

Chapter 2

Materials and Methods

Materials

Cell culture media, culture flasks, chemicals, reagents, kits, antibodies, membranes and transfection reagents were obtained from the following companies:

Applied Biosystems- Real time PCR reaction buffer, cDNA synthesis kit

BD Biosciences- PE-conjugated antibodies CD11b, CD114, IgG isotype control, FACS tubes

Bio-Rad- Bradford Reagent

Dharmacon- On-Target plus E6AP siRNA, scrambled siRNA, Dharmafect transfection reagent

Fermentas- Molecular weight markers, DNA ladder

GE Healthcare- Hyperfilm ECL, ECL Advance western blotting detection kit, pH strips for 1-D gel electrophoresis and GST-Sepharose beads

Gibco (Invitrogen)- Transfection reagent Lipofectamine-2000, Lipofectamine-LTX, DMEM high glucose, RPMI-1640 liquid and powder medium, Fetal Bovine Serum

Himedia- Agar powder, Luria Bertani broth

Eurofins MWG Operon- Primers for semiquantitative and quantitative PCR

Merck- Acetone, Isopropanol, Methanol, Ethanol

Millipore- Sterile filters, Protein Agarose A and G beads, Enhanced Chemi Luminescence, PVDF membrane

Roche- Real Time PCR kit and plates

Sigma-Aldrich- RPMI-1640 media, DMEM powder, Sodium bicarbonate, Sodium chloride, L-glutamine, Giemsa stain, Sodium acetate, Ethidium bromide, Bromophenol blue, Ammonium bicarbonate, PCR primers, Tris-saturated phenol, Gene elute plasmid miniprep kit, Coomassie brilliant blue R, Coomassie brilliant blue G, Ponceau stain, Acrylamide, Sodium dodecyl sulphate, Ammonium persulfate, TEMED, Trizma HCl, Trizma base, Glycine, Triton X-100, Imidazole, Calcium chloride, Chloroform, Glycerol, Sodium deoxycholate, BSA, DAPI, Ammonium chloride, Nonidet P-40, Paraformaldehyde, Formaldehyde, DTT, EDTA, Potassium chloride, Sodium di-hydrogen phosphate, Di-sodium hydrogen phosphate, DMSO, Protease

inhibitor cocktail, Phosphatase inhibitor cocktail I and II, Silver nitrate, Ampicillin, Chloramphenicol, Developer, Fixer, Rabbit, Mouse and Goat secondary HRP conjugated antibodies, Lysozyme and Agarose

SRL- Sodium dodecyl sulphate, Tris-base, Sodium acetate, Sodium chloride, Methanol, Sodium hydroxide, Glycine, Sodium bicarbonate, Boric acid

Tarson- 90 mm culture dishes, disposable sterile pipettes, 50 ml and 15 ml falcon tubes

Nunc- 25cm² culture flasks, 75cm² culture flasks, 96, 6, 12 and 24 well plates

USB- Sodium hydrogen phosphate, Urea, Disodium hydrogen phosphate, N,N-methylene- bis-acrylamide

Methods

2.1 Cell Culture

2.1.1 Culture Media

To prepare 1L of culture medium, 16.4g of RPMI-1640 was dissolved in 1L of distilled water. This incomplete culture medium was then filter sterilized with 0.22 μ of sterile filter. To prepare complete culture medium 10% heat inactivated fetal bovine serum was added along with 1X antibiotic solution. Cells were cultured in 5% CO₂ humidified incubator. HEK293T cells were cultured in Dulbecco's modified eagle medium (DMEM). DMEM was prepared by dissolving 16.4g of DMEM powder to 1L of distilled water and was further filter sterilized with 0.22 μ of filter unit. Complete culture medium was prepared by the addition of 10% heat inactivated FBS, 1X antibiotic solution and 2mM sodium pyruvate solution.

2.1.2 Cell Culture

HL60, K562 and U937 cells were cultured in the RPMI-1640 medium supplemented with 10% FBS and 1X antibiotic solution. Cells were grown at 37°C in 5% CO₂ humidified incubator. Cell were sub cultured after every fourth day by centrifuging at 1200rpm for 5min at RT. Cell pellet was washed once with sterile PBS and then again centrifuged to remove debris and old medium. Further, cells were dissolved in fresh medium and replated in 75cm² cell culture flask. HEK293T is an adherent cell line and was sub cultured after every third day. For sub culturing, old medium was decanted, cells were washed once with sterile PBS and trypsinized with 1X trypsin-EDTA solution. Cells were flushed up and down with sterile pipette and PBS until homogenous single cell suspension was achieved, this was followed by centrifugation at 1200rpm for 5min at RT. Cell pellet was dissolved in fresh medium and was plated in culture flask.

2.1.3 PBMCs (Peripheral blood mononuclear cell) isolation from blood samples

PBMCs were isolated from CML patient blood samples using histopaque-1077 (Sigma) (137). 10ml blood sample was mixed in equal volumes of MACS buffer (PBS containing 2% FBS and 2mM EDTA) and centrifuged at 400g for 10min. Supernatant was completely removed and dark red pellet was again washed with 25ml of MACS buffer. The dark pellet was diluted in MACS buffer in 1:1 ratio and was fractionated through Histopaque-1077 by centrifugation at 400g for

45min. Mononuclear cells were collected from the interface, washed twice with RPMI and were resuspended in RPMI-1640.

2.1.4 Cryopreservation of cells

Cells were cryopreserved in liquid nitrogen at -196°C so that they can be revived later for future use. Cryopreservation medium included 90% FBS and 10% DMSO. Cells were washed once with PBS and pelleted down. Cryopreservation medium was added to the cell pellet and this cell suspension was aliquoted in cryo vials and was placed in slow cooling isopropanol boxes which were first kept at -20°C for 12hrs, placed at -80°C for next 12hrs and then shifted to -196°C in liquid nitrogen for the long term storage.

2.1.5 Stable cell line preparation

β -estradiol inducible K562 stable cell line was prepared using pBABE puro p42 C/EBP α -ER plasmid construct. 1×10^5 cells/ml were plated in 6 well plates one day before transfection in phenol red free RPMI supplemented with 10% charcoal stripped FBS and 1X antibiotics. Next day 4h prior to transfection, medium of cells was changed with phenol red free RPMI supplemented with 1X antibiotics without FBS. Lipofectamine LTX reagent was used for the transfection. After transfection selection of cells was performed in $2.0 \mu\text{g/ml}$ puromycin supplemented medium for two weeks. Serial dilution of cells was carried out in 96 well plates to obtain single cell dilution. Cells were grown for 3 days and then were transferred to 6 well plates and later to 25cm^2 cell culture flasks. 6 clones were selected and after cell growth of nearly two weeks, western blotting was performed to verify the clones. Giemsa staining was performed for the detection of functional clones as K562-p42 C/EBP α -ER stable clones differentiate into neutrophils after estradiol induction.

2.1.6 Plasmids

Expression plasmids for pcDNA3.1-C/EBP α -HA (138), pcDNA3.1-E6AP (139) and pCAG-HA-E6AP-C843A (140), pGEX4T-GST-E6AP (141) were kind gifts from G. J. Darlington, Nihar Jana, Ikuo Shoji and Zafar Nawaz respectively; while pCDNA6- C/EBP α -His, pMT123HA-Ubi and p(C/EBP)2TK-luc are previously described (142). pBabe Puro-p42 C/EBP α -ER was kind gift from Alan D. Friedman (143).

2.2 MTT Assay

MTT method of cell determination is useful in the measurement of cell growth in response to any compound or cytotoxic agent in cytotoxicity studies and in the derivation of cell growth curves (144). Yellow MTT (3-(4, 5-Dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (usually between 500 and 600nm) by spectrophotometer. The absorption maximum is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced through the production of a dose-response curve. Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in colour. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in DMSO. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance.

1×10^4 cells per well in a 96 well plates were plated one day before start of experiment. Next day, the cells were induced with different concentrations of ormeloxifene (ORM) i.e. $1 \mu\text{M}$, $2.5 \mu\text{M}$, $5 \mu\text{M}$, $7.5 \mu\text{M}$, $10 \mu\text{M}$, $15 \mu\text{M}$, $20 \mu\text{M}$. DMSO was taken as a vehicle control since ORM was dissolved in it. HEK293 was used as a non-myeloid control cell. After 48 hrs of induction, MTT solution was added to the $1/10^{\text{th}}$ of the culture medium. Cells were incubated for 3h at 37°C in CO_2 incubator after addition of MTT solution (MTT solution was prepared in PBS to the working concentration of 5mg/ml). After 3h cells were centrifuged and culture medium was removed and the formazon crystals were dissolved in DMSO. The absorbance of the samples was measured with a specific enzyme-linked immunosorbent assay (ELISA) reader at 560nm . The percentage cytotoxicity was measured by the graph plotted with these readings from three independent experiments.

2.3 Apoptosis Assays

2.3.1 Annexin V-PI

Changes in the cellular morphology including cell shrinkage, nuclear condensation along with biochemical events leading to loss of mitochondrial membrane potential, loss of plasma membrane asymmetry and DNA cleavage between nucleosomes characterize apoptosis (145). The appearance of phosphatidylserine (PS) residues (normally hidden within the plasma membrane) on the surface of the cell is another parameter which can be used to detect and measure apoptosis (146). The presence of PS on the cell surface creates one of the specific signals for recognition and removal of apoptotic cells by macrophages. These PS changes can be detected with the anticoagulant, Annexin V, which has a high affinity for binding to PS (147). As the apoptosis progresses, cell membrane integrity is lost. Propidium iodide is an impermeable dye for live cells; however, in case of dead cells integrity of cell membrane is lost which allows PI to enter inside cells and nucleus where it can intercalate in between DNA bases. The amount of intercalated dye is directly proportional to no. of dead cells. Therefore, using DNA specific viability dyes like Propidium Iodide (PI), it is possible to distinguish between early apoptotic, late apoptotic, and dead cells.

K562, HL60 and U937 cells were induced with different ORM concentrations for the indicated time points. Annexin V-PI staining Kit (Sigma) as per manufacturer protocol was used for flow cytometry analysis of apoptotic cells. Briefly, 1×10^6 cells were washed with PBS. 5 μ l Annexin-V and 10 μ l PI solution was added in 1X binding buffer to prepare the buffer solution as per manufacturer's protocol for each tube. Cells were incubated in the AnnexinV-PI solution for 15min in dark at room temperature (RT) followed by addition of 400 μ l of binding buffer and subsequent analysis in Flow Cytometer (FACS Calibur, Becton Dickinson, USA).

2.3.2 TUNEL Assay

TdT-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) apoptosis detection system (Invitrogen) was used for the detection of apoptosis (148). During apoptosis, there is activation of nucleases that eventually degrade nuclear DNA into fragments of approximately 200 base pairs in length (145). DNA fragmentation exposes a large number of 3'-hydroxyl ends. These hydroxyl groups can then serve as starting points for terminal

deoxynucleotidyl transferase (TdT), which adds deoxyribonucleotides in a template-independent fashion. Addition of the deoxythymidine analog 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) to the TdT reaction serves to label the break sites. Once incorporated into the DNA, BrdU can be detected by an anti-BrdU antibody using standard immunohistochemical techniques. This method of labeling DNA breaks is referred to as Terminal Deoxynucleotide Transferase dUTP Nick End Labeling, or TUNEL.

Treated cells ($1-2 \times 10^6$) were washed with 1X PBS followed by fixing with 4% formaldehyde on ice for 15min. Further, cells were washed with 1XPBS and incubated in 50 μ l DNA labeling solution for 1h at 37°C. DNA labeling solution contains TdT enzyme, Brd UTP and reaction buffer as provided in kit. After DNA labeling, cells were washed twice with rinse buffer and incubated with 100 μ l dye labeled anti-BrdU antibody for 30min at RT in dark. Antibody staining solution is prepared by mixing 5.0 μ L of the Alexa FluorR 488 dye labeled anti-BrdU antibody with 95 μ L of rinse buffer. 0.5ml of iodide/RNase staining buffer was added to each sample and incubated in dark for 30min at RT. Subsequently TUNEL positive cells were analyzed in FACS flow cytometer. In addition, for the microscopic analysis, after incubation with antibody cells were cytocentrifuged and were visualized under fluorescent microscope.

2.3.3 JC-1 mitochondrial membrane potential assay

Mitochondria plays an important role during apoptosis (149). Mitochondrial membrane potential ($\Delta\psi$) is generated by mitochondrial electron transport chain which drives a proton flow from matrix through inner mitochondrial membrane to cytoplasm, thus creating an electrochemical gradient. This gradient in turn is responsible for the formation of ATP molecules by F_0-F_1 ATP synthase. For this reason $\Delta\psi$ is an important parameter for mitochondrial functionality and an indirect evidence of energy status of the cell. Changes in the mitochondrial membrane potential are measured by the flow cytometry using JC-1 dye (5, 5, 6, 6-tetrachloro 1, 1, tetraethyl benzimidazolocarbo-cyanine iodide). It is a cationic dye which accumulates in the mitochondria as J aggregate. Cells were treated as per indicated conditions and 30min prior to cytometric analysis, JC-1 was added to the cells (1×10^6 cells/ml) to a final concentration of 10 μ g/ml and incubated at 37°C in 5% humidified CO₂ incubator for 15min. 10,000 cells were examined for each samples FL-1 (530 nm) *versus* FL-2 (585 nm) dot plot on a Becton Dickinson Flow

Cytometer. JC-1 has dual emission depending on the state of the mitochondrial membrane potential.

2.3.4 Caspase glo Assay

The Caspase-Glo® 3/7 and 9 is homogeneous luminescent assay that measures caspases activity. This member of the cysteine aspartic acid-specific protease (caspase) family plays a key initiator role in the intrinsic apoptotic pathway of mammalian cells (150). Addition of this reagent results in the cell lysis followed by caspase cleavage of the substrate and generation of luminescent signal. The signal generated is proportional to the amount of caspase activity. Briefly, 10,000 cells per well were seeded in 96 well plates. After treatment with ormeloxifene, 1:1 ratio of caspase glow reagent (caspase 3/7 and caspase 9, Promega) to sample volume was added as per manufacturer's protocol and incubated for 1h at RT. Reading of luminescence was taken in luminometer. The graph was plotted for the relative luminescence produced in the treated sample as compared to the control.

2.4 Cell Cycle Analysis

2.4.1 DNA content analysis by FACS flow cytometry

Cell cycle analysis was done using propidium iodide (PI). Propidium iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4.5 base pairs of DNA. PI also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining. When bound to nucleic acids, the absorption maximum for PI is 535nm and the fluorescence emission maximum is 617nm. PI fluorescence is detected in the FL2 channel of flow cytometers.

For cell cycle analysis, K562 cells were treated with 7.5 μ M ORM for indicated time points. After treatment cells were washed once with PBS and then resuspended in 1ml PBS. Cell suspension was fixed with absolute ethanol to the final concentration of approximately 70%. Cells were incubated on ice for 15min and then washed once with PBS and resuspended in 500 μ l PI solution for 30min at RT in dark. After washing, cells were analyzed in Flow Cytometer (FACS Calibur, Becton Dickinson, USA) for the distribution of cells in different phases of cell cycle.

2.5 PCR Analysis

2.5.1 RNA isolation

Trizol reagent was used for the RNA isolation. Briefly, cells were centrifuged at 1200rpm for 5min at RT. Cell pellet was dissolved in 100 μ l PBS and then 1ml of trizol was added to it, mixed by vigorous shaking for 1min followed by incubation at RT for 5min. Further, 200 μ l of chloroform was added to each tube, mixed vigorously for 15sec and incubated at RT for 3min. After incubation, tubes were centrifuged at 16000rpm for 15min at 4°C. A colourless phase was obtained which was collected in a new tube, 1ml of isopropanol was added to it and incubated for 10min at RT. Tubes were centrifuged at 16,000rpm for 10min at 4°C. Pellet obtained was washed once with 1ml of 75% ethanol and centrifuged at 16,000rpm for 10min at 4°C. Supernatant was completely removed and pellet was air dried for 5min at RT. Pellet was redissolved in 20-25 μ l of nuclease free water and UV absorbance was taken at 260/280nm (151) to assess the purity of mRNA.

2.5.2 cDNA synthesis and Reverse Transcriptase PCR

cDNA synthesis was carried out from total RNA using cDNA synthesis kit (Applied Biosystems). PCR amplifications were carried out using *Taq* DNA polymerase for routine applications (Sigma). All the PCR reactions were carried out by standard procedures (152) using cDNA as template in a C1000TM Thermal Cycler (BioRad).

2.5.3 Quantitative Real time PCR

cDNA was used for the real time quantitative PCR analysis on Roche Light Cycler 480 using SYBR green master mix from Applied Biosystems. Statistical analysis was performed using $\Delta\Delta$ CT method.

Primer Sequences used in the Quantitative and Qualitative PCR analysis are given in the table 2.

Table 2: Primer Sequences

Primer		Sequences
c-myc	Left Primer	5'-TTCGGGTAGAAAACCAG-3'
	Right Primer	5'-CAGCAGCTCGAATTTCTTCC-3'
p21	Left Primer	5'-GACACCACTGGAGGGTGACT-3'
	Right Primer	5'-CAGGTCCACATGGTCTTCCT-3'
GAPDH	Left Primer	5'-CTTCAACACCCAGCCAT-3'
	Right Primer	5'-TAATGTCACGCACGATTTCC-3'

2.6 Biochemical Assays for cell staining

2.6.1 Hoechst Staining

Hoechst is a blue fluorescent dye used to stain DNA. This dye binds to all the nucleic acid but preferably to AT rich regions. Hoechst staining is done to stain the nucleus. 5×10^4 cells/2ml per well were plated a day before ORM treatment. Post ORM treatment, cells were washed and resuspended in 500 μ l PBS. Subsequently cells were cytospun on slides at 800rpm for 5min. Cells were stained with Hoechst stain (H6024, Sigma) (2 μ g/ml), washed with PBS to remove excess stain, air dried and analyzed under fluorescent microscope.

2.6.2 Giemsa staining

Giemsa stain is specific for phosphate group of DNA and it binds to the region of DNA rich in adenine and thymine. Gimesa is a mixture of methylene blue, eosin and azure B. K562p42-C/EBP α -ER cells were induced with 5 μ M estradiol for 5days. After induction, cells were cytospun on slides and air dried. Next day, cells were stained using May-Grunwald and Giemsa solution by placing in Coplin jar filled with May-Grunwald solution for 5min followed by washing with 1XPBS for 2min. Meanwhile, Giemsa solution was diluted 1:20 ratio in PBS and slides were stained in this diluted solution for 15-20 min. Cells were then washed in running tap water to remove the excess stain, air dried and subjected to microscopic examinations under light microscope and were photographed.

2.6.3 Immunofluorescence microscopy

HEK293T cells were plated in chamber slide one day before transfection. Next day cells were transfected with C/EBP α and E6AP expression plasmids. 24h post transfection, cells were washed with PBS, fixed in 4% paraformaldehyde for 10min, permeabilized with 0.5% Triton X-100 in PBS for 5min, washed with PBS, and then blocked with 1% BSA in PBS for 1h. Cells were then incubated with primary antibodies C/EBP α and E6AP (1:200) overnight at 4°C. Next day, cells were washed thrice with PBS, incubated with Alexa Flour 594 and 488 secondary antibodies (1:250 dilutions) for 1h; Again washed thrice with PBS followed by 4', 6-diamidino-2-phenylindole (DAPI) staining (Sigma). Cells were then mounted with vectasheild (vector) and visualized under confocal microscope (Leica) for analysis.

2.7 Western blot analysis for detection of protein expression

2.7.1 Protein lysate preparation and estimation

Protein lysates were prepared in the RIPA lysis buffer which contained 1% (w/w) NP40, 0.5% (w/v) Sodium Deoxycholate, 0.1% (w/v) SDS, 0.15M NaCl, 5mM EDTA and 1mM DTT. To this working solution, phosphatase inhibitors and protease inhibitors were added. For cell lysate preparation, cells were centrifuged at 1500rpm for 5min at 4°C and supernatant was removed. Cells were washed once with chilled PBS to remove the remaining medium and cell debris. Cells were again centrifuged and RIPA buffer was added to the cell pellets by repeated pipetting. Lysates were then placed on ice for 30min with repeated tapping after every 10min. Lysates were then centrifuged at 25,000rpm for 20min at 4°C. Supernatant was collected in a prechilled tube for further use. 5X Bradford assay reagent (BioRad) was used to quantify the concentration of proteins. Absorbance was taken at 595nm in spectrophotometer. A standard plot using BSA was prepared to calculate the protein concentration using absorbance reading.

2.7.2 SDS-PAGE and Western blotting

For western blotting, protein samples were prepared in 2X SDS sample loading buffer (125mM Tris pH 6.8, 4% SDS, 20% Glycerol, 10% 2-Mercapto-ethanol and pinch of bromophenol blue) and resolved on 10% SDS-polyacrylamide gel (142). Further, proteins were electroblotted on PVDF membrane (Millipore) in Tris-glycine buffer (48 mM Tris, 39 mM glycine, 0.04% SDS,

20% methanol). Blots were then blocked in 5% skimmed milk in PBS-T (PBS containing 0.5%v/v Tween-20) for 1h at RT followed by washing with PBS-T and incubation with required dilution of primary antibodies for overnight at 4°C. After three washes of 10min each with PBS-T, blots were incubated in HRP-conjugated secondary antibody for 1h at room temperature followed by three washing with PBS-T. Blots were developed using chemiluminescence substrate (ECL, Millipore) in LAS 4000. Primary antibodies used were Bax (Alexis), Cytochrome-c (Alexis), Caspase-3 (sc-7148), c-Abl (sc-23), CDK2 (sc-163), Bcl-2 (sc-492), p21 (sc-397), β tubulin (sc-9104), β actin (sc-47778), C/EBP α (sc-9314), ubiquitin (sc-8017), GST (sc-459), GAPDH (sc-13179) from Santacruz Biotechnology; PARP (9542), ERK1/2 (4370), c-myc (9402) from Cell signaling Technology and E6AP (E8655) from Sigma.

2.8 Protein-protein interaction assays

2.8.1 GST fusion protein purification and GST-pull down

For GST-pull down assay, GST-E6AP fusion proteins were expressed in *E. coli* after 0.2mM IPTG induction for 12h. Induced bacterial pellet was lysed in NETN buffer (150mM NaCl, 20mM Tris pH 8.0, 1mM EDTA pH 8.0, 0.1% NP40 and protease inhibitors), sonicated and centrifuged at 15000rpm. Supernatant was collected followed by subsequent protein purification using immobilised Glutathione Sepharose beads (Amersham Biosciences, USA). Sepharose beads bound with GST proteins were washed twice with NETN buffer on a rotating shaker at 4°C for 10min each and were then lysed in 2X SDS sample loading buffer (125mM Tris pH 6.8, 4% SDS, 20% Glycerol, 10% 2-Mercapto-ethanol and a pinch of bromophenol blue). Subsequently, it was resolved on 12% SDS PAGE and visualised by commassie blue staining for resolved proteins.

Cell lysates of C/EBP α over expressed HEK293T cells were prepared in RIPA buffer. For pull down experiments GST purified proteins were incubated with whole cell lysates in NETN buffer for 3h at 4°C on a rotating shaker. After pull down, protein bound GST sepharose beads were washed three times with NETN buffer. SDS loading dye was added to the beads and was resolved on 10%SDS-Polyacrylamide gel, immunoblotted with GST and C/EBP α antibody to confirm the interaction.

2.8.2 Co-Immunoprecipitation (CoIP) Assay

Co-immunoprecipitation is an *in-vitro* biochemical assay used to detect *in-vivo* interaction of two proteins. For this, HEK293T cells were cotransfected with C/EBP α and E6AP. 24h post transfection, cells were treated with MG132 (25 μ M) for 3h. Protein lysates after preclearing with IgG were incubated with C/EBP α antibody and Protein Agarose G beads (10 μ l) (Millipore) for 3h at 4°C in coimmunoprecipitation buffer (1% TBS, 0.5% NP40, Protease inhibitors). After incubation, beads were washed with IP buffer 3 times and bound proteins were eluted in laemmli buffer. Immunoprecipitated proteins were heated at 56°C for 90min in 2X SDS loading buffer and then boiled at 95°C for 5min. Samples were separated on 10% SDS-PAGE and were subsequently immunoblotted with C/EBP α and E6AP antibody.

2.8.3 *In-vivo* Ubiquitination Assay

HEK293T cells were transfected with C/EBP α , E6AP and ubiquitin constructs. 24h post transfection cells were harvested and RIPA lysates were prepared. Subsequently, co-immunoprecipitation was performed with 2 μ g of C/EBP α antibody using protein G Agarose beads (Millipore). After preclearing, protein lysates were incubated with antibody and beads for 3h in IP buffer. The co-immunoprecipitated proteins were then separated by 10% SDS-PAGE and probed with ubiquitin antibody. The same blot was stripped and reprobed with C/EBP α antibody to confirm the immunoprecipitation.

2.9 Luciferase Reporter Assay

1x10⁵ HEK293T cells/well were plated one day before transfections in 24 well plates. Next day cells were transfected with p(C/EBP)TK-luc promoter and expression plasmids for C/EBP α , E6AP and E6AP mutant (E6AP-C843A). 24h post transfection cell extracts were assayed for luciferase activity, using luciferase assay reagent (Gold Biotechnology). GFP expression was measured by multiplate fluorimeter. Luciferase activity values were normalized with GFP values and fold activity was calculated over untreated control. Data are presented as means of triplicate values obtained from representative experiments.

2.10 Fluorescence Activated Cell Sorting (FACS) Analysis

For the FACS analysis of differentiation surface markers on the K562-p42C/EBP α -ER stable cells were assayed. Cells were transfected with siE6AP and 48h post transfection, cells were induced with 5uM β -estradiol. It was followed by washing the cells once with PBS and subsequent resuspension in PE-conjugated cd11b antibody and the isotype IgG as control. Cells were analyzed under FACS flow cytometer (Becton Dickinson) for the expression of cd11b a myeloid differentiation marker.

Reagents and Buffers:

PBS: 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄. Adjust to a final pH of 7.4

Coomassie blue staining solution: 0.2% Coomassie blue, 7.5% Acetic acid, 50% Ethanol

Destaining solution: 50% (v/v) Methanol in water with 10% (v/v) Acetic acid

Laemmli Buffer (SDS loading buffer 2X): 125mM Tris pH 6.8, 4% SDS, 20% Glycerol, 10% 2-Mercapto β-ethanol and a pinch of Bromophenol blue

MACS Buffer: 2%FBS and 2mM EDTA in 1X PBS

Nuclear Extract Buffer A: 20mM HEPES, 10mM NaCl, 3mM MgCl₂, 1%NP-40, 10% Glycerol, 0.2mM EDTA, 1mM DTT and protease inhibitors

Nuclear extract buffer C: 20mM HEPES, 400mM NaCl, 20% Glycerol, 0.2mM EDTA, 1mM DTT, and Protease inhibitors

RIPA Lysis buffer: 1%NP40, 0.5%Sodium deoxycholate, 0.1%SDS, 0.15M NaCl, 5mM EDTA and 50mM Tris pH8.0

Urea Lysis buffer: 7M Urea, 2M Thiourea, 1% DTE, 4% CHAPS and 2.5mM EDTA

Running buffer (1X, 1L): 12g Tris base, 47.5g Glycine and 16g SDS in dH₂O

Transfer buffer (1X, 1L): 3g Tris base, 14.1g Glycine, 200ml Methanol and dH₂O

Blocking buffer: 5.0% milk in PBST

1X TBS: 20mM Tris Base, 150mM NaCl, pH 7.6

Co immunoprecipitation Buffer: 1% TBS, 0.5% NP40, Protease inhibitors

NETN Buffer: 150mM NaCl, 20mM Tris pH 8.0, 1mM EDTA pH 8.0, 0.1% NP40 and protease inhibitors

Stripping Buffer: 15g glycine, 1g SDS, and 10ml Tween 20; Adjust pH to 2.2. Volume maintained up to 1L with distilled water