A Secreted Disulfide Catalyst Controls Extracellular Matrix Composition and Function

Tal Ilani,[1]* Assaf Alon,[1] Iris Grossman,[1] Ben Horowitz,[1] Elena Kartvelishvily,[2] Sidney R. Cohen,[3] Deborah Fass[1]*

Disulfide bond formation in secretory proteins occurs primarily in the endoplasmic reticulum (ER), where multiple enzyme families catalyze cysteine cross-linking. Quiescin sulfhydryl oxidase 1 (QSOX1) is an atypical disulfide catalyst, localized to the Golgi apparatus or secreted from cells. We examined the physiological function for extracellular catalysis of de novo disulfide bond formation by QSOX1. QSOX1 activity was required for incorporation of laminin into the extracellular matrix (ECM) synthesized by fibroblasts, and ECM produced without QSOX1 was defective in supporting cell-matrix adhesion. We developed an inhibitory monoclonal antibody against QSOX1 that could modulate ECM properties and undermine cell migration.

Oxidation of cysteine side chains to form disulfide bonds is a fundamental event during protein folding in the endoplasmic reticulum (ER) (*1*). However, an additional disulfide catalyst, quiescin sulfhydryl oxidase 1 (QSOX1), is found downstream of the ER in the secretory pathway. QSOX1 is present in glandular secretions (*2*–*4*) and induced in (*5*) and secreted from (*6*) quiescent cultured fibroblasts. Despite detailed enzymological characterization (*7*–*9*) and tissue localization (*10*–*12*), the physiological role of QSOX1 remains to be determined (*1*). More generally, no biological process requiring a catalyst of de novo disulfide formation in the Golgi or extracellular environment is known.

Endogenous QSOX1 was Golgi-localized in various cell types (Fig. 1A and fig. S1A), as expected (*12*, *13*). QSOX1 secretion, however, was observed only for confluent fibroblasts (Fig. 1B and figs. S1B and S2). Sulfhydryl oxidase activity in fibroblast culture media correlated with QSOX1 transcript and protein levels (Fig. 1C), was not due to the paralogous QSOX2 (Fig. 1D), and reached maximal values comparable to 40 to 50 nM recombinant QSOX1 (rQSOX1) (Fig. 1E) (*14*).

To address the extracellular biological functions of QSOX1, we depleted the enzyme from WI-38 embryonic lung fibroblasts using small interfering RNA (siRNA) (fig. S3). The most evident outcome was a relative decrease in cell numbers in QSOX1-depleted fibroblast monolayers by day 4 after knockdown (Fig. 2A). While ruling out other causes (fig. S4), we noted the appearance of detached cells in the culture media after day 2 (Fig. 2A). Addition of 50 nM rQSOX1 to the culture media after knockdown both restored cell numbers and prevented cell detachment, but rQSOX1-AA, a catalytically inactive mutant (Fig. 2B and fig. S5), had no effect. By addition of catalase, we ruled out peroxide, the by-product of QSOX1-mediated disulfide formation (Fig. 2B), as the factor required for cell adherence (fig. S6).

Detached QSOX1-depleted cells were viable and readily reattached to control fibroblast monolayers (Fig. 2C), indicating that they retained functional cell adhesion components and pointing to a defective extracellular adhesion platform in QSOX1-knockdown cultures. Indeed, epithelial cells adhered poorly to QSOX1-depleted fibroblast cultures (Fig. 2D). Higher thiol content (i.e., less disulfide formation) was seen in extracellular material of QSOX1-knockdown compared with control cultures (fig. S7). Thus, QSOX1 extracellular disulfide catalysis is involved in a non–cell-autonomous adhesion mechanism.

Because quiescent fibroblasts produce copious extracellular matrix (ECM) (*15*), we examined the dependence of various ECM proteins on QSOX1 for proper assembly. Collagen IV, a major component of WI-38 ECM, appeared normal in quantity and morphology upon QSOX1 knockdown (fig. S8). The fibronectin matrix and a number of other prominent ECM proteins were also unaffected (fig. S8). In contrast, severe defects in ECM incorporation of the key basement membrane component laminin were observed. Specifically, soluble laminin appeared in culture media upon QSOX1 depletion (Fig. 3A), accompanied by a large decrease in laminin within the ECM (Fig. 3B and figs. S9 and S10). Addition of 50 nM rQSOX1, but not rQSOX1-AA, completely reversed the laminin-incorporation defect, demonstrating that sulfhydryl oxidase activity is required for laminin incorporation into the ECM. To locate sites of QSOX1 activity that may contribute to laminin incorporation, extracellular unpaired thiols in QSOX1-depleted cultures were labeled with maleimide-functionalized gold particles and visualized using scanning electron microscopy (SEM). Cultures lacking QSOX1 activity showed heavy gold labeling of defined ECM meshworks, whereas gold was nearly undetected in ECM of control cell cultures (Fig. 3C and fig. S11). Therefore, QSOX1 target cysteines are located in the ECM itself rather than on putative laminin scaffolding sites on the cell membrane.

[1]Department of Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel. [2]The Irving and Cherna Moskowitz Center for Nano and Bio-Nano Imaging, Weizmann Institute of Science, Rehovot 76100, Israel. [3]Department of Chemical Research Support, Weizmann Institute of Science, Rehovot 76100, Israel.

*Corresponding author. E-mail: tal.ilani@weizmann.ac.il (T.I.); deborah.fass@weizmann.ac.il (D.F.)

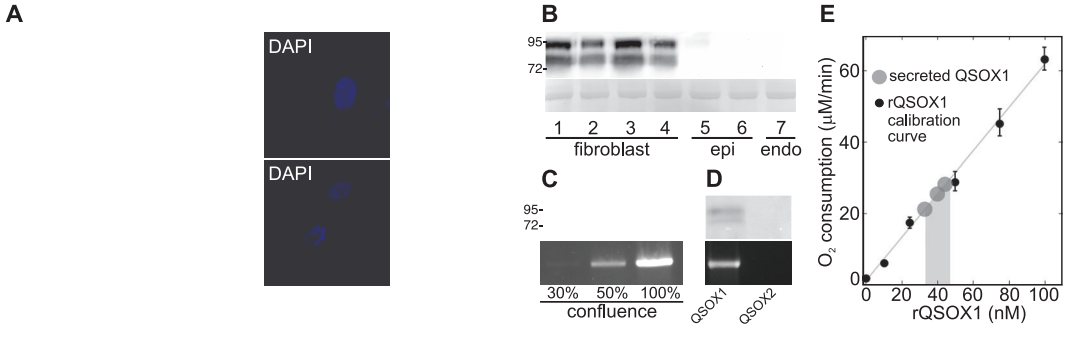

Fig. 1. Localization and secretion of QSOX1. (**A**) Subconfluent WI-38 fibroblasts immunostained with Golgi-specific (p115) or ER-specific (calnexin) antibody (red) and QSOX1 antibody (green). DAPI (4′,6-diamidino-2-phenylindole) staining (blue) indicates nuclei. Scale bar, 20 μm. (**B**) (Top) QSOX1 immunoblot of supernatants from confluent fibroblast, epithelial (epi), and endothelial (endo) cell cultures. 1, WI-38; 2, human foreskin fibroblasts; 3, pancreatic cancer–associated fibroblasts; 4, hTERT-immortalized WI-38 fibroblasts; 5, primary human mammary epithelial cells; 6, H460 large-cell lung carcinoma; 7, primary human umbilical vein endothelial cells. Molecular weight indicated in kD. (Bottom) Coomassie-stained load control. (**C**) QSOX1 immunoblot of WI-38 culture supernatants (top) and QSOX1 polymerase chain reaction (PCR) product from WI-38 total RNA (bottom) as a function of confluence. (**D**) Immunoblot of confluent WI-38 cell extracts (top) and PCR (bottom) demonstrate expression of QSOX1 but not QSOX2. (**E**) Sulfhydryl oxidase activity in culture supernatants measured by oxygen consumption. The calibration curve (black circles) was obtained using the indicated rQSOX1 concentrations. Gray circles are rates for three supernatant samples from confluent WI-38 cultures.

Laminin is secreted as a cross-shaped heterotrimer. Each laminin subunit, known as α, β, and γ, has multiple isotypes (*16*), combinations of which produce trimers with different assembly properties and perhaps functions (*17*). A significant fraction of unincorporated laminin from QSOX1-knockdown cultures was found in a trimeric complex (Fig. 3D), suggesting that QSOX1 is required not for forming laminin heterotrimers

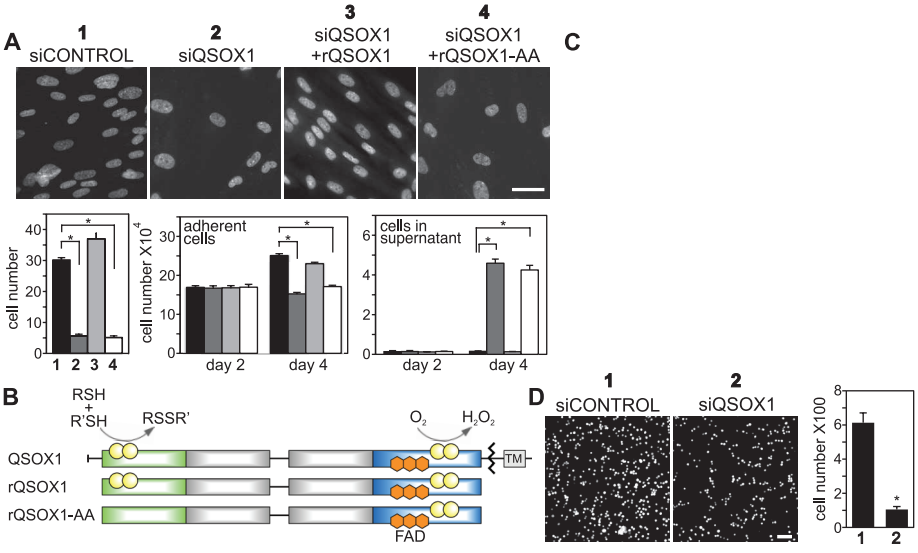

Fig. 2. Active QSOX1 is required for cell adherence to a monolayer. (**A**) (Top) DAPI staining of WI-38 cells after transfection with control (siCONTROL) or QSOX1-specific (siQSOX1) siRNA. Where indicated, siQSOX1 cultures were supplemented with 50 nM rQSOX1 or inactive mutant rQSOX1-AA. Scale bar, 20 μm. (Bottom left) Average cell numbers from 30 fields. (Bottom center and right) Total adherent and detached cells per well counted by flow cytometry. (**B**) Diagram of QSOX1 domain structure. Paired yellow balls represent redox-active disulfides. FAD, flavin adenine dinucleotide cofactor. Alternative splicing (jagged vertical line) generates either soluble or membrane-anchored (TM, transmembrane) QSOX1. H_2O_2 is a by-product of the disulfide formation mechanism. (**C**) Detached, red fluorescently labeled QSOX1-depleted fibroblasts readhered to control WI-38 monolayers. QSOX1 in the recipient monolayer is immunostained in green. Left panels highlight a readhered, QSOX1-depleted cell (arrows). Right panel demonstrates the general lack of QSOX1 in readhered cells, with exceptions indicated (arrowheads). Scale bars in left and right panels are 20 and 50 μm, respectively. (**D**) Fluorescently labeled HEK293 epithelial cells were layered onto control or QSOX1-depleted fibroblasts, and adherent cells were imaged. Scale bar, 50 μm. (Right) Average cell numbers from 11 fields.

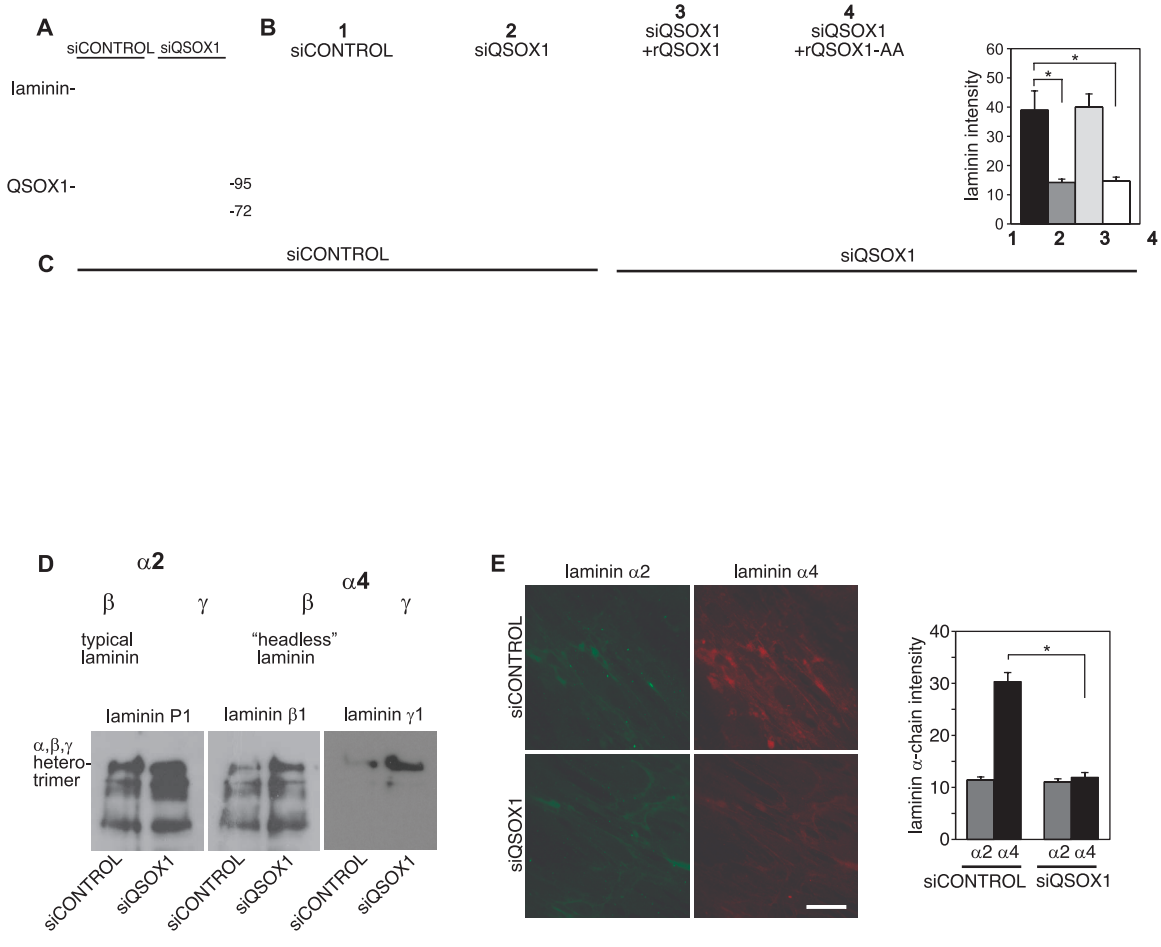

Fig. 3. QSOX1 is required for laminin incorporation into ECM. (**A**) Immunoblot of laminin (top) and QSOX1 (bottom) in WI-38 culture supernatants. Molecular weight is indicated in kD. (**B**) Immunostaining using P1 polyclonal antibody revealed a more substantial laminin matrix (green) in siCONTROL compared with siQSOX1 WI-38 cultures. Supplementation with rQSOX1, but not the rQSOX1-AA mutant, restored the thick laminin matrix. DAPI staining (blue) indicates nuclei. Scale bar, 20 μm. Right, quantification of laminin intensity. (**C**) SEM images of ECM in fibroblast monolayers labeled with maleimide-functionalized gold. Backscattering from gold (false colored in cyan) is superposed. Scale bar, 500 nm. (**D**) (Top) Illustration of laminin trimers containing α2 and α4 chains. (Bottom) Immunoblots of laminin in culture supernatants using P1 antibody or antibodies specific for laminin subunits β1 or γ1. (**E**) Immunostaining of siCONTROL and siQSOX1 WI-38 fibroblast cultures with laminin subunit-specific (α2 or α4) antibodies. Scale bar, 20 μm. (Right) Quantification of laminin chain intensity.

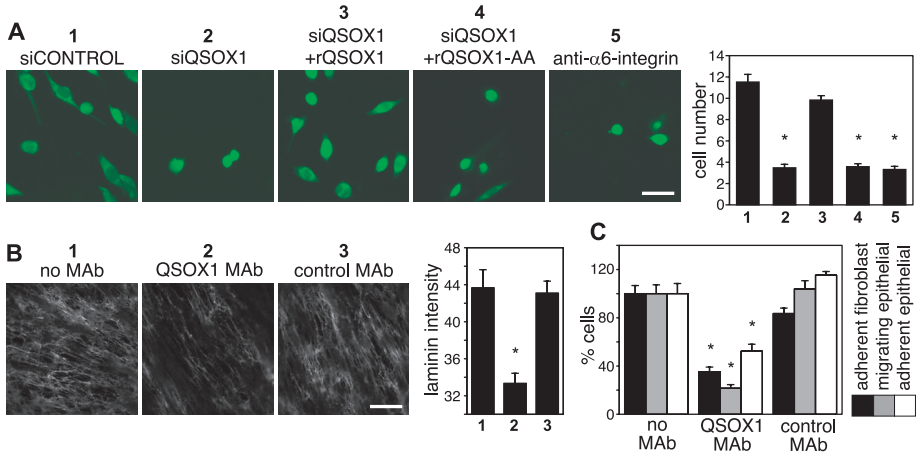

Fig. 4. QSOX1 inhibition blocks tumor cell migration. (**A**) (Left) Fluorescently labeled H460 lung cancer cells that crossed a preformed stromal layer of WI-38 fibroblasts subjected to the indicated treatments. Recombinant enzymes (rQSOX1 and rQSOX1-AA) were added after transfection of fibroblasts. As a positive control, H460 cells were treated with antibody that blocks activity of α6 integrin, a laminin receptor, before layering onto control fibroblasts. Scale bar, 20 μm. (Right) Quantification of migratory cells. (**B**) Immunostaining of laminin in untreated WI-38 fibroblasts or fibroblasts treated with QSOX1 inhibitory or control monoclonal antibody. Scale bar, 20 μm. (Right) Quantification of laminin intensity from 15 fields. (**C**) WI-38 fibroblasts in monolayer (black), epithelial cells that migrated through a preformed stromal layer of WI-38 fibroblasts (gray), and epithelial cells remaining adherent to a fibroblast layer after subjection to force (white) after pretreatment of fibroblasts with QSOX1 inhibitory or control antibody, as a percentage of control (no monoclonal antibody) values.

but for integration of assembled trimers into the ECM. Isotype-specific antibodies were selected based on the major laminin transcripts produced by WI-38 fibroblasts (table S1), and QSOX1 depletion was shown to result in disappearance of a meshwork containing α4 laminin, whereas α2 levels in the ECM were unchanged (Fig. 3E). Thus, multiple laminin matrices coexist within fibroblast ECM (figs. S12 and S13), differing in their requirement of QSOX1 for assembly.

The contribution of laminin to tumor progression (*18*), coupled with up-regulation of both the QSOX1 and LAMA4 (encoding laminin α4) transcripts in stromal cells surrounding invasive breast carcinomas (*19*), prompted us to investigate whether QSOX1 activity in ECM assembly contributes to tumor cell migration. We found that lung cancer cell migration across a preformed layer of WI-38 fibroblasts and their associated ECM (fig. S14A) was severely attenuated when QSOX1 was depleted during formation of the fibroblast layer (Fig. 4A). Subsequent tumor cell migration was re-established when QSOX1-depleted fibroblasts were supplemented with 50 nM rQSOX1, but not rQSOX1-AA, during ECM deposition. Similar results were obtained using paired pancreatic fibroblasts and epithelial cells (fig. S14B), indicating the generality of QSOX1 catalytic activity in construction of promigratory ECM. Although QSOX1-depleted fibroblast monolayers were more elastic than controls (fig. S15), suggesting greater penetrability, their failure to support migration is consistent with the known role of laminin in integrin-mediated adhesion, a prerequisite for tumor metastasis (*18*, *20*).

QSOX1 inhibition may therefore be a strategy to control ECM functionality. We developed an inhibitory monoclonal antibody against QSOX1 (fig. S16), designated MAb492.1. When MAb492.1 was supplied to WI-38 fibroblasts, less laminin was found in ECM produced by these cells (Fig. 4B), and fewer fibroblasts were present in the culture monolayer (Fig. 4C). Finally, a major drop in cell adhesion to and migration through the fibroblast layer were observed when the QSOX1 inhibitory antibody was added to the growing fibroblasts (Fig. 4C). Thus, blocking extracellular QSOX1 activity can modulate the architecture and properties of the ECM (fig. S17). Notably, peptides derived from QSOX1 are overrepresented in the serum of pancreatic cancer patients (*21*), suggesting an increase in extracellular QSOX1 in this disease state.

Here, we addressed the physiological function of QSOX1, a disulfide catalyst isolated more than three decades ago (*4*) and recently linked to human disease (*21*–*23*). Both QSOX1 and fundamental components of basement membrane ECM such as laminin arose near the base of the metazoan branch of the evolutionary tree (*24*, *25*). A role for secreted QSOX1 in laminin assembly suggests that QSOX enzymes may have contributed to the initial organization of multicellular organisms and may have acquired important functions during development in complex animals. In normal development, laminin guides cell migration (*26*), and under pathological conditions it lines metastatic niches and contributes to adhesive interactions required for tumor cell invasion (*20*, *27*). QSOX1 enzymatic activity potentially provides a handle with which to modulate ECM assembly and function in this context.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S17
Table S1
References (*28*–*35*)

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