Supporting Information

Live-cell video microscopy demonstrates copper chaperone Atox1 to be required for breast cancer cell migration

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Content:

www.pnas.org/cgi/doi/10.1073/pnas.1910722117

Extended Materials and Methods section

Cell lines, SiRNAs, and Reagents. MDA-MB-231 (*i.e.* MDA-231) human breast cancer cell lines were obtained from American Type Culture Collection (LGC standards). The cells were maintained at 37℃ and 5 % CO_2 in Dulbecco's Modified Eagle Medium (Gibco), supplemented with 10 % heat inactivated fetal bovine serum (FBS) (Invitrogen) (Invitrogen). Passage number was kept below 20, and the cells were tested for mycoplasma periodically. Control (siCtrl), Atox1 silenced (siAtox1) and ATP7A silenced (siATP7A) MDA-231 cells were generated by transient transfection with AllStars negative control siRNA or a pool of 4 different siRNA targeting Atox1 or ATP7A (Qiagen), respectively, using Lipofectamine RNAiMAX transfection reagent (Invitrogen). $CuCl_2$ was purchased from Sigma-Aldrich.

Boyden chamber assay. Single cell suspensions (50.000 cells/300 µL DMEM+1 % FBS) were seeded in the upper compartment (insert) and DMEM+10 % FBS (750 µL per well) was pipetted in the lower compartment of the Boyden chamber. After a 24 h incubation at 37℃ and 5 % CO_2, the cells were fixed and stained using the Crystal violet assay kit (Abcam) according the company´s recommendations. Shortly, the inserts were washed and stained with the crystal violet staining solution (with methanol). The cells that did not migrate and remained on the upper side of the membrane were carefully removed using cotton swabs. After an additional 4 washes, the inserts were dried and the stain was solubilized using solubilization solution. Finally, the staining solution was aliquoted into a 96-well plate (in triplicate) and its optical density (OD) was measured at 545 nm using a plate reader (FLUOstar Optima, BMG Labtech).

Western blotting. Proteins were extracted by lysis buffer containing 50 mM TrisHCl, 150 mM NaCl, 1 mM EDTA, 1 % Triton and 100 U/mL protease inhibitor cocktail (Roche). Protein concentrations were measured by the colorimetric BCA protein assay reagent (Alfa Aesar). Equal amounts of protein for each sample were subjected to SDS/PAGE and transferred onto PVDF membranes (Bio-Rad). Membranes were first probed with primary antibodies (rabbit anti-Atox1 antibody (ab154179, Abcam); rabbit anti-ATP7A antibody (ab125137, Abcam); rabbit anti-LOXPP antibody (NBP1-30327, Novus Biologicals); rabbit anti-IQGAP1 antibody (ab133490, Abcam); rabbit anti-beta actin antibody (ab8227, Abcam) and subsequently probed with HRP-conjugated goat anti-rabbit secondary antibody (Sigma). Protein bands were detected using the ECL plus Western blotting detection system (GE Healthcare) and ChemiDoc™ touch imaging system (Bio-Rad).

Video microscopy and cell tracking. Cells were seeded in 24-well plates with poly-L-lysine coated glass bottom (50.000 cells/1 mL per well). Upon cell adhesion (5 h post-seeding), the plate was positioned into a stage top incubator (Okolab) on an Eclipse Ti 2 inverted microscope (Nikon). Cells were automatically imaged using phase contrast microscopy at multiple positions per well using a 10X air objective (Nikon) during a 9 h time series with 10 min interval. Cell tracking was performed manually for all selected cells in ½ of the field-of-view (FoV) with size of 632,5 μm^2 (region-of-interest, ROI) at two different X-Y positions for at least 2 independent experiments by serial selection of the center of the cell nucleus at each time point during the time series using the Plugin “Manual Tracking” provided

in the ImageJ software. Single cells at the start of the time series, remaining within the ROI and not dividing during the time series were selected and tracked. Tracking analysis was performed by exported the tracks into Ibidi's "Chemotaxis and Migration" tool with generation of the Wind-Rose plots and determination of the migration parameters ´velocity´ (= accumulated distance/time) and ´Euclidean distance´ [1]. Statistical analysis was performed with the Mann-Whitney U test in SPSS (IBM SPSS statistics version 22).

Immunofluorescence microscopy. Cells seeded on poly-L-lysine coated coverslips were fixed during 20 min incubation with 4 % paraformaldehyde, blocked during 1 h with 10 % normal goat serum (Sigma-Aldrich) and permeabilized during 10 min with 0,1 % Triton-X-100 (Sigma-Aldrich). Samples were then incubated with the primary antibody diluted in 1 % blocking solution (mouse anti-Atox1 (H00000475-M01, Novus Biologicals), rabbit anti-ATP7A (ab125137, Abcam), rabbit anti-LOXPP (NBP1-30327, Abcam), rabbit anti-IQGAP1 antibody (ab133490, Abcam)) overnight at 4℃. Following, the samples were incubated with Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit secondary antibody (Invitrogen; A11001 or A11008, respectively) diluted in 1 % blocking buffer for 1 h. Samples were washed with PBS and mounted on microscope slides using ProLong™ diamond antifade mounting medium with DAPI (Invitrogen) and analyzed with an Eclipse Ti 2 inverted microscope (Nikon) equipped with a 60X/1,4NA oil immersion objective (Nikon).

Cu treatment of the cells. Cells were treated with standard culture medium (i.e. DMEM supplemented with 10 % FBS) or standard culture medium supplemented with 5 µM $CuCl_2$. Cell treatment was started at the time of cell seeding for video microscopy and 3h before further sample preparations for ICP-MS or PLA.

Cellular Cu analysis by ICP-MS. Metal-free ultrapure water and 0,9 % NaCl solution were prepared by 1 h treatment with Chelex (5 g/100 mL, Sigma-Aldrich). After cell treatment, the cells were collected after a wash with PBS via dissociation with TrypLE reagent, centrifuged at 600 g for 3 min, and washed with 0,9 % NaCl solution for three times. The cells were digested with concentrated (65 %) nitric acid (HNO_3, Merck Suprapure quality) overnight, incubated at 90 °C for 2 h. Before analysis, the samples were diluted 120X (0,025 mL to 3 mL) with 0.5 M HNO_3. Four standards (0; 0,1; 1 and 20 ppb Cu) were prepared from the stock solution (10000 ppb Cu, CPA Chem standard). The instrument Thermo iCAP Q was run in standard mode for the mass of 65. For each sample measurement, five replicate raw intensity readings were made to give average value with standard deviation. The instrument sensitivity drift during the analysis was monitored and compensated for with the addition of an internal standard of 2,5 ppb Sc to every sample and standard. Three independent biological experiments with two biological replicates having two technical replicates each for two of the experiments and with one biological sample having two technical replicates for one of the experiments. Statistical evaluation using the Student´s t-test.

In situ proximity ligation assay (PLA). PLA was performed on fixed MDA-231 cells using the Duolink® Detection Reagents Green kit (Sigma-Aldrich) according to the manufacturer's instructions.

Briefly, MDA-231 cells were fixed during 10 min at RT with 4 % PFA and permeabilized in 0,1 % Triton-X-100 for 10 min. Cells were incubated with blocking solution for 1 h at 37 °C and then with a pair of primary antibodies of different species (mouse anti-Atox1 (H00000475-M01, Novus Biologicals), mouse anti-ATP7A (ab131400, Abcam), rabbit anti-ATP7A (ab125137, Abcam), or rabbit anti-LOXPP (NBP1-30327, Abcam) overnight at 4 °C. The coverslips were washed, followed by incubation with the PLA probes (secondary antibodies against two different species bound to two oligonucleotides: anti-mouse PLUS and anti-rabbit MINUS) for 1 h at 37 °C. Cells were incubated with ligation solution for 30 min, and amplification solution for 100 min, both at 37 °C. The preparations were finally mounted with Duolink® *in situ* mounting medium with DAPI. Z-stack confocal images were collected through the entire cells at 2 µm interval on an Eclipse Ti 2 inverted microscope (Nikon), equipped with a Nikon 60X/1,4_S oil immersion objective. Maximum intensity projections representing approximately 30 % of the cell volume were generated, followed by manual setting of the image threshold at 120 to highlight the PLA dots and automatic counting of the PLA dots per image using the 'Analyze particles' function in Image J software [2]. In order to represent the number of PLA dots per cell, cell numbers per image were manually counted using the in parallel taken phase contrast images. Quantification was performed for two or three independent PLA experiments, whereby all cells were analyzed within 5 images per treatment condition. The average cell number per image is: 20 (SEM ± 1,63) and 21 (SEM ± 1,36) for MDA-231 siCtrl in standard and Cu-supplemented culture medium, respectively, 25 (SEM ± 3,37) and 30 (SEM ± 4,02) for MDA-231 siAtox1 in standard and Cu supplemented culture medium, respectively, and 27 (SEM ± 3,92) and 20 (SEM ± 3,32) for MDA-231 siATP7A in standard and Cu supplemented CM, respectively. Two-sided, unpaired *t*-test was used for statistical analysis.

LOX activity measurements. LOX activity was measured in the conditioned culture medium of control, Atox1 and ATP7A silenced cells using a LOX activity kit (based on fluorescence, Abcam) according to the company´s instructions. Fresh culture medium was incubated for 24 hours with the cells between 48 and 72 hours after transfection before collection of the conditioned culture medium for LOX activity measurement. In parallel, we performed crystal violet staining as a measure of cell density, and thus LOX activity measurements could be corrected for cell density variations. We performed 5 independent biological experiments with at least 2 technical repeats for each experiment. LOX inhibitor BAPN (3-aminopropionitrile fumarate salt, Sigma) was used as negative control and recombinant human lysyl oxidase homolog 2 (LOXL2) protein (R&D systems) as positive control. The level of silencing was tested in each experiment and average for data presented in Fig. 4 is 54 % for Atox1 and 80 % for ATP7A silencing.

Clinical association analysis. Clinical data and Atox1 mRNA levels (RNAseq, 1904 samples) were extracted from the publically available (www.cbioportal.org) METABRIC breast cancer study [3, 4]. Kaplan-Meier overall survival curve with log-rank test were generated using SPSS (IBM SPSS statistics version 22).

Table S1. ICP-MS for copper (Cu) for MDA-231 cells in standard culture medium (CM) and 5 µM Cu-supplemented CM (CM+5 µM Cu). Statistical analysis using Student´s t-test. *** for p<0.001 and **** for p<0.0001when comparison CM and CM+Cu. (n_{Exp}=3, $n_{replicates}$=10) (For experimental details, see Materials and Methods.)

	siCtrl		siAtox1		siATP7A	
	Cu per cell (fg)	**S.E.M.**	**Cu per cell (fg)**	**S.E.M.**	**Cu per cell (fg)**	**S.E.M.**
CM	3.93	1.50	4.00	0.74	2.74	0.53
CM +5µM Cu	14.07***	1.78	22.09 ***	2.97	18.17****	1.36

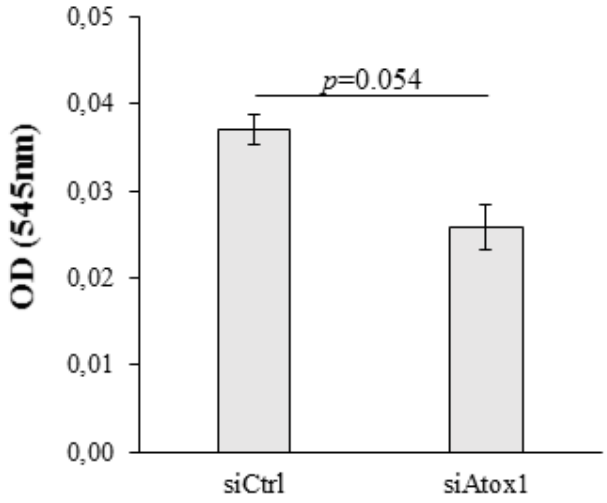

Fig. S1. MDA-231 cells with silenced Atox1 expression (50 % and 60 % silenced expression at time of cell seeding and at time of analysis, respectively) have reduced migration potential in the Boyden chamber migration assay. The column graph shows the average of optical density (OD) measurements obtained with plate reader after crystal violet staining of migrated control (siCtrl) and Atox1 silenced (siAtox1) MDA-231 cells (see section Materials and Methods for more details) (n_{Exp}=3, $n_{replicates/Exp}$=3). Error bars indicate standard error of the mean. P-value is calculated using the student´s *t*-test.

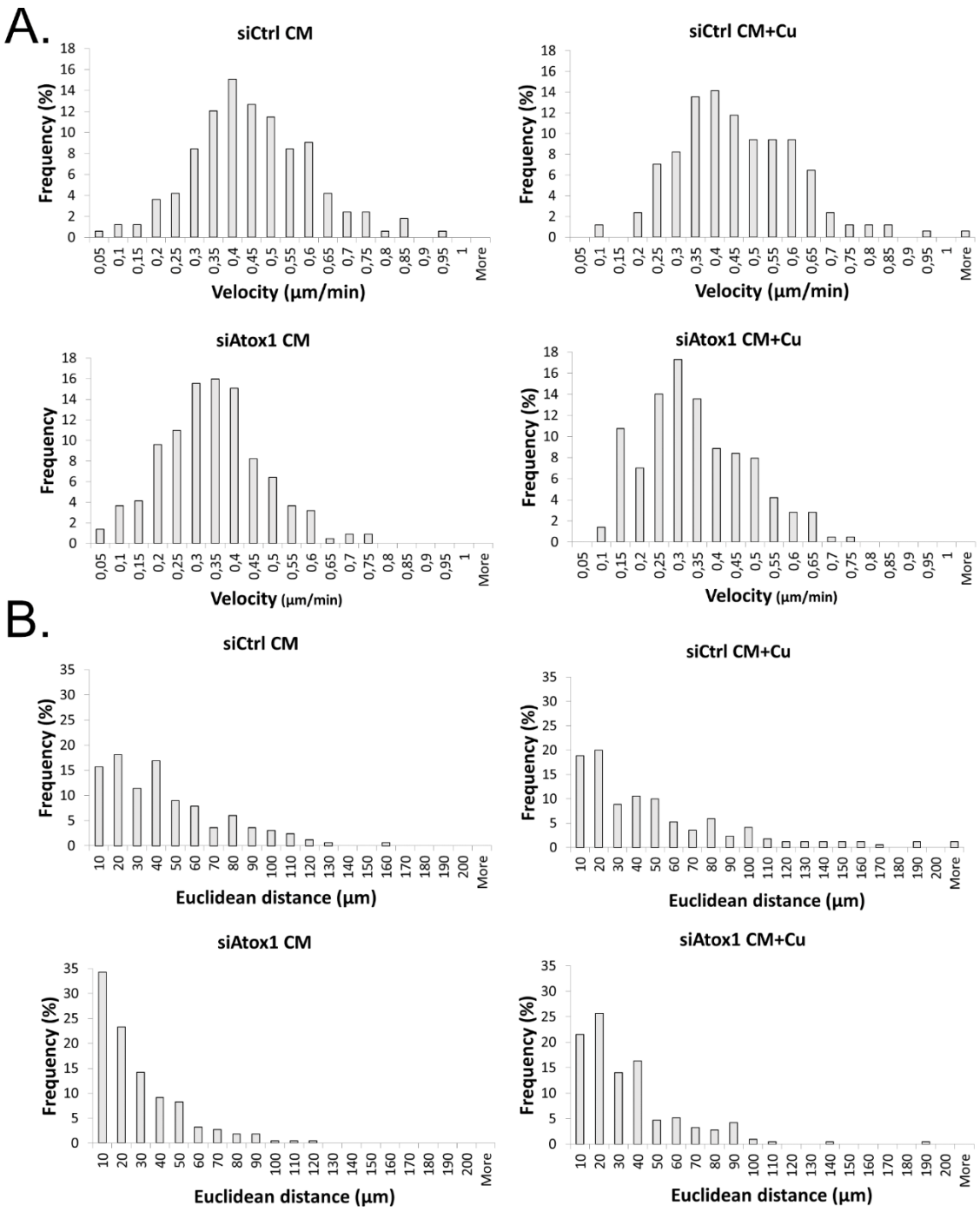

Fig. S2. Histograms showing distribution of data for (**A**) velocity and (**B**) Euclidean distance of cell tracks in control (siCtrl) versus Atox1 silenced (siAtox1) MDA-231 cells in standard culture medium (CM) or Cu-supplemented CM (CM+Cu) (n_{Exp}=3; $n_{ROI/Exp}$=2; $n_{tracked\ cells}$=166, 170, 219 and 214 for siCtrl CM, siCtrl CM+Cu, siAtox1 CM and siAtox1 CM+Cu, respectively).

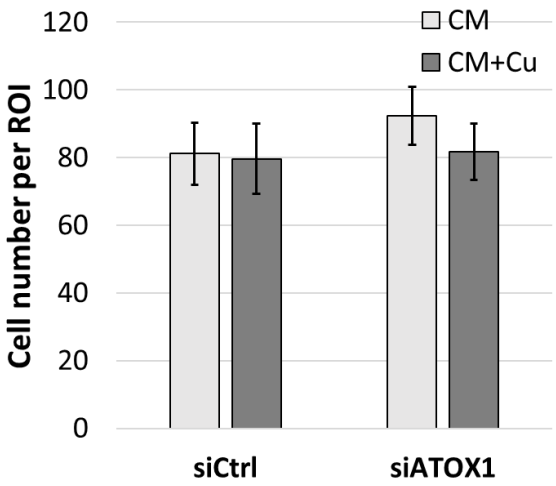

Fig. S3. Cell number per region-of-interest (ROI) (*i.e.* the image area used for cell tracking) is comparable for control (siCtrl) and Atox1 silenced (siAtox1) cells in standard (CM) and 5 µM Cu supplemented culture medium (CM+Cu). Error bars indicate the standard deviation of the mean (n_{Exp}=3, $n_{ROI/Exp}$=2.)

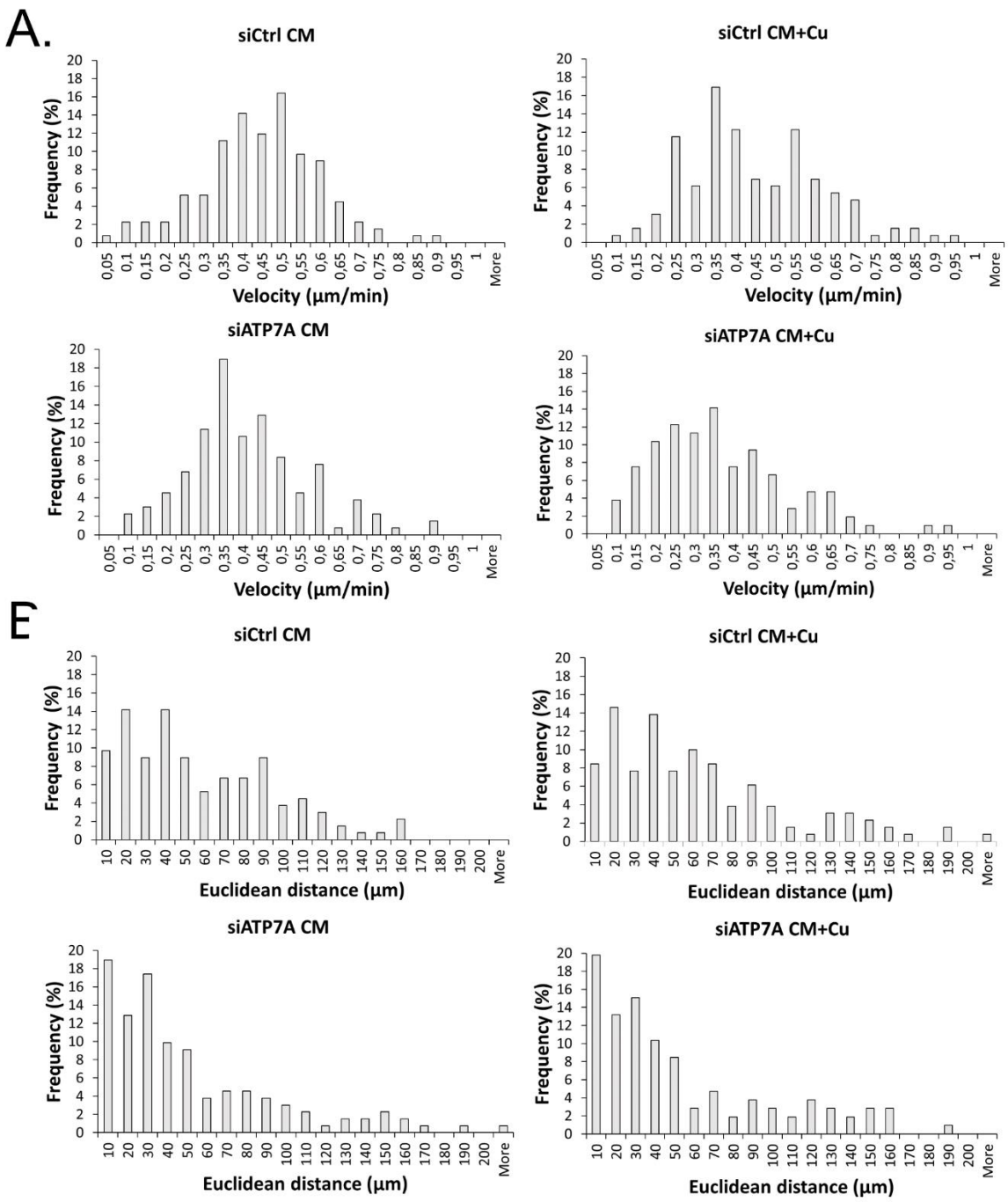

Fig. S4. Histograms showing distribution of data for (**A**) velocity and (**B**) Euclidean distance of cell tracks in control (siCtrl) versus ATP7A silenced (siATP7A) MDA-231 cells in standard culture medium (CM) or Cu supplemented CM (CM+Cu) (n_{Exp}=2; $n_{ROI/Exp}$=2; $n_{tracked\ cells}$=134, 130, 132 and 106 for siCtrl CM, siCtrl CM+Cu, siATP7A CM and siATP7A CM+Cu, respectively).

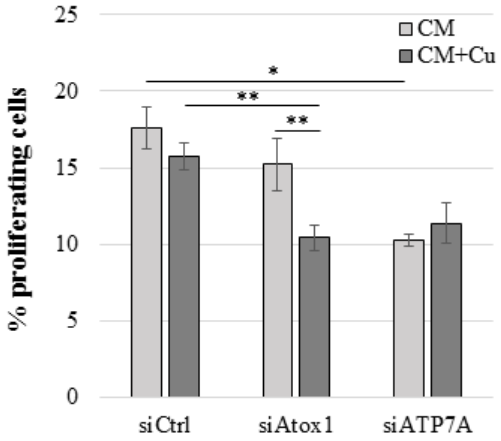

Fig. S5. Percentage of proliferative cells during the time series for control (siCtrl), Atox1 (siAtox1) and ATP7A silenced (siATP7A) MDA-231 cells cultured in standard culture medium (CM) and 5 µM Cu supplemented CM (CM+Cu). Cells that divided during the 9-h time series were considered proliferative (n_{Exp}=3, $n_{ROI/Exp}$=2). Error bars indicate standard deviation of the mean. Statistical analysis using Student´s *t*-test.

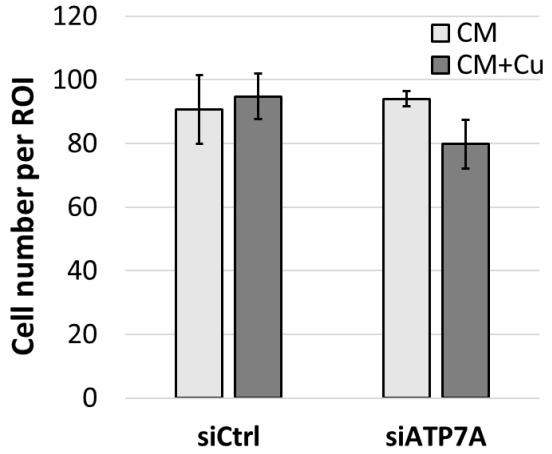

Fig. S6. Cell number per region-of-interest (ROI) (*i.e.* the image area used for cell tracking) is comparable for control (siCtrl) and ATP7A silenced (siATP7A) cells in standard (CM) and 5 µM Cu-supplemented culture medium (CM+Cu). Error bars indicate the standard deviation of the mean (n_{Exp}=2; $n_{ROI/Exp}$=2).

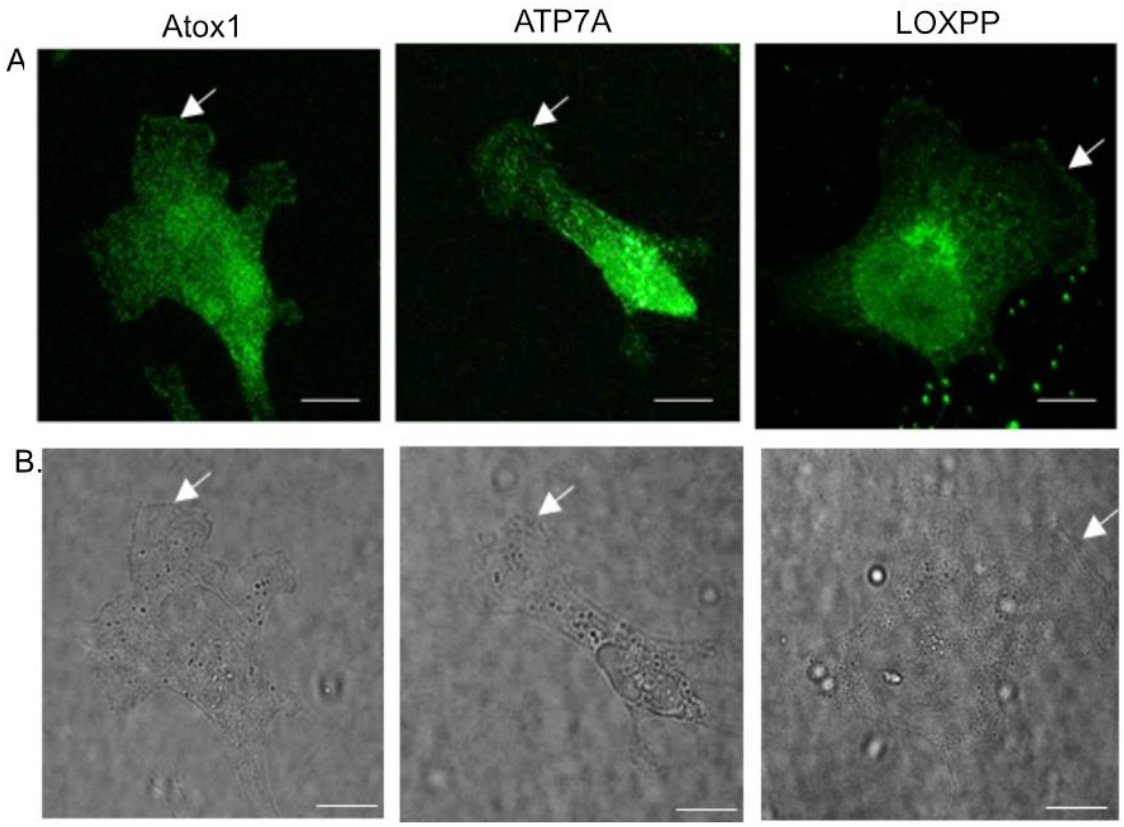

Fig. S7. Atox1, ATP7A and LOXPP proteins are in part localized at lamellipodia borders (indicated by white arrows). **A)** Representative maximum projection image of MDA-231 cells after immunofluorescent staining for Atox1, ATP7A, and LOXPP using specific primary antibodies and Alexa Fluor 488-conjugated secondary antibodies (green color). **B)** Phase contrast images in parallel to the fluorescent images shown in panel A. Scale bars indicate 20 µm.

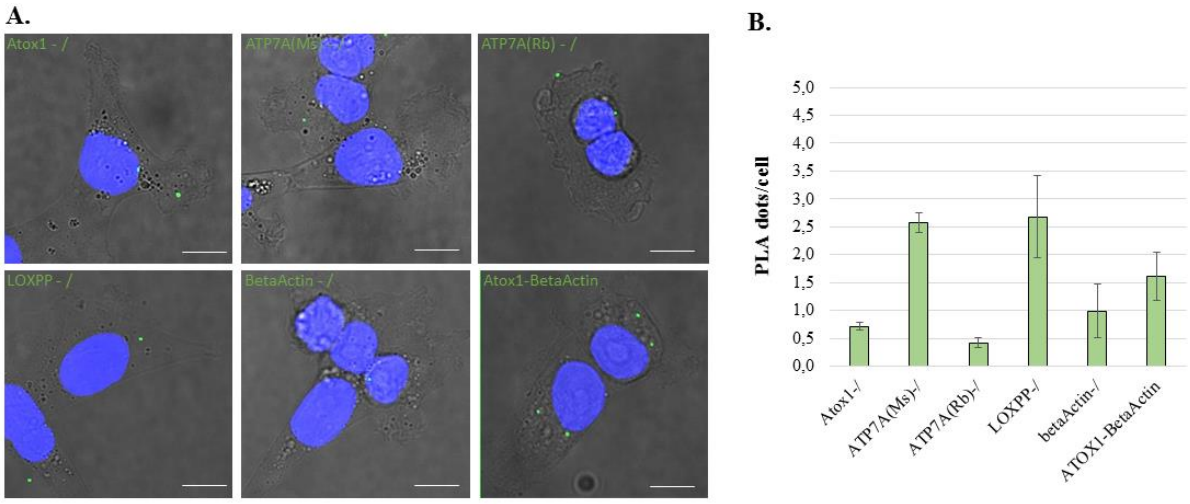

Fig. S8. Proximity ligation assays (PLA) technical (omit one of the primary antibodies) and biological (Atox1-BetaActin) negative controls. **A**) Representative maximum projection confocal micrographs (blue=DAPI, indicating nuclei; green=PLA dots) merged with phase contrast images. Scale bars indicate 10 µm. **B**) Quantitative results (n_{Exp}=2, $n_{ROI/Exp}$=3 for biological control, and $n_{ROI/Exp}$=2 for technical controls). Error bars indicate standard deviation of the mean.

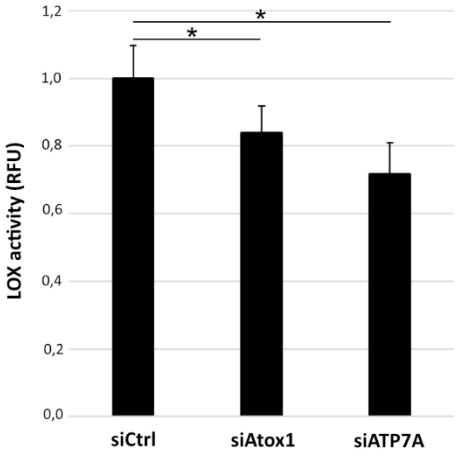

Fig. S9. Atox1 and ATP7A silencing result in lower LOX activity levels in culture medium of MDA-231 cells. The red fluorescent signal intensity (RFU), i.e. measure of LOX activity, in culture medium conditioned for 24 h with Atox1 (siAtox1) or ATP7A (siATP7A) silenced cells was significantly reduced (16 % and 28 %, respectively) in comparison to control (siCtrl) cells. Error bars indicate standard error of the mean (n_{Exp}=5; Student´s *t*-test)

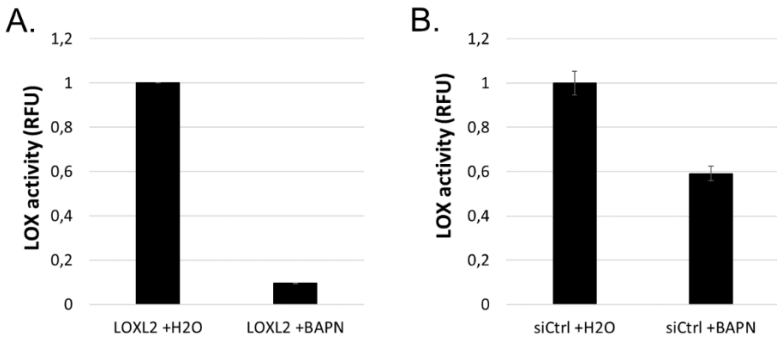

Fig. S10. Positive and negative technical controls for LOX activity measurements. LOX activity was measured in (**A**) fresh culture medium supplemented with 4 µg/mL recombinant active lysyl oxidase homolog 2 (LOXL2) protein with addition of H_2O (blanc) or 200 µM LOX inhibitor BAPN (3-aminopropionitrile fumarate salt), and (**B**) in conditioned 5 µM Cu supplemented culture medium of control silenced (siCtrl) MDA-231 cells with addition of H_2O (blanc) or 200 µM LOX inhibitor BAPN (3-aminopropionitrile fumarate salt). Error bars indicate standard deviation of the mean (n_{Exp}=1 with $n_{repeats/Exp}$=2 and 3, respectively).

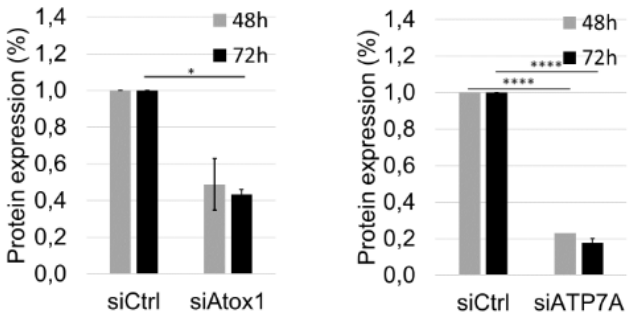

Fig. S11. Western blot results for Atox1 and ATP7A silencing in MDA-231 cells in parallel to LOX activity measurement. Quantitative results for Atox1 and ATP7A expression in control, Atox1 silenced (siAtox1) and ATP7A silenced (siATP7A) cells at 48h and 72h post-silencing, i.e. starting- and endpoint, respectively, of culture medium conditioning with the different cell conditions for LOX activity measurement.

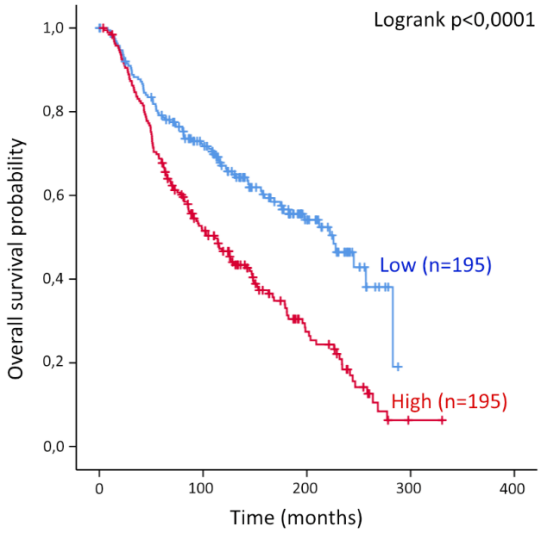

Fig. S12. Patient survival depends on Atox1 expression level. Overall survival Kaplan-Meier curve for breast cancer patients stratified by Atox1 expression. "Low" (blue) are the bottom 10 % and "high" (red) are the top 10 % of the patient cohort (n=1904) after sorting patients based upon Atox1 mRNA expression levels.

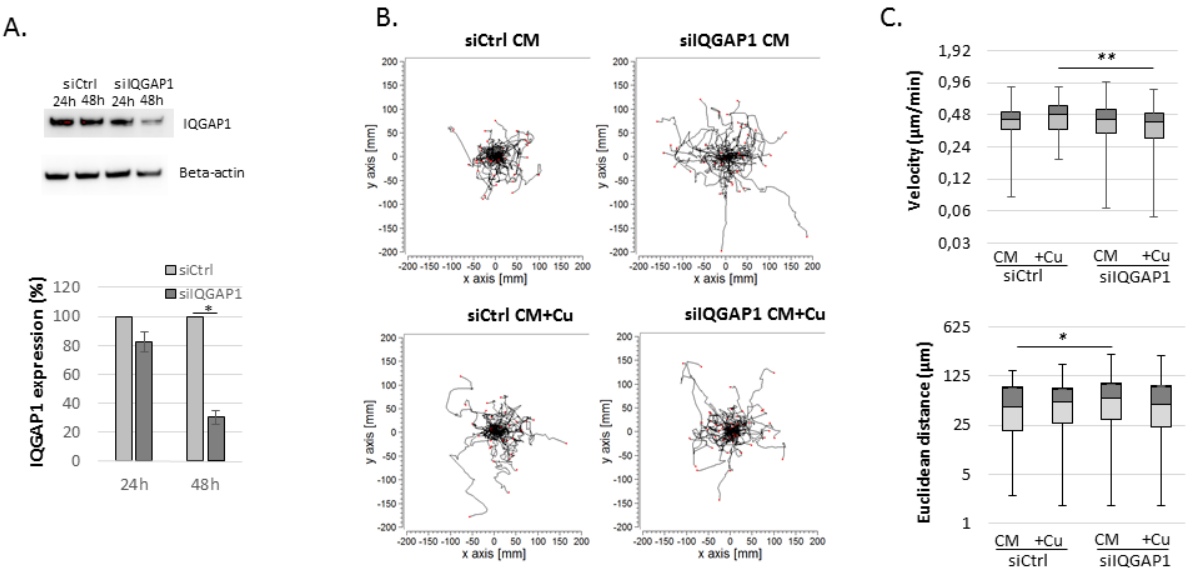

Fig. S13. IQGAP1 silencing reduces MDA-231 cell migration velocity in 5 µM Cu-supplemented culture medium (CM+Cu) and increases Euclidean distance in standard culture medium (CM). **A**) Western blot analysis of IQGAP1 expression at 24 h (20 %) and 48 h (70 %) post transfection with control (siCtrl) or IQGAP1-targeting siRNA (siIQGAP1). Beta-actin expression was determined as loading control. Error bars indicate standard deviation of the mean (n_{Exp}=3). **B**) Wind-Rose plots with tracks for siCtrl and siIQGAP1 cells in CM and CM+Cu (n_{Exp}=2, n_{ROI}=3 for CM and n_{ROI}=2 for CM+Cu). **C**) Box-whisker plots presenting migration velocity and Euclidean distance of the tracked cells (*Mann-Whitney U* statistical test; n_{Exp}=2, n_{ROI}=3 for CM and n_{ROI}=2 for CM+Cu; logarithmic scale in Y-axis).

Movies S1: Overlay of 2D phase-contrast microscopy video (9 h, 10 min interval) and tracks (ROI) of MDA-231 cells with control Atox1 expression levels treated in standard culture medium (siCtrl CM).

Movies S2: Overlay of 2D phase-contrast microscopy video (9 h, 10 min interval) and tracks (ROI) of MDA-231 cells with silenced Atox1 expression levels treated in standard culture medium (siAtox1 CM).

Movies S3: Overlay of 2D phase-contrast microscopy video (9 h, 10 min interval) and tracks (ROI) of MDA-231 cells with control Atox1 expression levels in 5 µM Cu-supplemented culture medium (siCtrl CM+Cu).

Movies S4: Overlay of 2D phase-contrast microscopy video (9 h, 10 min interval) and tracks (ROI) of MDA-231 cells with silenced Atox1 expression levels in 5 µM Cu-supplemented culture medium (siAtox1 CM+Cu).

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