involves a dual approach. On one hand the structure of the protein may be analysed to understand its function by comparing it with the function of other proteins with a similar structure. The other way is to identify the interacting proteins to pinpoint the pathway of its functioning. The crystal structure of HABP1 has already been solved (Jiang *et al.,* 1999). It was observed that this protein exists as a homo-trimer each monomer being composed of one N-terminal α helix (αA) followed by seven consecutive β-strands, designated β1 through β7, which form a highly twisted anti-parallel β-sheets, with β1 nearly perpendicular to β7 and ending with two C-terminal (αB and αC) α-helices. All the three helices are located on the same side of the β-sheet. Helix αB lies parallel to the β-sheet, with the helix axis perpendicular to the direction of individual strands The helix αB and the N-terminal portion (4 turns) of helix αC make extensive hydrophobic contacts with the β-sheet, which appear to be essential for the stability of the structure of the monomer. The N-terminal helix αA does not contact the β-sheet within the monomer, but forms an anti-parallel coiled coil with the C-terminal portion (5 turns) of αC. This coiled coil region is necessary for the protein-protein interactions required for homo-oligmerization as it forms extensive inter-molecular contacts. The helix αA packs with the anti-parallel αB of an adjacent monomer and the C-terminal region of αC packs against the back of the β-sheet. Overall the trimer has a doughnut shaped quaternary structure, with a sizeable central channel and an unusual asymmetric charge distribution on the surface. It looks as if the β-sheets form a hyperboloid shaped spool with the α-helices wrapped around it.

A HABP1 monomer does not possess a distinct domain and even though its structure is rather simple, the structure does not belong to and known protein fold as it has no similarity with any other protein in the Structure Database. However further structural analysis of the crystal revealed that the N- terminal sequence of HABP1 is similar to the WD 40 family of regulatory proteins.

The WD 40 family of proteins which constitute a class of regulatory proteins which has a signature sequence of G-H and W-D/E dipeptide usually separated by 25-27 amino acids. Such sequences predominantly forms a α helix and biochemical evidence suggests that this signature motif/fold are critical for protein-protein interactions.RACK1, RACK2, Calreticulin and a number of PKC regulatory proteins are prominent members of this family (Iwasaki *et al.,* 1995; Mochly-Rosen *et al.,* 1991a; Mochly-Rosen. et al 1991b).HABP1 has this WE signature motif at its N-terminal segment through which HABP1 has been reported to interact with other proteins like gC1q, GABA(A) receptor , the β subunit light chain of unphosphorylated myosin thus showing its involvement in protein-protein interactions.

So since HABP1 has much potential for protein-protein interactions, identification of interacting proteins is definitely one of the options to determine the functioning of HABP1 in *S. pombe* cells. As discussed earlier in Chapter 1, the morphology of the *S. pombe* cells producing HABP1, its changing cellular localization with time and the similarity of Schizosaccharomyces pombe with higher eukaryotes at the genetic level suggest the possible existence of multiple interacting proteins of HABP1.

This prompted us to first check whether any interacting proteins could be detected in *S. pombe.* This primary investigation was carried out in the BJ 7468 strain of *S. pombe.* The aberrant morphology in *S. pombe*

caused by the expression of HABP1 directed us to choose two main cycle regulatory proteins CDC 2 and CDC 25 for investigating their interaction with HABP1. These proteins were chosen as their loss of function phenotype resembled the aberrant morphology seen in *S. pombe* cells on the expression of HABP1. This study was executed in the JK 2423 strain of Schizosaccharomyces pombe. This strain was isogenic to the strain 972 h^- with only one difference. A 12 myc tag was affixed to the C-terminal of the cdc 25 gene in this strain. So the expression of cdc 25 gene in this strain could be detected by detecting the expression of this tag. Therefore, the expression of CDC25 in this strain could be easily monitored by using the commercially available myc antibody. At the same time, the cell cycle regulatory protein CDC2 having a specific motif PSTAIR can also be detected using commercial antibody against this motif.

5.B RESULTS

5.B.i ANALYSIS OF *S. Pombe* LYSATES SHOW THE PRESENCE OF MULTIPLE INTERACTING PROTEINS

S. pombe (BJ 7468) cells transformed with pRHP1 and pRep1 were grown at 29°C in EMM leu- Medium and their growth rate checked at 24, 48 and 72 hours respectively. Accordingly 10^8 cells were taken and processed for immunoblotting as described in Materials and Methods. After lysing the cells, the protein concentration of the lysates was determined and 200 μg of total protein per sample were resolved on a 12.5% SDS-PAGE. After transblotting onto a nitro-cellulose membrane probing was done with monoclonal anti-HABP1 antibody to check for the expression of this protein at different time points. As seen in figure 1 the expression of HABP1 was seen at all the three time points though the level of expression is minimum at 24 hours and maximum at 48 hours.

Then the same lysates were used to check for proteins interacting with HABP1. For this, 40 μgm of total lysate proteins from each time point were resolved on a 12.5% SDS-PAGE and probed with biotinylated recombinant HABP1. The results are shown in figure 2. Three proteins of roughly 22, 30 and 45 kDa were detected in all the lysates of pRHP1 and pRep1transformed cells. An additional band at 34 kDa was detected in the lysate of the pRep1 transformed cells of 72 hrs. The intensity of the 30 kDa band was the least in the 24 hr time point of the pRHP1 transformed cells while that of the 45 kDa band was the greatest at the 48 hr time point. These variations of band intensities along with the presence of certain bands at some time points and not in the others indicate a dynamic behaviour of HABP1 inside the pRHP1 transformed cells which appears to be time dependent. So from the above data it is observed that HABP1 indeed has multiple interacting proteins in these

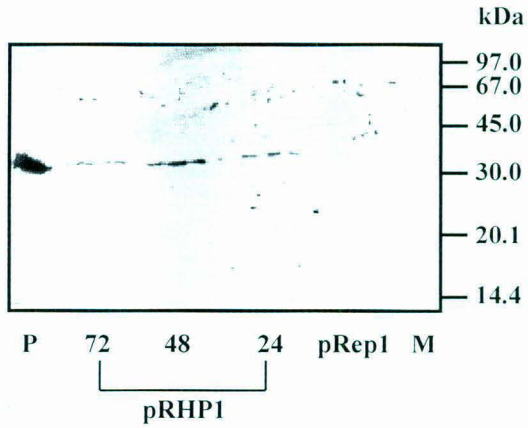


Fig 1: Expression of HABP1 at 24,48 and 72 hrs of growth of pRHP1 transformed *S.pombe* cells.P indicates purified rHABP1 protein, M indicates Marker.pRep1 indicates lysate from pRep1 transformed *S.pombe* cells.

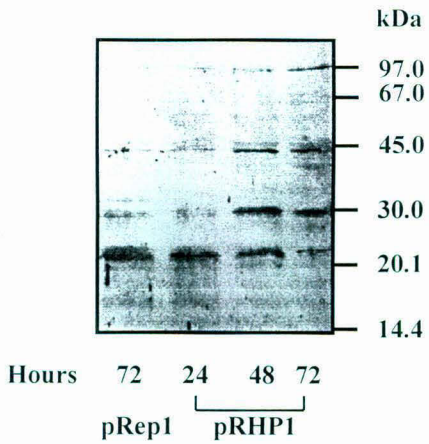


Fig 2: Far-Western Blot showing the proteins detected by biotinylated rHABP1 from lysates of pRHP1 and pRep1 transformed S.pombe cells from different hours.

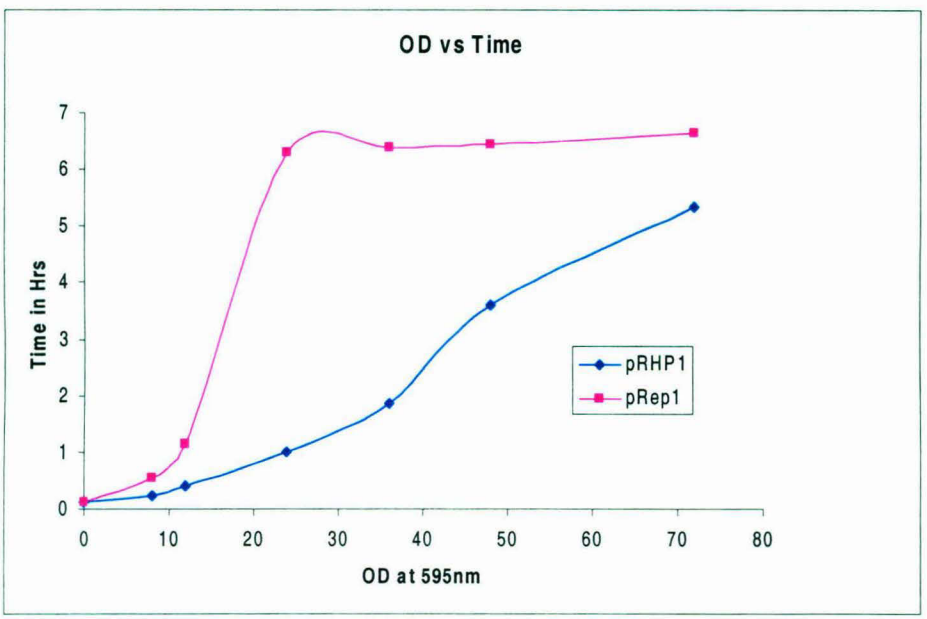


Fig 3: Cell Growth Curves of pRHP1 and pRep1 transformed *S.pombe* cells of the JK2423 cells. The pRHP1 transformed cells show growth inhibition compared to the pRep1 transformed cells.

Fig 4: Abnormal morphology of pRHP1 transformed *S. pombe* cells of the JK 2423 strain. Nuclei have been stained with Propidium Iodide (PI) and cell septum has been stained with Aniline Blue. Elongation, multinucleation and abnormal cell septum formation in pRHP1 transformed *S. pombe* cells at different time points are clearly visible.

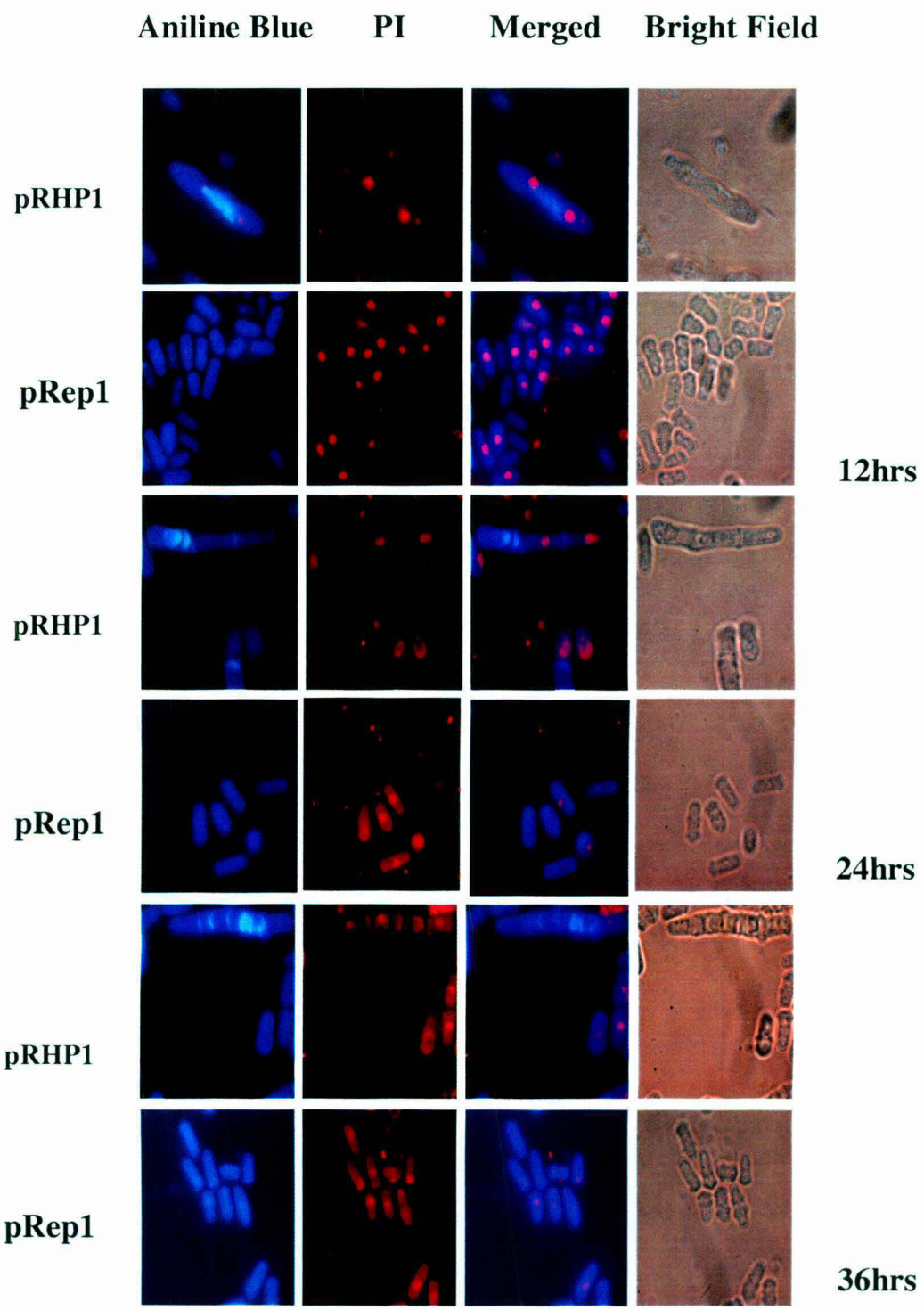

Aniline Blue
PI
Merged
Bright Field
pRHP1
pRep1
12hrs
pRHP1
pRep1
24hrs
pRHP1
pRep1
36hrs

Fig. 4

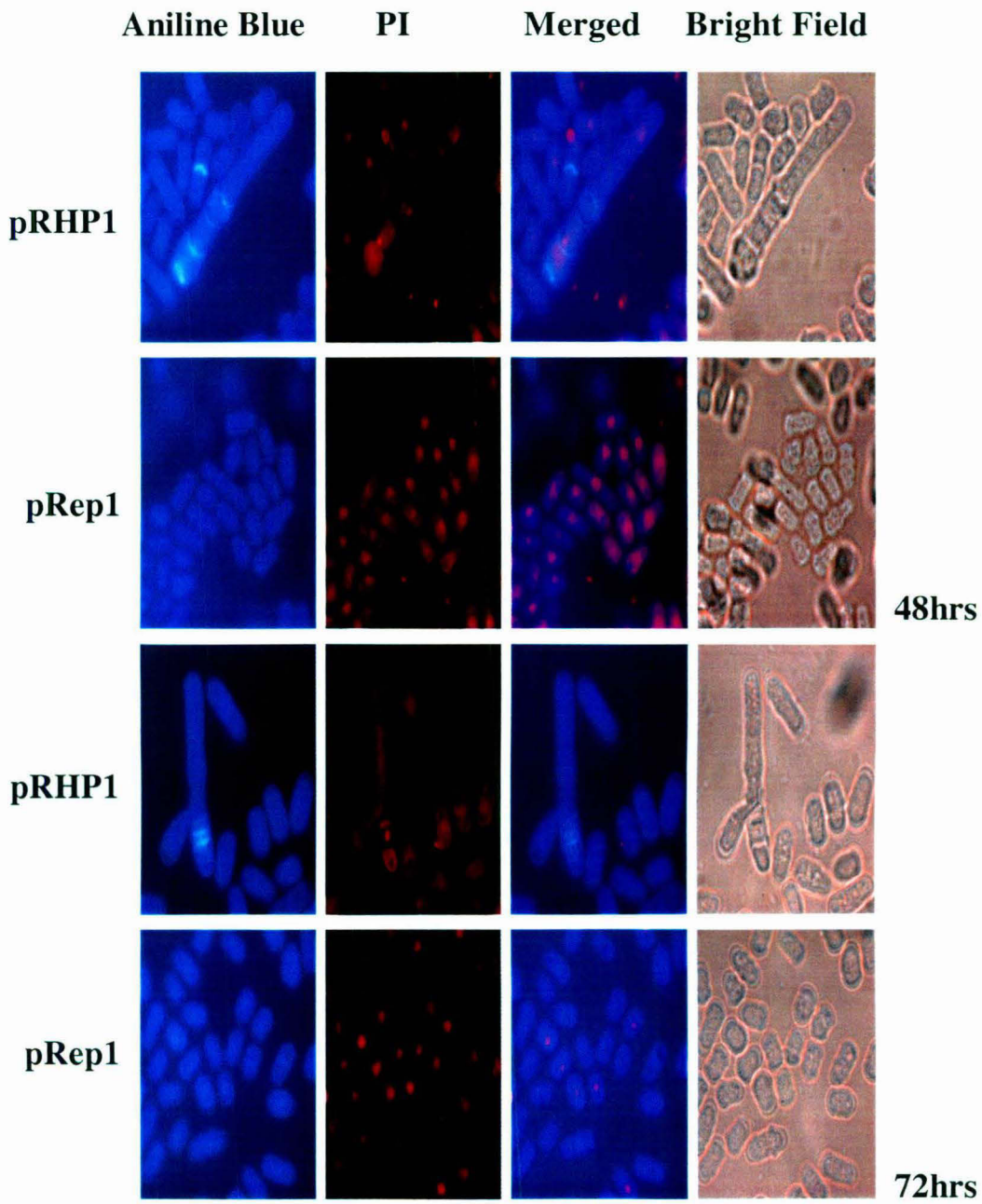

Aniline Blue
PI
Merged
Bright Field
pRHP1
pRep1
48hrs
pRHP1
pRep1
72hrs

Fig. 4 (contd.)

cells and this may be the reason why the aberrant morphology of the *S. pombe* BJ7468 cells are observed.

5.B.ii CELL GROWTH ASSAY OF TRANSFORMED JK2423 STRAIN OF *S. pombe*

The *S. pombe* JK 2423 strain was transformed with pRep1 and pRHP1 grown in EMM leu⁻ medium and the growth rates were plotted against time as seen in Fig. 3. It was observed that the cells bearing the plasmid pRHP1 grew significantly slower than the cells transformed with pRep1_vector.

5.B.iii *S. pombe* JK 2423 CELLS PRODUCING HABP1 DISPLAY IDENTICAL ABERRANT MORPHOLOGICAL FEATURES

JK 2423 cells transformed with pRHP1 and pRep1 were grown in EMM leu⁻ medium and at selected time points, their optical densities were checked and accordingly 10^7 cells were taken and ethanol fixed as per the procedure given in Material and Methods. Later the cells were rehydrated and a Propidium Iodide /Aniline Blue staining of the cells were done. PI (propidium iodide) stains the nucleus while aniline blue stains the septum. As seen in Figure 4 the cells bearing the plasmid pRHP1 show elongation, abnormal multiple cell septum and multinucleation as had been observed earlier in strains MBY 624 and BJ7468. So the morphological abnormality observed in this strain on expression of HABP1is identical to those of the others.

5.B.iv EXPRESSION OF CELL CYCLE REGULATORY PROTEIN CDC 2 AND CDC 25 ARE PERTURBED IN pRHP1 TRANSFORMED *S. pombe* JK2423 CELLS

S. pombe JK 2423 cells transformed with pRHP1 and pRep1 were grown in EMM leu⁻ medium at 29°C. Their optical densities were checked and accordingly 10^8 cells were collected at 12, 24, 36, 48 and 72 hours respectively. Later, they were lysed in ice-cold lysis buffer by vortexing with glass beads as given in Material and Methods and their protein

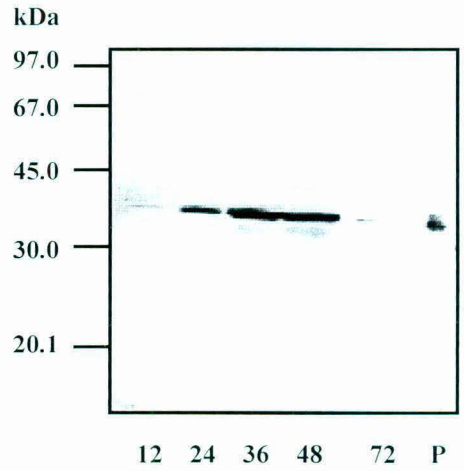


Fig 5:Immunoblot of lysates of pRHP1 transformed S.pombe cells at different time points with monoclonal anti-HABP1 anti-body.Lane marked P contains purified HABP1 as positive control. Maximum expression of HABP1 is seen at 36 and 48 hours.

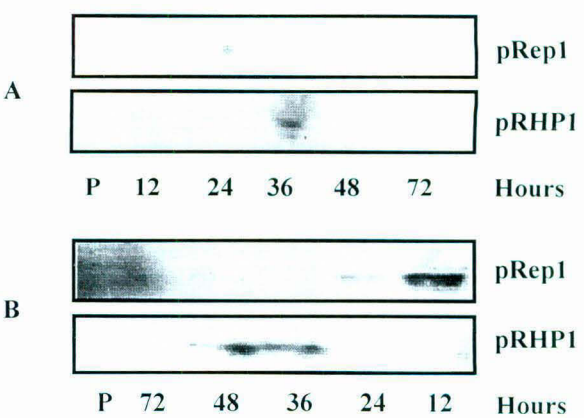


Fig 6: Differential expression of CDC2 (panel A) and CDC25 (panel B) at different time points in pRep1 and pRHP1 transformed *S.pombe* cells.The Lane P contains purified HABP1 as negative control.

concentration estimated by Bradford's Method. Then equal amounts (100 μg) of total lysate protein per lane were resolved in a 12.5% SDS-PAGE along with molecular weight marker and purified recombinant HABP1 and transferred onto a PVDF membrane and probed with monoclonal anti-HABP1 antibody. As seen in figure 5, HABP1 is seen to express at all time points but maximum expression is seen at 36 and 48 hours respectively.

Next equal amounts (100μg) of total lysate protein from different time points from pRHP1 and pRep1 transformed *S. pombe* JK 2423 cells were resolved on a 12.5 % SDS-Page and transferred onto a PVDF membrane and probed with anti-PSTAIR antibody for checking the expression of CDC2 at different time points. The results as displayed in Panel A of Fig. 6 shows the maximum expression of CDC2 at 24 hrs in pRep1 transformed cells while in pRHP1 transformed cells the maximum is seen at 36 hours. Thus CDC2 is differentially expressed *S. pombe* cells producing HABP1.

An identical experiment was performed to check the expression of CDC25 at different time points. Here the expression of CDC25 was examined by probing with anti-myc antibody. This was possible because the cdc 25 gene in this strain was C-terminally tagged with 12 myc. CDC25 expression was also seen to be different in pRHP1 transformed *S. pombe* cells. In pRep1 transformed *S. pombe* cells the maximum expression of CDC25 was seen at 12 hours while in pRHP1 transformed cells the maximum was at 36 and 48 hours just like the expression of HABP1 (Fig 6 Panel B).

Subsequently it was examined from pRHP1 transformed *S. pombe* lysates from 36 and 48 hours whether HABP1 could be co-immunoprecipitated with either CDC2 or CDC25. The anti-PSTAIR and anti-myc antibodies were utilized to pull down CDC2 and CDC 25

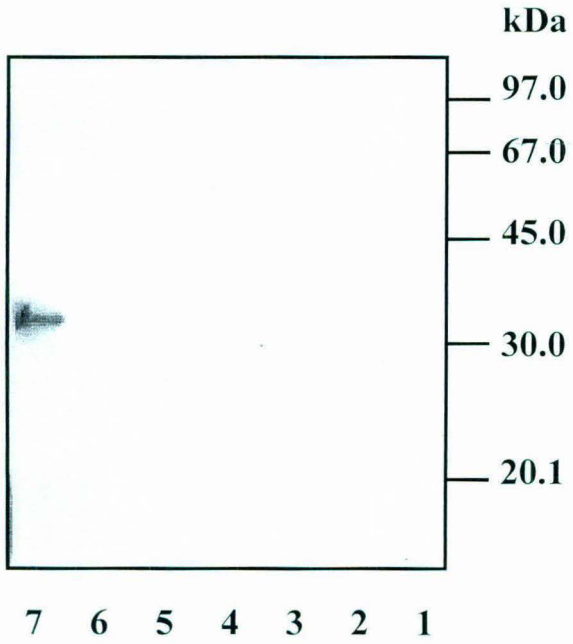


Fig 7: Co-immunoprecipitation of HABP1 with CDC2. Lanes 1,2 (beads in lysate), lanes 3,4 (anti-PSTAIR antibody incubated with lysate of pRep1 transformed cells from 36 and 48 hrs respectively). Lanes 5 and 6 (anti-PSTAIR antibody incubated with pRHP1 transformed S. pombe cells from 36 and 48 hrs respectively). Lane 7 (purified HABP1). Here just the purified protein has been detected by the monoclonal anti-HABP1 antibody.

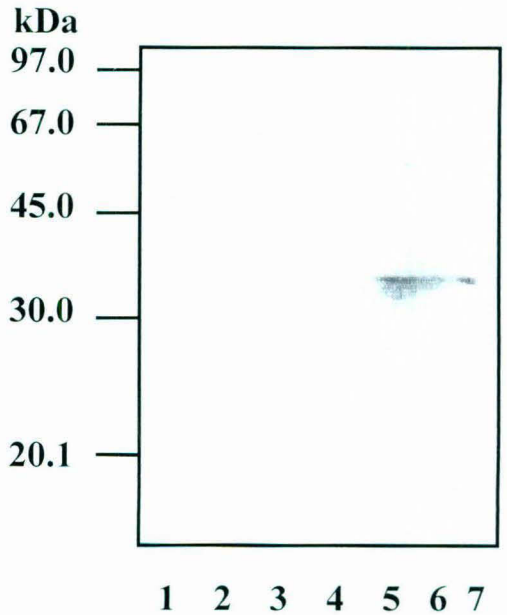


Fig 8: Co-immunoprecipitation of HABP1 with CDC25.Lanes 1,2 (beads in lysate), lanes 3,4 (anti-myc antibody incubated with lysate of pRep1 transformed cells from 36 and 48 hrs respectively. Lanes 5 and 6 (anti-myc antibody incubated with pRHP1 transformed S. pombe cells from 36 and 48 hrs respectively). Lane 7 (purified HABP1). Here the monoclonal anti-HABP1 antibody has detected the HABP1 being co-immunoprecipitated with CDC25 both at 36 and 48 hrs from pRHP1 transformed S. pombe cells along with the purified protein.

proteins in lysates onto pre-swelled Protein A beads. These beads were boiled in SDS-Page loading buffer and the eluates were resolved on a 12.5 % SDS-Page, transferred onto a PVDF membrane and probed with monoclonal anti-HABP1 antibody to detect the presence of HABP1. As seen in Fig 7, no HABP1 was detected to co-immuno precipitate with CDC2. However HABP1 was seen to co-immunoprecipitate with CDC25 at both 36 hrs and 48 hrs. This confirms the direct binding of HABP1 with CDC25 at these time points. No proteins were detected in pRep1 transformed cell lysates or in lysates where no primary antibody were used as negative controls in either of the blots confirming the specificity of the results. Purified HABP1 used as positive control was detected in both the blots.

5.C DISCUSSION

The morphological changes observed in *S. pombe* cells expressing HABP1 along with its changing localization within the cell with time suggested that these phenomenon were a result of multiple proteins interacting with HABP1. Accordingly the expression of HABP1 was checked at different time points to establish that it was present throughout the growth phase. Then the lysates of *S. pombe* cells transformed with pRHP1 and pRep1, from different time points probed with biotinylated HABP1 to see the possible presence of interacting proteins. The results as depicted in Fig. 2 show the presence of multiple interacting proteins at all the time points. The presence of three interacting proteins are observed in the lysates of pRHP1 transformed cells from 24,48 and 72 hours showing migrations at 20,30 and 45 kDa respectively. But the relative intensities of individual proteins seem to change. The intensity of the 30 kDa and 45 kDa proteins are least in the 24 hrs lysate while that of the 45 kDa protein is the highest in the 48 hour lysate. From the lysate of pRep1 transformed cell at 72 hrs along with these proteins an additional protein showing a migration at 34 kDa is observed. From this Far Western analysis, it becomes further evident that HABP1 interacts differentially with multiple proteins in time.

After the detection of multiple interacting proteins, the observed morphology of elongation and multinucleation prompted the choice of CDC2 and CDC25, two regulatory cell cycle proteins, for further study. For this the JK2423 strain of *S. pombe* was utilized. The C-terminal of the CDC25 gene of this strain is tagged with 12 myc. The rate of cell growth and morphology of this strain was checked and was observed to be identical to the other strains expressing HABP1. The expression of HABP1 was checked in pRHP1 transformed *S. pombe* cells at different time points and maximum expression was seen at 36 and 48 hours. The

expression of CDC2 was checked both in the pRep1 and pRHP1 transformed *S. pombe* cells at different time points. It was found that the maximum expression of CDC2 was seen at 24 hrs for pRep1 transformed cells and at 36 hrs for pRHP1 transformed cells. Next it was investigated whether HABP1 was being co-immunoprecipitated with CDC2 at 36 and 48 hours. It was found that HABP1 was not being co-immunoprecipitated with CDC2. Next the expression profile of CDC25 was checked in pRep1 and pRHP1 transformed *S. pombe* cells. It was observed that the maximum expression of CDC25 was seen at 12 hours for pRep1 transformed cells and at 36 and 48 hours for pRHP1 transformed cells. Next it was checked whether HABP1 co-immunoprecipitated with CDC25 at 36 and 48 hours. It was seen that both time points HABP1 is being co-immunoprecipitated with CDC25.

From these observations it becomes quite clear that expression of HABP1 causes differential expression of both CDC2 and CDC25 while it interacts directly with CDC25. The observed morphology of the *S. pombe* cells on expression of HABP1 can be partially explained by the interaction of CDC25 with HABP1. The binding of HABP1 with CDC25 would mean that the cell experiences a short supply of this protein. This would result in the cell getting arrested at the G2/M boundary (Daga and Zimenez, 1999). This implies that the smooth entry into cytokinesis is hampered. *S. pombe* cells have been observed to assume a multinucleated phenotype through a variety of pathways under such circumstances (Liu *et al.*, 2000).

As mentioned in the Introduction of this chapter, the N-terminal of HABP1 shows great similarity to the WD 40 family of regulatory proteins which entails its having a signature sequence of G-H and W-D/E di-peptide usually separated by 25-27 amino acids. Such sequences predominantly form a α helix and biochemical evidence suggests that this signature motif/fold are critical for protein-protein interactions. So

now the evidence that HABP1 does have interacting proteins makes it imperative that the importance of the N-terminal of this protein needs to be investigated.

Further, there are more proteins which interact with HABP1 which need to be identified before (/3Uit can be understood what's causing the abnormal formation of the multiple cell septa and the associated cell separation defect. For this the activity of proteins active in the cell separation pathway like eng1p (Martin-Cuadrado *et al.,* 2003) and Kic1 (Tang *et al.,* 2003) need to be checked for interactions with HABP1.

5.D Summary

So to summarise:

1) **HABP1 does have a number of interacting proteins in S.pombe which change over time indicating the dynamic nature of the protein-protein interactions.**

2) **Expressions of HABP1 in JK 2423 cells exhibit identical morphology and growth inhibition as seen in other strains under similar circumstances.**

3) **Expression of HABP1 in JK 2423 cells cause differential expression of major cell cycle regulatory proteins CDC2 and CDC25 at different time points.**

4) **HABP1 was not seen to co-immunoprecipitate with CDC2.**

5) **HABP1 was seen to co-immunoprecipitate with CDC25 implying direct binding of CDC 25 to HABP1.**

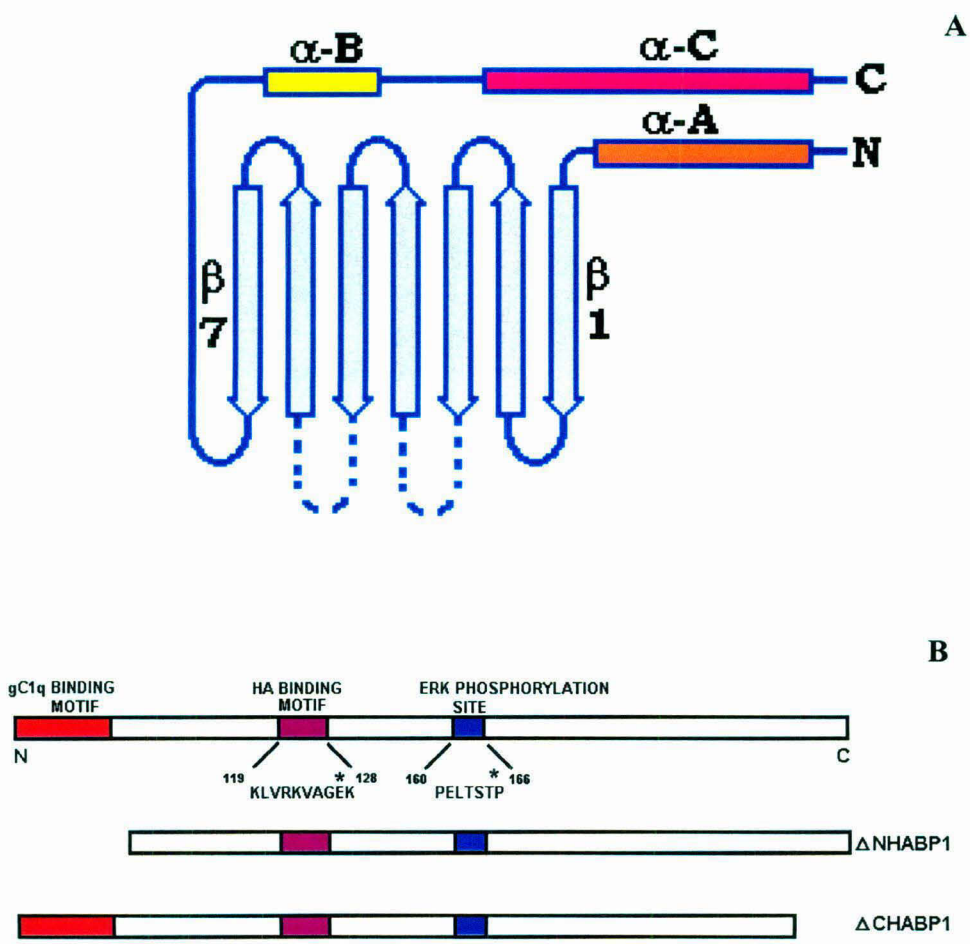
Chapter III:
Expression of Truncated Mutants of HABP1 in S. pombe Show Differential Morphological Characteristics

6.A INTRODUCTION

The function of any protein depends on the three dimensional *in vivo* structure of the protein along with the various post-translational modifications it may be undergoing. Important information about the structural aspects of the protein can be derived by the studies on the interaction of the protein in solution and by solving the crystal structure. The crystal structure of HABP1 has been solved and results shows it to be a trimeric protein with each monomer consisting of one N-terminal α (α_A) helix followed by seven anti-parallel β strands forming a highly twisted β sheet followed by the two C-terminal α helices (α_B and α_C) (Diag. 1A). All the three helices lie on the same side of β sheet with α_B being parallel to the β sheet but perpendicular to the orientation of the individual β strands. The helix α_B and the N-terminal portion (4 turns) of the helix α_C make extensive hydrophobic contacts with the β-sheet that seems to be important for the structural stability of the monomer. The N-terminal helix α_A does not contact the β sheet within the monomer but forms an anti-parallel coiled coil with the C-terminal portion of α_C. This region is important for protein-protein interactions and is required for oligomerization.

The trimer forms a doughnut shaped homo-trimer, with an unique non-crystallographic three fold axis of symmetry, a structure unobserved till date in any other protein (Jiang *et al.,* 1999). The crystal structure of this protein also displays a solvent exposed HA-binding motif in the trimer.

Recent studies have shown that the HABP1 trimer forms cysteine mediated dimer of trimers (Jha *et al.,* 2002). This oligomerization has functional implications as this makes the whole structure more compact which shows greater affinity for HA than a more relaxed structure. It is



Diag 1:(A) Schematic representation of a monomeric unit of the HABP1 crystal showing the α- helices and the β-strands making up the β-sheet. (B) Schematic representation of the allignments of the N-terminal and C-terminal deleted mutants with respect to the mature HABP1

also observed that there is asymmetric charge distribution in the protein– the positive charges are present on one face while the negative changes being present on the other. This allows for greater conformational flexibility to the protein, which could be the reason for multi-ligand interaction and multi-compartmental localization of HABP1.

Since both the monomeric and trimeric forms of HABP1 are active mainly through the interactions of the terminal α helices, truncated variants of HABP1 were generated to check their structural and functional relationships (Sengupta, 2003). For the generation of N-terminal deleted clone the amino acids 74 to 105 have been removed from the mature HABP1 which consists of the 74 to 282 amino acids. This deletes the N-terminal α_A helix and a part of the loop connecting the β1 strand. Since the N-terminal α_A seemed to be important for protein-protein interactions, it was hypothesized that elimination of this helix would substantially alter the structure and thus may affect the interaction of the truncated protein (ΔN.HABP1) with other proteins in *S. pombe* (Diag. 1B).

For the generation of C-terminal mutant clone, the amino acids 267 to 282 have been deleted thereby resulting in removal of half of the α_C helix along with the tail end of the C-terminal to generate the truncated protein ΔC.HABP1. The terminal helices of HABP1 were considered to be important for the structural integrity of HABP1 and hence for its functions. A schematic representation of the secondary structure of the HABP1 monomer along with the generation of the N-terminal and C-terminal deleted mutants are given in the panels A and B of Diagram 1 respectively. The N-terminal and C-terminal deleted mutants so generated (Sengupta., 2003) were subcloned into the shuttle vector pRep1 and the clones generated were named pRHΔN1 and pRHΔC1 respectively and were used to transform *S. pombe* cells.

It was hypothesized that such critical structural changes in HABP1 molecule would alter its behaviour in *S. pombe* and would help in interpreting the observed aberrant morphological changes and growth inhibition displayed by *S. pombe* cells on expression of HABP1.

6.B RESULTS

6.B.i SUBCLONING OF ΔN AND ΔC FRAGMENTS INTO THE SHUTTLE VECTOR pREP1

The gel purified ΔN and ΔC fragments (Sengupta, 2003) were ligated with gel purified Nde1, BamH1 double digested pRep1 vector. Since ΔN and ΔC were also cloned between the Nde1 and BamH1 sites, the sub-cloning would be directional. Ligation was done at 16°C for 16 hours with T4 DNA ligase (NEB) in the appropriate buffer. After the ligation step, the ligation mixture was used to transform competent *E. coli* DH5α cells and the colonies were screened for the proper clone. Cloning was confirmed by restriction digestion with Nde1 and BamH1 (Fig. 1) and the correct size of the insert was checked. The ΔN fragment is of 900 base pairs while the ΔC fragment was of 600 base pairs.

6.B.ii EXPRESSION OF THE TRUNCATED VARIANTS OF HABP1 IN *S. Pombe* MBY 624 CELLS

S. pombe transformed pRHP1, pRHΔN1, pRHΔC1 were grown to saturation and lysed. The extracted proteins are estimated and equal amounts (100 μgm) were resolved in a 12.5% SDS-PAGE, transferred onto a PVDF membrane and probed with monoclonal anti-HABP1 antibody (Fig. 2). As can be seen, the expression of the mature HABP1, ΔN. HABP1 and ΔC.HABP1 show a migration of 34, 27 and 32 kDa respectively on the immunoblot. This confirms the expression of HABP1, pRHΔN1 and pRHΔC1 in *S. pombe* (MBY 624) cells.

6.B.iii CELL GROWTH ASSAY OF THE pRHΔN1 AND pRHΔC1 TRANSFORMED *S. pombe* CELLS

pRHΔN1 and pRHΔC1 were used to transform *S. pombe* cells as described in Material and Methods. Following transformation the cells were grown in EMM leu-medium along with pRep1 and pRHP1 transformed cells and their growth rates measured at different time

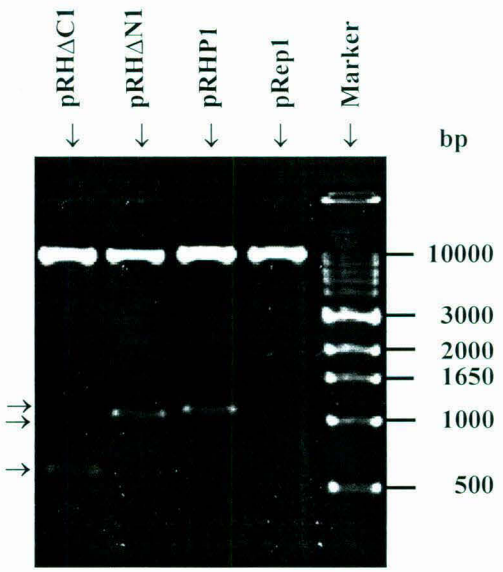


Fig 1: Shows the mature and truncated fragments, cloned in pRep1, with Nde 1 and BamH1 restriction endonucleases and resolved on a 0.7 % Agarose Gel.The fragment of HABP1 was 1030 BP in length,while that of ΔN was 900 BP and ΔC was 600 BP in length respectively.

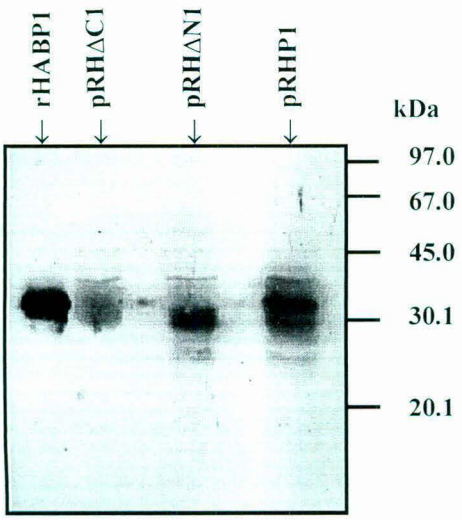


Fig 2:The mature protein and its truncated variants were immunodetected using monoclonal anti-HABP1 antibody after resolving them on a 12.5 % SDS-PAGE The truncated proteins can be detected by the anti-HABP1 antibody. The N-terminal deleted mutant shows a migration of approximately 28 kDa and the C-terminal shows a migration of 30 kDa in SDS-PAGE.

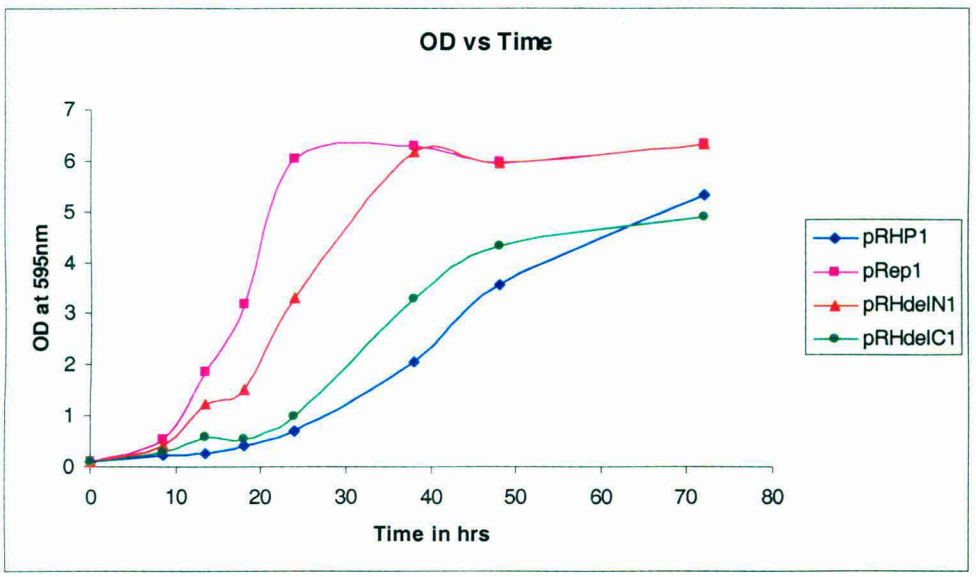


Fig 3:Cell Growth Curves of pRHP1,pRep1, pRHΔN1 and pRHΔC1 transformed S.pombe Cells of MBY 624 strain at different time points. The growth of cells transformed with the C-terminal deletant is akin to that of the cells transformed with pRHP1 and both show growth inhibition while the N-terminal deletant transformed cells do not show any growth inhibition just like the vector transformed cells.

Fig 4: Shows Propidium Iodide and Calcofluor Staining of pRHΔN1 and pRHΔC1 transformed *S. pombe* cells at different time points. The pRHΔN1 transformed cells show a morphology identical to the vector transformed cells and the pRHΔC1 cells elongation multinucleation and a cells separation defect along with some branching. This implies that the structural feature of HABP1 responsible for interacting with other proteins which causes the abnormal morphology in *S. pombe* is not preserved in the N-terminal deletant but is preserved is the C-terminal deletant.

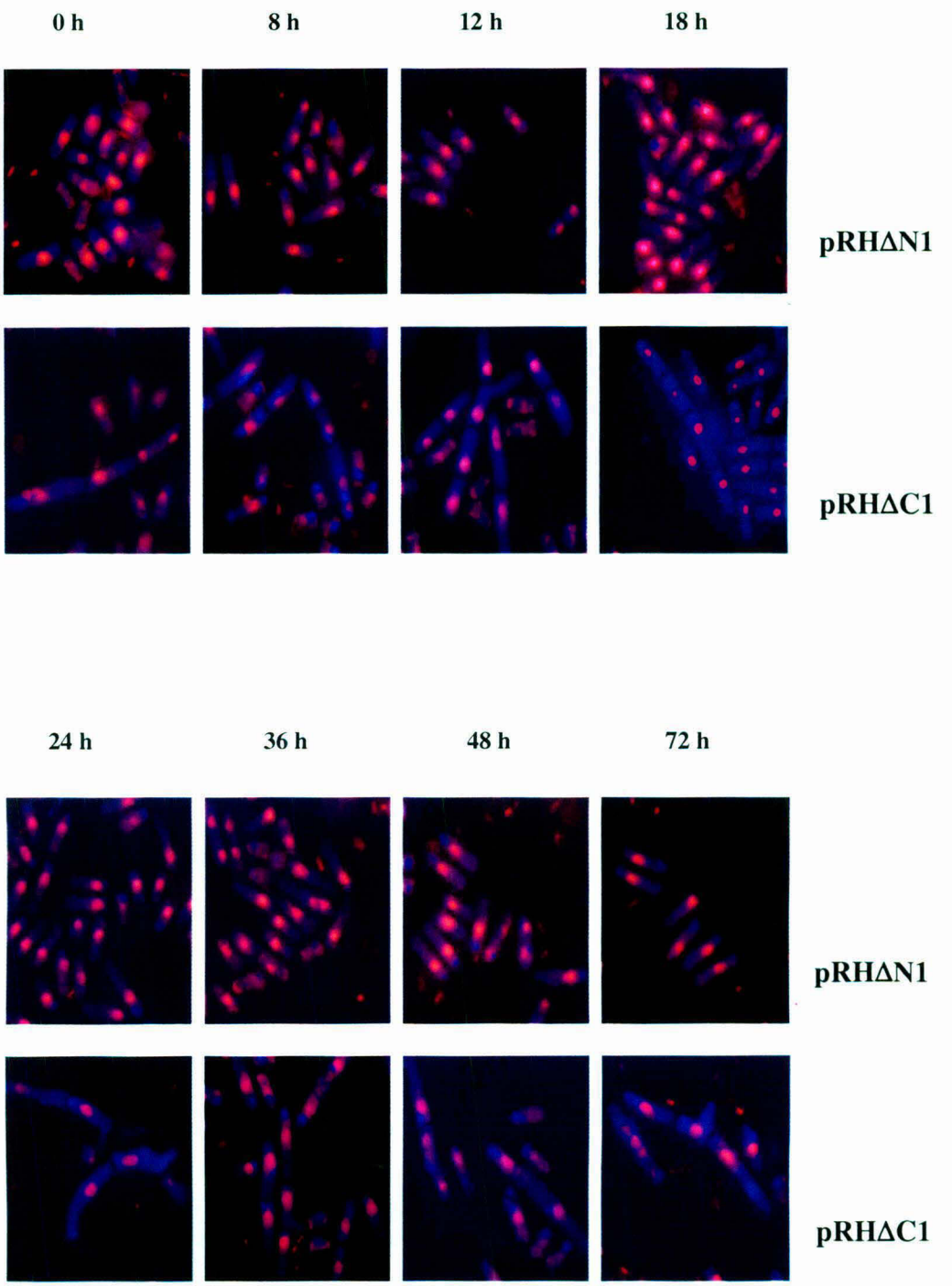


Fig. 3/4

Fig 5: Live cell staining of *S. pombe* MBY 624 strain transformed with pRHP1 and pRep1, pRHΔN1 and pRHΔC1 at different time points. The regulatory light chain (rlc) of myosin has been tagged with GFP in this strain and the nucleus has been stained with DAPI. The pRHP1 and pRHΔC1 transformed cells display elongation, multinucleation and abnormal cell septum formation. The pRep1 and pRHΔN1 cells show normal morphology. The cell septa in pRHP1 and pRHΔC1 transformed cells do not show the green fluorescence indicating it's the primary septum. This indicates that this is a cell separation defect.

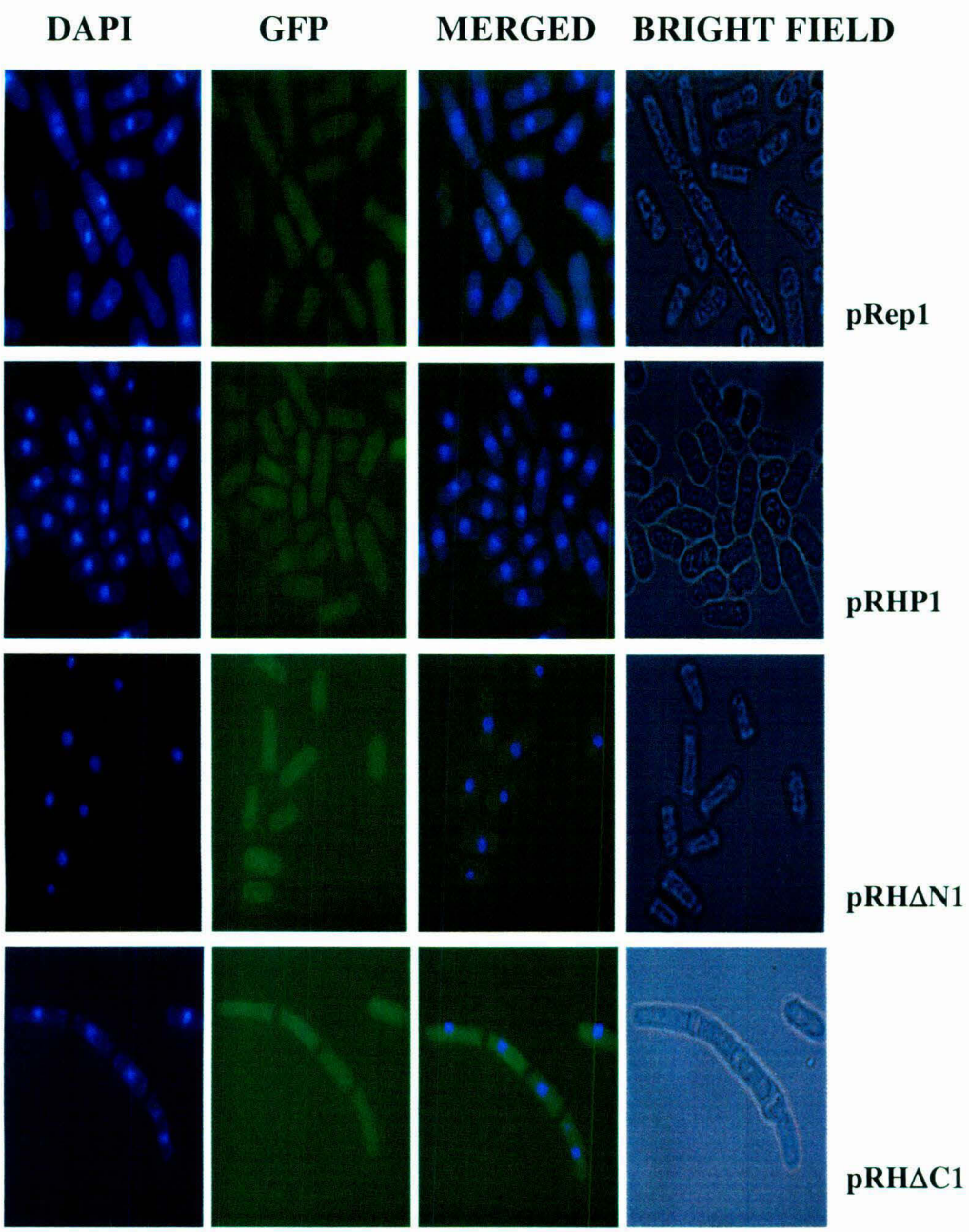

DAPI
GFP
MERGED
BRIGHT FIELD
pRep1
pRHP1
pRHΔN1
pRHΔC1
0 hrs

Fig 5

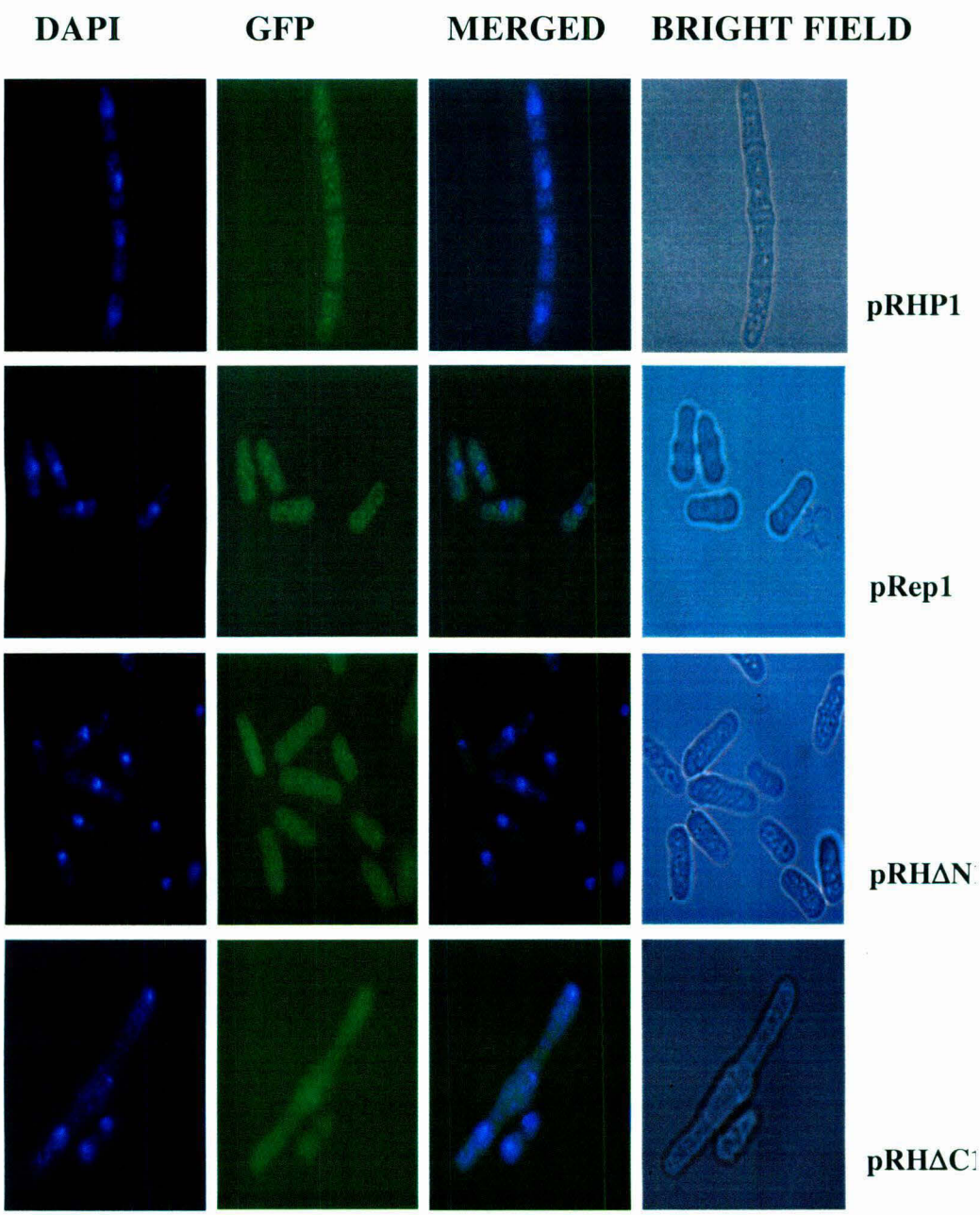


Fig 5(Contd.)

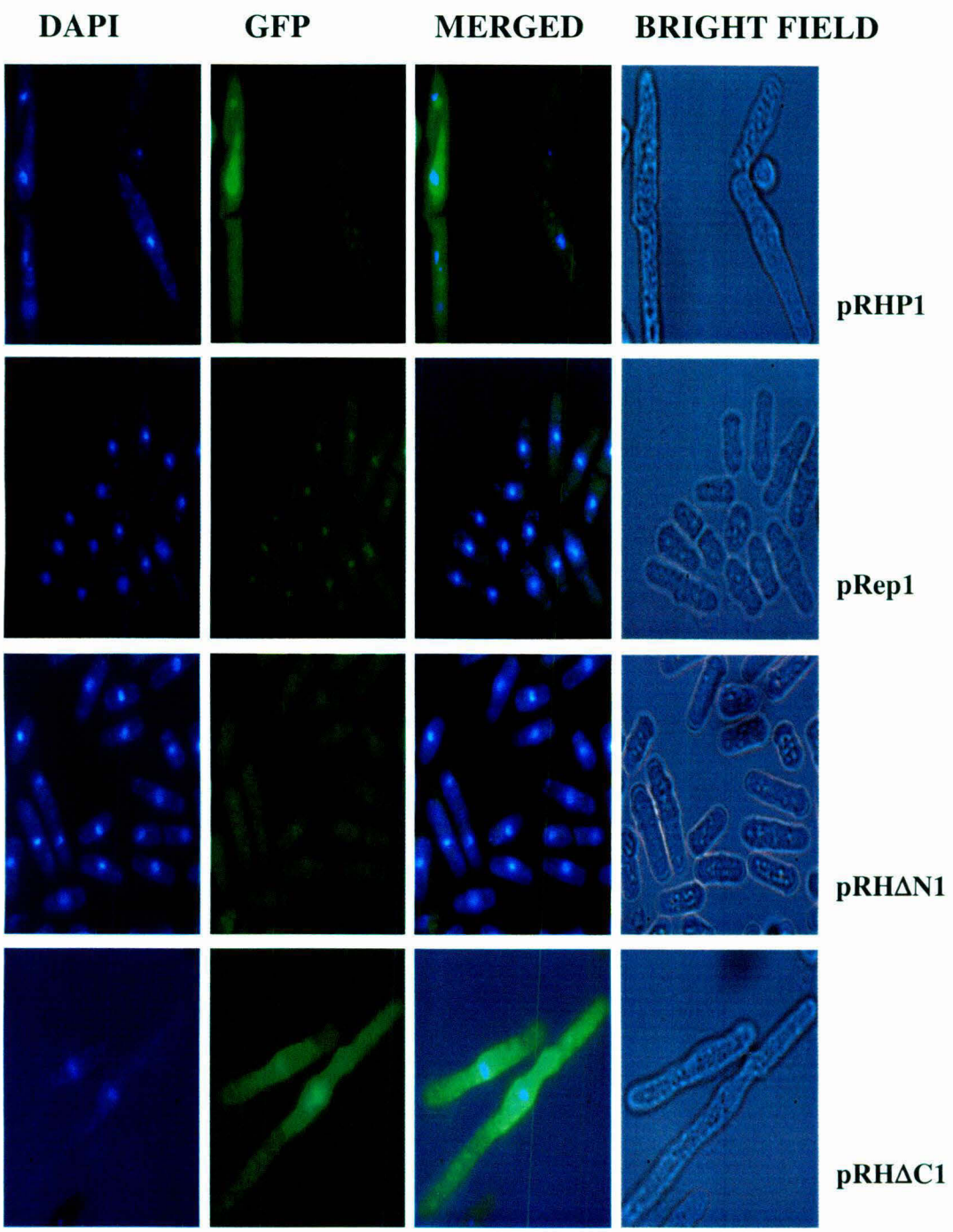

DAPI
GFP
MERGED
BRIGHT FIELD
pRHP1
pRep1
pRHΔN1
pRHΔC1
12 hrs

Fig 5(Contd.)

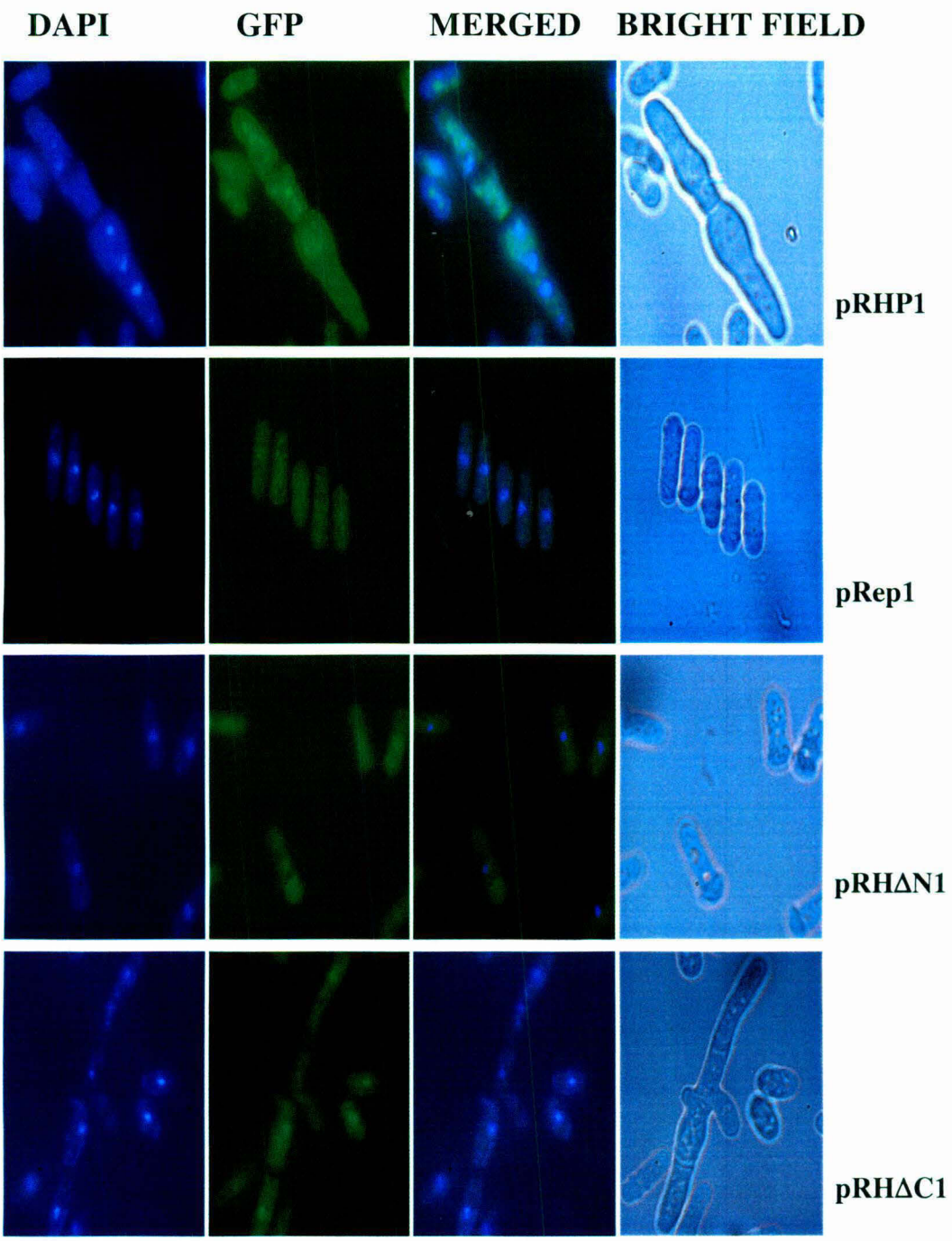
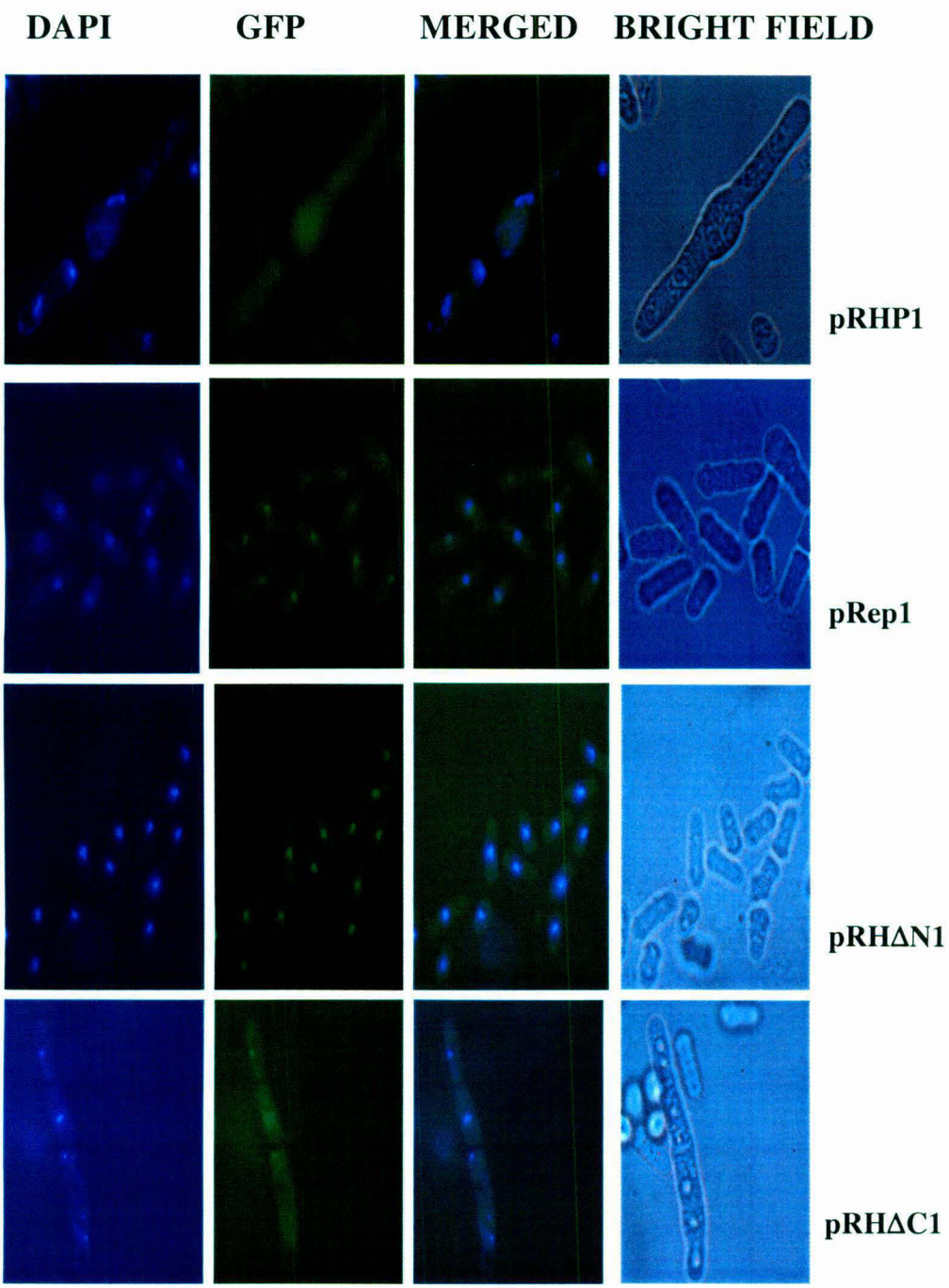
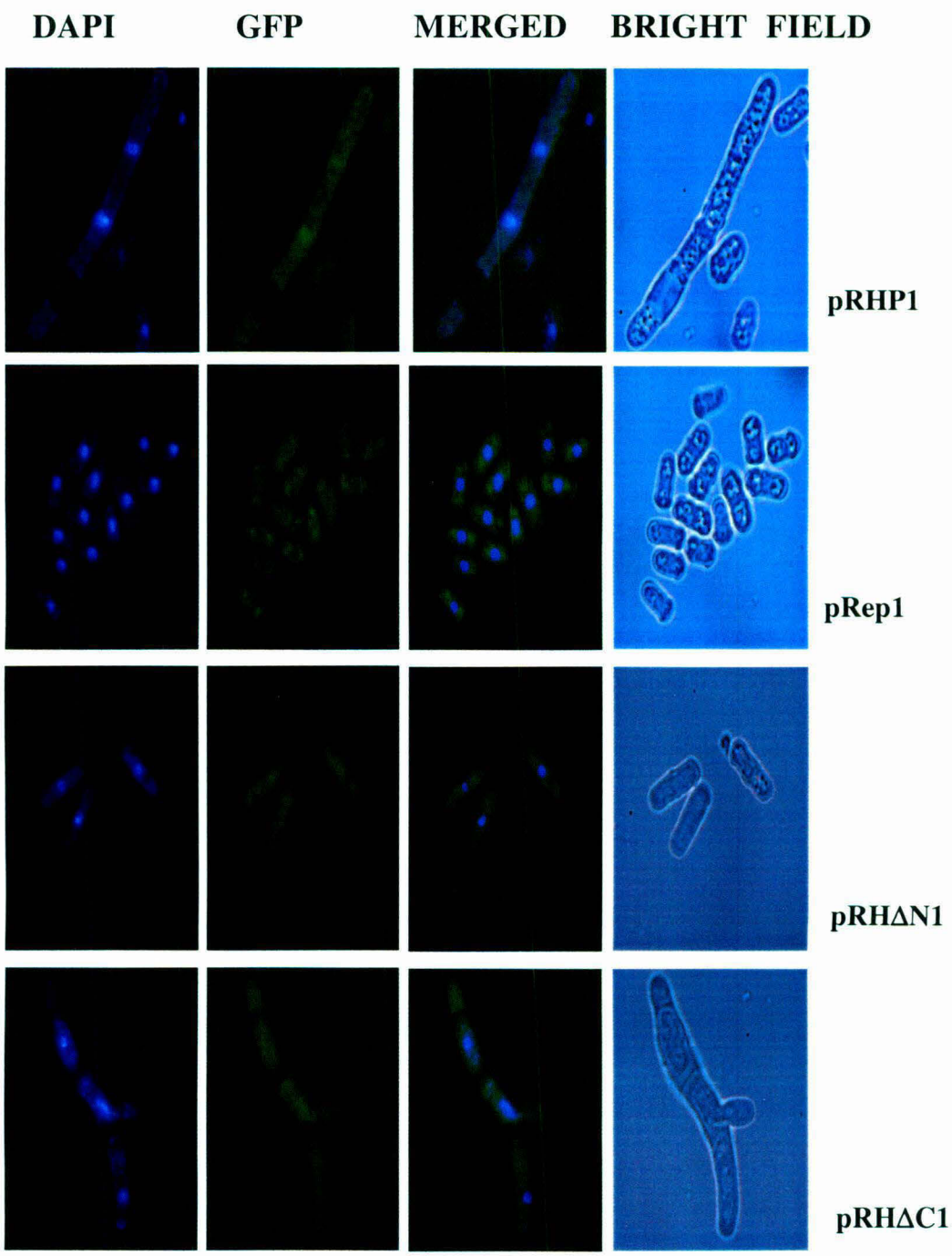

DAPI
GFP
MERGED
BRIGHT FIELD
pRHP1
pRep1
pRHΔN1
pRHΔC1
18 hrs

Fig 5(Contd.)



24 hrs

Fig 5(Contd.)



36 hrs

Fig 5(Contd.)

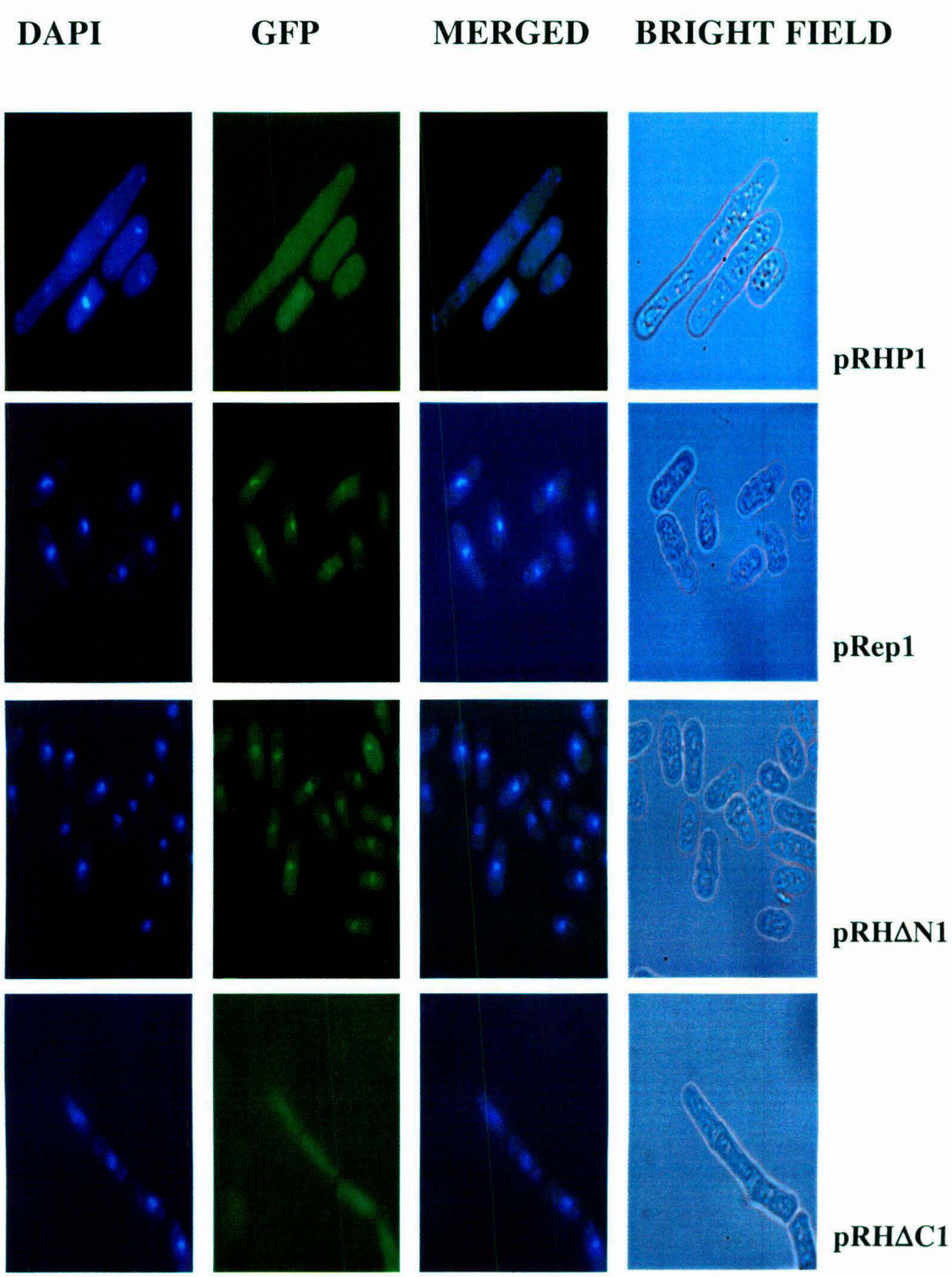

DAPI
GFP
MERGED
BRIGHT FIELD
pRHP1
pRep1
pRHΔN1
pRHΔC1
47 hrs

Fig 5(contd.)

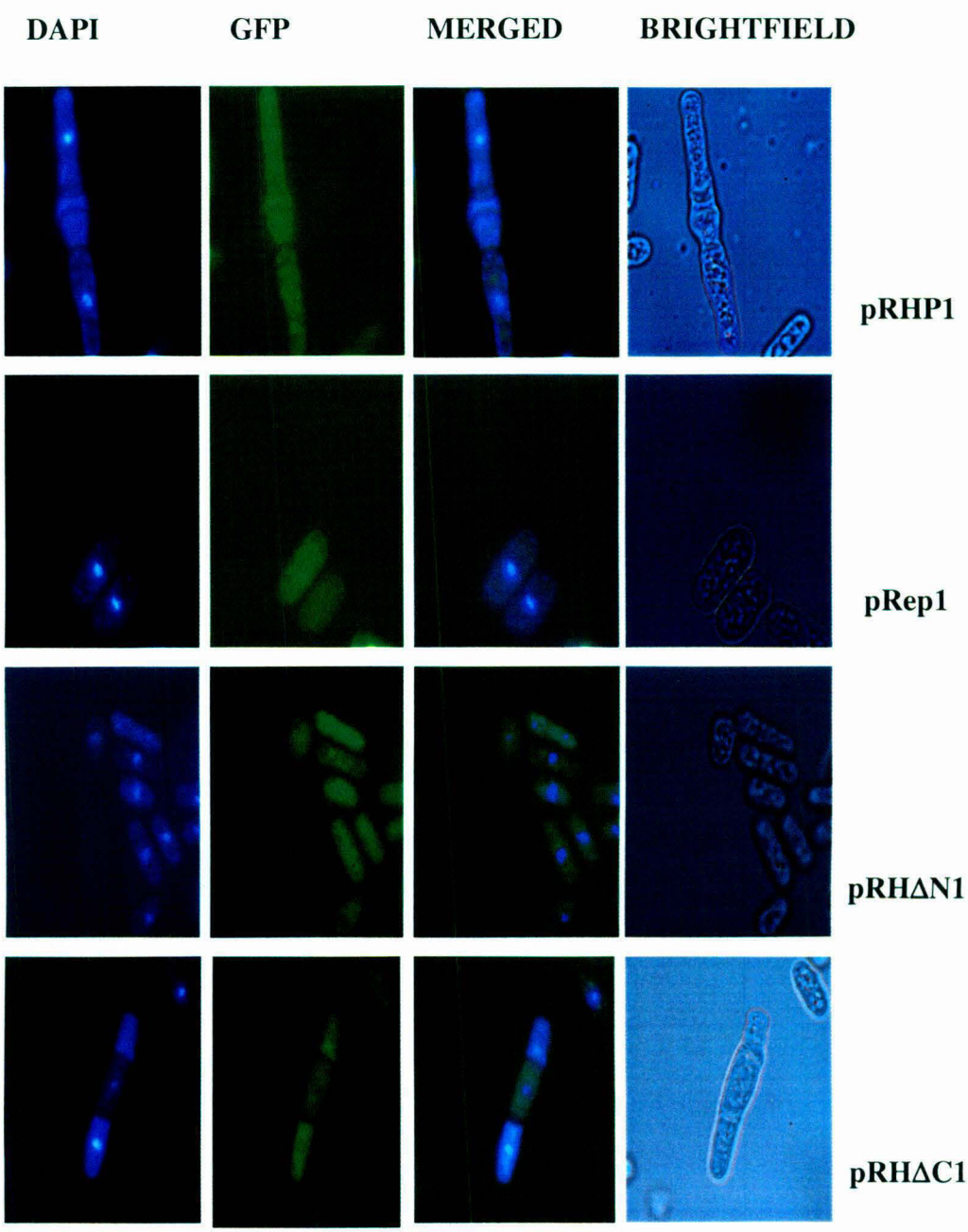

DAPI
GFP
MERGED
BRIGHTFIELD
pRHP1
pRep1
pRHΔN1
pRHΔC1
72 hrs

Fig 5h (contd)

points and plotted graphically. As seen in Fig. 3, the growth pattern of pRHΔN1 follows that of the vector transformed cells while that of pRHΔC1 strain is similar to that of pRHP1 transformed cells. Also, it was evident that the growth inhibition displayed by pRHΔC1 transformed cells is less than the pRHP1 transformed cells.

6.B.iv PROPIDIUM IODIDE/CALCOFLUOR STAINING OF *S. pombe* SHOW pRHΔN1 TRANSFORMED CELLS HAVING A NORMAL MORPHOLOGY WHILE pRHΔC1 TRANSFORMED CELLS WITH A MULTI-NUCLEATED MORPHOLOGY WITH SOME HYPHAL PROJECTIONS

pRHΔN1 and pRHΔC1 transformed *S. pombe* (MBY 624) cells are grown in EMM leu$^-$ medium and their growth rates measured at different time points (0, 8, 12, 18, 24, 36, 48, 72 hrs respectively) spectrophotometrically. Samples of 10^7 cells were accordingly collected at such time points and fixed with ethanol. Later these cells were rehydrated and were stained with Propidium iodide (for nucleus) and Calcofluor (for cell wall and medial septa). The results shown in figure 4 demonstrate that the pRHΔN1 transformed cells exhibit a morphology similar to that of pRep1 transformed cells (see Fig. 5, Chapter 1) while pRHΔC1 transformed cells show an aberrant morphological features like multiple nuclei, abnormal septa and in a few cases hyphal projections at all time points. This implies that the abnormal morphological features seen in HABP1 expressing cells are also present in pRHΔC1 transformed cells.

6.B.v LIVE CELL STAINING OF TRANSFORMED *S. pombe* STRAIN MBY 624

To eliminate the possibility of any artifact resulting due to the use of ethanol fixed cells for microscopic analysis, live cell staining at different time points (0,6,12,18,24,36,47 and72 hrs respectively) was performed with *S. pombe* MBY 624 cells transformed with pRHΔN1 and

Fig 6: Shows the ultrastructural analysis of pRep1, pRHP1, pRHΔN1 and pRHΔC1 transformed *S. pombe* cells at different time points. In the panel to the right the observed morphological abnormalities of multiple cell septum increase in vacuoles and elongation in pRHP1 transformed *S.pombe* cells are clearly visible vis-à-vis the pRep1 transformed cells. Similar abnormalities are also seen in pRHΔC1 transformed cells too in the right most panel. The pRHΔN1 transformed cells as seen in the second panel from the right display normal morphology like that of the pRep1 transformed cells shown in the second panel from the left.

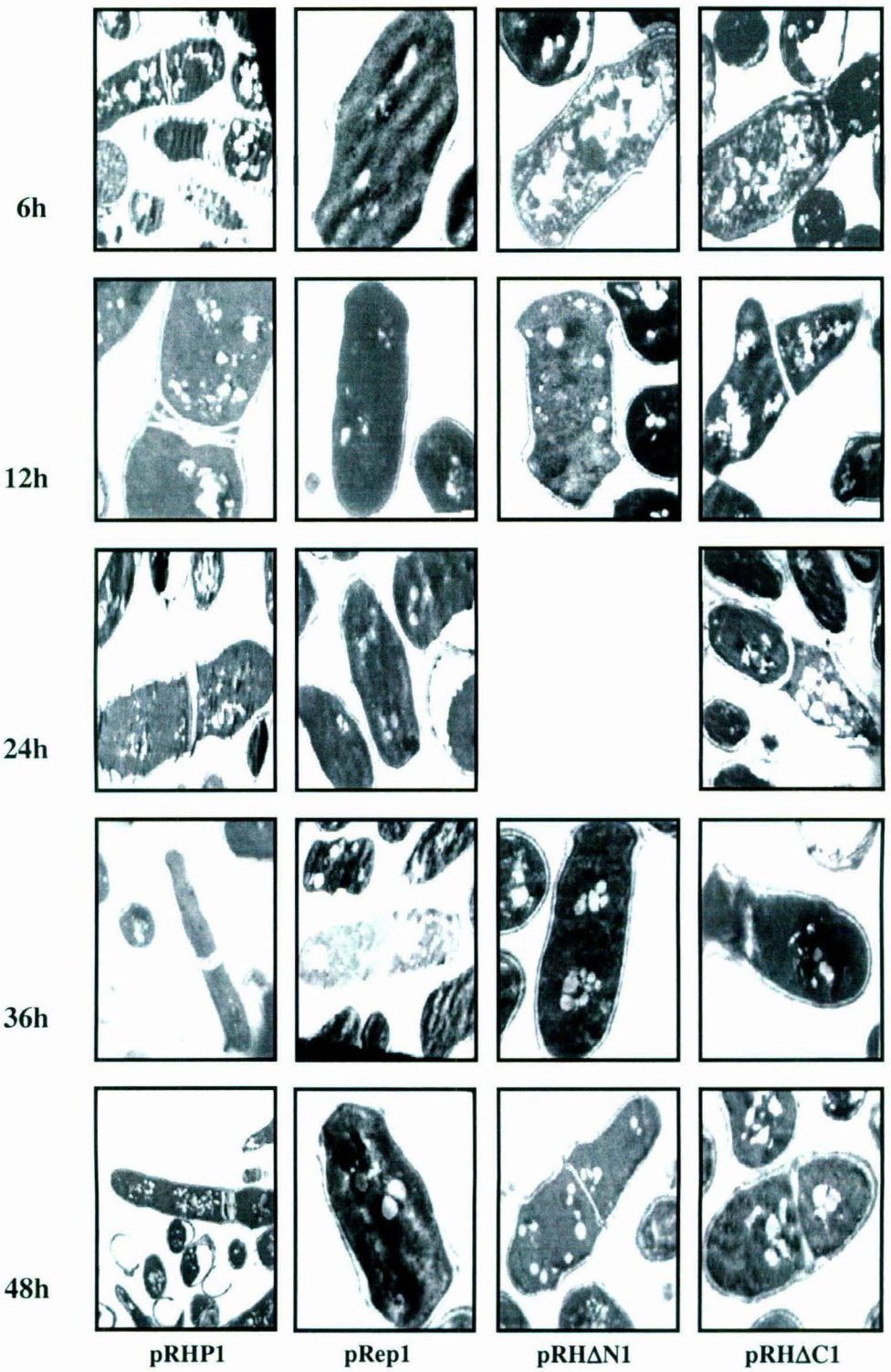


Fig 6

pRHΔC1. The regulatory light chain (RLC) of myosin is tagged with GFP in this strain. The nuclei of the living cells were stained with DAPI. The results (Fig. 5) show that pRHΔN1 transformed cells display a normal morphology just like pRep1 transformed cells while pRHΔC1 transformed cells show the same morphological abnormalities as pRHP1 transforming cells. Therefore the results obtained from ethanol fixed cells were in complete agreement with the results obtained using live cells ruling out the possibility that the abnormal morphology observed in ethanol fixed cells are artefactual in nature.

6.B.vi ULTRA MICROSCOPY OF pRHΔN1 AND pRHΔC1 TRANSFORMED CELLS

For studies with ultra microscopy *S. pombe* MBY 624 cells transformed with pRHP1, pRep1, pRHΔN1 and pRHΔC1 were grown and processed at different time points as described in Material and Methods. The morphological features of the cells were examined with an electron microscope. The results in figure 6 show that while pRHΔN1 transformed cells have complete similarity with the pRep1 transformed cells, pRHΔC1 transformed cells show a morphological changes that were similar to pRHP1 transformed cells.

6.C DISCUSSION

The results presented show that the growth inhibition shown by pRHΔN1 expressing cells is negligibly small and cells maintain a normal morphology. However the pRHΔC1 expressing cells show significantly high inhibition of cell growth similar to pRHP1 transformed cells. The morphological abnormalities exhibited by pRHΔC1 cells are quite identical to that shown by pRHP1 transformed cells. In fact as evident from the pictures, pRHΔC1 transformed cells cannot be differentiated from pRHP1 transformed cells. This observation suggests that the in *S. pombe* cells expressing ΔC.HABP1 are capable of retaining the necessary structure to discharge the same cellular functions as mature HABP1 albeit not as efficiently. Since the expression of ΔN.HABP1 does not have any significant effect on the growth and morphology of the *S. pombe* cells, it could be that the ΔN.HABP1 does not retain the necessary structure. However the biophysical analysis of recombinant HABP1, ΔN.HABP1 and ΔC.HABP1 indicate that the truncated mutants fail to trimerize but can form cysteine mediated dimers and does not display any change in their ability to bind HA. This implies that the structural attributes responsible for the HA binding property of HABP1 is not important for the rendering of its functions in *S. pombe*. Incidentally there are other structural changes due to the indicated truncations in HABP1 as have been observed by Circular Dichorism experiments (Sengupta, 2003), the functional implications of which are not yet fully understood.

The N-terminal of HABP1 shows great similarity to the WD 40 family of regulatory proteins which entails its having a signature sequence of G-H and W-D/E di-peptide usually separated by 25-27 amino acids. Such sequences predominantly form a α helix and biochemical evidence suggests that this signature motif/fold are critical for protein-protein interactions. Since in ΔN.HABP1 the entire α_A helix is

cleaved off. This destroys the coiled-coil interaction α_A has with α_B of an adjacent monomer, which is extremely important for protein-protein interactions. The hypothesis that the N-terminal α helix (α_A) is very important for protein–protein interactions in *S. pombe* is also borne out by the observed results as its seen that deletion of α_A just about abolishes the interaction of this truncated protein with other proteins in *S. pombe.* Also since the N-terminal sequence bears the WE signature motif, which is very significant for protein-protein interactions, the importance of the α_A cannot be over-estimated. However an alternative possibility is there that the loss of α_A reduces the structure of the N-terminal deleted mutant to a random coil. Further investigations are required to specify which is the correct explanation.

In case of the C-terminal deleted mutant, only half of the α_C helix has been removed. So it is possible that this deletion does not cause much change in the structure of the monomer and the α_A helix is also intact in this mutant. This may be the reason why it shows a morphology and growth patterns similar to that of pRHP1 transformed cells.

In conclusion, it is rational to state that the N-terminal helix of the HABP1 bearing WE signature is necessary for the changes it produces in *S. pombe* cells. Further studies need to be pursued to understand the structural differences between ΔN.HABP1 and ΔC.HABP1 and analyze why the two truncated proteins impart dissimilar response when expressed in *S. pombe* cells and whether that is by losing its ability to interact with other proteins like CDC25.

6.D. Summary

To summarize:

1) The two truncated mutants of HABP1 expressed in *S. pombe* have the same affinity for HA. Hence the HA binding property of

HABP1 may not be important for its causing the growth and morphological abnormalities in *S. pombe*.

2) **The N-terminal deleted mutant shows a growth pattern and morphology identical to the pRep1 transformed cells. The structure of HABP1 responsible for causing growth inhibition and morphological abnormalities is disrupted in the N-terminal deetant implying the great importance of the N-terminal α_A helix in protein-protein interactions of HABP1.**

3) **The C-terminal deleted mutant shows a growth pattern and morphology very similar to pRHP1 transformed cells implying that the structure of HABP1 responsible for causing the aberrant growth and morphology in *S. pombe* cells is intact in the C-terminal deletant.**

4) **The N-terminal α helix is important protein-protein interactions may be responsible for the abolition of abnormal behaviour caused by the expression of mature HABP1 in *S. pombe*.**

Summary and Conclusions

7.A Summary and Conclusion

Human Hyaluronan Binding Protein (HABP1) was identified in our laboratory and its role in cellular signaling was established. Molecular cloning of the gene encoding HABP1 further characterized its multifunctional nature as its sequence is identical with p32, the protein co-purified with mRNA alternate splicing factor, and the receptor of globular head of C1q, the complement protein. This gene seen to be present on the human chromosome 17p13.3 and has been represented in the human genome as the synonym: HABP1/p32/gC1qR. Although, HABP1 has been shown to interact with a few proteins by several groups and investigated its functional aspects, a lot of questions still remain to be answered. Thus in the present study to reveal its biological function/s by using the expression system of *Schizossacaromyces pombe,* the human gene HABP1 was subcloned in the shuttle vector pRep1under the strong promoter nmt1 (no message in thiamine). The simple eukaryote *Schizosaccharomyces pombe* was chosen as the expression system primarily due to its similarity with higher eukaryotes and secondly because it does not possess a homologue of HABP1. Thus, any deviation from the normal growth and morphological appearance if displayed by *S. pombe* expressing HABP1 will account for biological functions of HABP1. The expression of HABP1 in *S. pombe* cells of BJ7468 strain resulted in growth inhibition, elongation, multi-nucleation, increase in the number of vacuoles and multiple cell septum formation. These conclusions were made by studying the growth pattern, specific staining of the nucleus and cell septum and ultrastructural analysis.

It was further confirmed that the phenotypic changes, occurred, are only due to the consequence of translation of HABP1 gene as the presence of RNA transcript of the HABP1 gene does not induce any abnormality. Thus it could be due to interaction at protein level, but not

at the RNA level. The possibility of these observations being artefactual constructs due to the usage of fixed cells was ruled out by the use of the live cell staining of MBY624 strain. The specialty of this strain of *S. pombe* is that, the regulatory light chain (RLC) of myosin is tagged with green fluorescence protein (GFP) so it can be examined microscopically without fixing. Results of the live cell staining clearly supported our earlier observations that were made using fixed cells, thereby ruling out the possibility of occurrence of artefactual constructs in our case.

Surprisingly the localization of HABP1 appeared to change from the cytoplasm to the nucleus when the cells shifted from the lag phase to the log phase. It has to be mentioned here that HABP1 has already been shown to be an endogenous MAP kinase substrate which exhibits MAP kinase dependent nuclear translocation under mitogenic stimulation (Majumder *et al.,* 2002).

FACS analysis of HABP1 producing *S. pombe* cells too confirms the microscopically observed increase in DNA content of the fission yeast cells in the form of multinucleation. All the data presented here suggests that a defective cell cycle progression occur with the expression of HABP1 in *S. pombe*. Incidentally the growth inhibition and defective morphology has been already reported in *S. pombe* with the expression of several genes. Expression of pro-apoptotic protein like Bax produced a significant inhibition on cell growth in *S. pombe* which was seen to be rescued when the anti-apoptotic protein Bcl-XL was co-expressed (Jurgensmeier *et al.,* 1997). Interestingly it must be pointed out here that the overexpression of HABP1 in mammalian fibroblast cell line induces the expression of Bax (Meenakshi *et al.*, 2003). On the other hand, fission yeast cells also exhibit growth inhibition when the tumor suppressor protein p53 was expressed in it. The morphological changes like cell elongation, multi-nucleation and aberrant cell septum formation is also seen in several genetic backgrounds. An identical morphology is

seen in cell separation mutants like sep mutans and kic 1^+ and eng 1^+ disrupted strains (Zilahi *et al.,* 2000;Tang *et al.,* 2003; Martin-Cuadrado AB *et al.,* 2003). A similar elongation and multi-nucleation is seen in cdc 2 and cdc 25 disrupted strains (Daga and Jimenez., 1999). Thus our present data concludes that expression of HABP1 in *S. pombe* resulting in growth inhibition may be due to defective cell cycle regulation.

In continuation, to study if HABP1 can regulate cell cycle by interacting with the proteins that allows the cells to escape from one phase and to enter another, the JK2423 strain of *S. pombe* was used. The advantage of JK2423 is that the CDC25 tagged with 12 epitope of 'myc' was introduced. Therefore, the expression of CDC25 in this strain can be easily monitored by using the commercially available myc antibody. At the same time, the cell cycle regulatory protein CDC2 having a specific motif PSTAIR can also be detected using commercial antibody against this motif.

The JK2423 was chosen and after introducing HABP1 gene, initially the cell growth and the morphology of their strain was examined. After confirming the identical morphological changes and similar growth inhibition that occurred in other strains, the expression of CDC2 and CDC25 was monitored. The expression profile of both CDC2 and CDC25 differs in pRHP1 transformed *S. pombe* cells, compared with the vector transformed cells. The maximum expression of CDC2 or CDC25 was both delayed in HABP1 expressed cells. An interesting observation was made that when CDC25 expressed in a higher amount, HABP1 expression was also the highest and HABP1 was seen to co-immunoprecipitate with CDC 25 at those time points. As already discussed CDC25 is a cell cycle regulating protein that has the tyrosine phosphatase activity. CDC25 was been shown to dephosphorylate Tyr^{15} of CDC2, which in turn activates the Mitosis Activating Factor (MPF) which consists mainly of the heterodimer CDC2-CDC13. Thus

availability of CDC25 regulates the activation of MPF, allowing the cells to enter into mitotic phase. Most interestingly similar phenotypic changes as seen here are also observed in CDC25 deficient mutant. Thus it can be hypothesised that the differential expression of cell cycle regulatory proteins like CDC2 and CDC25 and the specific interaction of HABP1 with CDC25, regulates the availability of CDC25, results in the cell arrested of G2/M boundary. This causes the cells to elongate as has been observed in cdc 25 disrupted strains. At times multinucleation is also observed with possible complications with other proteins involved in cytokinesis along with this.

It is an well established fact that function of a protein depends on its three dimensional structure and by altering its structure, one can identify the sequence which are responsible for its function. The resolution of the crystal structure of p32 showed it to be trimeric in nature. However if its terminal regions are deleted, instead of the trimeric assembly, but it forms cysteine^{186} mediated dimer which intriguingly have identical affinity with hyaluronan (Sengupta, A., 2003). Thus in order to examine whether HABP1 has any special structural requirement to discharge its role in *S. pombe* cells, they were transformed with clones of the N and C terminal truncated mutants of the protein. Expression of both the N-terminal and C-terminal deleted proteins were confirmed in *S. pombe* by immuno-blotting with monoclonal anti-HABP1 antibody. Interestingly, it was noted that the N-terminal deleted clone showed absolutely normal morphology, identical to the cells transformed with the vector pRep1 while the cells transformed with the C-terminal deleted clone displayed an abnormal morphology very similar to the one shown by pRHP1 transformed cells.

Here it also needs to be pointed out that since both the deleted mutants have similar affinity towards HA, the perturbations produced in *S. pombe* cells on expression of HABP1 is not linked with the HA binding

activity of HABP1. Also HA is not known to be present in fission yeast cells.

However, the N-terminal α helix α_A (the part of the N-terminal that was deleted to generate the N-terminal deleted clone) seems to be very important since the absence of this helix abolishes the growth inhibition and abnormal morphology caused by HABP1 expression in *S. pombe*. As appeared from the crystal structure the N-terminal helix αA does not contact the β sheet within the same monomer, but forms an anti-parallel coiled coil with the C-terminal portion of α_C (the second C-terminal α helix) of an adjacent monomer. This coiled-coil region is not only responsible for homo-oligomerisation but also important for protein-protein interactions (Jiang *et al.,* 1999). Further structural analysis of the crystal indicate the N- terminal sequence is similar to the WD 40 family of regulatory proteins.

The WD 40 family of proteins which constitute a class of regulatory protein which has a signature sequence of G-H and W-D/E dipeptide usually separated by 25-27 amino acids. Such sequences predominantly forms a α helix and biochemical evidence suggests that this signature motif/fold are critical for protein-protein interactions.RACK1, RACK2, Calreticulin and a number of PKC regulatory proteins are prominent members of this family (Iwasaki *et al.,* 1995; Mochly-Rosen *et al.,* 1991a; Mochly-Rosen. *et al.,* 1991b).HABP1 has this WE signature motif at its N-terminal segment through which HABP1 has been reported to interact with other proteins like gC1q, GABA(A) receptor , the β subunit light chain of unphosphorylated myosin thus showing its involvement in protein-protein interactions. This also implies that the HABP1 functions in a compartment specific manner rather like a molecular chaperon. Thus the unperturbed growth and normal phenotype of *S. pombe* cells transformed with pRHΔN1 suggests the importance of of the N-terminal

segment with respect to protein-protein interactions even in *S. pombe.* Now if this is the case then the interaction of the N-terminal deleted mutant has to be investigated with CDC25 as the full length HABP1 has already been seen to interact with CDC 25. If the hypothesis that the N-terminal α helix being important for protein-protein interactions is true then there should be no interaction of ΔN.HABP1 with CDC 25.

However just this investigation would not explain all the morphological abnormalities witnessed upon expression of HABP1 in *S. pombe.* Interactions of HABP1 with proteins important for cell separation defects should also be seen. Though not much is known about exactly how the dissolution of the cell septum takes place after the end of mitosis, some proteins and protein complexes have been identified which seem to be responsible for cleavage of the primary cell septum. These include protens like sep 1 and eng 1p and the octameric exocyst complex. Interactions of HABP1 with these proteins and protein complexes need to be investigated.

Apparently though our present data does not throw any light on the mechanism of the observed abnormal septum formation a recent report may throw some light on how HABP1 may be involved in this process. p32/HABP1 was seen to co-purify a long time back with the human alternate splicing factor ASF/SF2, the significance of which was not understood then. Presently it is known that the functioning of alternate splicing factor ASF/SF2 depends greatly on its phosphorylation status and it is phosphorylated by CLK/STY a member of a family of dual specific serine threonine kinases implicated in the regulation of a number of cellular processes like cellular growth and differentiation. CLK/STY is known to phosphorylate the serine arginine rich area of ASF/SF2, which in turn regulates mRNA splicing (Prased *et al.*, 1999, 2003). There is another protein SRPK1 (human SR protein specific kinase 1) which also interacts with ASF/SF2 in a manner similar to CLK/STY,

phosphorylation being the main area of interaction (Colwill *et al.*, 1996). The interactions of CLK/STY and SRPK1 with ASF/SF2 seem to be competitive in nature. Now the interaction of p32/HABP1 with ASF/SF2 has been seen to be physiologically relevant. P32/HABP1 has been found to belong to a group of proteins which sequester ASF/SF2 and prevents it from interacting with mRNA thereby affecting mRNA splicing (Petersen-Mahrt *et al.*, 1999). Now the *S. pombe* homologue of SRPK 1 is a gene known as dsk 1^+ and a *S. pombe* homologue of CLK/STY has just been discovered to be a gene called kic1^+ (Tang *et al.*, 2000, 2003). The protein families of CLK/STY and SRPK1 are conserved across the species from fission yeast to humans and so the possibility of a functional interaction of a human protein with a member of these families cannot be ruled out. Now it has also been observed that kic1^+ has a close interaction with dsk 1^+ and together they regulate the cell surface and septum formation along with a late step in cytokinesis in *S. pombe*. Since expression of HABP1 in *S. pombe* creates abnormal cell septum formation and cell separation defect, it could be speculated that it sequesters kic 1^+ and dsk1^+ and causes the observed morphology. However the exact role of HABP1 specially keeping in mind the rescuing of the morphology on expression of the N-terminal mutant needs to be studied in details.

In fact availability of perturbed morphology along with growth inhibition of *S. pombe* cells due to the introduction of HABP1 and its rescue from these on expression of the N-terminal deleted HABP1 mutant gives us a model whereby the observed morphological and cell growth characteristics of *S. pombe* can be studied in depth at the molecular level.

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