Chapter 2

Materials and Methods

2.1 Composition of solutions

Cell lysis buffer (for nuclear protein extraction)

- 10 mM Tris-HCL pH 8
- 10 mM NaCl
- 0.2% Igepal (Sigma)
- 1% protease inhibitor cocktail (Sigma)

Extraction buffer (for nuclear protein extraction)

- 20 mM HEPES pH 7.9
- 0.2 mM EDTA
- 25% Glycerol
- 1.5 mM $MgCl_2$
- 0.42 M NaCl
- 0.001 M DTT (Invitrogen)
- 1% protease inhibitor cocktail (Sigma)

10 × dNTP mix (for DNA labelling of ChIP assays)

- 1 mM dCTP
- 2 mM each of dGTP, dTTP and dATP

Tecan hybridisation buffer

- 50% formamide (Fluka)
- 5% dextran sulphate
- 0.1% Tween 20 (BDH)
- 2 × SSC
- 10 mM Tris pH 7.4

PBS/0.05% Tween 20 (hybridisation wash solution 1)

PBS/0.05% Tween 20 for washing the arrays was prepared by dissolving the following salts in one litre of HPLC water.

- 7.33 g NaCl
- 2.36 g Na_2HPO_4
- 1.52 g $NaH_2PO_4 \cdot H2O$
- 500 µl Tween 20 (Sigma)

Cell lysis buffer (CLB)

- 10 mM Tris-HCl pH 8.0
- 10 mM NaCl
- 0.2% Igepal (Sigma)
- 10 mM sodium butyrate (Sigma)
- 50 µg/ml PMSF (Sigma)
- 1 µg/ml leupeptin

Nuclei lysis buffer (NLB)

- 50 mM Tris-HCl pH 8.1
- 10 mM EDTA
- 1% SDS
- 10 mM sodium butyrate (Sigma)
- 50 µg/ml PMSF (Sigma)
- 1 µg/ml leupeptin

IP dilution buffer (IPDB)

- 20 mM Tris-HCl pH 8.1
- 150 mM NaCl
- 2 mM EDTA
- 1% Triton X-100 (Sigma)
- 0.01% SDS

- 10 mM sodium butyrate (Sigma)
- 50 µg/ml PMSF (Sigma)
- 1 µg/ml leupeptin

IP wash buffer 1 (IPWB1)

- 20 mM Tris-HCl pH 8.1
- 50 mM NaCl
- 2 mM EDTA
- 1% Triton X-100 (Sigma)
- 0.1% SDS

IP wash buffer 2 (IPWB2)

- 10 mM Tris-HCl pH 8.1
- 250 mM LiCl
- 1 mM EDTA
- 1% Igepal (Sigma)
- 1% deoxycholic acid

IP elution buffer (IPEB)

- 100 mM $NaHCO_3$
- 1% SDS

TE (pH 8.0)

- 10 mM Tris base (pH 8.0)
- 1 mM EDTA

1 × PBS (for ChIP assays)

1 X PBS used for washing the cells in ChIP assay was prepared by dissolving the following salts in 1 litre of HPLC water and the pH was adjusted to 7.4 with HCl.

- 8 g NaCl
- 0.2 g KCl
- 1.44 g Na_2PO_4

- 0.24 g KH_2PO_4

20 × SSC

20 × SSC used in the washing steps in the TECAN hybridisation was prepared by dissolving the following salts in 1 litre of HPLC water.

- 175.3 g NaCl
- 88.2 g tri-sodium citrate

10 × Tris-buffered saline (TBS)

10 × TBS used in western blotting was prepared by dissolving the following salts in 1 litre of deionised water and the pH was adjusted to 7.6 with HCl.

- 24.4 g Tris-HCl
- 80 g NaCl

1 × Tris-buffered saline Tween 20 (TBST)

1 × TBST used in western blotting was prepared by diluting 100 ml 10 × TBS in 1 litre of deionised water and adding 1 ml of Tween 20.

12 × MES stock buffer

12 × MES stock buffer used in Affymetrix GeneChip hybridisations was prepared by dissolving the following salts in 1 litre of molecular biology grade water and filtered through a 0.2 μm filter.

- 64.61 g of MES hydrate
- 193.3 g of MES sodium salt

2× hybridisation buffer (for Affymetrix GeneChips)

- 2 × MES Stock Buffer
- 1 M NaCl
- 20 mM EDTA
- 0.01% Tween-20

Wash buffer A: non-stringent wash buffer (for Affymetrix GeneChips)

- 6 × SSPE
- 0.01% Tween-20

Wash buffer B: stringent wash buffer (for Affymetrix GeneChips)

- 2 × MES stock buffer
- 0.1 M NaCl
- 0.01% Tween-20

2X stain buffer (for Affymetrix GeneChips)

- 2 × MES stock buffer
- 1 M NaCl
- 0.05% Tween-20

2.2 Reagents

Antibodies

- Complete lists of antibodies, with company names and catalogue numbers, used in western blotting and chromatin immunoprecipitation assays are included in Appendix 3A and 3B respectively.
- anti-glycophorin A PE (BD Biosciences)
- biotinylated anti-streptavidin (Vector Laboratories)

siRNAs

- All siRNAs used in the RNAi assays were designed and synthesised by Eurogentec or Ambion. A complete list of siRNAs, manufacturers and sequences is included in Chapter 3.

Primer pairs

- All primer pairs used in the quantitative real time PCR assays were synthesised by Sigma. Complete lists of the sequences of the primer pairs are included in Appendix 1.

Enzymes

- proteinase K (Invitrogen)
- RNase A (ICN Biochemicals)
- RNase-free DNase I (Ambion)
- Klenow fragment (Invitrogen)
- SuperScript^{TM} II RNase H^{-} reverse transcriptase (Invitrogen)

Flourophores

- Cy3 dCTP (GE Healthcare)
- Cy5 dCTP (GE Healthcare)
- anti-glycophorin A PE (BD Biosciences)
- FITC conjugated GATA1 siRNA (Eurogentec)

2.3 Cell lines

Human erythroleukaemic cell line K562 (Lozzio and Lozzio 1975) was a gift from Professor Anthony Green, Department of Haematology, University of Cambridge, UK.

Human erythroleukaemic cell line HEL 92.1.7 (Martin and Papayannpoulou, 1982) was obtained from American Type Culture Collection (ATCC).

2.4 Tissue culture

2.4.1 Culturing and propagation of cell lines

Cell lines K562 and HEL 92.1.7 were cultured in RPMI 1640 (Sigma) supplemented with 10% v/v fetal calf serum (FCS) (Invitrogen) and 100 μg/ml penicillin/streptomycin (Sigma) (named thereafter 'supplemented media'). The cells were maintained at 37^{o}C and 5% CO_2 at a cell density of 0.5×10^6.

Sub-culturing was performed as follows:

1. Cells were counted every two days and spun down at 1200 rpm for 5 minutes.
2. Cells were resuspended in fresh warm supplemented media and maintained at a cell density of 0.5×10^6 in 75 cm^3 tissue culture flasks with vented caps (Corning) with a maximum of 50 ml of cells.
3. For addition sub-culturings, cells were counted and resuspended as above and maintained in 150 cm^3 flask with a maximum of 100 ml of media/cell suspension.

2.4.2 Cryopreservation of cell lines

For cryopreservation of cell lines, cells were counted and spun down at 1200 rpm for 5 minutes and resuspended in freezing media [FCS in 10% v/v dimethylsulphoxide (DMSO, Sigma)] at a density of 4×10^6 /ml. The cells were transferred to polypropylene cryotubes (Nunc) and cooled overnight at -70 ^{o}C at a rate of 1-2 ^{o}C per minute. The cryotubes were transferred to gas phase liquid nitrogen tank at -180 ^{o}C for long term storage.

To defrost frozen cell stock, cells were thawed rapidly in a 37 ^{o}C water bath, washed with 10 ml fresh media once and resuspended in 10 ml fresh supplemented media and maintained in a 25 cm^3 tissue culture flask with vented cap.

2.5 Transfection of siRNA

1. siRNAs for GATA1, SCL, E2A, LDB1, LMO2 and firefly luciferase were designed and synthesised by Ambion or Eurogentec (Chapter 3).
2. Media for K562 cells was changed one day before transfection and cells were maintained at a cell density of 0.5×10^6.
3. 5×10^6 cells were transfected with 2 µl of 100 µM of siRNAs in 100 µl of RPMI 1640 (10% v/v FCS and 100 µg/ml penicillin/streptomycin) using the Nucleofector^{TM} II system (Amaxa Biosystems) with programme T16.
4. Transfected cells were resuspended in 10 ml RPMI 1640 (10% v/v FCS and 100 µg/ml penicillin/streptomycin) in 25 cm^3 flask with vented cap and incubated at 37^{o}C and 5% CO_2. The final concentration of the siRNA was 20 nM.
5. 1×10^7 cells were transfected in two separate transfections (5 million cells in each) for each time point for each siRNA.
6. Cells were harvested at 12 hr, 24 hr, 36 hr and 48 hr after transfection for RNA and protein extraction as described in section 2.6 and 2.7.

2.6 RNA extraction

K562 cells were harvested (after transfection or without transfection) and total cellular RNA was extracted and purified as follows:

2.6.1 Total RNA extraction by TRIZOL

1. 1.5×10^6 of the transfected cells were harvested at 12 hr, 24 hr, 36 hr and 48 hr after siRNA transfection or 3×10^6 untransfected cells were harvested by centrifugation at 1200 rpm for 5 minutes.
2. Cell pellets were resuspended with 1 ml TRIZOL reagent (Invitrogen).
3. 0.2 ml of chloroform (Sigma) was added per 1 ml of TRIZOL reagent used.
4. The samples were mixed by shaking vigorously for 15 seconds and incubated at room temperature for 2-3 minutes.
5. After incubation, the samples were centrifuged at 12000 rcf for 15 minutes at 4°C.

6. The aqueous phases (upper layer) were transferred to new 1.5 ml microfuge tubes and 0.5 ml of isopropanol (Sigma) was added. The samples were mixed by inverting the tube a few times.
7. The samples were incubated at room temperature for 10 minutes and centrifuged at 12000 rcf for 10 minutes at 4°C. The RNA should now be visible as a pellet at the bottom of the tube.
8. The supernatants were removed and the pellets were washed once with 1 ml 75% ethanol (Sigma).
9. The samples were mixed by inverting the tube a few times and centrifuged at 12000 rcf for 5 minutes at 4°C.
10. The supernatants were removed and the pellets were air-dried.
11. The pellets were resuspended in 42 µl RNase-free water (Ambion) by pipetting until the pellets were completely dissolved.

2.6.2 DNase treatment for RNA samples

1. The RNA samples were subsequently treated with 6 units of RNase-free DNase I (Ambion) in a 50 µl reaction.
2. The samples were incubated at 37°C for 30 minutes.
3. The reaction mix was then heat inactivated at 100°C for 5 min and cooled on ice.

2.6.3 Phenol-chloroform extraction and ethanol precipitation

1. The RNA samples prepared as in section 2.6.2 were diluted to 100 µl with RNase-free water.
2. Equal volume of phenol-chloroform-isopropanol (Ambion) (100 µl) was added to the RNA samples.
3. The reaction mix was mixed thoroughly by vortexing and centrifuged at 13500 rpm for 5 minutes.
4. The aqueous phase (upper layer) of the centrifuged samples was transferred to a new 1.5 ml microfuge tube and 10 µl of RNase-free 3M sodium acetate pH5.2 (Ambion) and 250 µl 100% ethanol were added to each tube.
5. The RNAs were precipitated at -20°C for 1 hour.
6. RNAs were precipitated by centrifugation at 13500 rpm for 20 minutes at 4°C.
7. The supernatants were removed and the pellets were washed once with 500 µl 75% ethanol (Sigma).

8. The samples were mixed by inverting the tube a few times and centrifuged at 13500 rpm for 5 minutes at 4°C.
9. The supernatants were removed and the pellets were air-dried.
10. The pellets were resuspended in 30 - 50 µl RNase-free water (Ambion) by pipetting until the pellets were completely dissolved.
11. The total RNA extracted was quantified using Nanodrop ND-1000 spectrophotomic system (Labtech).
12. The RNA quality was assessed by electrophoresis of 500 ng of the sample on a 1% agarose/1 × TBE minigel (made up with RNase-free water).
13. 3 × volumes of 100% ethanol (Sigma) was added to the aqueous sample and the samples were then stored at -70°C.

2.7 Reverse transcription

First strand cDNAs were synthesised using SuperScript^{TM} II RNase H^{-} reverse transcriptase (Invitrogen) as follows.

1. 1 µg of RNA and 1 µl of 50 ng/µl random primers (Invitrogen) were mixed to a volume of 12 µl with RNase-free water.
2. The samples were incubated at 100°C for 1 minute and cooled on ice for 2 minutes.
3. 4 µl of 5X first strand buffer (Invitrogen), 2 µl of 0.1M DTT (Invitrogen) and 1 µl of 10 mM dNTPs (Invitrogen) were added to the samples.
4. 1 µl of SuperScript^{TM} II RNase H^{-} reverse transcriptase (Invitrogen) was added to the samples
5. The reaction mix was mixed gently by flicking and incubated at 42°C for 1 hour.
6. The samples were heat-inactivated at 100°C for 5 minutes and cooled on ice.
7. The resulting cDNAs were diluted to 10 ng/µl by TE pH 8.

2.8 Quantitative real-time PCR

2.8.1 Primer design

1. Primer pairs for all the real-time PCR assays were designed by using the Primer Express software version 2.0 (Applied Biosystems) or Primer 3 (http://frodo.wi.mit.edu/, Rozen and Skaletsky 2000) following the guidelines below:
    - primer length: 18-30 bases

- GC content: 40 – 60 %
- Tm: 64 °C
- dTm: 2°C
- amplicon length: 80 – 150 bp
- intron-spanning where possible

2. Primer pair sequences were checked for specificity by BLAST comparison with the entire human genome and *in silico* PCR (UCSC Genome Browser).
3. Primer specificity was confirmed by dissociation curve analyses in the real-time PCR. Only one peak was observed in all cases.

2.8.2 Quantitative real-time PCR

Quantitative real-time (qRT)–PCR was used to assess (i) the knockdown efficiency in the siRNA assays, (ii) to investigate expression of putative target genes in the siRNA assays and (iii) to confirm enrichment levels in the ChIP assays.

1. The SYBR green PCRs were set up in 96-well optical reaction plate (Applied Biosystems) in a 25 µl reaction in duplicate by mixing the following reagents on ice:

• water	variable
• 2 µM forward and reverse primer mix	5 µl
• 2 × SYBR green PCR mastermix (Applied Biosystems)	12.5 µl
• 10 ng/µl cDNA samples or 10 × diluted ChIP DNA samples	4 µl or 5 µl
Total volume:	25 µl

2. PCR was performed on a 7700 sequence detection system (Applied Biosystems) using the following conditions: 2 min at 50 ^{o}C; 10 min at 95 ^{o}C; 40 cycles of 95 ^{o}C for 15 s and 60 ^{o}C for 1 min.

2.8.3 Data analyses

Ct values were extracted using Sequence Detector 1.7a (Applied Biosystems) with the same threshold and data analyses were performed as follows:

<u>For expression assays (cDNA quantification):</u>

ΔCt = Ct (house-keeping gene) - Ct (gene of interest)

$\Delta\Delta$Ct = ΔCt (luciferase control) - ΔCt (siRNA knockdown)

Fold repression = $(1+\text{PCR yield})^{\Delta\,\Delta CT}$

% of mRNA remained after knockdown = 100/ Fold repression

For ChIP-PCR assays:

$\Delta Ct = Ct\ (input) - Ct\ (ChIP\ sample)$

$Fold\ enrichment = (1+PCR\ yield)^{\Delta CT}$

2.9 Protein extraction

2.9.1 Protein extraction

1. 8.5×10^6 of the transfected cells were harvested at 12, 24, 36 and 48 hour time points after siRNA transfection by centrifugation at 1200 rpm for 5 minutes.
2. The cell pellets were washed once with 10 ml ice-cold PBS followed by centrifugation at 1200 rpm for 5 minutes.
3. Cells were resuspended with 250 µl cell lysis buffer and incubated for 5 minutes on ice.
4. Nuclei were obtained by centrifugation at 11000 rcf for 1 minute at 4 ^oC.
5. Nuclei were washed once with 250 µl cell lysis buffer followed by centrifugation 11000 rcf for 1 minute at 4 ^oC.
6. Nuclei were resuspended in 70 µl of extraction buffer and snap frozen with dry ice in 100% ethanol.
7. Protein samples were stored at -80 ^oC.

2.9.2 Protein quantification

Nuclear proteins were quantified using the Bio-Rad Protein Assay Kit II.

1. 0.8 ml of filtered deionised water was added to 1.5 ml spectrophotometer cuvettes (Bio-Rad).
2. 0, 2, 5, 10, 15 or 20 µl of BSA (1 mg/ml) (Invitrogen) was added to the cuvettes in order to generate a standard curve.
3. 2 µl of nuclear protein extract was added to the cuvettes.
4. 200 µl of Dye Reagent Concentrate (Bio-Rad) was added to each cuvette.
5. The reaction mixtures were mixed well by pipetting.
6. The samples were incubated for 20 minutes at room temperature.
7. The absorbance of each sample at 595 nm was measured using the CECIL spectrophotometer CE2020 (CECIL Instruments).

8. The concentration of the nuclear proteins was calculated in Excel using the BSA standard curve in Excel.

2.10 Western blotting

2.10.1 Sample preparation

Nuclear protein samples were prepared under reducing or non-reducing conditions as follows:

For reducing samples:

- 10, 20 or 30 µg of nuclear proteins variable
- 4 × LDS loading buffer (Invitrogen) 7.5 µl
- sample reducing agent (Invitrogen) 3 µl
- deionised water variable

Total volume: 30 µl

For non-reducing samples:

- 10, 20 or 30 µg of nuclear proteins variable
- 4 × LDS loading Buffer (Invitrogen) 7.5 µl
- deionised water variable

Total volume: 30 µl

The samples were mixed by vortexing and denatured at 100 ^{o}C for 2 minutes.

2.10.2 SDS-PAGE

1. NuPAGE 4-20% Novex Bis-Tris gels (Invitrogen) were washed with distilled water and combs were removed and the wells were washed with 1 × MOPS running buffer (Invitrogen) with a syringe.
2. The gels were assembled in XCell SureLock^{TM} Mini-Cell (Invitrogen).
3. The inner and outer chambers of the Mini-Cell tank were filled with 200 µl and 500 µl of 1 × MOPS Running Buffer respectively (N.B. 500 µl of anti-oxidant (Invitrogen) was added to the inner chambers.)
4. The denatured proteins and 5µl of See Blue Plus Standard (Invitrogen) were loaded into the wells.
5. The proteins were electrophoresed for 1 to 1.5 hours at constant voltage of 200 V and a starting current of 125 mA/ gel at 4 ^{o}C.

2.10.3 Blotting

1. 1 × NuPAGE Transfer Buffer (500ml) was prepared as follows:
   - 20 × NuPAGE Transfer Buffer (Invitrogen) 25 ml
   - methanol 50 ml

- deionised water 425 ml

Total volume: 500 ml

2. The transfer buffer and 1 litre deionised water were kept in the fridge for at least 30 min before use.
3. The gels were dissembled after electrophoresis.
4. Blotting pads, filter papers and gels were equilibrated in cold transfer buffer for 10 seconds.
5. PVDF membranes (Millipore) were equilibrated in 100% methanol for 10 seconds and transferred to the cold transfer buffer.
6. The blot modules were assembled as follows:

For 1 gel	For 2 gels
• top (+) • 2 blotting pads • 2 filter papers • transfer membrane • gel • 2 filter papers • 2 blotting pads • bottom (-)	• top (+) • 2 blotting pads • 2 filter paper • transfer membrane • second gel • 2 filter paper • 1 blotting pad • 2 filter paper • transfer membrane • first gel • 2 filter paper • 2 blotting pads • bottom (-)

7. Any air bubbles in blotting pads and between the gel and membrane were removed.
8. The blot module was clipped together firmly and placed into a transfer tank.
9. The blot module was filled with transfer buffer until the gel/membrane sandwich was covered in transfer buffer.
10. The outer chamber was filled with cold deionised water to the top.
11. The blotting was performed at a constant voltage of 30 V for one gel and 35 V for 2 gels and a starting current of 170 mA/ gel at 4 ^{o}C for 2 hours.

2.10.4 Immunoblotting and detection

1. The membrane was blocked using blocking buffer (5% non-fat dry milk in tris-buffered-saline-tween 20 (TBST) at room temperature for 1 hour.
2. The membrane was then incubated with primary antibodies at the appropriate dilutions (Appendix 3A) in 10 ml blocking buffer at 4 ^{o}C overnight.

3. The membrane was then washed four times with TBST for 1 hour
4. The membrane was incubated with secondary antibodies at the appropriate dilutions (Appendix 3A) in 10 ml blocking buffer at room temperature for 1 hour.
5. The membrane was then washed again four times with TBST for 1 hour.
6. The membrane was incubated with ECL plus (GE Healthcare) for 5 minutes.
7. Signals were developed on ECL Hyperfilm (GE Healthcare).
8. The membrane was stained with 10 ml of water-diluted Dye Reagent Concentrate (Bio-Rad) at a dilution of 1:5 to visualise loading control.

2.11 Flow cytometry analysis for fluorescent oligo transfection

1. Media for K562 cells were changed one day before transfection and cells were maintained at a cell density of 0.5×10^6.
2. 5×10^6 cells were transfected in 100 µl of RPMI 1640 (10% v/v FCS and 100 µg/ml penicillin/streptomycin) using the Nucleofector™ II system (Amaxa Biosystems) with programme T16 with the following conditions:

   <u>0 hour time point:</u>

   - 100 µM FITC-labelled GATA1a siRNA 2 µl
   - 100 µM unlabelled GATA1a siRNA 2 µl

   <u>24 hour time point:</u>

   - 100 µM FITC-labelled GATA1a siRNA 2 µl
   - 100 µM unlabelled GATA1a siRNA 2 µl
3. Transfected cells were resuspended in 10 ml RPMI 1640 (10% v/v FCS and 100 µg/ml penicillin/streptomycin) in 25 cm^3 flask with vented cap and incubated at 37°C and 5% CO_2. The final concentration of the siRNA was 20 nM.
4. Cells were counted and 1×10^6 cells were harvested by centrifugation at 1200 rpm for 5 minutes.
5. Cells were washed with 10 ml PBS followed by centrifugation at 1200 rpm for 5 minutes.
6. Cells were resuspended in 1 ml PBS and filtered with 30um mesh filter (CellTrics®, Partec GmbH).
7. The resuspended cells were kept on ice and covered with aluminium foil.
8. Cells were flow-sorted using the Cytomics FC 500 flow cytometer (Beckman Coulter; Fullerton, CA) equipped with an air-cooled 20mW 488nm Argon laser.

9. Data were analysed with the WinMDI 2.8 software (http://facs.scripps.edu/software.html) and percentage of transfected cells were calculated.

2.12 Cell morphology studies

1. Media for K562 cells and HEL 92.1.7 cells was changed one day before transfection and cells were maintained at a cell density of 0.5×10^6.
2. 5×10^6 cells were either not transfected, sham transfected (placing the cuvette in the electroporator without pressing the button) or transfected with siRNAs or water in 100 µl of RPMI 1640 (10% v/v FCS and 100 µg/ml penicillin/streptomycin) using the Nucleofector™ II system (Amaxa Biosystems) with programme T16 as described in section 2.5.
3. The following transfections were set up and cells were harvested at corresponding time points:

Cell line	Transfection	Time points for harvest			
		0hrs	1hr	24hrs	48hrs
K562	nil	√			
K562	water		√	√	√
K562	LUC siRNA		√	√	√
K562	GATA1 siRNA		√	√	√
K562	E2A siRNA		√	√	√

4. Cells were counted and 1×10^6 cells were harvested by centrifugation at 1200 rpm for 5 minutes.
5. Cells were washed with 10 ml PBS followed by centrifugation at 1200 rpm for 5 minutes and cells were resuspended in 10 ml PBS and placed on ice.
6. 300 µl of the resuspended cells were placed into Cytofunnel disposable sample chambers (Shannon) on top of a microscope slide.
7. The samples were spun at 200 rpm for 5 minutes in Cytospin 3 (Shannon) to precipitate the cells on the slide.
8. The slides were allowed to air dry.
9. The slides were stained in Stain Quick-Staining Kit (Lamb).
10. The slides were washed with tap water to remove excessive stain and air dried at room temperature.
11. The dried slides were mounted in Depex Polystyrene (DPX) (BDH) and round cover slips (Shannon) and observed under a light microscope.

12. For a blinded test, the slides were blindly labeled and 100 cells were randomly scored with the following cell morphologies: cells with small blebs, cells with large blebs, cells with 2 nuclei, cells with > 2 nuclei and others.

2.13 Growth arrest studies

The growth patterns of K562 untransfected or transfected cells under different conditions were studied.

1. Media for K562 cells was changed one day before transfection and cells were maintained at a cell density of 0.5×10^6.
2. 5×10^6 cells were transfected in 100 µl of RPMI 1640 (10% v/v FCS and 100 µg/ml penicillin/streptomycin) using the Nucleofector™ II system (Amaxa Biosystems) with programme T16 as described in section 2.5.
3. The following transfections were set up and cells were harvested at corresponding time points:

Transfection	**Time points for harvest**			
	0 hr	**24 hr**	**48 hr**	**72 hr**
nil	√	√	√	√
Water	√	√	√	√
LUC siRNA	√	√	√	√
GATA1 siRNA	√	√	√	√

4. An aliquot of cells was taken at corresponding time points and stained with equal volume of Trypan Blue (Sigma) for 5 minutes at room temperature.
5. No. of viable cells were scored in two independent aliquots.

2.14 Glycophorin A expression detection in K562 and HEL 92.1.7

1. K562 and HEL 92.1.7 cells were cultured to confluence as described in section 2.4.1.
2. Cells were counted and 1×10^6 cells were harvested by centrifugation at 1200 rpm for 5 minutes.
3. Cells were washed twice with 10 ml PBS followed by centrifugation at 1200 rpm for 5 minutes.
4. Cells were resuspended in 100 µl of PBS and placed on ice.
5. Cells were stained by adding 20 µl of anti-glycophorin A PE (BD Biosciences) to the resuspended cells and incubating for 1 hour at 4 ^{o}C in the dark.
6. Stained cells were washed once with 10 ml PBS.

7. Cells were resuspended in 500 µl of PBS and analyzed by Cytomics FC 500 flow cytometer (Beckman Coulter; Fullerton, CA) equipped with an air-cooled 20mW 488nm Argon laser. Fluorescence emitted from PE was collected using 575BP (FL2) bandpass filter.
8. Alternatively, stained cells were resuspended in 500 µl of PBS with 3% formaldehyde for short term storage in the dark until ready for flow-sorting.
9. Percentage of cells expressing glycophorin A was calculated using WinMDI 2.8 software (http://facs.scripps.edu/software.html).

2.15 Differentiation study of K562

1. K562 cells were cultured to confluence as described in section 2.4.1.
2. Cells were counted and 1×10^6 cells were collected by centrifugation at 1200 rpm for 5 minutes and fixed in 500 µl of PBS with 3% formaldehyde.
3. Cells were counted and 10×10^6 cells were collected by centrifugation at 1200 rpm for 5 minutes.
4. Cells were resuspended with 20 ml of fresh supplemented media.
5. 40 µl of 25 mM hemin was added to the resuspended cells.
6. 1×10^6 cells were collected by centrifugation at 1200 rpm for 5 minutes and fixed in 500 µl of PBS with 3% formaldehyde at 24, 48, 72 and 96 hours.
7. Glycophorin A expression was quantified in all samples as described in section 2.14.

2.16 Affymetrix GeneChip expression analysis

Total RNAs extracted from siRNA-transfected K562 cells were subject to hybridisation to Affymetrix GeneChip Expression array. The RNAs were processed with the One-Cycle Target Labelling and Control Reagents provided by Affymetrix as follows:

2.16.1 Eukaryotic target preparation

Preparation of poly-A RNA spike controls

The poly-A RNA dilutions for 5 µg of total RNA were prepared with the Eukaryotic Poly-A RNA Control Kit as follows:

1. 2 µl of the Poly-A control stock was added to 38 µl of Poly-A control dilution buffer for the first dilution (1:20).

2. The samples were mixed thoroughly and spun down to collect the liquid at the bottom of the tube.
3. 2 µl of the first dilution was added to 98 µl of Poly-A control dilution buffer to prepare the second dilution (1:50).
4. The samples were mixed thoroughly and spun down to collect the liquid at the bottom of the tube.
5. 2 µl of the second dilution was added to 18 µl of Poly-A control dilution buffer to prepare the third dilution (1:10).
6. The samples were mixed thoroughly and spun down to collect the liquid at the bottom of the tube.
7. 2 µl of the third dilution was added to 5 µg of sample total RNA.

Double-stranded cDNA synthesis

RNA samples were subject to one-cycle cDNA synthesis with the One-Cycle cDNA synthesis kit.

1. RNA sample, diluted poly-A RNA controls, and T7-Oligo(dT) primer were mixed as follows:
   - RNA sample (5 µg) variable
   - diluted poly-A RNA controls 2 µl
   - T7 Oligo(dT) primer 50 µM 2 µl
   - RNase-free water variable

   Total volume: 12 µl
2. The tube was gently flicked a few times to mix, and then centrifuged briefly (~5 seconds) to collect the reaction at the bottom of the tube.
3. The reaction was incubated for 10 minutes at 70°C.
4. The sample was cooled at 4°C for at least 2 minutes and centrifuged briefly (~5 seconds) to collect the sample at the bottom of the tube.
5. In a separate tube, the first-strand master mix was assembled as follows:
   - 5 × 1st strand reaction mix 4 µl
   - DTT 0.1 M 2 µl
   - dNTP 10 mM 1 µl

   Total volume: 7 µl
6. The tube was gently flicked a few times to mix, and then centrifuged briefly (~5 seconds) to collect the reaction at the bottom of the tube.
7. 7 µl of first-strand master mix was transferred to each RNA/T7-Oligo(dT) primer mix for a final volume of 19 µl.

8. The tube was gently flicked a few times to mix, and then centrifuged briefly (~5 seconds) to collect the reaction at the bottom of the tube.
9. The reaction mix was incubated for 2 minutes at 42°C.
10. 1 µl of SuperScript II was added to each RNA sample for a final volume of 20 µl.
11. The tube was gently flicked a few times to mix, and then centrifuged briefly (~5 seconds) to collect the reaction at the bottom of the tube.
12. The samples were incubated for 1 hour at 42°C; then cooled for at least 2 minutes at 4°C.
13. In a separate tube, second-strand master mix was assembled as follows:

- RNase-free water 91 µl
- 5 × 2nd strand reaction mix 30 µl
- dNTP 10 mM 3 µl
- *E. coli* DNA ligase 1 µl
- *E. coli* DNA Polymerase 1 µl
- RNase H 1 µl

Total volume: 130 µl

14. The tube was gently flicked a few times to mix, and then centrifuged briefly (~5 seconds) to collect the reaction at the bottom of the tube.
15. 130 µl of second-strand master mix was added to each first-strand synthesis sample for a total volume of 150 µl.
16. The tube was gently flicked a few times to mix, and then centrifuged briefly (~5 seconds) to collect the reaction at the bottom of the tube.
17. The reaction mix was incubated for 2 hours at 16°C.
18. 2 µl of T4 DNA Polymerase was added to each sample and incubated for 5 minutes at 16°C.
19. After incubation with T4 DNA Polymerase, 10 µl of 0.5 M EDTA was added to the samples.

Cleanup of double-stranded cDNA

The cDNA samples were cleaned up using the Sample Cleanup Module as follows:

1. 600 µl of cDNA binding buffer was added to the double-stranded cDNA synthesis preparation and the samples were mixed by vortexing for 3 seconds.
2. 500 µl of the sample was applied to the cDNA Cleanup Spin Column sitting in a 2 ml collection tube, and the column was centrifuged for 1 minute at 10000 rpm.
3. The flow-through was discarded.
4. The spin column was reloaded with the remaining mixture and centrifuged as above.

5. The flow-through and collection tube were discarded.
6. The spin column was transferred into a new 2 ml collection tube.
7. 750 µl of the cDNA wash buffer was added onto the spin column.
8. The column was centrifuged for 1 minute at 10000 rpm. The flow-through was discarded.
9. The cap of the spin column was opened and centrifuged for 5 minutes at 13200 rpm. The flow-through and collection tube was discarded.
10. The spin column was transferred to a 1.5 ml collection tube, and 14 µl of cDNA elution buffer was added directly onto the spin column membrane. The resulting spin column was incubated for 1 minute at room temperature and centrifuge for 1 minute at 13200 rpm to elute. The recovered volume of cDNA was 12 µl.

Synthesis of biotin-labeled cRNA

Biotin-labeled cRNA was synthesised with the GeneChip IVT Labeling Kit as follows:

1. The reaction mixture was assembled at room temperature as follows:
   - template cDNA 12 µl
   - RNase-free Water 8 µl
   - 10 × IVT labeling buffer 4 µl
   - IVT labeling NTP mix 12 µl
   - IVT labeling enzyme mix 4 µl

   Total volume: 40 µl
2. The reagents were carefully mixed and the mixture was collected at the bottom of the tube by brief (~5 seconds) centrifugation.
3. The reaction was incubated at 37°C for 16 hours in an oven.

Cleanup and quantification of biotin-labeled cRNA

The cRNA samples were cleaned up using the Sample Cleanup Module as follows:

1. 60 µl of RNase-free water was added to the IVT reaction and the resulting sample was mixed by vortexing for 3 seconds.
2. 350 µl IVT cRNA binding buffer was added to the sample and the reaction mix was mixed by vortexing for 3 seconds.
3. 250 µl 100% ethanol was added to the lysate, and the reaction mix was mixed well by pipetting.
4. 700 µl of the sample was added to the IVT cRNA Cleanup Spin Column sitting in a 2 ml collection tube.

5. The column was centrifuged for 15 seconds at 10000 rpm. The flow-through and collection tube were discarded.
6. The spin column was transferred into a new 2 ml collection tube.
7. 500 µl of IVT cRNA wash buffer was added onto the spin column.
8. The column was centrifuged for 15 seconds at 10000 rpm. The flow-through was discarded.
9. 500 µl of 80% ethanol was added onto the spin column and the column was centrifuged for 15 seconds at 10000 rpm. The flow-through was discarded.
10. The cap of the spin column was opened and the column was centrifuged for 5 minutes at 13200 rpm. The flow-through and collection tube were discarded.
11. The spin column was transferred into a new 1.5 ml collection tube, and 11 µl of RNase-free water was added directly onto the spin column membrane. The column was centrifuged for 1 minute at 13200 rpm to elute.
12. 10 µl of RNase-free water was added directly onto the spin column membrane. The column was centrifuged for 1 minute at 13200 rpm to elute to the same tube.
13. An aliquot of the cRNA was diluted by 1:100 fold and quantified using Nanodrop ND-1000 spectrophotomic system (Labtech).
14. An adjusted cRNA yield was calculated to reflect carryover of unlabeled total RNA with the formula below:

    Adjusted cRNA yield = RNAm - (total RNAi) (y)

    RNAm = amount of cRNA measured after IVT (µg)

    Total RNAi = starting amount of total RNA (µg)

    y = fraction of cDNA reaction used in IVT

cRNA fragmentation

1. cRNAs were fragmented to 35 to 200 base fragments. The reaction mixture was assembled as follows:

    - cRNA (adjusted concentration) 20 µg
    - 5× fragmentation buffer 8 µl
    - RNase-free water variable

    Total volume: 40 µl

2. The reaction mix was incubated at 94°C for 35 minutes. The samples were placed on ice following the incubation.

2.16.2 Target hybridisation

1. A hybridisation cocktail was set up for each array by mixing the following reagents:

• fragmented cRNA (15 µg)	30 µl
• control oligonucleotide B2 (3 nM)	5 µl
• 20 × eukaryotic hybridisation controls (bioB, bioC, bioD, cre)	15 µl
• Herring sperm DNA (10 mg/ml) (Sigma)	3 µl
• Acetylated BSA (50 mg/ml) (Invitrogen)	3 µl
• 2 × hybridisation buffer	150 µl
• DMSO (Sigma)	30 µl
• RNase-free water	64 µl
Total volume:	300 µl

   N.B. The 20 × eukaryotic hybridisation controls and Herring Sperm DNA were heated to 65°C for 5 minutes to resuspend the cRNA or DNA before dispensing into aliquots.

2. The Affymetrix GeneChip® Human Genome U133 plus 2.0 probe array was equilibrated to room temperature immediately before use.

3. The hybridisation cocktail was heated to 99°C for 5 minutes in a heat block.

4. The array was wetted by filling it through one of the septa with 200 µl of 1 × hybridisation buffer using a micropipettor and appropriate tips.

5. The probe array filled with 1 × hybridisation buffer was heated at 45°C for 10 minutes with rotation of 60 rpm in the hybridisation oven.

6. The hybridisation cocktail which has been heated at 99°C was transferred to a 45°C for 5 minutes.

7. The hybridisation cocktail was spun at maximum speed in a microcentrifuge for 5 minutes to remove any insoluble material from the hybridisation mixture.

8. The buffer solution was removed from the probe array cartridge and the cartridge was filled with 200 µl of the clarified hybridisation cocktail.

9. The probe array was placed in the rotisserie box in the 45°C oven with rotation at 60 rpm for hybridisation for 16 hours.

2.16.3 Fluidics station setup

1. To wash, stain, and scan a probe array in the Fluidics station 450, an experiment was first registered in the GeneChip Operating Software (GCOS) with the following information:
   - Experiment Name
   - Probe Array Type
   - Array barcode
   - Sample Name

- Sample Type
- Project

2. The fluidics station was first primed. In the GCOS, Run → Fluidics → Protocol → Prime_450 were selcted.

3. The intake buffer reservoir A was changed to non-stringent wash buffer and intake buffer reservoir B was changed to stringent wash buffer.

4. All modules were selected to begin priming.

2.16.4 Probe array washing, staining and scanning

1. After 16 hours of hybridisation, the hybridisation cocktail was removed from the probe array and the probe array was filled completely with the 200 µl of non-stringent wash buffer (wash buffer A).

2. The staining reagents were prepared as follows:

   Streptavidin phycoerythrin (SAPE) stain solution (prepared immediately before use)

• 2 × MES stain buffer	600 µl
• 50 mg/ml acetylated BSA (Invitrogen)	48 µl
• 1 mg/ml Streptavidin Phycoerythrin (SAPE) (Molecular Probes)	12 µl
• RNase-free water	540 µl
Total volume:	1200 µl

   The solutions were mixed and aliquoted into two light-protected tubes of 600 µl each and used for stains 1 and 3.

   Antibody solution

• 2 × MES stain buffer	600 µl
• 50 mg/ml acetylated BSA (Invitrogen)	24 µl
• 10 mg/ml normal goat IgG (Sigma)	6 µl
• 0.5 mg/ml biotinylated anti-streptavidin (Vector Laboratories)	3.6 µl
• RNase-free water	266.4 µl
Total volume:	600 µl

3. The probe arrays were then washed with wash buffers A and B and stained in the Fluidic Station with protocol EukGE_WS2v5_450.

4. The resulting arrays were scanned in GeneChip® Scanner 3000 with AutoLoader.

2.16.5 Data analysis

1. The scanned arrays were analysed with two separate softwares: the Bioconductor package and the GeneSpring GX 7.3.1.

2. The quality of the probe arrays was accessed by the BioC Affy package of the Bioconductor. The overall perfect match signal intensity, 3' to 5' ration of housekeeping control genes,

uniformity of hybridisation and array-array correlation of signal intensity were calculated for each of the array. Arrays which differ significantly from others were discarded from further analyses. Criteria used for quality control were discussed in Chapter 4.

3. Signal intensities of all array elements were calculated using Robust Multichip Average (RMA) method in GeneSpring GX 7.3.1.
4. An independent experiment was created for each of the TFs under study in GeneSpring. For example, for GATA1, an experiment with all the biological replicates of luciferase siRNA transfected K562 and GATA1 siRNA transfected K562 was created.
5. Three levels of normalisation steps were performed for all arrays in the experiments:
   - Data Transformation: Values below 0.01 were set to 0.01.
   - Per Chip: Each measurement was divided by the 50^{th} percentile of all measurements in that sample.
   - Per Gene: Each gene was divided by the median of its measurements in all samples.
6. The normalised intensity values were extracted from GeneSpring as an Excel spreadsheet. TF/Luc ratios of normalised intensity were calculated and normalised to the median.
7. Two methods were used for the statistical analyses: the average method and the Venn method. In the average method, an average of the intensity values was taken in the three biological replicates. Ratios of these average values in the TF against the Luciferase negative control were calculated. Standard deviations of the median normalised ratios were calculated. Genes that are 2 standard deviations above or below the mean were selected to be the repressed or activated gene lists. In the Venn method, each biological replicate was treated independently, TF/Control ratios were calculated and activated or repressed genes were selected as in the average method. The activated or repressed genes were compared in each biological replicate and the overlapping genes were chosen as putative targets.
8. Gene Ontology classifications were done using GO Term Finder (http://go.princeton.edu/cgi-bin/GOTermFinder) (Boyle et al., 2004).

2.17 Chromatin immunoprecipitation on microarrays (ChIP-on-chip)

Chromatin immunoprecipitations were performed with wild type or transfected K562 and HEL cells as follows:

Wild type cells:

1×10^8 cells were cultured as mentioned above and media were changed one day before chromatin extraction. A 1×10^6 aliquot of cells was taken for flow-sorting to determine the proportion of dividing cells in the population (Cytomation MoFlo High Performance Cell Sorter, Dako Cytomation). Aliquot of cells was washed with 5 ml of PBS and fixed with 5 ml of 70% ethanol.

Transfected cells:

1. K562 cells were cultured as mentioned in section 2.4.
2. 60×10^6 of cells were transfected with siRNAs (5 million cells in each transfection) according to section 2.5.
3. Two transfections (10 million cells) were pooled and resuspended in 10 ml supplemented media and incubated in 25 cm^3 vented flasks for 24 hours. Six flasks were incubated in total.
4. A 1×10^6 aliquot of cells was taken for flow-sorting (Cytomation MoFlo High Performance Cell Sorter, Dako Cytomation).
5. Aliquot of cells was washed with 5 ml of PBS and fixed with 5 ml of 70% ethanol.
6. 2×10^6 cells were taken for RNA extraction while 7×10^6 cells were taken for protein extraction.
7. The remaining transfected cells (50×10^6) were harvested for chromatin extraction.

2.17.1 Chromatin preparation

1. Wild type or transfected cells were collected by centrifuging at 1200 rpm for 5 minutes, washed once with serum-free RPMI and resuspended in 50 ml serum-free RPMI in a glass flask.
2. DNA-protein interactions were cross-linked by adding 1.35 ml of 37% formaldehyde solution drop-by-drop (BDH AnalaR) (final concentration 1.0%) to the cells. Cross-linking was performed at room temperature with gentle agitation for 10 minutes and stopped by adding 3.15 ml of 2 M glycine (final concentration of 0.125 M) and incubated for 5 minutes at room temperature with gentle shaking.
3. The cells were transferred to a 50 ml Falcon tube on ice and centrifuged at 1200 rpm for 6 minutes at 4ºC. The pellet was washed with 10 ml ice-cold phosphate buffered saline (PBS) and centrifuged again at 2000 rpm for 5 minutes at 4ºC.

4. The cell pellet was resuspended in 3 ml of cell lysis buffer (CLB) and incubated for 10 minutes on ice. The nuclei were collected by centrifuging at 2500 rpm for 5 minutes at 4ºC.
5. Nuclei were lysed by resuspending in 1.2 ml of nuclei lysis buffer (NLB) and incubating on ice for 10 minutes. 0.72 ml of IP dilution buffer (IPDB) was then added to the nuclei and the content was transferred to a 5 ml Falcon tube.
6. The sample was sonicated using the Sanyo/MES Soniprep sonicator, with the settings as follows:

   Amplitude: 14 microns

   Number of bursts: 8

   Length of bursts: 30 seconds

   The sample was cooled for 1 minute in an ice/ethanol bath between each pulse. The DNA was sheared to approximately 300-1000 bp fragments.
7. The sheared chromatin was centrifuged at 13000 rpm for 5 minutes at 4ºC. The supernatant was transferred to a 15 ml Falcon and 4.1 ml of IPDB was added to each tube to bring the ratio of NLB:IPDB to 1:4. The chromatin was then snap frozen with liquid nitrogen and stored at -70 ºC.

2.17.2 Immunoprecipitation

1. Chromatin was precleared by adding 100 µl of normal rabbit IgG (Upstate Biotechnology) and incubated for 2 hours at 4ºC on a rotating wheel.
2. 200 µl of the homogeneous protein G-agarose suspension (Roche) was added and the chromatin was incubated for 5 hours at 4ºC on a rotating wheel.
3. The beads were centrifuged at 3000 rpm for 2 minutes at 4ºC. The supernatant was used to set up the following conditions in 2 ml tubes:
   - Control IgG: 0.675 ml chromatin + 0.675 ml $IPDB^{mod}$ (NLB+IPDB at a ratio of 1:4) + 10 µg IgG
   - Antibody under study: 0.675 ml chromatin + 0.675 ml $IPDB^{mod}$ + 10 µg of antibodies against the transcription factors under study
4. The samples were incubated overnight at 4ºC with rotation.
5. 270 µl of the chromatin was used to set up an input control and stored at -20ºC

6. The samples were centrifuged at 13000 rpm for 5 minutes at 4ºC. The supernatants were transferred to new 2 ml microcentrifuge tubes and 50 µl of the homogenous protein G-agarose suspension (25 µl bed volume) was added to each sample. The samples were incubated for 6 hours at 4ºC with rotation.
7. The protein G-agarose beads (Roche) were centrifuged at 13000 rpm for 20 seconds at 4ºC. The pellets were washed twice with 750 µl of IP wash buffer 1 (IPWB1), once with 750 µl of IP wash buffer 2 (IPWB2) and twice with 750 µl of TE pH8.0. For each wash, the samples were mixed briefly by vortexing and centrifuged at 7500 rpm for 2 minutes at 4ºC.
8. The DNA-protein-antibody complexes were eluted from the beads by adding 225 µl of IP elution buffer (IPEB) at room temperature followed by centrifugation at 7500 rpm for 2 minutes. This step was repeated and both elutions were combined in the same tube.

2.17.3 Reversal of crosslinking and DNA extraction

1. 0.2 µl of RNase A (ICN Biochemicals, 10 mg/ml) and 27 µl of 5 M NaCl (final concentration of 0.3 M) were added to each sample. 0.1 µl of RNase A (10 mg/ml stock) and 16.2 µl of 5 M NaCl were added to the input sample. The samples were incubated at 65ºC for 6 hours.
2. 9 µl of proteinase K (Invitrogen, 10 mg/ml) was added to each sample and the samples were incubated at 45ºC for overnight.
3. 2 µl of yeast tRNA (Invitrogen, 5 mg/ml) was added to each sample immediately before adding 500 µl of phenol/chloroform. The samples were mixed well by vortexing and centrifuged at 13200 rpm for 5 minutes at room temperature. The aqueous layer was transferred to new 2 ml microcentrifuge tubes. This step was repeated once with 500 µl of chloroform.
4. 5 µg of glycogen (Roche, 5 mg/ml), 1 µl of yeast tRNA (5 mg/ml stock) and 50 µl of 3 M sodium acetate pH 5.2 were added to each sample. The samples were mixed well and 1.25 ml of 100% ethanol was added. The DNAs were precipitated at -70ºC for 1 hour.
5. The samples were centrifuged at 13200 rpm for 20 minutes at 4ºC. The pellets were washed with 500 µl of ice-cold 70% ethanol.
6. The pellets were allowed to air-dry for 10 minutes and resuspended in 100 µl of HPLC water for the input and 50 µl of HPLC water for the other samples.

2.17.4 Labelling of ChIP DNA and input DNA with cyanine dyes

1. 20 µl of ChIP DNA was mixed with 60 µl of 2.5 × Random Primers Solution (BioPrime Labelling Kit, Invitrogen) and 50.5 µl of HPLC water.

2. 2 µl of input DNA for wild type cells (or 4 µl of input DNA for transfected cells) was mixed with 60 µl of 2.5 × Random Primers Solution and 50.5 µl of HPLC water.

3. ChIP and input DNAs were denatured in a heat block for 10 min at 100°C, and immediately cooled on ice.

4. The following reagents were added to the samples on ice and the contents were mixed thoroughly:

   - dNTP mix 15 µl
   - Cy3 (for ChIP samples) or Cy5 (for input samples) labelled dCTP (1 mM) (GE Healthcare) 1.5 µl
   - Klenow Fragment (BioPrime Labeling Kit, Invitrogen) 3 µl

5. The samples were incubated at 37°C overnight in darkness and the labelling reactions were stopped by adding 15 µl of stop buffer (BioPrime Labeling Kit, Invitrogen).

6. Unlabelled nucleotides were removed from DNA labelling reactions with G-50 microspin columns (GE Healthcare). Three columns (each column has only 50-60 µl maximum capacity) were required for each sample.

7. The resins in the G50 columns were resuspended by gentle vortexing. The cap was loosened by one-quarter turn and the bottom closure was snapped off.

8. The columns were placed in a 1.5 ml screw-cap microcentrifuge tube for support and centrifuged at 4000 rpm for 1 minute. The resins were washed once with 50 µl HPLC water and centrifuged at 4000 rpm for 1 minute.

9. The columns were placed in a new 1.5 ml tube and 50 µl of the labelling reactions was applied to the centre of the angled surface of the compacted resin bed of each of the columns. The columns were spun at 4000 rpm for 2 minutes. The flowthrough samples were retained and combined.

2.17.5 Hybridisation of the human transcription factor promoter array

1. Hybridisation DNA mixtures were prepared for precipitation as follows:

   - ChIP Cy3 labelled DNA ~180 µl
   - Input Cy5 labelled DNA ~180 µl
   - Human Cot1 DNA (Invitrogen) 135 µl
   - 3 M NaAc pH 5.2 55 µl
   - 100% EtOH (cold) 1200 µl

2. All the tubes were mixed gently, covered with aluminium foil and precipitated at -70°C for 60 minutes.

3. The precipitated DNAs were centrifuged for 15 minutes at 13000 rpm at 4 ^{o}C. The pellets were washed with 500 µl 80% EtOH, and centrifuged at 13000 rpm for 5 minutes. Supernatants were removed and tubes were re-spun at 13000 rpm for 1 minute. The pellets were air-dried
4. The DNA pellets were resuspended in 130 ųl of hybridisation buffer (2 × SSC, 50% deionised formamide, 10 mM Tris-HCl pH 7.4, 5% dextran sulphate, 0.1% Tween 20) and 3 ųl of yeast tRNA was added to each sample
5. The hybridisation DNAs were denatured for 10 minutes at 100°C and then immediately quenched on ice.
6. The samples were pulse spun. The hybridisation DNAs were incubated at 37°C for 60 minutes in the dark.
7. The TECAN automatic hybridisation/wash station was prepared, by placing the human transcription factor promoter array slides into the appropriate clean chambers, priming the wash buffer pumps and loading the appropriate hybridisation/ washing program protocol.
8. 110-120 ųl of hybridisation buffer was injected into a TECAN slide chamber containing the promoter array avoiding air bubbles.
9. The slides were allowed to prehybridise for 60 minutes at 37°C on a medium agitation setting, after which time the slides are automatically washed and dried in preparation for injection of the labelled hybridisation mixture.
10. The labelled hybridisation DNAs were pulse spun and 110-120 ųl was injected into a TECAN slide chamber containing the promoter array avoiding air bubbles.
11. The slides were allowed to hybridise for 45 hours at 37°C.

2.17.6 Slide washing, scanning and data analyses

1. The slides were washed in the TECAN station as follows, followed by drying with nitrogen gas:

- Ten washes in PBS with 0.05% Tween 20 at 37°C, each last for 1 minute with additional 30 seconds soak time for each wash
- Five washes in 0.1X SSC at 52°C, each last for 1 minute with additional 2 minute soak time for each wash
- 10 washes in PBS with 0.05% Tween 20 at 23°C, each last for 1 minute with additional 30 seconds soak time for each wash
- HPLC grade water at 23°C for 1 minute and 30 seconds

2. The Cy3 and Cy5 images were scanned with ScanArray 4000 XL scanner (Perkin Elmer) at 5 μm resolutions using a laser power of 100% and a photo multiplier tube (PMT) value of between 80%-85%.

3. Fluorescent intensities of each spot on the array were quantitated using the ScanArray Express software (Perkin Elmer) using the adaptive circle quantitation method and the total normalisation method. The spots representing the array elements were located automatically by the software and the mean signal intensity values against background were calculated for each channel. The mean ratios of the Cy5/Cy3 channels were reported in the resulting Excel datasheet.

4. Statistical analyses of the ChIP-on-chip data were performed in Microsoft Excel. Quality control for the hybridisation of the arrays was carried out by investigating the average signal intensity of the array and the signal/ noise ratios. Arrays with significantly lower signal intensity and signal/ noise ratios are discarded from further analyses.

5. All the 'unfound' spots on the array were not included in the statistical analyses.

6. Mean ratios, standard deviations (SDs) and coefficients of variation (CVs) were calculated for the two replicate spots representing each array element. The mean ratios were normalised against the median values of all the mean ratios.

7. As the positive control of ChIP, enrichments of elements on the SCL tiling array (included on the TF promoter array) were visualised by plotting the mean ratios of all array elements along the y-axis and the respective genomic positions along the x-axis. High quality ChIP should have significant fold enrichments in the +51 enhancer region of the SCL locus. Any ChIPs with low enrichments in this region were discarded from further analyses.

8. For the statistical analyses of the promoter elements, two methods were used: the average method (method B) and the Venn method (method A). In the average method, an average of the mean ratios of promoters was taken in the three biological replicates. These average values in the TF ChIPs were normalised with their corresponding negative control IgG ChIPs. Promoter elements which were 2 standard deviations above the mean were selected to be the enriched promoters. In the Venn method, each biological replicate was treated independently, IgG normalised and enriched promoters were selected as in the average method. The enriched promoters were compared in each biological replicate and the promoters enriched from all three biological replicates (overlapping in the Venn diagram) were chosen as putative targets.

2.18 Sequence analysis of promoters

2.18.1 Motif discovery for putative targets

Promoters of putative target genes co-regulated by SCL, GATA1 and E2A in Affymetrix expression analyses were selected for NestedMICA analyses (Down and Hubbard, 2005) for common regulatory motifs.

2.18.2 Conserved transcription factor binding sites identification

1. A 4 kb window (3 kb upstream and 1 kb downstream) around the transcription start sites of enriched promoters identified in ChIP-chip analyses was taken from Ensembl (http://www.ensembl.org/index.html).
2. TF binding sites were identified using TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess).
3. Local comparative genomic sequence alignments of the 3 kb window in across various species were obtained from UCSC Genome Browser (http://genome.ucsc.edu/).
4. Conserved TF binding sites were identified and ChIP-qPCR confirmation of TF binding was performed around these sites.

2.19 Transcriptional network generation

Network diagrams combining ChIP-on-chip and expression data were generated in BioTapestry software (http://www.biotapestry.org/) (Longabaugh et al., 2005).