ESM METHODS

Proteomic analysis

Biopsies were individually washed five times with ice-chilled solution containing phosphate-buffered saline and a protease inhibitor cocktail (Complete, Roche) to remove superficial blood traces. Each washed sample was then cut into small pieces, transferred in fresh Eppendorf tubes, suspended in a hypotonic buffer containing 50 mM HEPES pH 7.5, 1% Triton, complete protease inhibitor cocktail (Roche) and lysed by sonication on ice. To improve the tissue lysis, sample were frozen in liquid nitrogen and simultaneously disrupted through high-speed shaking using the TissueLyser II (Quiagen). Tissue lysates were cleared by centrifugation (14000 g, 10 minutes at 4°C). The pellet of insoluble debris was discarded while the proteins in the supernatant were precipitated overnight at -20°C with four volumes of cold acetone. Proteins were suspended in Tris-HCl 30 mM, Triton X-100 0.1% and protein content from each sample was quantified using micro BCA method (Pierce), following the manufacturer's instructions. Two pools of NH and RH were obtained by mixing equal quantities of proteins from healing and chronic ulcers. 150 µg of proteins from each pool were precipitated overnight at -20°C with cold acetone. Protein pellets were then suspended in 30 µl of iTRAQ dissolution buffer (triethylammonium bicarbonate, 0.5 M) to a final concentration of 5 µg/µl.

Proteomic analysis. iTRAQ labelling. Samples were reduced, alkylated and trypsin digested According to iTRAQ manufacturer's instructions (AB Sciex). To diminish any potential variation introduced by the labelling reaction, samples from RH and NH wounds were split in two aliquots of 60 µg to perform two technical replicates with tag swapping. Each peptide solution was labelled at room temperature for 1 h with one iTRAQ reagent vial (mass tags 114 and 115 for RH samples, tags 116 and 117 for NH samples). To verify the labelling efficiency, 1 µg of each labelled sample was individually analyzed by liquid chromatography – tandem mass spectrometry (LC-MS/MS) as specified below. Acquired data were searched against the Mascot database, setting iTRAQ labelling as variable modification. No unmodified peptides were identified from the search and all the peptides were correctly modified at the N-terminus and at each Lysine residue. Finally the four iTRAQ labelled samples were combined in a 1:1:1:1 ratio and the pool was vacuum dried in a SpeedVac system.

Strong Cation Exchange Fractionation. Strong cation exchange (SCX) was performed using a SCX cartridge (AB Sciex) following the manufacturer's protocol. Each iTRAQ labelled sample was suspended in 500 µl of equilibration buffer (5 mM KH_2PO_4, 25% acetonitrile, pH 2.9), adjusting the pH with 1 M H_3PO_4 and loaded onto a SCX cartridge with a syringe-pump system at a flow rate of 50 µl/min. Peptide elution was carried out in a step-wise mode, by using 500 µl of the following concentration of KCl in equilibration buffer: 40, 80, 120, 200, 350 mM. Each SCX fraction was then dried under vacuum, resuspended in 500 µl of 0.1% formic acid and desalted using C18 cartridges (Sep-Pack, C18, Waters) according to the manufacturer's instructions. Samples were dried again under vacuum and kept at -20 °C until MS analyses were performed.

LC-MS/MS analyses. Samples were dissolved in 30 µl of 0.1% formic acid, obtaining a final concentration of about 0.4 µg/µl. The MS analysis was performed on a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled online with a nano-HPLC Ultimate 3000 (Dionex - Thermo Fisher Scientific). About 1 µg of each fraction was loaded onto a 10 cm chromatographic column packed in-house into a pico-frit (75 µm I.D., 10 µm tip, New Objective) with C18 material (ReproSil, 300Å, 3 µm, Dr. Maisch). Peptides were eluted with a linear gradient of acetonitrile/0.1% formic acid from 3% to 50% in 90min at a flow rate of 250 nl/min. The instrument was programmed to perform a full scan at high resolution (60000) on the Orbitrap, followed by MS/MS scans on the three most intense ions acquired with CID fragmentation in the linear trap. A further HCD fragmentation was performed on the same ions with detection on the Orbitrap, to record the low molecular weight ions necessary for quantification. To improve the quality of the analysis and increase the number of quantified proteins, data were searched with Proteome Discoverer software (Thermo Fisher Scientific) as detailed below. All peptides identified with high (99%) or medium (95%) confidence were used to create a static exclusion list that was then included in the instrument method. All samples were analyzed again under identical conditions and all data obtained were merged into a MudPIT protocol and analysed.

Data analysis. Raw MS/MS files were analyzed using Proteome Discoverer 1.2 (Thermo Fisher Scientific). The software was connected to a Mascot Search Engine server version 2.2.4 (Matrix Science, London, UK) and to a Sequest Search Engine version 28.0 (Thermo Fisher Scientific). Data were filtered to exclude MS/MS spectra containing less than 5 peaks and with a total ion count lower than 50. Spectra were searched against the Uniprot Human database (35847 sequences; 20909407 residues). Peptide and fragment tolerances

were set to 10 ppm and 0.6 Da respectively. Enzyme specificity was set to Trypsin with up to 2 missed cleavages, methyltiocysteine, 4-plex iTRAQ at N-terminus and Lys were set as fixed modifications, and oxidation of methionine was selected as variable modification. False discovery rates (FDR) of 5% and 1% were calculated by Proteome Discoverer based on the search against the corresponding randomized database. Data were filtered considering as positive hits proteins identified with at least two unique peptides with individual medium confidence (FDR = 5 %) and only proteins quantified with at least 2 independent peptides. The final analysis was performed on a single list of peptides and proteins obtained by merging filtered MS/MS spectra before and after the application of exclusion lists. Peptides that could not be unequivocally attributed to a single protein were grouped into protein families to satisfy the principle of maximum parsimony. Proteins with at least a 1.5-fold change in their relative abundance between groups were considered significant.