Materials and Methods
Materials

Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum (FCS), all other chemicals used for cell culture medium, mammalian expression vector pcDNA3.1-Myc/His, Trizol Reagent and Superscript II reverse transcriptase kit were from Invitrogen Life Technologies (Carlsbad, CA). QIAexpressionist protein expression and purification kit including Ni-NTA-Agarose, gel purification kit and all primers used for amplification of c-DNA were from Qiagen (Valencia, CA). 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal), Protein A-agarose, Isopropyl-Thio-D-Galactopyranoside (IPTG), Ampicillin, Kanamycin, para-nitrophenyl phosphate (pNPP), diphenylene iodonium (DPI), N-acetylcysteine (NAC), L-N-monomethyl arginine, dephosphorylated casein, Myelin basic protein (MBP), Sodium pervanadate, Sodium fluoride, Aprotinin, Pepstatin, Leupeptin, Iodoacetamide were purchased from Sigma (St. Louis, MO). Bacto Tryptone, Yeast Extract and Bacto agar were from DIFCO, (San Diego, CA.). Immobilin dry-strips, dithiothreitol (DTT), and other chemicals used for 2D-gel electrophoresis were from Amersham Biosciences, (Uppsala, Sweden). Dichlorodihydrofluorescein diacetate (H$_2$DCFDA) and dihydroethedium (DHE) were from Molecular Probes, (Eugene, OR). All antibodies used for immunoprecipitation and western blottings and Enhanced Chemiluminescence (ECL) kit were from Santa Cruz Biotechnology (Santa Cruz, CA) eBiosciences (San Diego, CA, USA), BD Biosciences Pharmingen, (Palo Alto, California, USA), Cell Signaling Technology (Beverly, MA, USA), Molecular Probes Inc., (Oregon, USA), Sigma Chemicals, (St. Louis, USA). Cu/Zn SOD-encoding plasmid and EhCaBP plasmid were kind gifts from Dr. J Subramaniam of IIT, Kanpur and Dr. Sudhir Sopory, ICGEB, PMB Group, New Delhi respectively. All
other fine chemicals used were from Sigma (St. Louis, MO) or USB Corporation (Cleveland, OH). Restriction endonucleases and DNA modifying enzymes were from New England Biolabs (Inc. MA, USA). Promega Life Science (Madison, USA) and GIBCO-Invitrogen Corporation (New York, USA).

Composition of Common Media and Solutions

Bacterial growth media and solutions

**LB** (Luria-Bertani) medium (1000 ml): 10 g tryptone, 5 g yeast extract and 10 g NaCl (Sterilized by autoclaving).

**LB agar:** LB medium containing 15 g/liter agar (Sterilized by autoclaving).

**Kanamycin stock solution:** 25 mg/ml in double-distilled water, filter sterilized and stored in aliquots at -20°C until use.

**Ampicillin stock solution:** 100 mg/ml in double-distilled water, filter sterilized and stored in aliquots at -20°C until use

**Tris-EDTA buffer:** 10 mM Tris.HCl and 1 mM EDTA

Buffers and solutions for western blotting

**Lysis buffer:** 50 mM HEPES (pH 7.5), 10 mM EDTA, 10% (v/v) Glycerol, 0.5% Triton X-100 along with protease and phosphatase inhibitors as applicable.

**2X SDS-PAGE Buffer**

100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 4% β-mercaptoethanol, 0.01% bromophenol blue

**Transfer buffer:** 25 mM Tris base, 250 mM Glycine, 0.01% SDS (Sodium Dodecyl Sulfate) and 20% Methanol.
1X PBS (Phosphate-Buffered Saline): 13.7mM NaCl, 0.27mM KCl, 10mM di-
Sodium hydrogen phosphate (Na₂HPO₄) and 0.2mM Potassium di-hydrogen
phosphate (KH₂PO₄).

Blocking buffer: 5% (w/v) Bovine Serum Albumin in 1X PBS-T.

Wash buffer: 0.1% (v/v) Tween-20 in 1X PBS

Primary and secondary antibodies are prepared in wash buffer and blots were
developed by Enhanced Chemiluminescence (ECL) kit.

Buffers and solutions for 2D-gel electrophoresis

Lysis buffer: 8M Urea, 4% (w/v) CHAPS and 2% (v/v) Pharmalyte (pI 4 -7).

Rehydration buffer: 8M Urea, 2% (w/v) CHAPS, 0.28% (w/v) DTT, 0.1%
Bromophenol blue and 0.5% (v/v) Pharmalyte (pI :4 – 7).

Equilibration buffer: 50mM Tris-HCl (pH-8.8), 6M Urea, 30% (v/v) Glycerol,
2% (w/v) SDS and 0.1% Bromophenol blue. Prior to use DTT (10 mg/ml) and
Iodoacetamide (25 mg/ml) are added.

Buffers for phosphatase assay

Fractionation buffer: 10mM HEPES (pH 7.4), 4.5mM EGTA, 2.5mM EDTA
and protease and phosphatase inhibitors as applicable.

Extraction buffer: Fractionation buffer with 1% Triton X-100.

Assay wash buffer: 40mM HEPES, 10% (v/v) Glycerol, 5mM MgCl₂ and
0.001% (v/v) Triton X-100.

Assay buffer: 50mM MES (pH-6.4), 1mM EDTA, 0.001% (v/v). Triton X-100
and 20mM pNPP
Buffers for immunoprecipitation

**RIPA buffer:** 50mM Tris, 100mM NaCl, 1% (v/v) NP-40, 0.25% (w/v) Sodium deoxycholate, 1mM EDTA, 1mM EGTA along with phosphatase and protease inhibitors as applicable.

Buffers for ERK kinase assay

**Wash buffer:** 20mM Tris-HCl (pH 7.5), 20mM MgCl₂, 2mM DTT and 1mM pNPP along with 10µM sodium vanadate.

**Assay buffer:** 20mM Tris-HCl (pH 7.5), 20mM MgCl₂, 2mM DTT, 10µM ATP, 10µCi γ-³²P- ATP and 5µg MBP was added per reaction.

Buffers for Lyn kinase assay

**Wash buffer:** 25mM Tris-HCl (pH 7.5), 10mM MgCl₂, 0.5mM DTT and 0.1mM sodium vanadate.

**Assay buffer:** 25mM Tris-HCl (pH 7.5), 10mM MgCl₂, 0.5mM DTT and 0.1mM sodium vanadate. 50µM ATP, 10µCi γ-³²P- ATP and 5µg dephosphorylated casein was added per reaction.

Other buffer composition:

1x Protease cocktail: 16 µg/ml benzamidine, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml peptstatin A, 1 mM PMSF and 10 µg/ml phenanthroline

Fixing solution: 1 % formaldehyde, 0.2 % glutaraldehyde in PBS

Dye 25% Ficoll and 1% Orange G

PAGE Band Elution Buffer: 50 mM NH4HCO3 and 0.1 % SDS
PEG/LiAc solution: 10 mM Tris, pH 8.0, 1 mM EDTA, 100 mM LiAc, 40% PEG 3350

Phosphate-citrate buffer (0.05M): A- 25.7 ml of 0.2 M Na2HPO4
B-24.3 ml of 0.1 M citric acid final pH-5.0

Ponceau-S Stain: 0.2% w/v Ponceau-S, 3% w/v tri-chloroacetic acid, 3% (w/v) sulfosalicylic acid

Reagents for Silver Staining: Fixation solution (50:5:45 methanol : acetic acid : water), Sensitizing solution (0.02 % sodium thiosulfate), 0.1% AgNO3
Developing solution (0.04 % formaldehyde in 2 % sodium carbonate), Stopping Solution (1 % acetic acid)

Buffers for DNA Isolation

Solution I: 50 mM glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA
Solution II: 0.2 N NaOH and 1% SDS
Solution III: 4.8 Potassium acetate and 11.5% glacial acetic acid

Sonication Buffer: 50 mM sodium phosphate, pH 7.8, 300 mM NaCl

QBT Solution: 700 mM NaCl, 50mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X-100

QC Buffer: 1 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol

QF Buffer: 1.25M NaCl, 50 mM Tris-HCl, pH 8.5, 15% ethanol Sarkosyl Buffer
25 mM Tris, pH 8.0, 1.5 % Sodium-N-laurylsarcosine, 1 mM EDTA

TBE: 88 mM Tris-borate, 89 mM boric acid, 2 mM EDTA

TBST: 10 mM Tris, 150 mM NaCl, pH 7.5 and 0.05% Tween-20

Wash Buffer: 50 mM Sodium phosphate pH 6.0, 300 mM NaCl, 10 % glycerol
Dulbecco’s Modified Eagles Medium:

1 packet DMEM (for 1 litre), 3.7 gm sodium bicarbonate (NaHCO₃), 2.3 gm HEPES, 1.46 g L-Glutamine, 100 U/ml Penicillin and 100 μg/ml of Streptomycin sulfate.

Competent Cell Preparation and Transformation

A single colony of *E. coli* DH5α or BL21 (DE3) cells, was inoculated into 5 ml LB medium and was grown for 16 hours at 37°C with shaking at 200 rpm. When the optical density of the culture reached the 0.5-0.6, the cells were aseptically transferred to disposable, ice-cold 50 ml polypropylene tubes. The cell culture was chilled at 4°C for 10 min and pellet down at 3,000 rpm at 4°C. The cell pellet was re-suspended in 25 ml of 100 mM CaCl₂ and stored on ice for 30 min. The cells were then pellet down and re-suspended in 1.5 ml of 50 or 100 mM CaCl₂. For higher efficiency, cells were kept on ice in the cold room for another 24 hrs. The cells were made 20% in glycerol and aliquots were made in sterile microfuge tubes and were stored at -70°C for future use. The competent cells were used for transformation and propagation of various DNA constructs.

Transformation was carried out as follows:

About 5-20 ng of DNA or ligation mixture in a volume of 10 μl was added into 100 μl of competent cells in 1.5 ml microfuge tubes and incubated on ice for 30 min. The tubes were transferred to a 42°C water bath for 1.5 min followed by 10 min on ice. To this, 800 μl of LB broth without antibiotic was added and the cells incubated at 37°C with shaking for one hour. The cells were then pellet down, media discarded and the pellet obtained re-suspended in 50 μl of fresh LB medium. Finally, half of the total volume was plated on LB agar plates containing the appropriate antibiotic.
**DNA Isolation**

Miniprep DNA was isolated by alkaline lysis method, as described in Sambrook et al. or with Qiagen Columns as per manufacturer's instruction with few modifications as described below.

**a). Medium scale isolation and purification of plasmid DNA by Qiagen-Tip column (Qiagen GMBH).**

Fifty ml (for high copy number) or 100 ml (for low copy number) of overnight grown culture of bacterial cells was harvested by centrifugation at 5,000 rpm at 4°C. The pellet was re-suspended in 4 ml solution I. The cells were lysed by adding 4 ml of freshly prepared solution II and gently mixed by inversion and kept at room temperature for 5 min. Following this, 4 ml of chilled solution III was added, mixed by inversion and incubated on ice for 20 min. The mixture was centrifuged at 13,000 rpm at 4°C for 30 min. The cleared supernatant was loaded onto the Qiagen tip-100 column, pre-equilibrated with QBT solution and allowed to pass by gravity. The Qiagen tip-100 was washed twice with 10 each ml of buffer QC. Plasmid DNA was eluted with 5 ml of buffer QF and was precipitated with 0.6 volume of isopropanol at room temperature. Following centrifugation at 12,000 rpm for 30 min, the plasmid pellet was washed with 70% ethanol, air-dried and dissolved in Tris-EDTA (TE) buffer.

**b). Small-scale isolation and purification of plasmid DNA (Alkaline lysis method).**

Five ml of an overnight grown culture of bacterial cells was harvested by centrifugation at maximum speed for 30 sec. The pellet was re-suspended in 100 μl of ice-cold solution I and vortexed. The cells were lysed by adding 200 μl of freshly prepared solution II and gently mixed by inversion. To this, 150 μl of
chilled sol III was added, mixed well and incubated on ice for 3-5 min. The supernatant was collected by centrifugation at maximum speed for 5 min at 4°C and extracted once with equal volume of phenol : chloroform : isoamyl alcohol (25:24:1). followed by a chloroform extraction. The DNA was precipitated by adding 1 ml of ethanol and kept at -70°C for 2 hr. After incubation, the DNA was recovered by centrifugation at maximum speed for 15 min at 4°C. The plasmid DNA pellet was washed with 70 % ethanol, air-dried and dissolved in TE buffer.

c). Cesium Chloride (CsCl)-gradient DNA Preparation

DNA constructs used for mammalian cell transfection were either prepared by the Qiagen midi-DNA extraction kit or by CsCl gradient centrifugation as follows:

5 ml LB medium was inoculated with a single colony from a freshly streaked plate and grown overnight at 37°C with vigorous shaking. Subsequently 500 ml of Luria Broth containing appropriate antibiotic was inoculated with 5 ml of the overnight grown culture and incubated for 14-16 hours with shaking. The cells were then pellet by centrifugation at 4°C. The pellet was re-suspended in 40 ml Solution I and kept on ice for 10-20 min. Then, 80 ml of Solution II was added, mixed and kept at room temperature for 5 min. To this, 60 ml of Solution III was added, mixed well and incubated on ice for 20-30 min followed by centrifugation at 8,000 rpm at 4°C for 15 min in a GS3 rotor. The supernatant was filtered through 8-layered cheese cloth and transferred into a fresh bottle. The DNA was precipitated with 0.7 volumes of isopropanol. The DNA pellet was dried and re-suspended in 4 ml of TE mixed with 4 g of CsCl and dissolved completely. Ethidium bromide (50 μl; 10 μg/μl) was poured into 5 ml Beckman Quick-Seal tubes and the tubes filled with the DNA-CsCl mixture. After balancing
and sealing the tubes, the samples were centrifuged at 55,000 rpm in VTi 65 rotor in Beckman ultracentrifuge for 16 hrs. The plasmid DNA bands were collected using a 5 ml syringe fitted with an 18 gauge needle. To remove ethidium bromide from DNA, an equal volume of water-saturated butanol was added, mixed well and the upper phase was removed repeatedly until the DNA solution became clear. The DNA was then diluted 4-5 times with TE pH-8.0 followed by 3-4 rounds of ethanol precipitation. The DNA concentration was estimated and kept as a 1 mg/ml working solution. The quantity and purity of nucleic acids in solution was determined by measuring the absorbance at 260 and 280 nm. The concentration of nucleic acids was calculated by taking 1 OD$_{260}$ = 50 µg/ml for DNA, 40 µg/ml for RNA and 33 µg/ml for single stranded oligonucleotides respectively. The purity of nucleic acids was checked by their absorbance A$_{260}$/A$_{280}$ ratio.

**Purification of DNA Fragments from Agarose Gel**

DNA samples were resolved by electrophoresis on 0.8-1.5% of agarose: low melting agarose gel. The desired fragment was identified using standard molecular weight markers (1 kb ladder) and purified using one of the following techniques.

- **a). Phenol freeze-fracture method**

  The agarose gel piece with the desired DNA containing band was cut into pieces and macerated by passing through an 18-gauge needle attached to a syringe. To this an equal volume of Tris-HCl, pH 8.0 saturated phenol was added, vortexed and frozen at -80°C for 1 h. The samples were then thawed and centrifuged at 13,000 rpm for 5 min at room temperature. The upper aqueous phase was collected and subjected to two rounds each of chloroform and
chloroform-isoamyl alcohol (24:1) extraction. The DNA fragment was then precipitated by adding 0.1 volume of 3 M Na-acetate (pH 4.8) and 2.5 volume of ethanol and kept for 1 hour at −70°C. The DNA was collected by centrifugation at 14,000 rpm for 15 min at 4°C, washed with 70% alcohol, air dried and dissolved in TE buffer.

b). GFX gel extraction column

To the cut pieces of agarose gel containing the desired DNA fragment, 350 μl of capture buffer, as supplied with GFX PCR DNA and Gel band purification kit (Amersham Pharmacia Biotech Inc. Piscataway NJ, USA) was added. The gel pieces were dissolved by heating at 55°C for 10 min. The mixture was loaded onto a GFX spin column and spun briefly at 10,000 rpm. The flow through was discarded and the column was washed once with wash buffer at 10,000 rpm for 1 min followed by a dry spin. The purified DNA fragment was eluted with 30 μl of 10 mM Tris-HCl, pH 8.0 or water.

Polymerase Chain Reaction (PCR).

Rapid amplification of the DNA was carried out using Taq DNA polymerase and a set of convergent primers. Fifty pmoles each of the forward and reverse primers along with 200 μM of each dNTP and 2.5U of Taq DNA polymerase was used for PCR. The reaction conditions for PCR included denaturation at 94°C for 1 min, primer annealing at 55°C (or 5°C less than the lower Tm of the two primers), for 1 min and extension at 72°C for 1 min for each kb length of the expected product, for 25-38 cycles in 50 μl of reaction volume. An aliquot from the mix was resolved on 1% agarose gel to check for amplification.
PCR primers used in this work

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’ → 3’)</th>
<th>Reverse Primer (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human PRDX1</td>
<td>ATA GCG GCC GCA AGA TGG CTT CAG GAA ATG CTAA</td>
<td>GCT AAG CTT CTT CTG CTT GGA GAA ATA TTC T</td>
</tr>
<tr>
<td>Mouse SOD2</td>
<td>ATA GCG GCC GCA TAA GGA GTA TGT GTC GGG CAG TGT GTA TCT</td>
<td>GCT AAG CTT CTT CTT GCA AGC TGT GTA TCT</td>
</tr>
<tr>
<td>Human Catalase</td>
<td>ATA GCG GCC GCC ACG TTA TGG CGA CGA AGA TGG CTA TGG CTG ACA GC</td>
<td>GCT AAG CTT CAG ATT TGC CTT CTC CCT TG</td>
</tr>
<tr>
<td>Human SOD1</td>
<td>ATA GCG GCC GCC GAG TTA TGG CGA CGA AGA TGG CTA TGG CTG ACA GC</td>
<td>GCT AAG CTT GGC GAT CCC AAT TAC ACC AC</td>
</tr>
<tr>
<td>EhCaBP</td>
<td>ATA GCG GCC GCA TGG CTT TTA AGA GGA TCC ATA ATG GTG AGA GGT ACC GAG GAA AAC</td>
<td>AGA GGT ACC GAG GAA AAC TCA AGG AAT TCT TC</td>
</tr>
<tr>
<td>Duox&lt;sub&gt;1&lt;/sub&gt; (for RT)</td>
<td>CTT ACT GTT CAC TGA GCC AC</td>
<td>GGA AAA CAT AAA GGA GAT GCT G</td>
</tr>
<tr>
<td>Duox&lt;sub&gt;1&lt;/sub&gt; (To Clone)</td>
<td>ATA GCG GCC GCG TTA TGG GGT TCC ACT TAG CTC</td>
<td>AGA GGA TCC ATA ATG GTG AGA AAA ATG AGT CC</td>
</tr>
</tbody>
</table>

DNA Sequencing

Purified plasmid DNA (10 μg) was denatured with 0.2 M NaOH and 0.2 mM EDTA at 37°C for 10 min. The denatured DNA was then precipitated using 3 M Potassium acetate (pH 5.2) and ethanol. After two washes with 70% ethanol, the dried DNA was dissolved in 10 μl of dH2O. Annealing of the primer to denatured DNA was done by incubating the DNA, primer and sequencing buffer at 95°C for 5 min and then at 37°C for 5 min followed by 5 min at room temperature. The sequencing reactions were performed essentially as described in
the T4 DNA Sequencing Kit instruction manual (USB, USA) in the presence of

\(^{35}\)S-dATP. The samples were boiled then chilled on ice and separated on a 6% polyacrylamide gel containing 8M urea. Electrophoresis was carried out at constant power (75 watts) to maintain the gel temperature at 55°C. Two loadings were done to resolve maximum nucleotide sequence. After completion of the run, the gel was transferred on to Whatmann 3 MM paper, dried in a gel dryer and subjected to autoradiography.

**Primers used for sequencing Sequencing primer Sequence**

T7 Promoter 5'-TAA TAC GAC TCA CTA TAG GG3'

SP6 promoter 5'-ATT TAG GTG ACA CTA TAG AA3'

The other primers used for sequencing are described elsewhere in the text. Some of the clones were sequenced commercially using automated sequencing.

**Cell lines and primary cells**

A20, CH-1, WEHI-231, HeLa, and J774 cells were cultured in Dulbecco’s modified Eagle’s Medium (DMEM) or RPMI supplemented with 10% fetal bovine serum (FBS) and 1X penicillin-streptomycin, and were subcultured on reaching 90% confluence. The THP-1 were cultured in RPMI 1640 supplemented with 10 % FBS and 1X penicillin-streptomycin and subcultured as required.

**DNA Transfection**

a) Electroporation

A20 cells were grown in DMEM and 10 % FBS. At 75% confluence, the cells about 4-7 million cells were trypsinized and transfected with 10 μg of either proviral DNA or plasmid constructs in a 300 μl total volume of RPMI 1640 containing 10 mM glucose, 2% DMSO by electroporation. The electroporator (Bio-Rad, Hercules, USA) was set to 260 V and 975 μF. The cells were then
plated into the wells of a six-well tissue culture dishes in 3 ml of RPMI (or DMEM) supplemented with 10% FBS containing 1X Penicillin-Streptomycin. Transfection was measured by a reverse transcriptase (RT) assay or by Western Blot analysis.

b) Lipofection

Mouse NIH3T3 cells were grown in DMEM containing 10% FBS in a 5% CO2 incubator. The cells were seeded at about 30-50% confluency in 60 mm dishes one day before transfection. On the following day, cells were transfected with CsCl gradient purified or column purified (Qiagen, Germany) DNA using Lipofectin (Invitrogen, USA). To 90 µl Opti-MEM (Invitrogen), 10 µl Lipofectin was added and incubated for 45 min (Solution A). To this was added 5-10 µg DNA in 95 µl of Opti-MEM (Solution B). At the end of incubation, Solution A and Solution B were mixed and incubated further for 15 min at room temperature to allow formation of a DNA-lipid complex. Meanwhile, the cells were washed once with PBS and once with Opti-MEM. Finally, to each plate containing 1.8 ml Opti-MEM, 200 µl of the DNA-lipid mixture was added and incubated at 37°C. After six hours of incubation, the Opti-MEM was replaced with complete medium containing DMEM and 10% FBS. At about 48 h post-transfection, the cells were harvested and used for various experiments.

Cloning and Sequencing of PCR Products

The amplified fragments were cloned into the pGEM-T Easy vector (Promega) or UA (Qiagen) cloning vector and amplified in *Escherichia coli* DH5α according to the manufacturer’s specifications. Following confirmation by
restriction analysis or colony PCR the inserts from 15-20 single bacterial colonies were first subjected to manual sequencing.

**Purification of mouse splenic B-cells**

6-8 weeks old BALB/C mice were dissected and the spleen was isolated and washed in HBSS. The tissue was minced between two frosted slides to make a suspension of cells. RBC was depleted off the cells by treating the resulting cell suspension with RBC Lysis Buffer containing 8.3 g/l of ammonium chloride in 10 mM Tris-Cl (pH 7.5). 4 ml of RBC Lysis Buffer was added per spleen, kept for 5 mins at room temperature and then 15 ml of HBSS was added to it before pelleting. Cells were washed thoroughly with HBSS and finally resuspended in RPMI media containing 20% FCS. Adherent cells were removed by panning on plastic surface tissue culture plates at 37°C for 1 hr. T-cells were removed from the obtained cell suspension by incubating with a mixture of magnetic beads coated individually with anti-CD90 (Thy1.2), anti-CD4 and anti-CD8 antibodies. Some anti-CD11c and anti-CD11b antibodies were also added. These were finally separated with MACS (magnet assisted cell sorter) columns. The resulting cell suspension obtained is rich in B-cells.

Resting B-cells were purified from the above cell preparation by discontinuous percoll density gradient described as follows. 2.5 ml each of 70%, 66%, 60% and 50% percoll were over layered in a 15 ml falcon and topped with 2.5 ml of isolated spleenic B-cell suspension in 10% RPMI and centrifuged in a swinging bucket rotor at a speed of 2500 rpm for 30 mins at 4°C. High density cells at the interface of 66% and 70% were collected and washed thoroughly with HBSS and cultured in 10% RPMI. The resting nature of these cells can be
monitored by surface staining with anti-mouse anti-IgD FITC antibodies and monitoring by flowcytometry. These have 96-98% sIgD.

**Preparation of cell lysate**

Transfected, untransfected controls or infected cells were grown in 60 mm dishes in an appropriate medium (DMEM or RPMI-1640) containing 10% FBS, unless stated otherwise. Where required, serum starvation was carried for 4-6 h in serum-free medium. The labeling of cells with [35S]-methionine/cysteine was as described earlier. For harvest, cells were washed with ice-cold PBS, and unless stated otherwise, the monolayer was dissolved in 750 µl of radioimmunoprecipitation buffer (RIPA) containing 1x protease inhibitor cocktail. The lysate was vortexed for 1 min to shear the genomic DNA and then centrifuged at 13,000 rpm at 4°C for 15 min in a Biofuge 17RS (Heraeus, Germany).

**Immunoprecipitation**

For immunoprecipitation, the protein concentration was assayed with the Bradford reagent (Sigma) and lysate containing equal amounts of protein (~500-600 µl) were taken. To this, a 1:100 dilution (5 to10 µl) of the required antibody was added for 1 hr and mixed on a Nutator at 4°C. This was followed by the addition of 100 µl of a washed 10% suspension of protein A/G Sepharose and mixed again for 1 h at 4°C. The immunoprecipitate was washed 4-5 times with RIPA buffer and analyzed further by SDS-PAGE and fluorography.

**Polyacrylamide Gel Electrophoresis of Proteins**

Polyacrylamide gel electrophoresis (PAGE) was performed according to the protocol of Laemmli (1970). Gels were prepared as described elsewhere (Molecular cloning A Laboratory Manual; Sambrook, Fritsch and Maniatis) and run in the presence of 0.1% SDS (denaturing). Protein samples were prepared by
mixing with equal volume of 2X SDS-PAGE sample buffer and boiling in a water bath for 5 min. Gels were run at a constant voltage of 100 V. Following the run, gels were either electroblotted onto the nitrocellulose membrane (Hybond-C, Amersham, UK) or stained with 0.25% Coomassie blue R-250 in 50% methanol and 10% acetic acid as described by Laemmli (1970).

**Western Blotting**

Western blotting was done according to Towbin et al (1979). Mini Trans-blot Electrophoretic Cell (Hoefer) was used to transfer the proteins from gel onto nitrocellulose membrane. The apparatus for electroblotting was assembled according to the manufacturer’s instructions. Electroblotting was performed in the presence of 39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol at a constant voltage of 50 V for 1 h at 4°C. Transfer of proteins onto the membrane was checked by staining with Ponceau S stain. The membrane was rinsed briefly in TBST and then incubated in blocking solution containing 5% nonfat milk in TBST for 1 h with gentle shaking at 37°C. The blocking solution was replaced with an appropriate dilution of primary antibody in TBST with 1% BSA and incubation was continued at 37°C for another 1 h with gentle shaking. Thereafter, the blots were washed thrice with TBST for 5 min each. After washing, the blots were incubated with horseradish peroxidase conjugated secondary antibody solution (1:5,000 dilution in TBST having 1% BSA) at 37°C for 1 h. The blots were washed as described above and processed using diaminobenzidine (DAB) as a substrate or Enhanced Chemi-luminescence (ECL).

**Radioactive Labelling of cells**

The cells were cultured in phosphate free (or Cysteine, Methionine free) DMEM or RPMI for 3-5 hrs after washing properly followed by addition of $^{32}$P-
labelled orthophosphoric acid (1mCi/ml) or (35S-labelled Methionine/Cysteine, 0.5mCi/ml). for another 2-5 hrs depending upon the radioactive counts in the supplied radioactive compound

2-Dimensional Gel Electrophoresis and analysis

A20 cells (2 x 10⁷) were placed in phosphate-free DMEM supplemented with 1% FCS for 2 hr, following which [³²P]-orthophosphoric acid (0.5 mCi/ml). was added and the culture continued for an additional 4-5 hr. They were then stimulated with the F(ab)₂ fragment of goat anti-mouse IgG at a final concentration of 25 µg/ml for the indicated times. Where necessary, the appropriate inhibitor was added at 10 min prior to stimulation (EGTA and TMB-8 at final concentrations of 3mM and 100µM respectively). The cytoplasmic fractions of cell lysates were then resolved by 2-DE as previously described (Gorg et al. 2000). Resolution in the first dimension was achieved using 13 cm IPG dry strips (pH, 4-7), and a 12% SDS polyacrylamide gel was used to resolve in the second dimension. Comparable loading in all the groups was ensured through silver staining of the resultant gels. Dried gels were then exposed to X-ray films.

Phosphoproteins were visualized by autoradiography and digitized on a Molecular Dynamics computing densitometer using the ImageQuant software (Amersham Biosciences). Only those spots with an area greater that 75 pixels were considered, and the minimum intensity surrounding the spot on the film was taken as its background and subtracted to give the true intensity. Relative quantification was achieved by normalizing against three distinct spots that were unaffected upon anti-IgG stimulation of cells. Calibration for the Mw and pI was done on the basis of standard markers that were run on parallel gels.
Silver Staining of polyacrylamide gels

After the gel has been run, the protein was fixed by incubating the gel slab in fixation solution for 20-30 minutes. The gel slab was rinsed with water (2 changes, two minutes per change) and then left it further in water for one hour on a shaking platform. Extended washing was done to eliminate yellowish background usually observed after long developing of the gel. The gel was sensitized with sensitizing solution for 1-2 minutes and the solution discarded with a quick rinse of the gel slab with two changes of water (10 seconds each). The gel is covered with chilled 0.1% AgNO₃ for 30 minutes at 4°C (fridge). Silver nitrate solution was discarded and the gel was quickly rinsed with two changes of water (30 seconds per each change). The gel was developed with developing solution and as soon as it turns yellow, it is replaced with a fresh solution. When a sufficient degree of staining has been obtained, staining is quenched by discarding the developing solution and replacement with 1% acetic acid. The gel is washed with 1% acetic acid several times and store in the same solution.

Colloidal Coomassie Staining of polyacrylamide gels

a). Dye stock solution:

0.1 % (w/v). Coomassie Brilliant Blue G250, 2 % (w/v) ortho-phosphoric acid
10 % (w/v). ammonium sulfate

b). Dye working solution: 80 % (v/v) dye stock solution, 20 % (v/v) methanol

c). Staining procedure:

The gel was fixed with 40% (v/v) ethanol, 10% (v/v) acetic acid for at least 60 min. It was then washed with water for 2 x 10 min (gel should be completely covered). Stain the gel in dye working solution at least over night. The gel was then transferred into new box with 1% (v/v) acetic acid and wash with 1%
acetic acid until all Coomassie particles were removed (background should be clear; several changes of 1 % acetic acid is required for good resolution).

**RNA isolation**

RNA was isolated using manufacturer’s protocol. Briefly, 1ml of TRizol Reagent (GIBCO BRL) was added per 10 million of cells or 1mg of tissue for cell/tissue lysis and mixed properly by pipetting. 200μl of chloroform was added after 10 mins and mixed well with vigorous shaking for 1 min. After 5mins, the contents were centrifuged at 13000 rpm for 15 mins at 4°C. The aqueous phase was taken and RNA was precipitated by adding 500 μl of isopropanol alcohol per ml of TRizol used. This was followed by wash of the RNA pellet with 70% ethanol in DEPC treated water. Finally, RNA was dissolved in appropriate volume of DEPC treated water and quantification was done using spectrometric absorption at 260nm.

**RT-PCR**

Two microgram of RNA from each group was subjected to reverse transcription (RT) using oligo (dT)$_{20}$ and Superscript II Reverse Transcriptase following the manufacturer’s protocol. The cDNAs were amplified by PCR with appropriate primers. The PCR cycle used for amplification was as given below:

- **Hot start** 94°C for 5 minutes (Taq polymerase was added after hot start).
- **PCR cycles**
  - Denaturation: 94°C, 1 minute,
  - Annealing: 55°C, 1 minute,
  - Extension: 72°C, 1 minute

32 to 38 PCR cycles were run as applicable for different genes followed by one cycle of extension of incomplete strand at 72°C for 10 minutes. PCR products were analyzed on 1% agarose gel electrophoresis. For single step RT-PCR, only
the specific primers were used and the first strand synthesis was done for 45 min at 50°C using manufacturer's protocol

**Northern Blotting**

Northern blotting was done using standard protocol.

**Fluorescence Microscopy**

For fluorescence microscopy, the cells were grown on coverslip-bottomed dishes, transfected, and fixed *in situ* at various times with 3% paraformaldehyde for 15 min. This was followed by permeabilization with 0.1% saponin for 30 min and blocking with 1% BSA for 1 h. The cells were then stained on the coverslips with primary reagents for 1 h, followed by washing and secondary reagents were appropriate. Confocal images were acquired on a Bio-Rad MRC-1024 confocal microscope (Bio-Rad Microsciences, Hemel Hampstead, UK) with factory-set dichroics and a Krypton-Argon laser, using Lasersharp acquisition software (Bio-Rad). Images were processed in MetaMorph (Universal Imaging, Downingtown, PA) and Adobe Photoshop (Adobe, San Jose, CA) softwares.

To examine internalization kinetics of cell surface molecules, transfected cells were surface-labelled with the primary reagents, and incubated at 37°C for various times before being fixed, permeabilized and stained with appropriate secondary reagents, followed by blocking and staining for organelle marker molecules.

For reactive oxygen species (ROS) measurement, cells on coverslips were labelled with 5μM DCFDA-AM dye for 30 mins and washed twice in plain RPMI. After that, the cells were stimulated with appropriate stimuli for specific time points to monitor the kinetics of ROS generation.
**In-vitro Kinase Assay**

Lyn (or any kinase) was first immunoprecipitated from the various groups (5 x 10⁷ cells/group/time point) as described above. The immunoprecipitates were washed, and then incubated in the kinase reaction buffer (25 mM Tris, pH 7.5 containing 0.5 mM DTT, 0.1 mM orthovanadate, 50 µM unlabeled ATP, 10 mM MgCl₂, and 10µCi/tube of [³²P]-ATP). The reaction was initiated through the addition of a 5µM final concentration of the synthetic peptide substrate (sequence: AEEEEIGFEAKKKK) or dephosphorylated casein or myelin basic protein (MBP) as substrate. The reaction was terminated by centrifugation, followed by spotting 20 µl of the supernatant onto Whatman-81 chromatography paper. After extensive washing, the radioactivity associated with the spots was determined by scintillation counting. To ensure that the results obtained do not reflect variations in the amount of Lyn in the different groups, parallel sets of immunoprecipitates were also analyzed by Western blot using anti-Lyn antibodies. When casein or MBP were used as substrates, the reaction was terminated by direct addition of 2X SDS dye and boiling it for 5 mins followed by SDS-PAGE and exposure of the dried radioactive gels to photographic films.

**In-vitro Phosphatase Assay**

This was done essentially as described earlier, using para-nitrophenyl phosphate as the substrate (Hua et al. 1998; Yoshida and Kufe 2001). That comparable amounts of phosphatase (either SHP-1 or B220) present in the various tubes was again ensured through Western blot on parallel sets of immunoprecipitates.
Reactive Oxygen Species (ROS). **Measurement by Spectroflouirimetry**

For ROS measurement 5 x 10^6 cells were suspended in 1 ml of serum-free DMEM, and labeled with 1μM of Dichlorodihydrofluorescein diacetate (H₂DCFDA) for 15 min at 37°C. Cells were washed twice with medium and resuspended in 750μl of medium. 100μl of the suspension was pipeted in each well of the 96-well plate supplied by the manufacturer. Stimulants were pumped into the wells at the desired times. The fluorescence was measured by Floustar Optima spectrofluorimeter of BMG laboratories. The filters used for fluorescence measurement has an absorption spectrum of 480 nm and emission spectrum of 520 nm.

**Bacterial Expression and Purification of the mouse Duox1 Protein**

One colony of the pET28a construct in BL21(DE3) E.coli cells was inoculated into 5 ml LB medium containing 100 μg/ml of ampicillin and grown overnight at 37°C with shaking. The culture was diluted 100-fold into fresh 50 or 100 ml M9-NZM medium containing 25 μg/ml ampicillin and grown at 37°C with shaking to an A₆₀₀ of 0.6 to 0.8. The bacteria were induced with 1 mM IPTG for 4hours at 37°C with constant shaking. The cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C in a GS3 rotor (Sorvall). The E. coli pellet was washed twice with PBS and either kept frozen or processed further. The cells were resuspended in sonication buffer containing 10 μg/ml lysozyme and 1 mM PMSF and lysed by 5 cycles of freeze-thaw followed by sonication 4-5 times with 1 min pulses, and centrifuged at 12,000 rpm in SA600 rotor for 15 min. To the clarified lysate, a final concentration of 50 mM of imidazole was added and was used to bind to Ni-NTA agarose pre-equilibrated with sonication buffer for 1 h at room temperature. The column was washed with five bed volumes of wash buffer. The
puriﬁed protein was eluted using a step gradient of 0.1-0.5 M imidazole in steps of 0.1 M in wash buffer. Most of the proteins came out in the 0.1-0.4 M imidazole fractions. Fractions containing the protein were pooled, dialyzed against PBS, made 20% in glycerol and kept frozen at -70 °C till further study.

**Band Purification of Protein from SDS-Polyacrylamide gel**

One mg of Ni-NTA puriﬁed His-Duox protein was loaded on a 15 % preparative SDS-polyacrylamide gel. After 2 h of electrophoresis, a strip of gel was sliced out and stained with 0.3 M CuCl₂. The sliced piece was aligned with the original gel and the band corresponding to the Duox protein was sliced out and crushed using a 10 ml syringe. About 10 ml of elution buffer was added to the crushed gel pieces. The sample was then boiled 10 min and kept on a Nutator overnight at room temperature. Later, the gel pieces were ﬁltered out using glass wool in a 10 ml syringe. The eluant was lyophilized and dissolved in 1ml of 1xPBS. The SDS was partially removed by keeping the sample on ice and centrifuging at maximum speed. Protein estimation by the Bradford assay showed about 80% recovery.

**Flow cytometry**

**a) Flow cytometry for surface molecules**

For surface ﬂow cytometric analyses, cells were ﬁrst washed with serum free RPMI and then with FACS buffer containing 0.5% BSA and 0.01% sodium azide in 1X PBS. The Fc receptors on them were blocked by incubating them with anti-CD16/anti-CD32 antibodies. This is important so as to prevent non speciﬁc binding with these receptors and transducer negative signaling. Cells were then stained with primary reagents on ice for 30 min, followed by washing and incubation with appropriate secondary reagents. Following this, cell s were ﬁxed
with FACS Fixing Solution containing 0.01% sodium azide and 0.1% paraformaldehyde.

**b) Flow cytometry for intracellular molecules**

For intracellular staining, cells were permeabilized with 0.03% saponin. Stained cells were fixed with 1% paraformaldehyde and analyzed on a FACS Calibre (BD Biosciences, San Jose, CA) flow cytometer.

**c) Flow cytometry for calcium measurement**

For intracellular calcium measurement, 3x10⁶ cells were incubated with 1-2μM Fluo3-AM dye and incubated in 10% RPMI for 30 mins. Following this the cells were washed in serum free RPMI thrice and finally re-suspended in 700μl of media. For flow cytometric analysis, 100μl aliquots of these cells were taken and diluted to 1ml with RPMI. Cells were stimulated in the process of sample acquisition with standard settings of the parameters for calcium measurement as recommended by manufacturer.

**Confocal microscopy**

For confocal microscopy, the cells were grown on coverslip-bottomed dishes, transfected, and fixed *in situ* at various times with 3% paraformaldehyde for 15 min. This was followed by permeabilization with 0.1% saponin for 30 min and blocking with 1% BSA for 1 h. The cells were then stained on the coverslips with primary reagents for 1 h, followed by washing and secondary reagents were appropriate. Confocal images were acquired on a Bio-Rad MRC-1024 confocal microscope (Bio-Rad Microsciences, Hemel Hampstead, UK) with factory-set dichroics and a Krypton-Argon laser, using Lasersharp acquisition software (Bio-Rad). Images were processed in MetaMorph (Universal Imaging, Downingtown, PA) and Adobe Photoshop (Adobe, San Jose, CA) softwares.
To examine internalization kinetics of cell surface molecules, transfected cells were surface-labelled with the primary reagents, and incubated at 37°C for various times before being fixed, permeabilized and stained with appropriate secondary reagents, followed by blocking and staining for organelle marker molecules.

Liquid β-galactosidase assay

The liquid β-galactosidase activity was determined using the substrate chlorophenol red-β-galactopyranosidase (CPRG). The culture was grown in 5 ml SD medium for 16 hrs at 30°C. The cells were vortexed and 1 ml of inoculum was transferred to 4 ml of fresh medium. The cells were incubated at 30°C with shaking at 200 rpm for 3-5 hrs till the cells reached the mid-log phase (OD₆₀₀=0.8-1.0/ml). Equivalent amounts of culture (1.0-1.5 ml) were taken in microfuge tubes such that the numbers of cells for all cultures were the same. The cells were harvested by centrifugation and washed with 1 ml of buffer I. The cells were then suspended in 300 μl of buffer I from which 100 μl suspension was transferred into a fresh microfuge tube. Consequently cells were permeabilized by 5 cycles of freeze thaw in liquid nitrogen for 2 min followed by a 37°C water-bath for the same time. To the tube 0.7 ml of buffer II was added and mixed by vortexing. One ml of buffer II was kept aside to be used as blank. After 1 hr, 0.5 ml of 3 mM ZnCl₂ was added to all the tubes to stop the reaction. The tubes were centrifuged at 14,000 rpm and the supernatant was transferred to freshly labeled tubes. The absorbance at at 578 nm was recorded for each sample. Only those readings that lie in the linear range of 0.2-1.8 absorbance units were considered. Relative enzymatic activity was calculated as a mean of five independent transformants.
siRNA assay

A combination of two 21-mer siRNA duplexes specific for the annotated cDNA sequence of DUOX1, with 2-nucleotide (dT). 3’ overhangs were used to knockdown the expression of Duox1 (target sequences: TTGAGACAAAGTGAGAATTAA and GAGCACTGTTTAAGAACTATA). Each siRNA duplex was used at 10 μg/5 x 10^6 cells, and transfection was achieved using the RNAiFect kit (Qiagen) and strictly following the protocol recommended by the manufacturer. The negative control siRNAs used were of scrambled RNAs. After 2 days in culture, cells were harvested and then analyzed for the various parameters described in the text.