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Growth Arrest and Induction of Apoptosis Due to Downregulation of Happl by Gene Disruption in HeLa Cells
Introduction:

Hyaluronan Binding Protein 1 (HABP1), an important multifunctional hyaladherin, was first reported, long time back as a naturally occurring, HA-binding protein in rat liver [D’souza and Datta, 1985]. It is a ubiquitously expressed, highly negatively charged, multifunctional mammalian glycoprotein, being identical with p32/gC1qR [Deb and Datta, 1995; Das and Datta, 1996] and named as HABP1 by Hugo Nomenclature Committee. It is synthesized as a 282 amino acid long pro-protein, which is post-translationally cleaved at the N-terminus into a 209 amino acid long mature protein [Honore et al., 1993]. However, the pro-protein form of HABP1 has not been detected in any tissue except the germ cells [Bhardwaj et al, 2002], as it is present in a very labile state in most other tissues. The pro-protein form of HABP1/p32/gC1qR has a mitochondrial localization signal which is cleaved off in mature protein after its translocation into mitochondria. Major part of endogenous HABP1 is localized in the mitochondria, and mitochondrial matrix. [Muta et al, 1997; Matthews and Russell, 1998].

Studies accumulated over the decades provide evidence that HABP1 is a ubiquitously expressed, multifunctional chaperone protein with significant role multiple cellular pathways. Its direct or indirect role has been implicated in vastly different physiological processes like cell adhesion, migration and proliferation, tumorigenesis, inflammatory reactions, blood coagulation, cell-growth arrest, apoptosis, autophagy etc. HABP1 is a ligand of hyaluronan and it is well documented that hyaluronan and its ligands have critical role in morphogenesis, cell-growth and differentiation, cell signaling and carcinogenesis. HABP1 is also known to interact with numerous, apparently unrelated, intra- and extra-cellular regulatory proteins and other biological molecules that serve as its ligands at various cellular localizations and it may be postulated that the interaction of HABP1 with a vast array of proteins and ligands accounts for its diverse functional role in various cellular pathways and determines its specific functions at different cellular compartments.
However, diverse cellular responses of HABP1 and numerous interacting cellular molecules, essentially indicate its distributed presence at extra-mitochondrial locations in different cell types. HABP1/p32/gC1qR has been reported to be localized on the cell surface [Gupta & Datta, 1991], where it interacts with HA, D-glycosylated Mannose (DMA) and proteins like C1q, h-kininogen, Factor-XII, vitronectin, fibrinogen, cC1qR or calreticulin homologue, MT1-MMP and several plasma proteins that mediate the role of HABP1 in cell adhesion, anchorage and spreading, in inflammatory reactions, in procoagulant activity. Intracellular location of HABP1 in the cytosol [Dedio et al, 1998]) and mitochondria [Muta et al, 1997] reportedly interacts with MAPK, Hrk, PKC and its isoforms, Cytochrome b, α1B-AR and some viral proteins. The interaction of HABP1 with these cytosolic and mitochondrial proteins provides a clue to its possible role in regulating Hrk-induced apoptosis, compartmentalization of PKC and regulating its kinase activity and so on. Recently, HABP1/p32/gC1qR was reported to interact physically with both human and murine smARF, the inducer of type II autophagic cell death. smARF, though a short lived protein, is stabilized by its interaction with HABP1/p32/gC1qR and subsequent translocation to mitochondria wherein it initiates p53 and Bcl-2 independent, autophagic cell death [Reef et al., 2006]. Functional interaction of HABP1 with several viral proteins like adenovirus core protein, EBNA-1, HCV-core protein, HIV-I tat and HIV-I-Rev suggests its possible role in pathogenesis. Of special interest is the mounting evidence of localization of HABP1 in the nucleus under different pathological and non pathological conditions as reported from our laboratory as well as by other researchers.

HABP1/p32/gC1qR was initially reported as a subunit of human pre-mRNA splicing factor SF2 and was copurified with ASF/SF2 from HeLa cell extracts [Krainer et al., 1991]. Subsequently, cDNA analysis established complete homology between p32 and HABP1 [Deb and Datta, 1995]. Interaction of HABP1 with ASF/SF2 blocks its phosphorylation which is
essential for stable RNA binding during the formation of splicesosome. HABP1 prevents stable interaction of ASF/SF2 with RNA thus inhibiting its function as a splicing factor [Xiao and Manely, 1997; Peterson-Mahrt et al., 1999]. HABP1 has been reported to interact with Lamin B receptor, an inner nuclear membrane protein that forms an in vivo complex with the nuclear lamins A and B, a nuclear envelope kinase (p58), and two other nuclear proteins with apparent molecular mass of 18kDa (p18) and 34kDa (p34) [Simos and Georgatos, 1992]. p32/HABP1 forms a complex with LBR/p58 and functions as a linking component between the nuclear membrane and the intranuclear structure involved in RNA splicing. The mammalian CCAAT-binding factor (CBF), also called nuclear factor Y (NF-Y), consisting of three different subunits, CBF-A, CBF-B and CBF-C, is a strong transcriptional activator, that forms a complex with promoter DNA containing the CCAAT motif. HABP1/p32 was copurified with CBF complex from the HeLa cell extracts and later on was found to interact specifically with CBF-B subunit inhibiting CBF-mediated transcription by recombinant as well as endogenous CBF. The functional interaction between HABP1 and CBF may also regulate signalling between the mitochondria and the nucleus [Chattopadhyay et al., 2004].

Previous reports from our laboratory have provided convincing evidence of involvement of HABP1 in various signalling pathways. HABP1, in association with HA is implicated in cellular functions like cell adhesion and tumor invasion [Gupta and Datta, 1991], spermatocyte interaction [Ghosh et al., 2003], spermatogenesis and sperm motility [Ranganathan et al., 1995] and cellular signaling [Rao et al., 1996]. It has been shown that there occurs an enhancement in the phosphorylation of HABP1 upon HA and PMA stimulation suggesting its critical role in cell signaling and formation of second messenger [Rao et al, 1996, 1997]. Further, we have demonstrated that HABP1 is an endogenous substrate of ERK and MAPK. The utility and versatility of MAP kinase cascade in determining the balance between cell
growth and apoptosis is well established. HABP1 acts as an integral part of the MAP kinase cascade and is translocated to the nucleus upon mitogenic stimulation. [Majumdar et al, 2002]. Subsequent studies have elucidated a critical role of HABP1 during cell cycle regulation in organisms ranging right from yeast to mammals. It has been documented that overexpression of HABP1 in mammalian fibroblast (F111) cell-lines causes perturbed cell growth and extensive vacuolation in these cells and arrests these cells at G1/S transition, subsequently leading to apoptosis. However serum replenishment allows a 5% increase in the number of cycling cells entering from G0/G1 to S-phase of growth cycle [Meenakshi et al., 2003]. We have also reported the sub-cellular localization of HABP1 in the golgi network and its distribution pattern during mitosis in COS1 cells [Sengupta et al., 2005] This study shows that during interphase, HABP1 is dispersed throughout the cell and is also localized in the nucleus. In subsequent stages of mitosis, however, it continues to be dispersed throughout the cell, but is excluded from the space occupied by the DNA or the de-condensing nuclei. The N-terminal and C-terminal truncated variants of HABP1 induce autophagic vacuolation and disruption of f-actin network in mammalian cell lines [Sengupta et al., 2004] It has also been reported from our laboratory that constitutive expression of HABP1 in S. pombe disrupts cell cycle regulation, causing growth retardation and induces morphological aberrations like multi-septa formation by interacting with regulatory proteins like Cdc25 through its N-terminal α-helix [Mallick and Datta, 2005]. Constitutive expression of HABP1 in normal murine fibroblast (F111) cells induces excessive ROS generation causing mitochondrial dysfunction thereby leading to retarded cellular growth and morphological abnormalities which ultimately induces apoptosis by the intrinsic pathway [Chowdhury et al., 2007]. Interestingly, the stable upregulation of HABP1 in HeLa cells which is an aggressive human cervical cell line also induces growth perturbations and abnormal phenotype such as decreased nuclear/cytoplasmic ratio, increased Bax expression and subdiploid
population along with two fold increase in ROS (Anupama thesis, unpublished observation). The enhanced level of HABP1 has been shown to be critically associated with cisplatin mediated apoptosis in HeLa cells [Komal and Datta, 2006].

With this background, we proceeded to investigate if the downregulation of HABP1 has any effect on cell growth in HeLa cells and thus establish the physiological significance of the expression level of HABP1 in these cells in maintaining its survival and cell proliferation. For this we employed vector based gene disruption of HeLa cells by siRNA mediated downregulation of HABP1 in HeLa cells. The siRNA clones pSilHABP1-570 and the scrambled RNA clone pSilHABP1-SC were generated and kindly given by Dr. A. Komal.

RESULTS:

[1] Confirmation of downregulation of HABP1 with pSil/HABP 570 in HeLa cells:

HeLa cells were cultured and maintained in high glucose DMEM as described in Materials and methods. HeLa cells were grown in 60 mm culture dishes for 24 hours following which the complete DMEM was replaced with serum free DMEM and the cells were transiently transfected with the silencing RNA for HABP1. All transfections were done using Lipofectamine according to the manufacturer’s protocol. pSil/HABP1 570 was used as the silencing RNA for HABP1 and pSil/HABP1 SC, with the scrambled sequence, was used as the control vector. Appropriate controls in the form of HeLa cells transfected with Lipofectamine only and untreated HeLa cells were also maintained simultaneously. The cells were replenished with DMEM containing 10 % FBS after 8 hours of transfection. The transfected and untransfected cells were collected after 36 hours of transfection, lysed and resolved on 12.5% SDS-PAGE and transblotted onto PVDF membranes as
Fig 3: Confirmation of downregulation of HABP1 in HeLa cells by gene disruption of HeLa using vector based RNAi technique (A) Immunoblot analysis with polyclonal anti-HABP1 confirms reduced expression of HABP1 in HeLa cells transiently transfected with HABP1 siRNA, pSil-HABP1-570, while the scrambled RNA pSil-HABP1-SC and the reagent control had negligible effect, with respect to untransfected HeLa. Purified recombinant HABP1 was also loaded as a positive indicator. Equal loading was ascertained by immunoblotting with polyclonal anti-GAPDH antibody (B) and fold change was calculated with respect to untreated HeLa cells.
described in materials and methods. Immunodetection of these blots with polyclonal anti-HABP1 (Fig. 3A) showed a reduced expression of HABP1 in HeLa cells transiently transfected with HABP1 siRNA, pSil-HABP1-570), while the scrambled RNA pSil-HABP1-SC and the reagent control had negligible effect, with respect to untransfected HeLa. Equal loading of lysates in each lane was ascertained by immunoblotting with polyclonal anti-GAPDH antibody (Fig 3B). The HABP1-bands were normalised against corresponding GAPDH bands and fold change was calculated, which revealed approximately 0.5 fold reduction in levels of HABP1 in the HeLa cells transfected with pSil/HABP1 570 with respect to the untransfected HeLa. Thus, downregulation of HABP1 in HeLa cells by gene disruption of HeLa using vector based RNAi technique was confirmed.

[2] Growth retardation in HeLa cells with downregulation of HABP1

In order to analyse the growth profile of HeLa cells upon downregulation of HABP1, non-radioactive cell proliferation assay was performed for HeLa cells transiently transfected with HABP1 siRNA, pSil/HABP1-570; with scrambled siRNA, pSilHABP1-SC; reagent control and untransfected HeLa at various time points from the time of transfection as described in the previous section. The observations reflected in the growth curve (Fig. 4) depict that HeLa transfected with pSilHABP1-570 showed a progressively reduced cell growth as compared to the control and untransfected HeLa and the difference in cell viability is maximum at 36 hours after transfection.

[3] Insignificant ROS generation in HeLa cells on downregulation of HABP1

The growth retardation in the HABP1 disrupted HeLa cells prompted us to check the generation of ROS in cells. With the use of redox responsive fluorescent dye H2DCFDA, the ROS level inside the cells was measured
Fig 4: Reduced viability of HeLa cells upon downregulation of HABP1. Non-radioactive cell proliferation assay was performed for HeLa cells transiently transfected with HABP1 siRNA (pSilHABP1-570), with scrambled siRNA (pSilHABP1-SC), reagent control and untransfected HeLa at various time points from the time of transfection, as indicated. HeLa transfected with pSilHABP1-570 showed a progressively reduced viability and the maximum reduction in survival was observed at 36h. Each value represents means ± SD for three independent experiments.
Fig. 5: Detection of internal ROS generated in the HABP1 disrupted HeLa cells. Very nominal level of ROS was generated in HeLa cells upon downregulation of HABP1 (~1.5/mg protein) with respect to untransfected HeLa or HeLa transfected with the scrambled control or the reagent only.
using fluorescence assay. However, fluorimetric data revealed that there occurs only a nominal level of ROS generation (~1.5fold) in HeLa cells in which HABP1 had been downregulated with respect to the untransfected HeLa or the HeLa cells transfected with the scrambled control or the reagent only (Fig 5).

[4] Cellular localization of HABP1 with its downregulation in HeLa cells:
After confirming the downregulation of HABP1 in HeLa cells, we proceeded to examine the localization pattern of HABP1 in the transiently transfected HeLa cells. Cells were seeded on separate coverslips 24 hours prior to transfection, following which, Lipofectamine™ mediated transfection was done as described. Cells were transfected with pSil/HABP1 570, the silencing RNA for HABP1 and the scrambled sequence pSil/HABP1 SC was used as the control vector. Appropriate controls in the form of HeLa cells transfected with Lipofectamine only and untreated HeLa cells were also maintained simultaneously. After 36 hours of transfection, immunostaining of these cells was performed according to the protocol described for detecting the expression profile and localization of HABP1 inside as well as on the cell surface. Immunofluorescence data (Fig 6A) revealed that HABP1 was more or less uniformly distributed in the entire cell i.e. both in the cytosol and nucleus, in case of untransfected HeLa cells and in control transfectants. Interestingly, in case of HeLa cells in which HABP1 was downregulated by vector based RNAi technique, nuclear exclusion of HABP1 is observed along with its reduced cytoplasmic level. However, membrane localization profile of HABP1 in HeLa cells showed uniform distribution with decreased expression in both the silenced and control cells as depicted in fig. 6B.
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Fig 6A: Intracellular localization profile of HABP1 in HeLa cells upon silencing HABP1: Immunocytochemical analysis showing reduced expression of HABP1 (green fluorescence) and its absence from the nucleus upon silencing of HABP1 gene using RNAi technique as compared to HeLa cells transfected with scrambled RNAi vector or the reagent only or the untransfected HeLa.
**Fig 6B: Membrane localization profile of HABP1 in HeLa cells upon silencing HABP1:** Immunofluorescence data showing uniform membrane distribution of HABP1 in HeLa cells upon HABP1 downregulation by silencing HABP1 gene using RNAi technique as well as in normal HeLa cell line and cells transfected with scrambled RNAi vector or the reagent only.
[5] **Morphological changes in the HeLa cells following downregulation of HABP1:**

Further, we examined the morphology of the HeLa cells upon the downregulated expression of HABP1. Cells seeded on coverslips were transfected with pSil/HABP1 570, pSil/HABP1 SC and Lipofectamine™ and untransfected HeLa was also maintained. After 36 hours of transfection, haematoxylin-eosin staining was done to check the resultant phenotype. The nucleus of the HeLa cells was visibly smaller following HABP1 downregulation with respect to the control and untransfected HeLa cells (Fig.7). Statistical analysis revealed a significant change in the nuclear/cytoplasmic ratio of pSilHABP1_570 which is 0.384 as against 0.801, the mean of ratios of the three different control cell lines.

[6] **Confirmation of subdiploid population and variation in Bax levels upon downregulation of HABP1:**

To examine the role of HABP1 on induction of apoptosis, pSil/HABP1-570 and pSil/HABP1 SC was transiently transfected in HeLa cells along with appropriate controls. After 36 hours of transfection, FACS analysis of the propidium iodide stained showed an increase in the percentage of subdiploid population from ~5 % in case of the control and untransfected HeLa cells to ~20 % in the HeLa cells in which HABP1 had been downregulated (Fig. 8), thus indicating a possible involvement of HABP1 in apoptosis.

To substantiate our observation, lysates of HeLa cells transiently transfected with HABP1 siRNA pSilHABP1-570, the scrambled RNA pSilHABP1-SC, reagent control and untransfected HeLa cells resolved on SDS-PAGE were immunodetected with monoclonal anti-Bax confirmed enhanced Bax expression in HeLa cells following downregulation of HABP1 (Fig 9A). The equal amount of protein in lysates was confirmed by immunoblotting with polyclonal anti-GAPDH (Fig 9B) and fold change was
Fig 7: Morphology of HeLa cells following downregulation of HABP1 in HeLa by vector based RNAi technique. HeLa cells were transiently transfected with HABP1 siRNA, pSilHABP1_570 showed altered morphology with respect to cells transfected with the scrambled sequence pSilHABP1_SC. The bar represents 10μm. Each experiment was performed in triplicate.
Fig. 8: FACS Analysis of the HABP1 silenced HeLa cells: Flowcytometry data of the HeLa cells transfected with psilHABP1-570 shows a distinct increase in the subdiploid population by psilHABP1-570 with respect to the scrambled and untransfected control. The result of FACS analysis is graphically represented in fig. 8B.
Fig 9: Upregulation of Bax in HeLa cells upon transient downregulation of HABP1 indicates induction of apoptosis. Lysates of HeLa cells transiently transfected with HABP1 siRNA pSilHABP1-570 (lane 1), the scrambled RNA pSilHABP1-SC (lane 2), reagent control (lane 3) and untransfected HeLa cells (lane 4) resolved on SDS-PAGE were immunodetected with monoclonal anti-Bax (A). The equal amount of protein in lysates was confirmed by immunoblotting with polyclonal anti-GAPDH (B) and fold change was calculated.
Fig 10: Enhanced expression Bax in HeLa cells upon silencing HABP1:
Immunofluorescence data revealed upregulation and peri-nuclear localization of 
Bax in cells in which HABP1 is downregulated using RNAi technique as 
compared to normal HeLa cells (panel 4) and cells with reagent control and 
scrambled control.
calculated. Statistical analysis revealed approximately two fold increase in Bax expression following HABP1 downregulation in HeLa cell. Immunocytostaining of similarly transfected HeLa cells was performed using anti-Bax antibody and Mitotracker green. Immunofluorescence data showed profuse expression of Bax, with predominant perinuclear localization in HeLa cells transfected with pSil/HABP1 570 (Fig 10). Although there's an increase in Bax expression in HABP1 downregulated HeLa cells, but it is not colocalized with Mitotracker suggesting the involvement of the extrinsic pathway in the induction of apoptosis.

[7] Confirmation of apoptosis induction by analyzing the expression of p53, Mdm 2, p21:

In an attempt to confirm the induction of apoptosis and analyse the probable pathway, we proceeded to analyse the expression level and localization profile of p53 and p21 in these HeLa cells.

To begin with, lysates of HeLa cells transiently transfected with HABP1 siRNA pSilHABP1-570, the scrambled RNA pSilHABP1-SC, reagent control and untransfected HeLa cells were resolved on SDS-PAGE and immunodetected with polyclonal anti-p53 (Fig 11A.), anti-Mdm2 (Fig11B.) or anti-p21 (Fig11C). Equal loading in each blot was ascertained by immunodetection with anti-GAPDH antibody and fold change was calculated. Taken altogether, it was established that downregulation of HABP1 in HeLa cells leads to a significant increase, around 3 folds, in the expression level of p53 and simultaneously in the expression level of p21, around 2 folds, along with downregulation of Mdm2 proteins, thus indicating p53 dependent cell-growth arrest and apoptosis in HeLa cells upon downregulation of HABP1.

Immunofluorescence data of HeLa cells transiently transfected with pSil/HABP1-570 along with appropriate controls, revealed distinct upregulation and nuclear translocation of p53 in HeLa cells in which HABP1 had been silenced (Fig 12). p21 which in case of control and untransfected
Fig 11: Enhanced expression of p53 and simultaneous downregulation of MDM2 in HeLa cells upon downregulation of HABP1 confirms induction of apoptosis. (A) Lysates of HeLa cells transiently transfected with HABP1 siRNA pSilHABP1-570 (lane 1), the scrambled RNA pSilHABP1-SC (lane 2), reagent control (lane 3) and untransfected HeLa cells (lane 4) resolved on SDS-PAGE were immunodetected with polyclonal anti-p53. Equal loading was ascertained by immunodetection of the same blot with polyclonal anti-GAPDH and fold increase was calculated, showing 2.6 fold enhanced expression. Each experiment was performed in triplicate. (B) Lysates of HeLa cells transiently transfected with pSilHABP1-570, with pSilHABP1-SC and untreated HeLa cells were further immunodetected with anti-Mdm2.
Fig 11 (contd.): Enhanced expression of p21 in HeLa cells upon downregulation of HABP1 confirms induction of apoptosis. (A) Lysates of HeLa cells transiently transfected with HABP1 siRNA pSilHABP1-570 (lane 1), the scrambled RNA pSilHABP1-SC (lane 2), reagent control (lane 3) and untransfected HeLa cells (lane 4) resolved on SDS-PAGE were immunodetected with polyclonal anti-p21. (B) Equal loading was ascertained by immunodetection of the same blot with polyclonal anti-GAPDH and fold increase was calculated.
Fig 12: Enhanced expression of p53 and its translocation to nucleus following downregulation of HABP1 in HeLa cells. Immunocyto staining with anti-p53 revealed enhanced expression of p53 and its nuclear translocation in case of HeLa cells transiently transfected with HABP1 siRNA (pSilHABP1-570) with respect to the HeLa cells transiently transfected with scrambled RNA (pSilHABP1-SC) or reagent control or the untransfected HeLa cells. Each experiment was done in triplicate.
Fig 13: Enhanced expression of p21 and its accumulation in nucleus following downregulation of HABP1 in HeLa cells. Immunocytostaining with anti-p21 revealed enhanced expression of p21 and its nuclear translocation in case of HeLa cells transiently transfected with HABP1 siRNA (pSilHABP1-570) with respect to the HeLa cells transiently transfected with scrambled RNA (pSilHABP1-SC) or reagent control or the untransfected HeLa cells. Each experiment was done in triplicate.
HeLa cells was mainly localized around the nuclear membrane and the nucleolus was found to accumulate in the nucleus of the HeLa cells in which HABP1 had been downregulated using the HABP1 siRNA (Fig. 13)

[8] Expression level of proteins of survival pathway changes on HABP1 downregulation:

Finally to substantiate our observations, we proceeded to analyse the status of survival proteins in HeLa cells upon downregulation of HABP1. HeLa cells were transfected with pSil/HABP1 570, pSil/HABP1 SC and Lipofectamine™ alone and untransfected HeLa was also maintained. Corresponding cell lysates prepared after 36 hours of transfection were resolved on SDS-PAGE and immunodetected with polyclonal anti-Akt (Fig. 14A.) and polyclonal anti-p-ERK (Fig. 14B). A distinct reduction was observed in the level of expression of Akt and in the phosphorylation of ERK following downregulation of HABP1 with respect to the controls, thus confirming reduced viability in the HeLa cells in which HABP1 had been silenced. Equal loading was ascertained by immunodetection of the same blot with polyclonal anti-GAPDH (Fig. 14C) and fold reduction was calculated.
Fig 14: Reduced expression of survival pathway proteins in HeLa cells following downregulation of HABP1. Lysates of HeLa cells transiently transfected with HABP1 siRNA pSilHABP1-570 (lane 1), the scrambled RNA pSilHABP1-SC (lane 2), reagent control (lane 3) and untransfected HeLa cells (lane 4) resolved on SDS-PAGE were immunodetected with (A) polyclonal anti-Akt and (B) polyclonal anti-pERK. Distinct reduction was observed in the level of expression of Akt and pERK following downregulation of HABP1 with respect to the controls. Equal loading was ascertained by immunodetection of the same blot with polyclonal anti-GAPDH (C) and fold reduction was calculated. Each experiment was done in triplicate.
Discussion:

In the present study, we have made an attempt to identify the specific functional role of HABP1 and the significance of physiological levels of this protein in mediating apoptosis in a mammalian cancer cell line by silencing the HABP1 gene in HeLa cells. Disruption of HABP1 gene in HeLa cells using vector based siRNA technique leads to approximately 50% silencing of HABP1 in these cells, as confirmed by immunoblotting and statistical analysis. We observed a progressive reduction in growth rate of HeLa cells upon transient downregulation of HABP1, and the maximum difference in cell viability occurred 36 hours after transfection. Immunofluorescence data reconfirmed downregulation of HABP1 in HeLa cells and also revealed the interestingly the conspicuous absence of HABP1 from the nucleus while it was uniformly distributed throughout the cytosol and nucleus in the scrambled control and untreated HeLa. Moreover, no visible change was observed in the membrane localization pattern of HABP1 in the experimental and the control cells. In addition, there occurred noticeable change in the morphology of HeLa cells upon silencing HABP1 gene as indicated by reduced nucleo-cytoplasmic ratio. FACS analysis revealed a distinct increase in subdiploidy in HeLa cells upon disruption of HABP1 gene. This suggested arrest of cell growth leading to apoptosis, which was confirmed by enhanced expression of Bax and its profuse extramitochondrial localization in the HABP1 disrupted HeLa cells. Further, there occurred a significant increase in expression level of p53 pathway proteins, p53 and p21, along with their nuclear translocation while the p53 regulatory protein Mdm2 was visibly downregulated. This was concurrent with reduced expression of Akt and deregulation of ERK activation following downregulation of HABP1, thus conclusively confirming the reduced growth rate along with loss of cell viability and induction of apoptosis upon disruption of HABP1 gene in HeLa cells.
HABP1/p32/gC1qR is a ubiquitously expressed multifunctional protein that exhibits diverse cellular localization including nucleus especially in HeLa cells. Its interaction with numerous other proteins and ligands at different cellular compartments has been hypothesized to mediate its functional role in various cellular and physiological pathways. The regulatory role of HABP1 in cell cycle and apoptosis has long been postulated based on the fact that HABP1 exhibits nuclear localization and interacts with critically important nuclear proteins which are involved in cell cycle regulation and apoptosis. Various reports from several laboratories, including ours provide strong evidence in support of this hypothesis. p32/HABP1 was initially copurified with human ASF/SF2 form HeLa cell extracts and was found to regulate RNA splicing by the splicing factor -2 by blocking its phosphorylation and thereby inhibiting its RNA-binding capacity [Krainer et al., 1991; Peterson-Mahrt et al, 1999]. We have reported earlier that constitutive overexpression of HABP1 in fibroblast cells leads to cell cycle arrest in G1/S transition, extensive vacuolation and ultimately induces apoptosis in fibroblast cells [Meenakshi et al., 2003]. We have further reported that stable overexpression of HABP1 in HeLa cells induces cell death in HeLa cells and is involved in mediating cisplatin induced apoptosis as the HABP1 disrupted stable transfectants showed resistance to apoptosis [Komal and Datta, 2006].

In the present study, we have downregulated HABP1 by gene disruption of HeLa cells using vector based RNAi technique. HeLa cells were transiently transfected with the HABP1 siRNA pSilHABP1-570 and a negative control containing the scrambled RNA sequence, pSilHABP1-SC. This method resulted in ~50% silencing of HABP1 in these cells as confirmed by immunodetection of the corresponding lysates with anti-HABP1. The downregulation of HABP1 in HeLa, resulted in growth inhibition and arrested cell cycle progression as reflected in the growth curve, which also indicated that maximum inhibition in growth of HABP1 gene disrupted in HeLa cells occurred 36 hours post transfection, which is explained by the fact that
mRNA expression of the transfected DNA of SiRNA reaches its peak at approximately 36 hours after transfection. HABP1 exhibited a differential cellular localization profile upon downregulation. HABP1 is known to be localized in the nucleus of HeLa cells where it interacts with several important proteins involved in RNA splicing, such as ASF/SF2 [Peterson-Mahrt et al., 1999] and LBR/p58 [Simos and Georgatos, 1992] and with proteins involved in regulation of transcription and cell cycle progression such as transcription factor IIIB [Yu et al., 1995] and B subunit of CBF-NF-Y [Chattopadhyay et al., 2004]. We observed that HABP1 was diffused throughout the cytosol and was present in modest amounts in the nucleus in the untransfected and negative control HeLa cells, but it was conspicuously absent from the nucleus in case of HeLa cells in which the HABP1 gene had been silenced. However, it was seen to be uniformly distributed on the cell surface, cytosol and mitochondria in the HABP1 disrupted as well as untreated HeLa cells.

In course of a normal cell cycle progression, cells replicate their genome during S-phase of the cycle and hence are in a 4n state accompanied by enlarged nucleus and increased nucleo/cytoplasmic ratio prior to mitosis. The downregulation of HABP1 in HeLa cells resulted in a distinctly altered phenotype as compared to the negative control or untransfected HeLa cells. The HABP1 gene disrupted HeLa had a visibly smaller nucleus and hence reduced nucleo/cytoplasmic ratio compared to the negative control, which possibly indicates arrest of cell cycle prior to genome replication at the G1/S transition.

Increase in subdiploidy, which may result from nuclear fragmentation is an indicator of induction of apoptosis. HeLa cells in which HABP1 gene was silenced exhibited a marked increase in subdiploidy as revealed from the FACS analysis data. This was further corroborated with the enhanced expression of Bax which is a known apoptotic marker having a significant involvement in the apoptotic pathways. Bax levels were visibly increased in
HeLa cells following HABP1 downregulation. Further profuse localization of Bax in the perinuclear region was also observed which together establish induction of apoptosis in these cells. This observation prompted us to check the level of ROS generation in the HABP1 disrupted cells, which was found to be nominally elevated in the HeLa cells upon silencing HABP1. This possibly indicates that downregulation of HABP1 does not affect the mitochondrial function drastically and might not lead to mitochondrial dysfunction, suggesting that the apoptosis in induced independent of mitochondria.

The level of the tumor suppressor protein p53, which is dormant under normal conditions, is upregulated by stress stimuli and DNA damage resulting in growth arrest or apoptosis depending upon cellular conditions and extent of DNA damage [Volgstein et al, 2000]. The ability of p53 to bind DNA and activate transcription has been shown to be required for p53-dependent growth arrest, which occurs in both G1 and G2 phases of the cell cycle [el-Deiry, 1998; Vogelstein et al., 2000]. However, activation of the cyclin-dependent kinase inhibitor p21 gene is important for p53-dependent arrest in G1 phase [el-Deiry, 1998; Vogelstein et al., 2000]. Loss of the guardianship function of p53 allows continued replication of cells with damaged DNA, and in turn leads to an accumulation of genetic changes that contribute to malignant progression [Dranton, 1998].

Mdm2 is a p53 regulatory protein that binds to p53 protein and inhibits p53-dependent transcription [Momand et al., 1992]. This association mediates shuttling of p53 out of the nucleus into the cytoplasm [Roth et al., 1998] and subsequent proteosomal degradation [Haupt et al., 1997; Kubbutat et al., 1997]. This multifaceted regulation of p53 by Mdm2 is controlled by another tumor suppressor, p14ARF of the pRB regulatory pathway [Weber et al., 1999]. Inhibition of Mdm2 by p14ARF leads to release of p53 from Mdm2 resulting in phosphorylation, stabilization and nuclear accumulation of p53 which drives expression of its downstream effector p21 that leads to G1 arrest.
p21 is an important downstream effector of the p53 pathway, 21-kDa protein product of approximately 166 amino acids encoded by the ras gene (chromosome 6p21) and it has been reported to be located in both the nucleus and cytoplasm of cells [Rotchell et al., 2001]. However, only the nuclear form of p21 protein functions as a CDKI. p21 is a direct transcriptional target of p53, and is strongly induced by wild-type p53 in response to DNA damage. It mediates the growth suppression effects of p53 by arresting the cell cycle at the G1/S checkpoint and by inducing apoptosis [el-Deiry et al., 1994]. In addition, p21 has also been demonstrated to be involved in cellular senescence, terminal differentiation, and apoptosis through p53-independent mechanisms. [Sato et al., 2002; Zeng et al., 1996]. Higher concentrations of p21 lead to an increase in its stoichiometry in p21-cylin D1-cdk4 complexes, which results in the inhibition of cyclinD1-cdk4 activity [Zhang et al., 1994; Harper et al., 1995].

In the present study, we report downregulation of Mdm2 and subsequent upregulation of p53 along with its nuclear accumulation in HeLa cells upon silencing HABP1 gene. This is concomitant with strongly enhanced expression and increased nuclear localization of p21, which possibly mediates G1 arrest, as evident from the reduced nucleo/cytoplasmic ratio in the HABP1 disrupted HeLa cells with respect to the negative control or untreated HeLa. This conclusively confirms that HABP1 downregulation by gene disruption induces cell-cycle arrest in the G1 phase which subsequently leads to programmed cell death, while the control and the untreated HeLa visibly avoids cell cycle arrest and continues with normal cell proliferation.

The Extracellular signal-Regulated Kinase, (ERK), a member of the MAPK family is a key regulator that ensures cell cycle progression through G1 phase and sustained ERK activation stimulates G1/S transition [Meloche et al., 1992; Roovers et al., 1999]. We have earlier reported that HABP1 is an endogenous substrate of ERK and is an integral part of the MAPK cascade which
translocated to the nucleus upon mitogenic stimulation leading to MAPK activation [Majumdar et al., 2002]. We report here that upon silencing HABP1 gene in HeLa cells, there occurs a visible downregulation of the survival pathway proteins, Akt and p-ERK, which subsequently leads to cell cycle arrest and reduced viability in the HABP1 disrupted HeLa. It maybe noted that in stable fibroblasts and HeLa transfectant, there’s an excess generation of ROS indicating that apoptosis induction maybe mediated through mitochondrial dysfunction. But on downregulation of HABP1 in HeLa cell line, there’s no accumulation of HABP1 in the mitochondria and no increased ROS generation. Apparently, apoptosis induction in HeLa cells with HABP1 downregulation is mediated through p53 dependant cell cycle arrest.

Taken together, our observations convincingly conclude that downregulation of HABP1 in HeLa cells by vector based RNAi technique leads to blockage of cell proliferation and induces programmed cell death by p53-dependent pathway. This, in view of the fact that HABP1 upregulation is associated with cisplatin mediated apoptosis in HeLa cells, indicates that the level of HABP1 is crucial for maintaining normal cell survival as it possibly dictates the balance between cell survival and programmed cell death.