LETTERS

# Disulphide-isomerase-enabled shedding of tumour-associated NKG2D ligands

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**Tumour-associated ligands of the activating NKG2D (natural killer group 2, member D; also called KLRK1) receptor—which are induced by genotoxic or cellular stress—trigger activation of natural killer cells and co-stimulation of effector T cells, and may thus promote resistance to cancer[1–6]. However, many progressing tumours in humans counter this anti-tumour activity by shedding the soluble major histocompatibility complex class-I-related ligand MICA, which induces internalization and degradation of NKG2D and stimulates population expansions of normally rare $NKG2D^+CD4^+$ T cells with negative regulatory functions[7–9]. Here we show that on the surface of tumour cells, MICA associates with endoplasmic reticulum protein 5 (ERp5; also called PDIA6 or P5), which, similar to protein disulphide isomerase, usually assists in the folding of nascent proteins inside cells[10]. Pharmacological inhibition of thioreductase activity and *ERp5* gene silencing revealed that cell-surface ERp5 function is required for MICA shedding. ERp5 and membrane-anchored MICA form transitory mixed disulphide complexes from which soluble MICA is released after proteolytic cleavage near the cell membrane. Reduction of the seemingly inaccessible disulphide bond in the membrane-proximal α3 domain of MICA must involve a large conformational change that enables proteolytic cleavage. These results uncover a molecular mechanism whereby domain-specific deconstruction regulates MICA protein shedding, thereby promoting tumour immune evasion, and identify surface ERp5 as a strategic target for therapeutic intervention.**

NKG2D-mediated tumour rejection can be effective at early stages of tumour growth[1–3,5]. However, sustained surface expression and shedding of soluble MICA (sMICA) by late-stage human tumours negatively imprint on the local and systemic immune response, thus promoting tumour immune evasion[7–9]. The significance of this relationship is highlighted by beneficial effects of neutralizing anti-MICA antibodies that were induced as a result of immunotherapy in a clinical trial[11]. These findings are supported by a mouse model study that confirmed systemically impaired natural killer cell and CD8 T-cell functions, accompanied by increased tumour susceptibility, as a result of NKG2D downmodulation by locally sustained NKG2D ligand expression[12]. However, mice lack sequences that are orthologous to human *MICA* and the closely related *MICB*, and shedding of naturally expressed NKG2D ligands has not been observed in mice[6].

Early studies of the interactions between NKG2D and its ligands used randomly oligomerized recombinant MICA or ULBP family ligands produced as immunoglobulin fusion proteins, all of which bound exclusively to NKG2D-expressing lymphocytes[13,14]. On testing MICA and ULBP2 tetramers, we confirmed that these high-avidity reagents stained the NKL natural killer cell line and that binding was entirely accounted for by NKG2D (Fig. 1a). However, during the course of screening ~40 cell lines by flow cytometry, we observed that MICA, but not ULBP2, tetramers stained cell types lacking NKG2D. The highest fluorescence intensities were recorded with U266 myeloma cells and all of the ten epithelial tumour lines tested, and correlated with relatively large amounts of cell-surface MICA. Only monocytic U937 cells were identified as negative for tetramer binding (Fig. 1a). The MICA tetramers were prepared using glycosylated protein secreted by transfected 293T cells. However, tetramer binding was not reduced after cleavage of cell-surface polypeptide-linked carbohydrates but was inhibited in the presence of unglycosylated recombinant MICA (Fig. 1a). Thus, these results revealed an interaction involving MICA—but not NKG2D ligands in general—and an unidentified surface protein.

Candidate MICA-binding proteins were purified from U266 and negative control U937 outer cell membranes using MICA-coupled sepharose beads. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and silver staining revealed two sets of protein bands that were detected with U266 but not U937 cells (Fig. 1b). By mass spectrometry, two protein bands in the 76–78-kilodalton (kDa) molecular mass range corresponded to heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) (HSPA5, also known as BiP). A major protein band of 50 kDa was identified as ERp5 (ref. 10), and two additional proteins of about 47 and 48 kDa shared similarities with thioredoxin family members. Because all of these proteins are typically intracellular, we scrutinized their outer cell membrane localization. Using the same purification protocol and surface-biotinylated cells, immunoblots probed with streptavidin-horse radish peroxidase (HRP) or polyclonal antibodies demonstrated the presence of HSPA5, and more prominently ERp5, on the surface of U266 but not U937 cells, which was confirmed by staining for ERp5 (Fig. 1c, d). ERp5 is related to protein disulphide isomerase. Both proteins contain two thioredoxin-like domains, each with a pair of active site cysteines in CXXC motifs, and mediate the intracellular formation of nascent polypeptide disulphide bonds; however, they have also been implicated in extracellular disulphide exchange[10,15–17].

In exploring a functional relationship between MICA and ERp5, we were guided by the epithelial tumour-associated expression that is characteristic of MICA but not the ULBP family of NKG2D ligands[6,9]. This idea was encouraged when freshly isolated tumour cells displayed similar patterns of tetramer and anti-ERp5 and anti-MICA antibody binding, and matched serum samples were positive for sMICA (Fig. 2a). We thus tested for a role of ERp5 in MICA shedding by exposing U266 cells and tumour lines HeLa, A375 melanoma, and HCT116 and LoVo colon carcinoma to DTNB (5,5-dithiobis-(2-nitrobenzoic acid)) or PAO (phenylarsine oxide), which impair

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protein disulphide isomerase function by forming disulphide and coordination bonds, respectively, with thiol groups in its catalytic sites[18]. Both inhibitors reduced the production of sMICA at titred non-toxic concentrations without affecting surface expression of MICA (Fig. 2b and data not shown). Treatment with PAO also diminished tetramer binding, suggesting that MICA interacts directly with an ERp5 catalytic site (Fig. 2c). However, these inhibitors are relatively nonspecific and may have pleiotropic effects. Therefore, and to preclude an involvement of thiol isomerases other than ERp5, we expressed short interfering (si)RNA constructs targeting two regions of *ERp5* messenger RNA in A375 cells. As measured by real-time reverse transcription PCR (RT–PCR), *ERp5* mRNA was reduced by ~70–80% as a result of the siRNA targeting (Fig. 3a). As a consequence, ERp5 surface expression, MICA tetramer binding and sMICA shedding decreased, although the amount of MICA surface protein was not noticeably changed (Fig. 3b, c). Thus, the cumulative evidence indicated that surface ERp5 function is required for MICA shedding.

The functional association between ERp5 and MICA was biochemically analysed. Initial failure to co-immunoprecipitate these proteins from lysates of surface-biotinylated HeLa cells implied that ERp5 and MICA maintain no stable complexes after solubilization. However, ERp5 co-immunoprecipitated with MICA when HeLa cells were treated with trichloroacetic acid (TCA), which traps mixed disulphide polypeptides and quenches thiol interchange (Fig. 4a, lane 1)[19]. Sulphydryl groups in subsequent cell lysates were alkylated and immunocomplexes deglycosylated with *N*-glycanase. This procedure was modified by using HeLa cells grown in the presence of denatured and reduced RNase (dRNase), which served as excess substrate, shifting ERp5 equilibrium towards the reduced state[20] and thereby favouring disulphide exchange with MICA. Analysis by SDS–PAGE and immunoblotting showed that increasing the concentration of dRNase resulted in larger amounts of ERp5 co-immunoprecipitating with MICA (Fig. 4a, lanes 1–4). Concomitantly, the MICA polypeptide of 38 kDa (shortened in HeLa cells owing to homozygous cytoplasmic tail deletion[21]) disappeared, and proteins with molecular masses of 31 and 34 kDa emerged. The 34-kDa protein corresponded to truncated sMICA, as determined by secondary precipitation from dissociated immunocomplexes and comparison to sMICA isolated from HeLa cell culture media (Fig. 4a, lanes 3, 4, 9 and 12). The 31-kD protein may represent another substrate or co-factor that was recruited into ERp5–MICA complexes. Similar data were obtained using anti-ERp5 for immunoprecipitations (Fig. 4a, lanes 5 and 6). None of those biochemical changes was observed when cells were grown in the presence of native RNase (Fig. 4b). Thus, these results

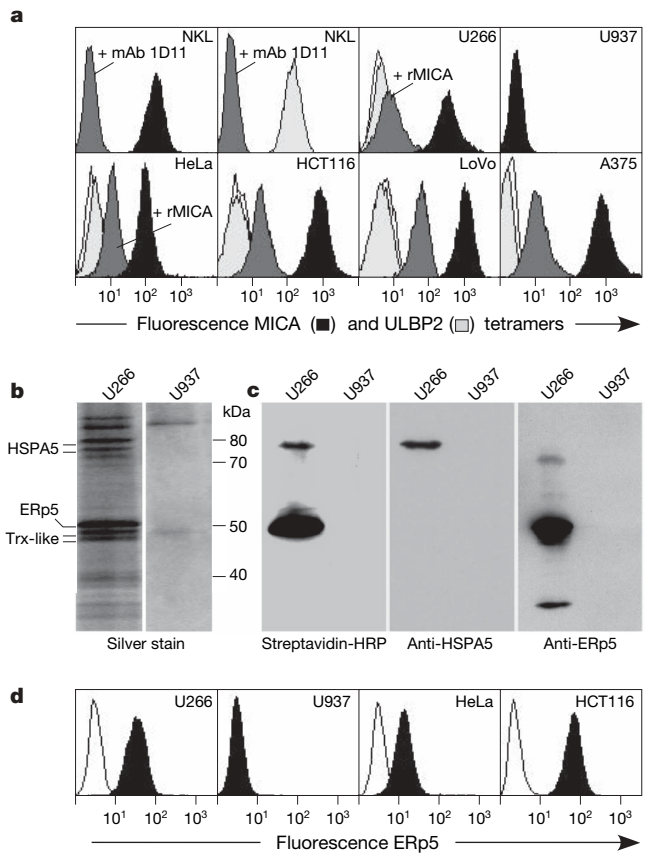


**Figure 1 | Surface interactions of MICA with ERp5 and HSPA5. a**, Flow cytometry confirms MICA (black shading) and ULBP2 (light-grey shading) tetramer binding to NKL cells and inhibition by anti-NKG2D monoclonal antibody 1D11 (dark-grey shading). MICA but not ULBP2 tetramers bind to NKG2D-negative U266, HeLa, HCT116, LoVo and A375 tumour cells, and binding is inhibited by bacterial recombinant MICA (dark-grey shading). U937 cells are negative for MICA tetramer binding. Open profiles are control IgG stainings. **b**, Silver staining of U266 and U937 outer cell membrane proteins enriched for binding to MICA beads. Trx, thioredoxin. **c**, Probing of MICA bead-purified proteins from surface biotinylated cells with streptavidin-HRP or specific antisera. Two additional bands in the anti-ERp5 lane are cross-reactive. **d**, Anti-ERp5 stainings (black shading). Open profiles are IgG controls.

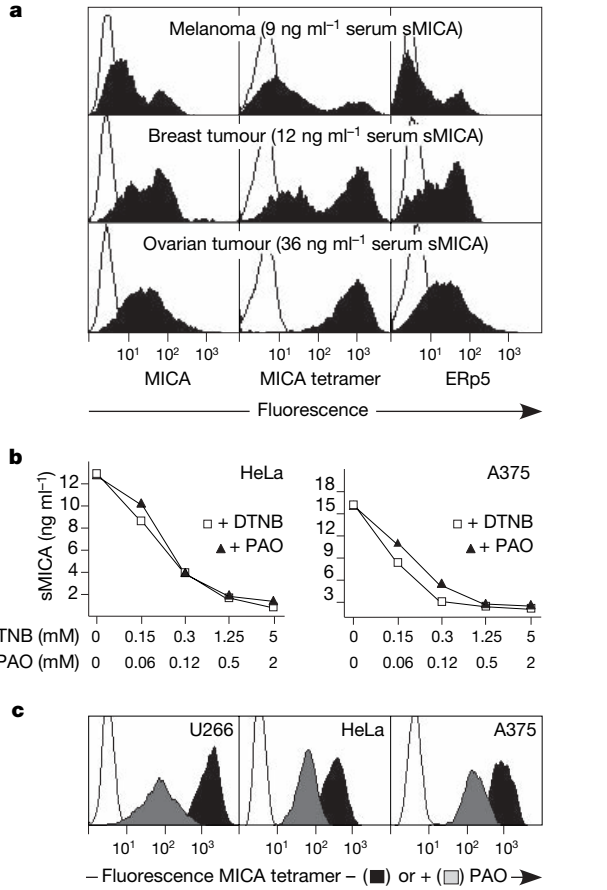


**Figure 2 | Tumour-associated ERp5 surface expression and pharmacological inhibition of sMICA shedding. a**, Freshly isolated melanoma, breast and ovarian tumour cells are positive for MICA, tetramer binding and surface ERp5 (black shading; open profiles are IgG control stainings for anti-MICA and anti-ERp5, and phycoerythrin-streptavidin controls for MICA tetramer). Matched patient peripheral blood serum samples contain the indicated amounts of sMICA. Data are representative of five matched sample pairs. **b**, DTNB and PAO reduce shedding of sMICA by HeLa and A375 cells in a dose-dependent manner, as determined by ELISA. Similar results were obtained with HCT116 and LoVo cells. **c**, PAO interferes with MICA tetramer binding. Open profiles are phycoerythrin-streptavidin controls.

demonstrated dynamic interactions between ERp5 and MICA that were closely tied to the production of sMICA, which was corroborated by large increases of sMICA in dRNase-treated HeLa and A375 cell cultures (Fig. 4c).

To demonstrate ERp5-mediated MICA disulphide bond reduction and explore substrate and domain specificities, bacterially produced recombinant proteins were mixed and incubated in the absence of reducing agents and thus under oxidizing conditions. Non-reducing gel electrophoresis and comparison to β-mercaptoethanol (β-ME)-treated samples showed gradual reduction of MICA (Supplementary Fig. 1). This was remarkable as ERp5 affected an intact, properly folded, substrate protein isoenergetically and in the absence of any other factor in solution. A similar result was obtained with the closely related MICB (data not shown). In contrast, ERp5 did not affect unrelated proteins with relatively accessible intra-chain (siderocalin) or intra-chain and inter-chain (KLRD1–NKG2A) disulphide bonds (Supplementary Fig. 1). No synergistic effect was observed when MICA was exposed to ERp5 together with HSPA5. Of the two ERp5 thioredoxin-like domains, which were expressed as two separate polypeptides (amino acid residues 1–118 and 135–421; Fig. 5a), only the amino-terminal domain displayed functional activity (Fig. 5b and data not shown). As with protein disulphide isomerase, ERp5 uses a catalytic mechanism whereby one active site cysteine invades the target disulphide, transiently forming a disulphide-linked heterodimer that is resolved by disulphide exchange with the second active site cysteine[10,15]. Of two ERp5(1–118) mutant fragments, the C36S point mutation showed no activity on MICA substrate whereas C39S formed a trapped disulphide-linked intermediate, thus confirming the role of C36 as the invading and C39 as the resolving cysteine in this reaction (Fig. 5c). By size-exclusion chromatography, intact ERp5 was a trimer in solution whereas the two individual domains behaved as monomers (data not shown). Thus, ERp5 multimerization was not required for MICA reduction.

Similar to conventional major histocompatibility complex class I molecules, MICA contains three intra-chain disulphide bonds located between amino acid residues 36 and 41, 96 and 164, and 202 and 259 in the α1, α2 and the C-type immunoglobulin-like α3 domain, respectively[22]. To identify the target disulphide, the α1α2 platform and α3 membrane-proximal domains were expressed and tested separately. ERp5 displayed no activity with the α1α2 domain (Fig. 5d). Because we were unable to electrophoretically resolve reduced and non-reduced forms of the relatively small α3 domain, we used the ERp5(1–118) polypeptide fragment with the C39S mutation for analysis. Gel electrophoresis revealed a large protein band shift corresponding to a mixed disulphide heterodimer (Fig. 5e). Thus, the disulphide bond targeted by ERp5 was in the MICA α3 domain.

Proteolytic cleavage of MICA is thought to be mediated by metalloproteinases[23]. However, with HeLa and A375 cells we observed no reduction in sMICA shedding by metalloproteinase inhibitors, suggesting that diverse proteases may have the ability to cleave MICA. To determine the MICA cleavage site, we purified sMICA from cultures of transfectant C1R-MICA cells, which express modest amounts of ERp5 but proliferate vigorously in serum-free media. Carboxy-terminal sequencing by mass spectroscopic analysis of tryptic peptide fragments revealed ragged MICA C termini defined by several neighbouring amino acid residues at or near the transmembrane boundary.

Our results suggest that MICA cleavage occurs in complex with ERp5 before mixed disulphide resolution, which in all likelihood results in immediate 'snap-back' oxidization of the MICA disulphide bond. ERp5 escape from intracellular retention is probably independent of MICA, as intracellular interactions have not been observed[24]. Precedent for biological functions of surface thiol isomerases includes

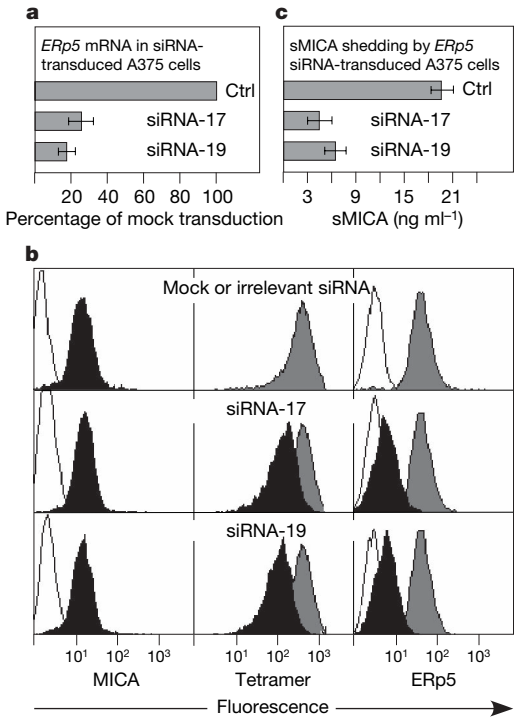


**Figure 3 | ERp5 is required for sMICA shedding.** **a**, Expression of siRNA constructs 17 or 19 in A375 cells results in ~70–80% reduction of *ERp5* mRNA, as measured by real-time RT–PCR. **b**, Knockdown of *ERp5* mRNA decreases MICA tetramer binding and ERp5 surface expression (black shading in centre and right columns). MICA expression (black shading in left column) is unchanged; open profiles represent IgG control stainings. **c**, Knockdown of *ERp5* mRNA diminishes sMICA shedding as determined by ELISA. Control bars in **a** and **c** and grey-shaded control profiles in **b** represent mock-transduced cells or cells expressing irrelevant siRNA. Error bars in **a** and **c** represent deviations among three experiments.

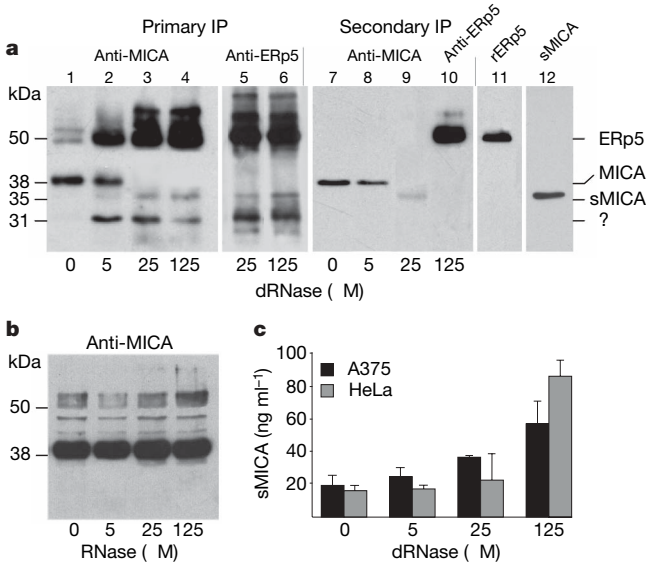


**Figure 4 | ERp5–MICA disulphide exchange enables MICA cleavage.** **a**, Treatment of surface-biotinylated HeLa cells with TCA before lysis, MICA immunoprecipitation, SDS–PAGE and membrane transfer reveals MICA–ERp5 complexes (lane 1). Protein identities are confirmed by secondary precipitations (lanes 7, 8, 10), primary precipitations of ERp5 (lanes 5, 6) and by recombinant ERp5 (lane 11). After cell culture in the presence of dRNase, co-immunoprecipitated ERp5 increases (lanes 2–4), full-length MICA disappears and sMICA emerges (lanes 3, 4). sMICA identity is confirmed by secondary precipitation (lane 9) and comparison to sMICA from cell culture media (lane 12). **b**, Control experiment with cells grown in the presence of native RNase. **c**, dRNase promotes sMICA shedding. Error bars represent deviations among three experiments.

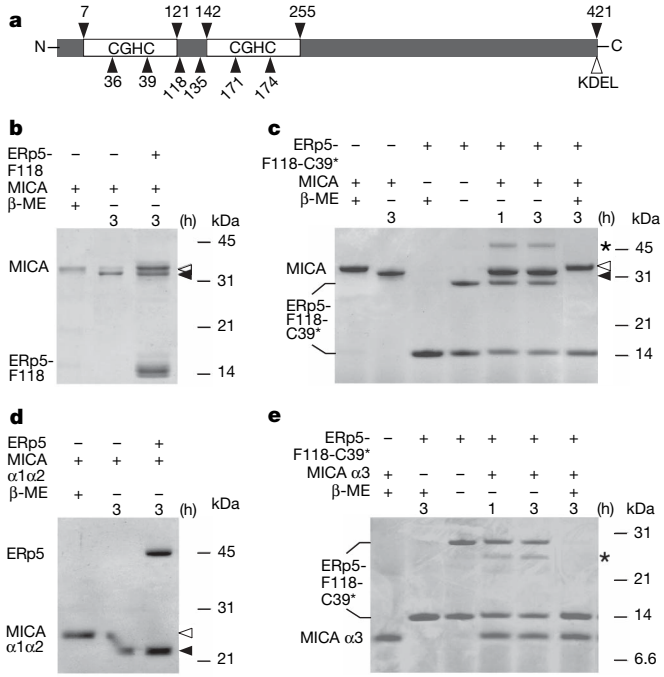


**Figure 5 | ERp5 exhibits specificity for the MICA α3 domain. a**, ERp5 organization with CGHC motifs within thioredoxin domains (open boxes). The top row of numbers identifies amino acid positions at domain boundaries; the bottom row of numbers identifies cysteine positions and truncation sites of expressed ERp5 fragments. **b**, MICA is partially reduced by ERp5(1–118) (ERp5-F118). **c**, MICA and the C39S mutant (C39*) of ERp5(1–118) form mixed disulphide heterodimers (asterisk) that are resolved by β-ME. The C39S mutant of ERp5(1–118) partially forms homodimers (see also **e**). **d**, ERp5 has no effect on the MICA α1α2 domain. The partial reduction in lane 2 is owing to bleeding of β-ME from lane 1. **e**, The C39S mutant of ERp5(1–118) reduces the MICA α3 disulphide bond as indicated by unresolved heterodimers (asterisk). Filled and open arrowheads in **b**–**d** mark the positions of non-reduced and reduced forms, respectively, of MICA substrates.

alteration of integrin affinity states, CD4 homodimer formation by inter-chain disulphide exchange, which enables HIV-1 T-cell infection, and switching of cell-surface tissue factor functional states between activation of coagulation and G-protein-coupled signalling[16,17,25–27]. The function of ERp5 demonstrated here enables tumour immune evasion and may influence autoimmune diseases through sMICA-mediated T-cell modulation[6].

## METHODS SUMMARY

**Tetramers, antibodies, protein identification and ELISA for sMICA.** Tetramers were prepared from recombinant proteins, which were expressed in transfected 293T cells and purified using Invitrogen methodology, by BirA enzymatic biotinylation and conjugation with phycoerythrin-streptavidin. Anti-NKG2D (monoclonal antibody 1D11) and anti-MICA (monoclonal antibody 2C10) antibodies have been described[13,24]. Rabbit anti-ERp5 and anti-HSPA5 were from Affinity BioReagents and Stressgen, respectively. MICA binding proteins were purified from U266 cells by dounce-homogenization, dextran-PEG partitioning, Triton X-114 phase separation, and affinity chromatography using MICA-conjugated sepharose beads, followed by analysis of separated protein bands by mass spectrometry. For immunoblotting, MICA-binding proteins were prepared after cell-surface biotinylation with EZ-Link Sulfo-NHS-LC-Biotin (Pierce). The enzyme-linked immunosorbent assay (ELISA) for sMICA has been described[7].

**siRNA expression and real-time RT–PCR.** Retroviral transduction using pBABE-GFP constructs and Phoenix amphotropic packaging cells were used for *ERp5* siRNA expression. As with real-time RT–PCR[9], oligonucleotide sequences and further details are given in the Methods section.

**Immunoprecipitations and MICA cleavage.** Denatured and reduced RNase A was prepared as described[20]. HeLa cells were exposed to dRNase for 16 h, washed and surface biotinylated, incubated in 10% (w/v) TCA in phosphate-buffered saline (PBS) for 30 min on ice, washed, and lysed with immediate pH neutralization in standard NP-40 lysis buffer containing protease inhibitors and *N*-ethylmaleimide. Immunoprecipitated protein complexes were treated with *N*-glycanase and subjected to SDS–PAGE and membrane transfer. C-terminal truncation analysis of sMICA was performed by mass spectrometry at the Harvard University Microchemistry Facility.

**ERp5 activity assays.** All proteins, domains and the C36S and C39S mutants (made by Stratagene Quick Change methodology) were expressed in bacteria and purified as described[28]. ERp5 and substrate proteins were incubated at room temperature in PIPES buffer and resolved in Tris-glycine or Bis-Tris NuPAGE (Invitrogen) gels.

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

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

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## METHODS

**Tumour samples and cell lines, antibodies, tetramers, pharmacological inhibitors, and ELISA for sMICA.** The source of tumour cell suspensions has been reported previously[7]. Cell lines were from the American Type Culture Collection. Anti-NKG2D (monoclonal antibody 1D11) and anti-MICA (monoclonal antibody 2C10) antibodies have been described[13,24]. Rabbit polyclonal anti-ERp5 and anti-HSPA5 antibodies were from Affinity BioReagents and Stressgen, respectively. Recombinant MICA*001 (residues 1–276) and ULBP2 (residues 1–202) were produced in transfected 293T cells and purified from culture supernatant using Invitrogen methodology. Tetramers were prepared by BirA enzymatic biotinylation and conjugation with phycoerythrin-streptavidin. Cells were stained with saturating tetramer concentrations for 1 h at 4 °C and examined by flow cytometry. Non-glycosylated MICA was expressed in bacteria and purified as described[22]. Cells were exposed to DTNB or PAO (Sigma) for 24 h at the indicated concentrations. For inhibition of tetramer binding, cells were grown for 24 h in the presence of 0.5 μM PAO. Metalloproteinase inhibitors GM 6001 and MMP inhibitor III were from Calbiochem. The ELISA for sMICA has been described[7].

**Identification of MICA-binding surface proteins.** U266 and U937 cells (each $5 \times 10^9$) were dounce-homogenized in 10 mM Tris-HCl (pH 7.6), 0.5 mM $MgCl_2$, 1 mM PMSF, 1 μg ml$^{-1}$ leupeptin, and 1 μg ml$^{-1}$ pepstatin. Membrane fractions were isolated from cleared supernatants by dextran-PEG partitioning, washed (8% sucrose, 5 mM Tris-HCl (pH 7.4)), and dissociated in lysis buffer (50 mM Tris-Cl (pH 7.4), 1% Triton X-114, 150 mM NaCl, 5 mM EDTA, 5 mM iodoacetamide, protease inhibitors). Cleared supernatants were warmed to 37 °C and proteins partitioned during Triton X-114 phase separation. Proteins in aqueous fractions were affinity purified using MICA conjugated to cyanogen-bromide-activated sepharose beads, visualized by SDS–PAGE and silver staining, and analysed by MALDI-TOF at the FHCRC Mass Spectrometry Facility. For immunoblotting, MICA-binding proteins were prepared after cell surface biotinylation with EZ-Link Sulfo-NHS-LC-Biotin (Pierce).

**siRNA expression and real-time RT–PCR.** Oligonucleotide pairs for siRNA-17 and siRNA-19 targeting *ERp5* (disulphide isomerase-related protein P5; GenBank accession number D49489) mRNA at positions 316–338 and 556–567 were GATCTTGTTGTCAAAGTTGGTGCAGTTGTCTTCTTCTCAACTG-CACCAACTTTGACAACATTTTTG and AATTCAAAAATGTTGTCAAAG-TTGGTGCAGTTGAGAAGAAGACAACTGCACCAACTTTGACAACAA, and GATCTTGATAGTTCAAGTAAGAAGGATGTCTTCTTCTCATCCTTCTTA-CTTGAACTATCATTTTTG and AATTCAAAAATGATAGTTCAAGTAAGA-AGGATGAGAAGAAGACATCCTTCTTACTTGAACTATAA, respectively (all 5′–3′; internal hairpin sequence, 3′-end termination signal, and *Bgl*II and *Eco*RI overhangs are underlined). An irrelevant oligonucleotide pair with no homology to any human gene was GATCTTATGTCAAGTTGTATAGTTA-TTCAAGAGATAACTATACAACTTGACATATTTTTG and AATTCAAAAA-TATGTCAAGTTGTATAGTTATCTCTTGAATAACTATACAACTTGACATAA. Annealed primers were ligated into retroviral vector pBABE-GFP and constructs were sequenced. Virus was produced in Phoenix amphotropic packaging cells and culture supernatant used for infection of A375 cells, which were sorted for GFP expression. Real-time RT–PCR was performed as described[9], using primer sets TGCGGCACGCTGCAGGGCT and TTGACAGTGACCACACCATGGAGCATA for *ERp5* complementary DNA, and GGAACGGAAAGGACCTCAGGATG and CTGGGAGCTCCTGGTGCTGTTG for *MICA* cDNA, and SYBR Green reagents (Molecular Probes).

**Preparation of dRNase and capturing of ERp5–MICA complexes.** RNase A was denatured and reduced in 0.1 M Tris-OH (pH 8.6), 6 M guanidine hydrochloride, and 0.15 M dithiothreitol for 24 h at room temperature, and desalted using D-Salt Dextran columns (Pierce) equilibrated with PBS[20]. $2 \times 10^6$ semi-confluent HeLa cells were exposed to the indicated concentrations of dRNase or native RNase for 16 h, washed, and surface biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce). Labelled cells were incubated in 0.5 ml 10% (w/v) TCA in PBS for 30 min on ice, washed sequentially in 10% and 5% TCA in PBS, and lysed in 50 mM Tris (pH 7.4), 1% Surfact-Amps NP-40 (Pierce), 150 mM NaCl, 5 mM EDTA, 40 mM *N*-ethylmaleimide (Sigma), 1 mM PMSF, leupeptin (1 μg ml$^{-1}$), and pepstatin (1 μg ml$^{-1}$). Lysate pH was adjusted to 7.0 with 1 M Tris-OH (pH 9.5). Protein complexes were precipitated with monoclonal antibody 2C10 (anti-MICA) or ERp5 polyclonal antibody, treated with *N*-glycanase, and processed for SDS–PAGE. For sequential precipitation, monoclonal antibody 2C10 immunocomplexes were dissociated in 150 mM Tris (pH 7.4), 0.5% SDS and 10 mM dithiothreitol, diluted tenfold with lysis buffer containing 25 mM iodoacetamide, incubated for 1 h at room temperature for dithiothreitol neutralization and sulphydryl alkylation, and re-precipitated with anti-MICA monoclonal antibody BAMO-1 (Axxora) or anti-ERp5. For determination of sMICA C-terminal cleavage, supernatant from C1R-MICA transfectants grown in Opti-MEM (Gibco) was concentrated using Amicon Ultra-15 centrifugal filters (Millipore). Immunoprecipitated sMICA was treated with *N*-glycanase, isolated by SDS–PAGE, and subjected to peptide fragmentation analysis by MALDI mass spectrometry at the Harvard University Microchemistry Facility.

**ERp5 activity assays.** Ectodomain-only MICA, siderocalin and KLRD1–NKG2A were expressed in bacteria and purified as described[28]. We similarly produced ERp5 (residues 1–421 of the mature protein), the ERp5 fragments 1–118 and 135–421, the C36S and C39S mutants (made by Stratagene Quick Change methodology) of ERp5(1–118), and the isolated MICA α1α2 platform (residues 1–180) and α3 domains (residues 187–274)[22]. All *ERp5* sequences were fused to N-terminal hexahistidine tracts and included a C-terminal stop codon to prevent expression of the adjacent hexahistidine in pET22(b). Recombinant proteins were purified by metal affinity (BD Talon, Clontech) and size-exclusion chromatography (Superdex 200, Pharmacia). For testing of functional activity, ERp5 or derivative proteins (2 μg) were incubated at room temperature with MICA substrates or control proteins (1.5 μg) in PNEA (25 mM PIPES (pH 7), 150 mM NaCl, 1 mM EDTA, and 0.02% sodium azide) in a total volume of 5 μl per time point sample, mixed with 2x SDS–PAGE sample buffer (5 μl) with or without β-ME, and resolved in 15% Tris-glycine or 12% Bis-Tris NuPAGE (Invitrogen) gels.