Methods and Materials

Tissue collection

Non-pregnant (NP) myometrial samples were taken from the uteruses removed from women undergoing hysterectomy from benign gynaecological conditions. Pregnant, not in labour samples (P) were obtained from the lower segment of the uterus from women undergoing elective caesarean section at term. Written consent was obtained from all women, and ethical approval was granted by the North Tyneside Health Authority Ethics Committee.

Treatment of myometrial strips and cell cultures

Myometrial strips were dissected from either NP or P myometrium in cold-HBSS. Both, myometrial strips or myometrial cell cultures (established from non-pregnant (NP) myometrial tissue samples as described previously (20)) were treated in MEM D-Val (PromoCell, Germany)/10%FCS/ Penicilin/ Streptamycin at 37˚C in 5 % CO_2 with the following: Forskolin (FSK, 10µM), Compound 2 (100µM), TSA (3.3µM), or H89 (10µM) for the duration stated in the individual experiments. Control samples were incubated with vehicle either EtOH or DMSO.

Protein lysate preparation

Protein lysates for immunoprecipitations (IPs) were prepared by mechanical homogenisation of myometrial strips in sucrose buffer (25mM Tris base, pH 7.6, 25mM sucrose, 1mM EDTA, inhibitors of proteases, phosphatases (Sigma, UK) and deacetylases (Active Motif Europe, Belgium) using an Ultra-Turrax mixer **(**T25 basic**,** IKA Labortechnik**,** Germany) at 24000 rpm, followed by centrifugation at 250xg for 2 min. The supernatants were homogenised by passing through small gauge needles and centrifuged at 24100xg for 2 min. The supernatants were used for immunoprecipitation (IP) assays. Protein lysates for Western

blotting (WB) were prepared by mechanical homogenization of myometrial cells or strips in WB buffer (62.5mM TRIS-Cl pH 6.8, 2% SDS, 10% Sucrose/inhibitors as detailed above).

Immunoprecipitation (IP) assays and Western blotting

IPs was performed as described previously (19) with 1 mg of protein extracts in HEPES Co-IP buffer (20mM HEPES, pH 7.9, 75mM KCl, 2.5mM $MgCl_2$, and 0.1% NP-40) / inhibitors of proteases, phosphatases and deacetylases (as detailed above). Proteins were pre-cleared with 2µg of rabbit or mouse IgG (Table 1) and 15µl of protein A or G-coated magnetic beads (Invitrogen, UK) for 45 min at 4°C. Pre-cleared proteins were incubated with the respective primary antibodies (Table 1) for 3h at 4°C. For acetylated lysine immunoprecipitation a mixture of three antibodies was used: a rabbit polyclonal and mouse monoclonal (Cell Signalling) and a rabbit polyclonal (ImmuneChem, Table 1). Protein/Ab complexes were recovered with 25µl of protein A or G protein-coated magnetic beads and washed 4X with HEPES-IP buffer. Proteins were retrieved by boiling for 5 min in 20µl of loading buffer (250mM TRIS-Cl pH 6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol) and subjected to Western blotting. Here, Westerns were carried out as described previously (36) using primary antibodies detailed in Table 1 and the respective secondary HRP-conjugated antibodies (1:5000, DAKO, UK). Where indicated ECL immunodetected bands were scanned and quantified using the Intelligent Quantifier software package (BioImage, Ann Harbor, USA). Membranes were re-probed with an antibody against GAPDH as a loading control.

Generation of the Hsp20-Ac-Lysine^{160} antibody

The Hsp20-Ac-Lysine^{160} antibody was raised against a peptide sequence containing amino acids 155-160 of human Hsp20 (accession number AAH68046) that encompassed acetylated

lysine^{160}. Both the acetylated peptide and antibody were produced by Cambridge Research Biochemicals, UK. Rabbits were immunised 4 times with 1mg of the following [C]-Ahx-Ahx-PPAAAK^{160}-acetylated)-peptide coupled to KLH and immunised over 77 days when the terminal bleed took place. The antibody was affinity purified against the acetylated cognate peptide and subsequently used at 1:3,000 dilution. The cognate acetylated peptide was used at 40μg/ml for 1 hour in antibody neutralising experiments to define specificity for acetylated Hsp20 in the myometrial tissues.

Sub-cellular protein extraction of myometrial tissues

NP and P myometrial tissue strips were dissociated into single cell suspensions using ProteoExtract Tissue dissociation buffer kit following the manufacturer`s instructions (Calbiochem, UK) with 1 g of each tissue. 10 mg of collagenase type II (ProteoExtract Collagenase Set, Novagen, UK)/EDTA-free protease inhibitor cocktail was used for this procedure. The cell suspension was strained using a 70 μm cell strainer (Dutscher Scientific, UK) and was used subsequently for sub-cellular protein extraction with the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem). This procedure allowed the separation of the following fractions: a) a cytosolic protein, b) a cytoskeletal membrane and membrane organelles, c) a cytoskeletal actin and d) a nuclear protein fraction as detailed by Waltregny et al (13). The same procedure was used for NP myometrial cells from cell cultures collected by scraping in PBS.

In vitro HDAC8 activity assay

In vitro activity of TSA and Compound 2 in inhibiting HDAC8 was measured using the HDAC8 Fluorometric Drug Discovery Kit (Enzo Life Sciences, UK) according to the

accompanying protocols. Samples were measured with excitation at 360nm and emission at 460nm using the Luminescence Spectrometer LS50B (Perkin Elmer, USA).

Myometrial contractility studies

P longitudinal myometrial strips were mounted for isometric recording under 2 mN tension in organ baths as previously described (18, 21) and allowed to spontaneously contract or augmented with oxytocin (OT, 2nM). Strips were allowed 4-5 initial contractions before treatment with indicated reagents. The measurements from these contractions were used as control for the contractions after treatment. TSA (3.3μM) or Compound 2 (100μM) were added for up to 2 hours. Control samples were treated with vehicle (DMSO). A time control, (no treatment) was included in each experiment. The effects of TSA and the respective controls were assessed by calculation of the integral from selected areas for each 30 min interval and expressed as a percentage of the integral obtained in the 30 min period prior to treatment, using the PowerLab hardware unit and Chart v4.2 software (AD Instruments, Hastings, UK). After contractility measurements, myometrial strips were frozen in liquid nitrogen after either 3 hour or 24 hour (maintained in buffer ± TSA or Compound 2). Extracted proteins were Western-blotted for expression levels of acetylated Histone H3 (aH3) and acetylated-α-tubulin.

Fluorescent Immunocytochemistry

Myometrial cell cultures were fixed for 5 min in -20°C cold MetOH and washed. Primary antibodies were incubated over night in 1% BSA/TBS at 4°C (for concentrations see Table 1). Then were washed in TBS and secondary antibodies conjugated to either Alexa 484 or 568 (Invitrogen, UK) were added for 1 hour. Cells were washed in TBS and cell nuclei

stained with DAPI for 5 min. Cells were mounted in Fluorescent Mounting Medium (DACO, USA).

RNA isolation and Gene Chip microarray analysis

P myometrial strips were incubated in the presence or absence of TSA (3.3μM) or Compound 2 (100μM) for 24 hour and RNA isolated using the RNAqueous-Kit (Ambion, USA). Gene microarray was performed by ServiceXS (Netherlands) on the 12 sample HumanHT-12 format of Ilumina BeadStation Platform. Changes in gene expression profiles with TSA and Compound 2 compared to untreated controls were analysed using GeneSpring GX 11 (Agilent). Probes were considered to have a present call for detection values >0.8 and an absent call for detection values <0.6. Raw signals were thresh-holded to a value of 1 and a quantile normalisation applied to log transformed data. For each probe the median of the log summarized values from all the samples was calculated and subtracted from each of the samples. Probes were filtered by flags. To pass the filter probes with flag values of Present or Marginal must be present in all samples of at least one experimental condition (control, TSA or Compound 2). Genes differentially expressed between conditions were determined with ANOVA ($p<0.05$ with Benjamini-Hochberg FDR applied) and a Tukey-HSD post-hoc test. To generate heat-maps, genes with a corrected p-value of <0.05 and a fold change of >2 between comparisons was used. The microarray data (GSE27124) derived from these experiments was submitted to the data repository GEO (http://www.ncbi.nlm.nih.gov/geo).