LETTERS

TRPC channel activation by extracellular thioredoxin

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Mammalian homologues of *Drosophila melanogaster* transient receptor potential (TRP) are a large family of multimeric cation channels that act, or putatively act, as sensors of one or more chemical factor[1,2]. Major research objectives are the identification of endogenous activators and the determination of cellular and tissue functions of these channels. Here we show the activation of TRPC5 (canonical TRP 5) homomultimeric and TRPC5–TRPC1 heteromultimeric channels[3–5] by extracellular reduced thioredoxin, which acts by breaking a disulphide bridge in the predicted extracellular loop adjacent to the ion-selectivity filter of TRPC5. Thioredoxin is an endogenous redox protein with established intracellular functions, but it is also secreted and its extracellular targets are largely unknown[6–9]. Particularly high extracellular concentrations of thioredoxin are apparent in rheumatoid arthritis[8,10–12], an inflammatory joint disease that disables millions of people worldwide[13]. We show that TRPC5 and TRPC1 are expressed in secretory fibroblast-like synoviocytes from patients with rheumatoid arthritis, that endogenous TRPC5–TRPC1 channels of the cells are activated by reduced thioredoxin, and that blockade of the channels enhances secretory activity and prevents the suppression of secretion by thioredoxin. The data indicate the presence of a previously unrecognized ion-channel activation mechanism that couples extracellular thioredoxin to cell function.

TRPC5 is markedly activated by extracellular lanthanide ions[4,14,15]. The effects of these ions depend on a glutamic acid residue at position 543 (ref. 14) in the predicted extracellular loop adjacent to the ion pore (Supplementary Figs 1 and 2). This structural feature may therefore have functional importance in enabling extracellular factors to activate the channels. Because lanthanides are unlikely to be physiological activators, we were interested in alternatives and developed a hypothesis based on amino acid sequence alignment, which showed two cysteine residues near glutamic acid 543 that are conserved in TRPC5, TRPC4 and TRPC1 (Supplementary Fig. 2), a subset of the seven TRPC channels[1–5]. TRPC5 and TRPC4 have similar functional properties[4] and both form heteromultimers with TRPC1 (refs 3–5), a subunit that has weak targeting to the plasma membrane when expressed in isolation[3,16].

Pairs of cysteine residues may be covalently linked by a disulphide bridge that can be cleaved by reduction. We therefore applied the chemical reducing agent dithiothreitol (DTT) to HEK-293 cells expressing TRPC5 (refs 15, 16). There was channel activation with the characteristic current–voltage (*I–V*) relationship of TRPC5 and blocking by 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of TRPC5 (ref. 5) (Fig. 1a, b, d). Current recovered on wash-out of DTT (data not shown). Similarly, the membrane-impermeable disulphide reducing agent Tris (2-carboxyethyl) phosphine hydrochloride (TCEP; Fig. 1c, d) activated TRPC5, whereas the thiol reagent [2-(trimethylammonium) ethyl]methanethiosulphonate bromide (MTSET) had no effect (Fig. 1d). TRPC5 was inhibited by cadmium ions only after pretreatment with DTT (Fig. 1e, f), which is consistent

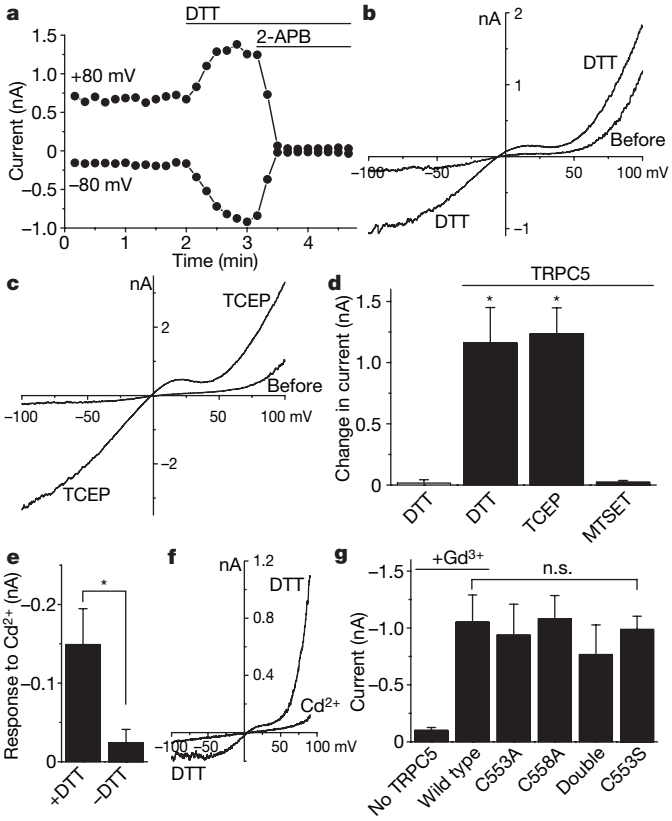

Figure 1 | Functional disulphide bridge in TRPC5. Whole-cell recordings from HEK-293 cells. **a**, In a cell expressing TRPC5, response to bath-applied 10 mM DTT and 75 μM 2-APB. **b**, *I–V* relationship from **a**. **c**, As for **b** but with 1 mM TCEP. **d**, Currents at −80 mV evoked by 10 mM DTT ($n = 8$), 1 mM TCEP ($n = 5$) or 5 mM MTSET ($n = 6$) in cells expressing TRPC5. DTT had no effect without TRPC5 ($n = 5$). **e**, Inhibition of current at −80 mV by 0.1 mM Cd^{2+} in TRPC5-expressing cells with and without DTT treatment. **f**, As for **e** but typical *I–V* relationships. **g**, Currents at −80 mV after transfection with green fluorescent protein (GFP) alone (no TRPC5, $n = 8$) or GFP plus wild-type TRPC5 ($n = 7$) or the TRPC5 mutants C553A ($n = 11$), C558A ($n = 6$), C553A + C558A (double, $n = 3$) or C553S ($n = 6$). Gd^{3+} (100 μM) activated wild-type TRPC5 but had no effect on mutants. All currents were blocked by 2-APB (see, for example, Supplementary Fig. 5). Where error bars are shown, results are expressed as means and s.e.m. Asterisk, $P < 0.05$; n.s., no significant difference.

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with the metal ions acting by re-engaging cysteine residues[17]. Other TRP channels lacking the cysteine pair in a similar position were unresponsive to DTT (Supplementary Figs 2 and 3). The data support the hypothesis that the cysteine pair in TRPC5 normally engages in a disulphide bridge that constrains the channel in a state of limited opening probability, enabling enhanced channel activity when the bridge is broken.

To test the hypothesis further, we expressed TRPC5 mutants containing alanine in place of cysteine. Such mutants were constitutively active and were not stimulated by reducing agent or lanthanide (Fig. 1g and Supplementary Figs 4 and 5). Ionic currents for the single mutants (C553A, C553S or C558A) and double mutant (C553A + C558A) were not significantly different, suggesting that the two cysteine residues have a joint role (Fig. 1g). Expression of wild-type TRPC1 together with the TRPC5 double mutant led to smaller constitutive currents that were not affected by DTT or lanthanide, which is consistent with TRPC1 suppressing the current amplitude but not conferring a functional effect of reducing agents (Supplementary Fig. 6). Dimers of TRPC5 were not detected under non-reducing conditions, suggesting an intra-subunit rather than inter-subunit disulphide bridge (Supplementary Fig. 7).

Thioredoxin is an important redox protein with established biological roles including those in cancer, ischaemic reperfusion injury, inflammation and ageing[8]. It is both an intracellular and secreted protein[6–9]. It is reduced by the NADPH-dependent flavoprotein thioredoxin reductase and in this form has the capability of breaking disulphide bridges[8]. Extracellular reduced thioredoxin (rTRX) acts similarly to DTT, causing TRPC5 activation (Fig. 2a, b). We therefore proposed that rTRX is a previously unrecognized endogenous extracellular regulator of TRPC5. In taking this idea forward we also considered TRPC1 because many cells endogenously expressed TRPC5 and TRPC1 together, leading to TRPC5–TRPC1 heteromultimers[3,5,16,18]. The TRPC5–TRPC1 channel is also activated by rTRX or DTT (Fig. 2c). Consistent with previous reports[3,16] was our observation that the TRPC5 and TRPC5–TRPC1 channels had distinct 'fingerprint' *I–V* relationships (for example Fig. 3b and Supplementary Fig. 14).

Thioredoxin concentrations up to a mean of 0.41 $\mu g\,ml^{-1}$ (maximum 1.2 $\mu g\,ml^{-1}$) have been detected in serum and synovial fluid from patients with rheumatoid arthritis[8,10–12]. Furthermore, reducing capability of thioredoxin exists in serum; thioredoxin reductase occurs in human joints and its activity is correlated with disease

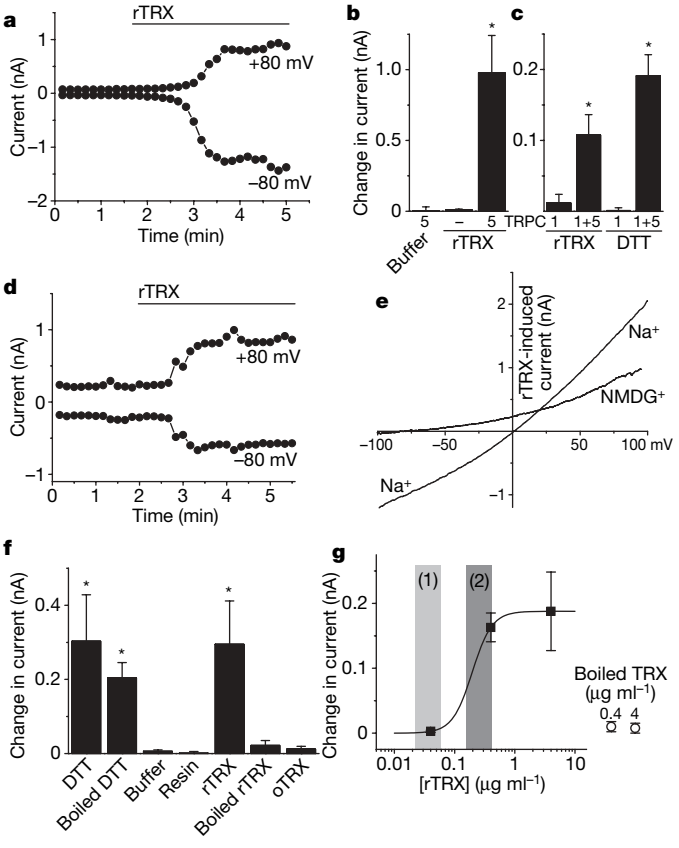

Figure 2 | Ionic current induced by rTRX. **a**–**c**, Whole-cell current data from HEK-293 cells expressing TRPC5 alone (**a**, **b**), TRPC1 alone or TRPC5 plus TRPC1 (**c**). **a**, Effect of 4 $\mu g\,ml^{-1}$ rTRX. **b**, Current at −80 mV in response to elution buffer diluted 1:100 ($n = 4$) or rTRX with ($n = 8$) and without ($n = 3$) TRPC5 expression. **c**, Responses to rTRX or 10 mM DTT ($n = 5$ for each). **d**, Effect of rTRX on a human FLS cell. **e**–**g**, Data for rabbit FLS cells. **e**, rTRX induced *I–V* relationships in standard bath (Na^+) or *N*-methyl-D-glucamine ($NMDG^+$) solution (see Supplementary Fig. 9). **f**, Currents evoked at −80 mV. **g**, Data for human rTRX with a fitted Hill equation (concentration giving half-maximal response 0.20 $\mu g\,ml^{-1}$, slope 2.64). Open symbols are control data and shaded areas are the concentrations of TRX in patients without arthritis (1) or with osteoarthritis (1) or rheumatoid arthritis (2). In **f** and **g**, $n = 5$ per data point. Where error bars are shown, results are expressed as means and s.e.m. Asterisk, $P < 0.05$.

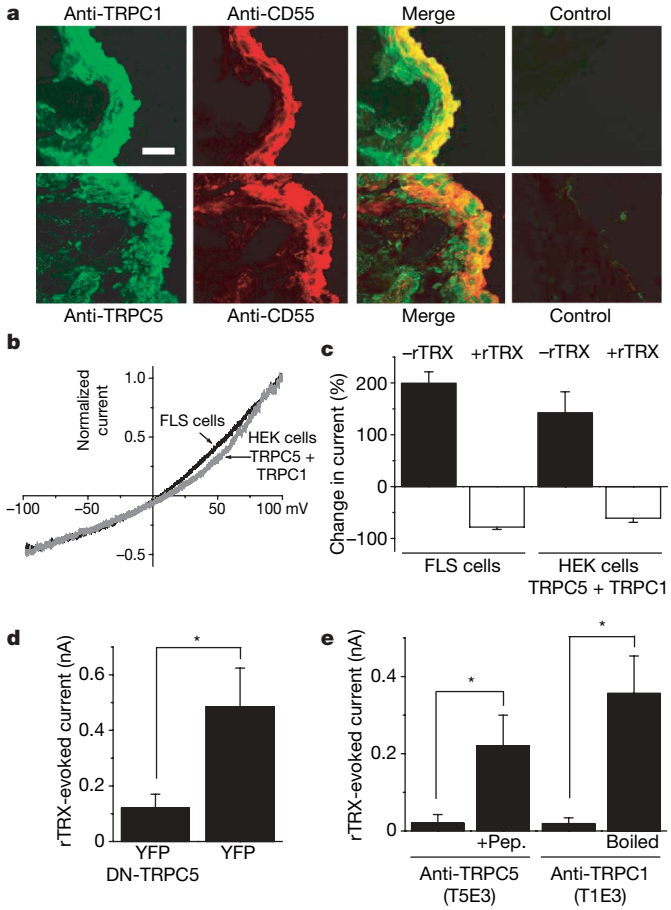

Figure 3 | Endogenous TRPC expression and function. **a**, Tissue sections from joints of patients with rheumatoid arthritis stained with T1E3 or T5E3 (green) or anti-CD55 (red) antibodies. Controls were omission of anti-CD55 antibody, and T1E3 or T5E3 preadsorbed on its antigenic peptide. **b**, Normalized rTRX-evoked *I–V* relationships for rabbit FLS cells ($n = 3$) and HEK-293 cells expressing TRPC5 and TRPC1 ($n = 5$). **c**, Changes in currents at −80 mV in response to 10 $\mu M\ La^{3+}$ before or after treatment with 4 $\mu g\,ml^{-1}$ rTRX (FLS cells, $n = 6$; HEK cells, $n = 4$). **d**, Current at −80 mV in FLS cells transfected with dominant-negative (DN) TRPC5 plus yellow fluorescent protein (YFP) or YFP alone ($n = 5$ for each). **e**, As in **d** but showing the effects of anti-TRPC antibodies ($n = 5$ for each). Pep., antigenic peptide. Where error bars are shown, results are expressed as means and s.e.m. Asterisk, $P < 0.05$.

severity[10,19,20] (see also Supplementary Results and Supplementary Discussion). We therefore considered whether the activation of TRPC5 by rTRX is relevant to the cells that secrete synovial fluid, the CD55-positive fibroblast-like synoviocytes (FLS cells). CD55-positive FLS cells (Supplementary Fig. 8) showed a non-selective cationic current in response to DTT or rTRX (Fig. 2d–g and Supplementary Figs 9 and 10). The mean current evoked by rTRX at −80 mV in FLS cells from the knee joint of patients with rheumatoid arthritis was -0.85 ± 0.42 nA (mean ± s.e.m.; $n = 14$). Oxidized TRX (oTRX) had no effect (Fig. 2f). The effective concentrations of rTRX indicate a possible relevance to rheumatoid arthritis (Fig. 2g). Nitric oxide is an alternative endogenous regulator of cysteine residues[21]; however, it failed to evoke current in FLS cells, even at a concentration 100-fold that required to evoke vasorelaxation (Supplementary Fig. 10, Supplementary Results and Supplementary Discussion).

There have been no previous reports on the expression of TRPC channels in synovial joints, so we explored synovial tissue biopsies from patients with rheumatoid arthritis. TRPC5 and TRPC1 proteins were detected and localized together with CD55 (Fig. 3a). Similarly, the FLS cells used in our electrophysiological experiments expressed messenger RNAs encoding TRPC5 and TRPC1, western blotting indicated the presence of TRPC5 and TRPC1 proteins, and immunolabelling revealed TRPC5 and TRPC1 at the cell surface (Supplementary Figs 8, 11 and 13).

The *I–V* relationship of the rTRX-evoked current in FLS cells was similar to that of the TRPC5–TRPC1 heteromultimeric channel (Fig. 3b). Furthermore, experiments with lanthanum ions showed unusual and striking similarity between the endogenous current and the current of overexpressed TRPC5–TRPC1: in the absence of a reducing agent, lanthanum ions stimulated current in both HEK-293 cells (exogenously expressing TRPC5–TRPC1) and FLS cells, whereas after the induction of current by rTRX, lanthanum ions were inhibitory in both cases (Fig. 3c and Supplementary Fig. 9). Also consistent with the involvement of TRPC channels were the observations that the rTRX-evoked current of FLS cells was blocked by 2-APB and that the inward current was suppressed when most of the extracellular Na^+ was replaced by the bulky and impermeant cation *N*-methyl-D-glucamine (Fig. 2e and Supplementary Fig. 9). As a further test of the involvement of TRPC5 and TRPC1, FLS cells were transfected with a dominant-negative ion-pore mutant of TRPC5 that inhibited native channels capable of interacting with TRPC5 (refs 16, 22). The mutant suppressed current evoked by rTRX (Fig. 3d).

Further evidence that TRPC5 and TRPC1 contribute to the endogenous rTRX-responsive channel of FLS cells came from studies with anti-TRPC5 (T5E3) and anti-TRPC1 (T1E3) antibodies, which target the predicted extracellular loop region and specifically block the functions of TRPC5 and TRPC1, respectively[23–25]. T5E3 and T1E3 antibodies labelled unpermeabilized FLS cells, unlike antibody targeted to the intracellular carboxy terminus of TRPC5, which labelled only permeabilized cells (Supplementary Figs 8 and 11), indicating that TRPC5 and TRPC1 are transmembrane proteins with extracellular epitopes. Like dominant-negative mutant TRPC5, T5E3 or T1E3 suppressed rTRX-evoked current (Fig. 3e). Antibody targeted to CD55, which is a membrane protein unrelated to TRP, had no significant effect ($n = 7$; data not shown). Gene expression, electrophysiology, pharmacology, recombinant DNA and antibody studies therefore yielded data consistent with the carrying of rTRX-evoked current in FLS cells by a channel containing TRPC5 and TRPC1.

One of the functions of FLS cells is to secrete matrix metalloproteinases (MMPs), which are associated with tissue remodelling and the progression of arthritis[26]. The use of zymography to detect gelatinase activities of MMP-2 and MMP-9 secreted from rabbit FLS cells (Supplementary Fig. 12) revealed that T5E3 and T1E3 antibodies have large stimulatory effects (Fig. 4a, b). Human FLS cells showed greater MMP-2 secretion than that of MMP-9 (compare Supplementary Fig. 12 with Fig. 4a). Enzyme-linked immunosorbent assays (ELISAs) for human MMP-2 enabled the quantification of the absolute concentration of total MMP-2 secreted; again, either T5E3 or T1E3 antibody had a profound stimulatory effect (Fig. 4c). Similarly, knockdown of expression of the genes encoding TRPC1 and TRPC5 by RNA-mediated interference enhanced the secretion of MMP-2 (Supplementary Fig. 13). Inhibition of MMP secretion by the addition of exogenous reducing TRX was lost in the presence of T5E3 (Fig. 4d). Similar data were obtained for pro-MMP-1 secretion from human FLS cells (Fig. 4e, f) and MMP-9 measured by zymography in rabbit FLS cells ($n = 6$; data not shown). The data therefore reveal constitutive and rTRX-evoked activity of TRPC5 and TRPC1 channels that inhibits the secretion of MMP from FLS cells.

The data of this study indicate that secreted TRX is a type of ion channel agonist that acts through its reduced form to break a restraining intra-subunit disulphide bridge between cysteine residues in TRPC5, thereby stimulating the channel either as a homomeric assembly or as a heteromultimer with TRPC1. A transduction mechanism is therefore revealed that can directly couple cell activity to extracellular reduced thioredoxin. This mechanism may have particular relevance in conditions such as rheumatoid arthritis, in which TRX concentrations are strongly elevated, but the broad distributions of TRX and the channels indicate that the mechanism could be widely used.

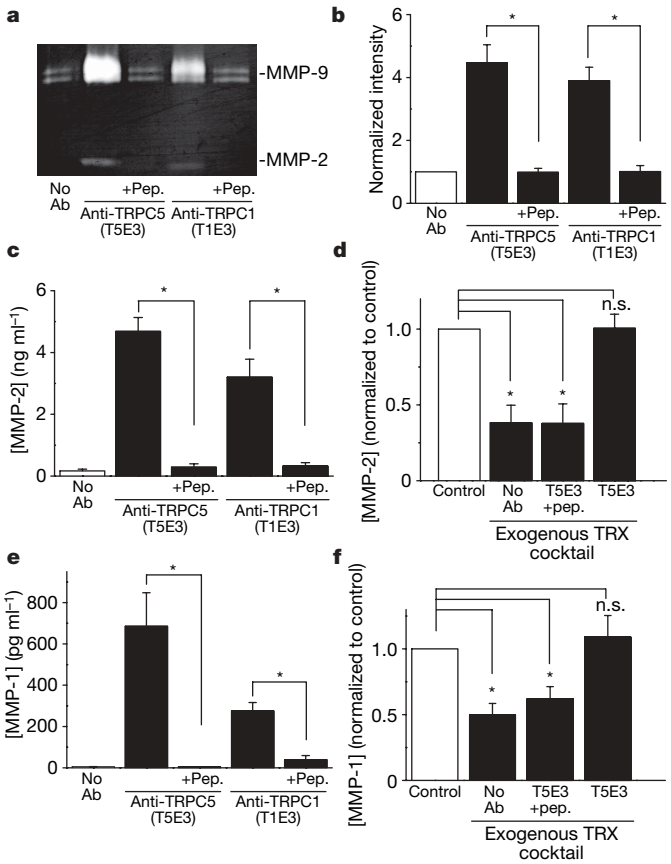

Figure 4 | Relevance to secretion from FLS cells. **a**, Zymogram showing MMP-9 (pro and active) and MMP-2 from rabbit. Ab, antibody. **b**, As for **a** but mean data after normalization of rabbit MMP-9 band intensity to the control group without antibody ($n = 3$ for each). **c**, ELISA data for human MMP-2 ($n = 4$). **d**, Effect of T5E3 ($n = 4$) on inhibition of human MMP-2 secretion by exogenous TRX cocktail. For each group, secretion in TRX was normalized to that in its absence (control). **e**, **f**, As for **c**, **d**, but for secretion of human pro-MMP-1. Results are expressed as means and s.e.m. Asterisk, $P < 0.05$; n.s., no significant difference.

METHODS SUMMARY

Cells. Synovial tissue biopsies were obtained with informed consent from patients diagnosed with rheumatoid arthritis at the Academic Unit of Musculoskeletal Disease, Chapel Allerton Hospital, Leeds. Ethical approval was given by the local ethics committee. Human synovial tissue biopsies were washed with PBS and digested in 0.25% type 1A collagenase for 4 h at 37 °C, after which FLS cells were cultured in DMEM/F-12 + Glutamax (Gibco). HEK-293 cells were grown in DMEM-F12 (Gibco) and rabbit FLS cells (HIG82; ATCC) were grown in Ham's F12 (Gibco). Culture media contained 10% fetal calf serum, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air and replated on coverslips or 24-well plates before experiments.

Electrophysiology. Whole-cell patch-clamp recordings were performed[15,16] at 21 ± 2 °C using patch pipette solution containing (in mM): 115 CsCl, 10 EGTA, 2 $MgCl_2$, 5 Na_2ATP, 0.1 NaGTP, 10 HEPES, 5.7 $CaCl_2$; the pH was adjusted to 7.2 with CsOH. The standard bath solution contained (in mM): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.2 $MgCl_2$ and 1.5 $CaCl_2$; the pH was adjusted to 7.4 with NaOH.

Data analysis. Ionic currents are shown as positive values when they increased in response to a treatment, and as negative values when they decreased. Data are expressed as means and s.e.m., where n is the number of individual experiments. Data sets were compared by using paired or unpaired Student's t-tests, with a significant difference indicated by $P < 0.05$ (asterisk) and no difference by n.s. All human tissue or cell data are derived from, or are representative of, at least three independent experiments on samples from three patients.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 6 July; accepted 24 October 2007.

1. Flockerzi, V. An introduction on TRP channels. *Handb. Exp. Pharmacol.* **179,** 1–19 (2007).
2. Nilius, B., Owsianik, G., Voets, T. & Peters, J. A. Transient receptor potential cation channels in disease. *Physiol. Rev.* **87,** 165–217 (2007).
3. Strubing, C., Krapivinsky, G., Krapivinsky, L. & Clapham, D. E. TRPC1 and TRPC5 form a novel cation channel in mammalian brain. *Neuron* **29,** 645–655 (2001).
4. Plant, T. D. & Schaefer, M. TRPC4 and TRPC5: receptor-operated Ca^{2+}-permeable nonselective cation channels. *Cell Calcium* **33,** 441–450 (2003).
5. Beech, D. J. Canonical transient receptor potential 5. *Handb. Exp. Pharmacol.* **179,** 109–123 (2007).
6. Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. & Sitia, R. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J. Biol. Chem.* **267,** 24161–24164 (1992).
7. Arner, E. S. & Holmgren, A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* **267,** 6102–6109 (2000).
8. Burke-Gaffney, A., Callister, M. E. & Nakamura, H. Thioredoxin: friend or foe in human disease? *Trends Pharmacol. Sci.* **26,** 398–404 (2005).
9. Schwertassek, U. *et al.* Selective redox regulation of cytokine receptor signaling by extracellular thioredoxin-1. *EMBO J.* **26,** 3086–3097 (2007).
10. Maurice, M. M. *et al.* Expression of the thioredoxin–thioredoxin reductase system in the inflamed joints of patients with rheumatoid arthritis. *Arthritis Rheum.* **42,** 2430–2439 (1999).
11. Yoshida, S. *et al.* Involvement of thioredoxin in rheumatoid arthritis: its costimulatory roles in the TNF-α-induced production of IL-6 and IL-8 from cultured synovial fibroblasts. *J. Immunol.* **163,** 351–358 (1999).
12. Jikimoto, T. *et al.* Thioredoxin as a biomarker for oxidative stress in patients with rheumatoid arthritis. *Mol. Immunol.* **38,** 765–772 (2002).
13. Smolen, J. S., Aletaha, D., Koeller, M., Weisman, M. H. & Emery, P. New therapies for treatment of rheumatoid arthritis. *Lancet* advance online publication doi:10.1016/S0140-6736(07)60784-3 (13 June 2007).
14. Jung, S. *et al.* Lanthanides potentiate TRPC5 currents by an action at extracellular sites close to the pore mouth. *J. Biol. Chem.* **278,** 3562–3571 (2003).
15. Zeng, F. *et al.* Human TRPC5 channel activated by a multiplicity of signals in a single cell. *J. Physiol. (Lond.)* **559,** 739–750 (2004).
16. Xu, S. Z. *et al.* A sphingosine-1-phosphate-activated calcium channel controlling vascular smooth muscle cell motility. *Circ. Res.* **98,** 1381–1389 (2006).
17. Elliott, D. J. *et al.* Molecular mechanism of voltage sensor movements in a potassium channel. *EMBO J.* **23,** 4717–4726 (2004).
18. Riccio, A. *et al.* mRNA distribution analysis of human TRPC family in CNS and peripheral tissues. *Brain Res. Mol. Brain Res.* **109,** 95–104 (2002).
19. Lemarechal, H. *et al.* High redox thioredoxin but low thioredoxin reductase activities in the serum of patients with rheumatoid arthritis. *Clin. Chim. Acta* **367,** 156–161 (2006).
20. Lemarechal, H. *et al.* Impairment of thioredoxin reductase activity by oxidative stress in human rheumatoid synoviocytes. *Free Radic. Res.* **41,** 688–698 (2007).
21. Yoshida, T. *et al.* Nitric oxide activates TRP channels by cysteine S-nitrosylation. *Nature Chem. Biol.* **2,** 596–607 (2006).
22. Strubing, C., Krapivinsky, G., Krapivinsky, L. & Clapham, D. E. Formation of novel TRPC channels by complex subunit interactions in embryonic brain. *J. Biol. Chem.* **278,** 39014–39019 (2003).
23. Xu, S. Z. & Beech, D. J. TrpC1 is a membrane-spanning subunit of store-operated Ca^{2+} channels in native vascular smooth muscle cells. *Circ. Res.* **88,** 84–87 (2001).
24. Xu, S. Z. *et al.* Generation of functional ion-channel tools by E3 targeting. *Nature Biotechnol.* **23,** 1289–1293 (2005).
25. Xu, S. Z., Boulay, G., Flemming, R. & Beech, D. J. E3-targeted anti-TRPC5 antibody inhibits store-operated calcium entry in freshly isolated pial arterioles. *Am. J. Physiol. Heart Circ. Physiol.* **291,** H2653–H2659 (2006).
26. Burrage, P. S., Mix, K. S. & Brinckerhoff, C. E. Matrix metalloproteinases: role in arthritis. *Front. Biosci.* **11,** 529–543 (2006).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements This work was supported by Wellcome Trust grants to D.J.B. and A.S., and a Physiological Society Junior Fellowship to C.C. P.S. has an Overseas Research Scholarship and University Studentship, J.N. has a Biotechnology and Biological Sciences Research Council PhD studentship, Y.M. a university studentship and Y.B. a scholarship from the Egyptian Ministry of Higher Education.

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METHODS

Complementary DNA clones, mutagenesis and cell transfection. HEK-293 cells stably expressing tetracycline-regulated human TRPC5 have been described[15]. Expression was induced by 1 μg ml^{-1} tetracycline (Tet^{+}; Sigma) for 24–72 h before recording. Non-induced cells without addition of tetracycline (Tet^{-}) were controls. Human TRPC1 cDNA was expressed transiently from the bicistronic vector pIRES EYFP[16]. Point mutations in human TRPC5 were introduced by using QuikChange site-directed mutagenesis (Stratagene) and appropriate primer sets. Dominant-negative TRPC5 is a triple alanine mutation of the conserved LFW sequence in the ion pore[16,22] (Supplementary Fig. 2). The mutations were confirmed by direct sequencing of the entire reading frame. cDNAs were transiently transfected into HEK-293 cells or synoviocytes with FuGENE 6 transfection reagent (Roche) or Lipofectamine 2000 (Invitrogen) 48 h before recording. cDNA encoding GFP or YFP was cotransfected to identify transfected cells.

Electrophysiology. A salt-agar bridge was used to connect the ground Ag–AgCl wire to the bath solution. Signals were amplified with an Axopatch 200B patch clamp amplifier and controlled with pClamp software v. 6.0 (Axon) or Signal software v. 3.05 (CED). A 1-s ramp voltage protocol from −100 mV to +100 mV was applied at a frequency of 0.1 Hz from a holding potential of −60 mV. Current signals were filtered at 1 kHz and sampled at 3 kHz. Patch pipettes were made from borosilicate tubing which, after fire-polishing and filling with pipette solution, had a resistance of 3–5 MΩ. The osmolarity of the pipette solution was adjusted to ~290 mosM with mannitol and the calculated free Ca^{2+} was 200 nM. ATP and GTP were omitted when recording from cells expressing TRPC5 alone. When we were studying TRPC5 in HEK-293 cells, gadolinium chloride (Gd^{3+}, 1–5 μM) was included in the bath solution to block background currents[15], which evoked submaximal TRPC5 current in some recordings before other agents were applied. The effect of reducing agents was not dependent on the presence of Gd^{3+}. The recording chamber had a volume of 150 μl and was perfused at a rate of about 2 ml min^{-1}. Recordings from human FLS cells used the Patchliner (Nanion) planar patch-clamp system with rapid bath solution exchange. For antibody treatment experiments, cells were treated with one of T1E3 at 1:500 dilution (ref. 23), T5E3 at 1:100 dilution (refs 24, 25), boiled (10 min) T1E3 at 1:500 dilution, T5E3 antibody at 1:100 dilution preabsorbed on its antigenic peptide (10 μM) or anti-CD55 antibody (see below), which were diluted in F12 Ham's medium and incubated with cells for 2–3 h at 37 °C before patch-clamp recording.

Immunostaining. Sections 4 μm thick were obtained from snap-frozen synovial tissue biopsy samples of patients suffering from rheumatoid arthritis, fixed with acetone and stored at −80 °C until use. Staining was in accordance with standard protocols. In brief, sections were incubated with primary antibody overnight at 4 °C and with secondary antibody (goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma) and donkey anti-mouse IgG conjugated with Cy3 (Jackson)) for 1 h at 21 ± 2 °C. For cell labelling, FLS cells adhered to coverslips were fixed for 13 min in 4% paraformaldehyde and, unless indicated, permeabilized for 2 h with 0.1% Triton X-100 in 1% BSA. Incubation in primary antibody was overnight at 4 °C and with secondary antibody (goat anti-rabbit IgG-FITC) for 2 h at room temperature. For control experiments, antibodies were preabsorbed on their antigenic peptide (10 μM) or omitted, as specified. Slides were mounted with 4,6-diamidino-2-phenylindole hardest mounting medium (Vector Labs) and analysed with a Zeiss confocal microscope. T1E3, T5E3, T5C3, anti-CD55 (Serotec) and CD68 (Dako) antibodies were used at 1:500, 1:100, 1:500 and 1:200 dilutions, respectively.

Secretion assays. FLS cells were cultured for 24 h in 24-well plates and serum-starved for 24 h; fresh serum-free medium was then added that contained a TRX cocktail, which included TRX (0.4 μg ml^{-1}), NADPH-dependent flavoprotein TRX reductase (0.5 μg ml^{-1}) and NADPH (2 μg ml^{-1}) for 12 h. Omission of TRX was the control. Incubations with antibodies occurred for 2 h before addition of the TRX cocktail and were maintained in the presence of the TRX cocktail. Supernatants were collected, frozen and analysed by zymography or ELISA. For zymography the supernatant was mixed with 2× non-reducing SDS–PAGE sample buffer and resolved through a 7.4% polyacrylamide gel impregnated with 1.5 mg ml^{-1} gelatin. After electrophoresis, gels were washed, incubated and stained as described previously[27]. The relative density of gelatinolytic bands was determined from scanned images of gels by using ImageQuant software (Amersham). MMP-2 or MMP-1 concentrations in supernatants from human cells were quantified with Quantikine Human total MMP-2 and pro-MMP-1 ELISA kits in accordance with the manufacturer's instructions (R&D Systems).

Chemicals. All salts and reagents were from Sigma or BDH. Gadolinium (Gd^{3+}) chloride, lanthanum (La^{3+}) chloride, cadmium (Cd^{2+}) chloride, DTT, 2-APB, NADPH and NADPH-dependent flavoprotein thioredoxin reductase (*Escherichia coli*) were from Sigma. Recombinant thioredoxin (TRX; Sigma) was from *E. coli* (unless specified) or human (no differences in effect were observed compared with *E. coli* TRX) and purchased from Sigma. MTSET was from Toronto Research Chemicals and TCEP was from Pierce Biotech. MTSET, TCEP and NADPH were prepared fresh for each experiment. Collagenase was from StemCell Technologies Inc. 2-APB (75 mM) stock solution was in 100% dimethylsulphoxide. To prepare reduced thioredoxin (rTRX), TRX (1 mg) was dissolved in 1 ml of the binding buffer (10 mM HEPES, 1 mM EDTA, 50 mM NaCl, pH 7.0) and 0.25 ml of this was mixed with 2.5 μl of 1 M DTT, incubated at room temperature for 30 min and then added to 0.25 ml of pre-equilibrated resin (DEAE-Sephadex; Sigma). The mixture was centrifuged for 30 s and then washed three times with binding buffer to remove DTT completely. Elution buffer (0.25 ml; 10 mM HEPES, 1 mM EDTA, 1 M NaCl, pH 7.0) was added and centrifuged to harvest the supernatant. The final TRX concentration was determined by Bradford assay. rTRX was diluted from cold stocks (on ice) immediately before use.

27. Porter, K. E. *et al.* Simvastatin inhibits human saphenous vein neointima formation via inhibition of smooth muscle cell proliferation and migration. *J. Vasc. Surg.* **36**, 150–157 (2002).