Materials and Methods
Materials:

All chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO, USA) unless otherwise mentioned. Plasmid DNA was purified with the help of Qiagen Midi Kit (GmbH, Germany). Low molecular weight SDS-PAGE marker & PVDF membranes were obtained from Pharmacia Biotech Inc. (Uppsala, Sweden).

Primary as well as secondary antibodies were purchased from Santa Cruz Biotechnology Inc., (USA) and Cell Signaling Tech. (USA). Antibody to HABP1/gC1qR was generated in our laboratory as described in Deb and Datta [Deb and Datta, 1996]. MitoTracker™ Green and 5,6-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate (H2DCFDA) Molecular Probes Inc. (Eugene, OR).

All reagents used for cell culture were purchased from Hyclone and Transfection reagent Lipofectamine™ 2000 was purchased from Gibco Brl., (Rockville, MD, USA). Reagent lipofectamine™ was from Invitrogen. Cell culture plastic ware was obtained from Corning-Costar Inc. (Corning, NY, USA). 0.45 μM membrane filters for filtering media and other reagents were obtained either from Millipore (MA, USA) Filteration unit and isopropanol cryobox were purchased from nalgene (Nalge Nunc International Corporation, Rochester, NY, 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).
Methodology:

[1] Maintenance of mammalian cell-culture:

    Cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and 100 μg/ml Pen-Strep and 50 μg/ml fungizone and grown at 37°C in a humidified 5% CO₂ incubator.

[2] Silencing HABP1 gene in HeLa by vector based RNAi technique:

    Knockdown of HABP1 in HeLa cells was achieved by disrupting the HABP1 gene by transiently transfecting HeLa with siRNA of HABP1. The plasmid pSil/HABP1 570, which was used as the silencing plasmid and pSil/HABP1 SC with the scrambled sequence which was used as a control vector were generated and kindly given by Dr A. Kamal [Kamal and Datta, 2006]. The plasmid with the scrambled sequence produces a non specific RNA. All transfections were carried out using Lipofectamine-2000™ according to the manufacturer’s protocol. Briefly, the cells were seeded in 60mm cell culture dishes or on coverslips in 6-well or 12-well culture plates 24 hours prior to transfection and maintained in complete DMEM. The required amount of DNA (0.5μg-2μg), pSil/HABP1 570 and pSil/HABP1 SC were diluted in 50μl-200μl of serum-free DMEM, mixed gently and incubated at room temperature for 5 minutes. Simultaneously, required amount of Lipofectamine 2000™ was diluted in same volume of SFM and also incubated at room temperature for 5 minutes. Appropriate control, i.e., the reagent alone was also incubated in SFM for same time point. Thereafter the two solutions were mixed gently and co-incubated at room temperature for another 20 minutes to allow formation of plasmid DNA-Lipofectamine™ complex. Meanwhile, the complete growth medium of the cells was removed and the cells were incubated in SFM for 20 minutes following which the DNA-Lipofectamine complex was added to the corresponding wells. Cells in one well or dish was simultaneously maintained in SFM alone as the untreated control. The cells thus transfected
were incubated for 4-6 hours at 37°C in a CO₂ incubator following which the SFM was replaced with complete DMEM and the cells were grown for 36 hours and then processed for immunodetection or other biochemical analysis. The subsequent reduction in expression level of HABP1 was confirmed by immunodetection of the lysates and immunocytochemical analysis with anti-HABP1.

[3] Synchronization of randomly growing mammalian cell culture:

In a synchronously growing culture, all or most of the cells perform similar biochemical functions simultaneously. A synchronized or partially synchronized culture of mammalian cells is of potential usefulness for investigation of various cellular properties, cell cycle dependent events and control mechanisms during cell cycle progression as these require a large quantity of cells at some specific stage of the cell-cycle.

The randomly growing mammalian cell cultures were synchronized according to the modified protocol of Cao [Cao et. al., Exp Cell Res., 1991] by blocking the cell cycle using thymidine and hydroxyurea. TdR block results from an inhibition of the enzymatic mechanism for the synthesis of deoxycytidine triphosphate from cytidine 5’monophosphate due to excess thymidine [Xeros, 1962]. Hydroxyurea has both inhibitory and cytotoxic action on cells [Sinclair, 1965, 1967]. HU is selectively lethal to S-phase cells and reversibly blocks DNA synthesis, thus preventing G₁ cells from entering S-phase.

5mM thymidine was added to a randomly growing monolayer cell culture in complete medium and incubated for a period longer than the sum of G₂ + M + G₁ portion of the cell cycle, which sums up to 14 h in HeLa. The culture was released from thymidine block by reculturing in fresh complete medium for a period exceeding S-phase (9.5 h). This procedure was repeated for a second cycle of blockage. Following which, cells were cultured with 2mM HU and incubated for a period longer than the sum of
G₂ + M + G₁ portion of the cell cycle. HU block was then released and the cells were sampled at different time points, i.e., 5 h (for S phase), 9.5 h (for G₂ phase) and 14 h (for G₁ phase) analyzed by flowcytometry for degree of synchronization.

[4] FACS analysis of propidium iodide stained cells:

Flowcytometry was used to determine the relative DNA content of cells to check the degree of cell-synchronization as well as the percentage of subdiploidy in he transfected cells. Monolayer cultured cells grown in fresh medium after release of HU block or 36 h post transfection were rinsed with filtered 1X PBS (37°C) and mildly trypsinized. The cells were then harvested in chilled filtered PBS (pH=7.2), pelleted and gently resuspended in 200μl filtered chilled PBS and incubated in ice for about 15 minutes. To this cell suspension, 2ml ice-cold ethanol was added while vortexing gently and slowly. The cells were then incubated for at least 30 min at -20°C. Fixed cells were washed twice and allowed to re-hydrate in filtered PBS for 15 min. Prior to FACS analysis, the cells were incubated with the DNA staining solution (PBS with 40 μg/ml propidium iodide and 100μg/ml RNase A) for 30 min. The DNA content of the cells was analysed with cell quest software on a flowcytometer (Becton-Dickinson, BDLSR):

[5] Blocking of synchronized mammalian cell culture at different checkpoints during the cell cycle, using varying concentration of cell-cycle blockers:

Synchronized culture of mammalian cells was arrested at different stages of cell cycle, using varying doses of cell cycle blockers such as Hydroxyurea, Apigenin, 5-Fluorouracil, Nocodazole, etc. in order to study certain stage-specific biochemical events.

Apigenin, a plant flavonoid has been reported to selectively induce G₂/M arrest by causing significant decrease in cyclins A & B and Cdk1,
thus inhibiting Cdk1 kinase activity [Wang et al, 2004]. Apigenin also induces p-53 dependent apoptosis in a time and dose-dependent manner after causing G₂/M arrest [Gupta et al, 2001].

5-FU is a chemotherapeutic agent known to cause inhibition of thymidylate synthetase (TS), and hence thymidine triphosphate (dTTP) synthesis, resulting in alteration of the balance of deoxynucleotide (dNTP) pools and disruption of DNA. Thus it leads to accumulation of cells in S-phase. [Elstein, 1997]

Nocodazole induces reversible depolymerization of microtubules and destabilization of the spindle apparatus leading to mitotic arrest in the late metaphase [Murata-Hori et al, 2004; Kasas et al, 2005].

[6] Determination of growth kinetics:

0.2x10⁵ cells were seeded in each well of a 24 well culture cluster 24 h prior to transfection or synchronization procedure. After the treatment the samples were collected at different time points in triplicates. 60 µl of MTT dye (5 mg/ml in DMSO) was added to each well and incubated at 37°C for four hours in a humidified CO₂ incubator. The precipitate formed was solubilised in the DMSO and the absorbance was recorded when all samples were collected. The colored formazan product is stable at 4°C for several days. The absorbance was recorded at 570 nm.

[7] Hyaluronan stimulation:

Monolayer culture of mammalian cells was treated with varying concentrations/doses of hyaluronan to check its effect on cell cycle progression and expression level of endogenous HABP1 with respect to HA stimulation.
[8] Preparation of whole-cell lysate for immunoblot analysis:

Lysates of transfected or synchronized HeLa cells were prepared in RIPA lysis buffer or 1X Laemmli buffer at ice-cold conditions. The lysates were then centrifuged and the supernatant was then collected and processed for protein estimation and immunodetection.

[9] Protein estimation

Protein content was estimated using Bradford [Bradford, 1976] method, depending on the quantity of protein present in the sample and other experimental constraints. Bovine serum albumin (BSA) was used as the standard.

[10] Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Linear slab gel electrophoresis under the 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 4% acrylamide, 0.106% N,N'-methylene bisacrylamide, 0.125 M Tris-HCl, pH 6.8, 0.01% temed and 0.1% ammonium per sulphate (APS). The protein samples were electrophoresed in running buffer containing 0.025 M Tris-base, 0.192 M glycine, pH 8.3 and 0.1% SDS. The protein samples were boiled in lammeli buffer containing 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and with 100mM DTT or 5% β-mercaptoethanol (β-ME). Standard molecular weight marker was also electrophoresed alongside to calculate the subunit size of the protein.

[11] Immunodetection to check the level of expression of a protein:

Lysates, thus obtained from transfected or synchronised cells arrested at certain stage were resolved on 12.5% SDS–PAGE and transblotted on PVDF membrane. Proteins were immunodetected with required antibodies, i.e., anti-HABP1, anti-GAPDH, anti-p53, anti-Mdm2,
anti-Bax, anti-Erk or anti-pERK and then visualized by nitro-blue tetrazolium/5-bromo-4-chloro-3 indolyl phosphate (NBT/BCIP) detection system using alkaline phosphatase conjugated secondary antibody or by enhanced chemiluminescence using peroxidase conjugated secondary antibody.

[12] Immunocytochemical analysis and immunocytostaining of mammalian cells to detect the localization pattern of HABP1 and other proteins during different stages of the cell cycle:

Coverslips with 60-70% cells were washed with 1X PBS, pH 7.2 and the cells were then fixed either in chilled (4°C) p-formaldehyde for 10 minutes at 37°C, to maintain the plasma-membrane in impermeable state or fixed and permeabilized in chilled methanol-acetone solution (1:1 by volume) for five minutes. Thereafter the coverslips were rinsed thoroughly but gently in 1X PBS, pH 7.2, blocked in 3% BSA-PBS for 1 hr at 37°C and incubated with α-HABP1 or any other required antibody with suitable dilution in 1% BSA-PBS for 1-2 hr at 37°C in a humidified chamber. After proper washing with 1X PBS, pH 7.2, the cells were co-incubated with appropriate secondary antibody suitably diluted in 1% BSA-PBS for an hour at 37°C. To detect mitochondria, the fluorescent mitochondrial marker MitoTracker™ Green (100nM) was added to the cells and incubated for 10-15 minutes followed by thorough but gentle washing. To analyze the nuclear morphology, appropriately diluted Hoechst 33352 (from 1 mg/ml stock solution) was added to each coverslip and incubated for another 10 minutes at 37°C. The images of MitoTracker™ were captured at 490nm excitation and 516nm emission filter after washing properly with phosphate buffer saline. Fluorescence images were monitored using an Axioscope microscope (Carl Zeiss, Germany) equipped with epifluorescence and Axiocam camera system coupled with Axio Vision software (Carl Zeiss, Germany)