Supplemental Information

Materials and Methods

Bioinformatics

The alignment in Fig. S1B was produced with T-Coffee (1) using default parameters, slightly refined manually and viewed with the Belvu program (2). The limits of the protein sequences included in the alignment are indicated by the residue positions provided at each side. The amino acid colouring scheme indicates average BLOSUM62 scores (which are correlated with amino acid conservation) for each alignment column: black (greater than 3.9), gray (between 3.9 and 3) and light gray (between 3 and 0.5). Protein sequences were obtained mainly from UniProt, GenBank and JGI databases, but were supplemented by manually assembled ESTs and FGENESH+ predicted gene models (3).

Plasmids and cloning

Human HDAC1 (NM_004964.2) cDNAs were generated from IMAGE consortium clones 2820260 and 5142020 by PCR (KOD Hot Start DNA polymerase, Novagen), adding flanking BamHI and NotI sites. Human SIN3A (NM_015477.2) was PCR amplified from EST 9021096 as a BglII-XhoI fragment and the coding region of FAM60A (NM_021238) was obtained as a BamHI-NotI fragment by RT-PCR (Superscript III, Invitrogen) of human ovary RNA (Promega). Full-length products so obtained were cloned into either pSC-A or pSC-B holding vectors (Strataclone) and sequence verified prior to subcloning into derivatives of the mammalian expression vectors pCMV5 and pcDNA5 FRT/TO (Invitrogen) and the bacterial expression vector pGEX6P-1.A silent C>A change was introduced

to SIN3A to render the flanking BglII site unique prior to use. Truncations of SIN3A were subsequently PCR amplified as BglII NotI fragments and cloned into pGEX6P-1 for bacterial expression. All site-directed mutagenesis was carried out using QuickChange method (Stratagene) and KOD Hot Start DNA polymerase (Novagen). Oligonucleotides were obtained from Invitrogen Life Sciences. All clones were verified by sequencing both strands of each construct, performed by The Sequencing Service, College of Life Sciences, University of Dundee (www.dnaseq.co.uk). The cyclin D1 promoter luciferase plasmid was a kind gift from from Richard Pestell (Thomas Jefferson University, Philadelphia, USA).

Cell culture and generation of stable cell lines

HEK293 cells, HeLa cells and mouse embryonic fibroblasts (MEF) from C57/Bl6 mice were grown in DMEM media supplemented with 10% (v/v) foetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamate (GIBCO, Invitrogen). U2OS cells were kept in McCoy's 5A growth medium (Invitrogen) supplemented as before. Embryonic stem (ES) cells from C57/Bl6 mice isolated by Dr. Dennis Castor (JR lab) were cultured on 0.1% gelatine coated plates and grown in DMEM containing 15% (v/v) foetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamate, 1% sodium pyruvate, 1% non-essential aminoacids, 0.1 mM 2-mercaptoethanol and 1000 U/ml LIF (Leukemia inhibiting Factor).

For the selection and maintenance of stable cell lines expressing tetracycline-inducible GFP-FAM60A or GFP-SIN3A made from pCDNA-FRT-TO-GFP-FAM60A, and pCDNA-FRT-TO-GFP-SIN3A respectively, culture media was complemented with the addition of 100 µg/ml hygromycin and 15 µg/ml blasticidin. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Antibodies

Primary antibodies used in this study are the following: FAM60A antibody (sheep S381C, second and third bleeds) was raised in sheep against full-length bacterially expressed FAM60A fused to GST at the Scottish Antibody Production Unit (Carluke, Lanarkshire) and was affinity purified with immobilized antigen. The sheep polyclonal antibody against GST has been described before (4). HDAC1 (A300-713A), HDAC2 (A300-705A), SIN3A (A300-724A), BRMS1L (A300-154A), RBBP46/RBBP7 (A300-958A), CO-REST (A300-130A), CHD4/MI2 BETA (A301-081A) and RBBP1L1/ARID4B (A302-233A) were all from Bethyl; SAP30 (C-18, sc-8471) and ING2 (S-20, sc-67646) were from Santa Cruz; cyclin D1 (DCS6), PLK1 (4535), cyclin B1 (V152) and cyclin A (BF683) were all from Cell Signaling; anti-HA (12CA5) and anti-GFP were from Roche; anti-FLAG M2 was from Sigma and anti-GAPDH (6C5, AB8245) was from Abcam. Anti-FLAG (M2) agarose and rabbit IgG agarose were from Sigma. GFP-Trap beads were

from Chromotek. Protein G-sepharose and gluthathione-sepharose 4B were from GE Healthcare.

Purification of GFP-FAM60A from HEK293 cells

FlpIn T-Rex HEK293 cells (Invitrogen) stably expressing tetracycline-inducible GFP-FAM60A were generated according to the manufacturer's instructions. Cells stably expressing GFP-FAM60A or parental HEK293 Flp-In cells (Invitrogen) were incubated with tetracycline (1 μg/ml) for 24 hr and harvested. A total of 400 mg of protein extracts per cell line were pre-cleared with 500 μl of protein G-sepharose and then incubated with 150 μl of GFP-Trap beads for 2 h at 4 °C. Beads were washed five times in lysis buffer and twice with a buffer containing 50 mM Tris 7.4 and 0.27 M Sucrose. GFP-Trap beads were boiled in LDS-PAGE sample buffer (Invitrogen) containing 5% (v/v) 2-mercaptoethanol and resolved in a 4%–12% SDS-PAGE gradient gel (Nupage, Invitrogen). The gel was fixed and stained with colloidal Coomassie, and the gel lanes were cut into 18 different slices. Proteins present in each band were identified by tryptic digest followed by mass fingerprinting, essentially as described before (4).

Protein depletion using siRNA

For siRNA knockdowns, cells were transfected with the relevant siRNA duplexes (100 nM) using the HBS/ calcium phosphate precipitation method for HEK293 and HeLa cells and HiPerFect (Qiagen) for U2OS cells following manufacturers'

instructions. Cells were then incubated at 37°C for 12–48 hr, as indicated. Control (CON) siRNA was described before (5) and corresponded to the jellyfish GFP sequence. Two separate FAM60A-specific siRNAs were used: FAM60A-1 and FAM60A-2. All siRNA oligonucleotides were from MWG Biotech, Germany. The siRNA sequences used are the following:

SIN3A: 5'-GGUCUAAGAGCUUACUCAA-3'

FAM60A-1: 5'-GUCAGAUGACGGCUCAGAU-3'

FAM60A-2: 5'-CAGUAAACUGCAGAAGGAA-3'

HDAC1: 5'-GUUAGGUUGCUUCAAUCUA-3'

Chromatin immunoprecipitation (ChIP)

ChIP was carried out based on previously-reported protocols (6). Cells were cross-linked with formaldehyde for 10 min at which point 0.125 M glycine was added, and cells washed with phosphate-buffered saline. Cells were lysed with lysis buffer (1% SDS, 10mM EDTA, 50 mM Tris-HCL, pH 8.1, 1 mM PMSF, 1 mg/ml leupeptin, 1mg/ml aprotonin), followed by sonication and centrifugation. The supernatant was precleared with sheared salmon sperm DNA and protein G-Sepharose beads (Sigma). The supernatant was incubated with specific antibodies overnight, and then with protein G-Sepharose beads for 1 h. After an extensive wash step, the complexes were eluted with buffer (100 mM $NaHCO_3$, 1% SDS) and incubated with proteinase K. DNA was purified using NBS polymerase chain reaction purification kit (NBS). For re-ChIP experiments,

protein-DNA complexes were released from the beads by incubation with dilution buffer containing 10 mM DTT, for 30 min at 37C. The sample was then diluted 40 times in dilution buffer and immunoprecipitations, washes and elution were performed as before.

PCR was performed using the following primers:

Cyclin D1: FOR-AGTCCGTGTGACGTTACTGTTGT

Cyclin D1: REV-CTCCCGCTCCCATTCTCT

GAPDH: FOR-CGGTGCGTGCCCAGTTG

GAPDH: REV-GCGACGCAAAAGAAGATG

Aldh6a: FOR-CCAATCGGAGTTCCAGGTTA

Aldh6a: REV-TCCTGGGGATAAGTGATTGG

TGFβ1: FOR- GATGGCACAGTGGTCAAGAGC

TGFβ1: REV- GAAGGATGGAAGGGTCAGGAG

Antibodies used in ChIP were:

Acetyl-H3 K9/K14, 1*μ*g (06-599, Millipore), FAM60A 2*μ*g, GFP 2*μ*g (Roche), HDAC-1 2*μ*g (06-720, Millipore), Sheep/Rabbit/Mouse IgG 2*μ*g. (DSTT, Sigma), Polymerase II CTD (sc-47701, Santa Cruz Biotechnology).

qPCR was performed using a Stratagene Mx3005 QPCR machine and Brilliant II SYBR Green QPCR Low ROX Master Mix (600830, Stratagene). Antibody enrichment/promoter occupancy was measured by real-time quantitative PCR for

the indicated promoters. qPCR values were normalized to the input material. Control conditions were set as “1” and relative mRNA values were calculated relative to this.

qPCR, PCR and primers

RNA was isolated using the RNeasy Microkit from Qiagen. Total RNA was reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen), and real-time PCR carried out using a Stratagene Mx3005 QPCR machine and Brilliant II SYBR Green QPCR Low ROX Master Mix (600830, Stratagene). PCR primers are as follows:

Cyclin D1: FOR- GTGCTGCGAAGTGGAAACC (7)

Cyclin D1: REV- ATCCAGGTGGCGACGATCT

Actin: FOR-CTGGGAGTGGGTGGAGGC (8)

Actin: REV-TCAACTGGTCTCAAGTCAGTG

Cyclin A2: FOR- CACTCACTGGCTTTTCATCTTC

Cyclin A2: REV- CAGAAAACCATTGGTCCCTC

Antibody enrichment/promoter occupancy was measured by real-time quantitative PCR for the indicated promoters. qPCR values were normalized to the input material. Control conditions were set as “1” and relative mRNA values were calculated relative to this.

Cell cycle synchronization

Low passage, low confluency cultures of asynchronously growing U2OS cells were treated with 2.5 mM thymidine (Sigma-Aldrich) for 20 hours, washed and released for 16 h in fresh media and treated again with thymidine for another 20 h. This is referred to as a double-thymidine block. After the second treatment, plates were washed extensively with fresh media and transfected with siRNA. Six hours later nocodazole (Sigma-Aldrich) was added to plates at a final concentration of 100 ng/ml and cells were incubated for a further 20 h before being extensively washed and released in fresh media without nocodazole. For chromatin immunoprecipitation (ChIP) experiments, RO3306 (Cdk1 Inhibitor IV, Calbiochem) was used instead of nocodazole for 20 hours at a final concentration of 9 μM.

EdU cell proliferation assay.

Cells synchronized as described above were labeled with 10 μM EdU for one hour prior to harvesting. Cells were trypsinized, washed with PBS, fixed in 4% paraformaldehyde for 15 minutes and kept at 4 °C until processed. EdU positive cells were measured using the Click-iT EdU Flow Cytometry kit (Invitrogen) following manufacturer instructions. DNA was subsequently counterstained with propidium iodine and cells were FACS sorted as above. Single cells were gated using forward scatter and side scatter parameters (linear scale), DNA was detected in the FL2-H channel (linear) and fluorescence was detected in the FL4-H channel.

Supplementary figure legends

Fig. S1. Representative multiple sequence alignment of the FAM60A putative GATA domain. The FAM60A family sequences are named according to their abbreviated binomial names. Database of origin and species names are: *H.sapiens*, UniProt:Q9NP50, *Homo sapiens; M.musculus,* UniProt:Q8C8M1, *Mus musculus; G.gallus,* UniProt:F1NPQ3, *Gallus gallus; X.laevis,* UniProt:Q4V7W6, *Xenopus laevis; D.rerio,* GenbankESTs:CT629784 CT712272 CK687956, *Danio rerio; B.floridae,* GenBank:XM_002201663, *Branchiostoma floridae* (lancelet)*; S.kowalevskii,* UniProt:UPI0001CBA551, *Saccoglossus kowalevskii* (Hemichordata); *S.purpuratus,* UniProt:UPI0000E48FC8, *Strongylocentrotus purpuratus* (echinoderm); *L.gigantea,* JGI-Genome&FGENESH+, *Lottia gigantea* (mollusc); *C.teleta*, JGI-Genome&FGENESH+, C*apitella teleta* (annelid); *D.pulex*, JGI-Genome&FGENESH+, *Daphnia pulex* (Crustacea); *L.vannamei,* GenBankESTs:FE090686 FE083281, Litopenaeus vannamei (Crustacea); I.scapularis, UniProt:B7PE63, *Ixodes scapularis* (tick); *A.mellifera*, UniProt:UPI0000DB7511, *Apis mellifera* (bee); N.vitripennis, UniProt:UPI00015B51D4, Nasonia vitripennis (wasp); H.saltator, UniProt:E2BPF7, *Harpegnathos saltator* (ant); *P.humanus*, UniProt:E0W0Q1 *Pediculus humanus corporis* (body louse); *T.castaneum,* UniProt:D6WE57, *Tribolium castaneum* (beetle); *D.melanogaster,* UniProt:A8JUX7, *Drosophila melanogaster*; *D.pseudoobscura*, UniProt:Q29GZ3, *Drosophila pseudoobscura; N.vectensis,* UniProt:A7SKQ3, *Nematostella vectensis* (cnidarian); *S.mansoni*, UniProt:C4QDN3, *Schistosoma mansoni* (platyhelminth).

(**B**) Cell cycle regulation of FAM60A mRNA. U2OS cells were synchronized in mitosis by release from a double thymidine block into nocodazole for 20 h. The cells were then released from mitotic arrest and lysed at the times indicated. Cyclin D1 and FAM60A mRNA levels were measured by qPCR.

Fig. S2. Binding of FAM60A to gene promoters requires the conserved GATA-type zinc finger.

(**A**) Quantitative PCR analysis of total mRNA from U2OS cells transfected for 48 h with FAM60A-1 siRNA, SIN3A siRNA or a control siRNA (GFP) was carried out with primers specific for cyclin D1 or cyclin B1.

(**B**) U2OS cells were transfected with plasmids expressing GFP, GFP-FAM60A wild-type, GFP-FAM60A C16A C19A or GFP-FAM60A C53A C56A. Cells were lysed and subjected to ChIP analysis with anti-GFP antibodies. qPCR was carried out on DNA extracted from the precipitates with primers specific for the cyclin D1 promoter.

(**C**) HEK293 cells were transfected with plasmids expressing GFP, GFP-FAM60A wild-type, GFP-FAM60A C16A C19A or GFP-FAM60A C53A C56A. Extracts were subjected to immunoprecipitation with anti-GFP antibodies and precipitates were probed with the indicated antibodies.

Fig. S3. Overexpression of SIN3A or depletion of FAM60A does not affect association of HDAC1 with, or H3 acetylation at, the TGF-β promoter.

U2OS cells expressing GFP-SIN3A in a tetracycline-inducible manner, transfected with two separate FAM60A siRNAs or with a control GFP-specific siRNA, were incubated, or not, with tetracycline. Cell extracts were subjected to chromatin immunoprecipitation with the indicated antibodies; anti-GFP antibodies ("IgG") were used as control. Quantitative PCR was carried out on DNA extracted from the precipitates with primers specific for the TGF-β promoter.

Fig. S4. Cell cycle synchronization and release. U2OS cells were released from a double-thymidine block into medium containing nocodazole to arrest them in mitosis. After 20 h cells were released from nocodazole into fresh medium and samples were taken at the indicated times.

(**A**) Schematic diagram of the arrest and release procedure. Where relevant, cells were transfected with siRNAs after release from double thymidine block, before mitotic arrest.

(**B**) Quantitation of the proportion of cells in G1, S and G2/M phases of the cell cycle in (A) based on propidium iodide staining of cells followed by FACS analysis.

Table S1

Identification of proteins that co–immunoprecipitate with GFP-tagged FAM60A.

Supplementary References

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