



# Copper chaperone Atox1 plays role in breast cancer cell migration



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## ABSTRACT

Copper (Cu) is an essential transition metal ion required as cofactor in many key enzymes. After cell uptake of Cu, the metal is transported by the cytoplasmic Cu chaperone Atox1 to P<sub>1B</sub>-type ATPases in the Golgi network for incorporation into Cu-dependent enzymes in the secretory path. Cu is vital for many steps of cancer progression and Atox1 was recently suggested to have additional functionality as a nuclear transcription factor. We here investigated the expression level, cellular localization and role in cell migration of Atox1 in an aggressive breast cancer cell line upon combining immunostaining, microscopy and a wound healing assay. We made the unexpected discovery that Atox1 accumulates at lamellipodia borders of migrating cancer cells and Atox1 silencing resulted in migration defects as evidenced from reduced wound closure. Therefore, we have discovered an unknown role of the Cu chaperone Atox1 in breast cancer cell migration.

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## 1. Introduction

Copper (Cu) is found in the active sites of proteins that participate in cellular reactions such as respiration, antioxidant defense, neurotransmitter biosynthesis, connective-tissue biosynthesis and pigment formation [1–3]. To avoid toxicity of Cu, the intracellular concentration of Cu is regulated via dedicated proteins that facilitate its uptake, efflux as well as distribution to target Cu-dependent proteins and enzymes [4–6]. In humans, the 68-residue Cu chaperone Atox1 [7] picks up Cu that has entered the cell via the membrane-bound Cu importer Ctr1 and delivers the metal to cytoplasmic metal-binding domains in ATP7A and ATP7B, two homologous multi-domain P<sub>1B</sub>-type ATPases located in the trans-Golgi network. Many human Cu-dependent enzymes acquire Cu in the Golgi before reaching their final destination [4–6]. Intriguingly, Atox1 was recently reported to have additional functionality as a Cu-dependent transcription factor (TF) promoting the expression of *Ccd1* (cyclin D1) [8], *SOD3* [9,10] and *NADPH oxidase* [11].

Since Cu is a key component of many essential enzymes [12–14], it is not surprising that Cu is required for at least three characteristic phenomena involved in cancer: proliferative immortality, angiogenesis and metastasis. Roles in cancer metastasis of the Cu-dependent proteins *LOX* [15], *SPARC* [16], *Mek1* [17] and *MEMO*

[18] have been reported. Cancer metastasis consists of a cascade of processes that depends sensitively on cell migration ability. Upon local tissue invasion, cancer cells migrate through the tumor stroma and blood vessels to reach and grow at distant metastatic sites [19]. This requires cells to adopt to, interact with, and modify their surrounding extracellular matrix. Local cell invasion and migration are achieved by the formation of actin-rich plasma membrane protrusions at the leading edge known as lamellipodia, filopodia, and invadopodia. Intriguingly, in models of angiogenesis and neurogenesis, 80–90% of the total Cu stores in intracellular compartments was reported to be re-located to filopodia protrusions and across the cell membrane of endothelial cells [20]. The accumulated Cu in and outside the filopodia protrusions was proposed to be protein-bound but no target protein was identified. Despite the apparent importance for Cu and Cu-dependent enzymes in cancer processes, the roles of Cu transport proteins, such as Atox1, are unknown. To assess the putative role of Atox1 in breast cancer progression, we here used aggressive MDA-MB-231 breast cancer cells to investigate the functional role of Atox1 in cell migration.

## 2. Materials and methods

### 2.1. Cell lines

MCF7 and MDA-MB-231 human breast cancer cell lines were obtained from American Type Culture Collection (LGC standards). Both cell lines were maintained at 37° and 5% CO<sub>2</sub> in Eagles MEM (Sigma-Aldrich), supplemented with 10% heat inactivated fetal

Abbreviations: Cu, copper; TF, transcription factor; Ccd1, cyclin D1.

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bovine serum (Invitrogen) and 1% penicillin/streptomycin cocktail (Invitrogen).

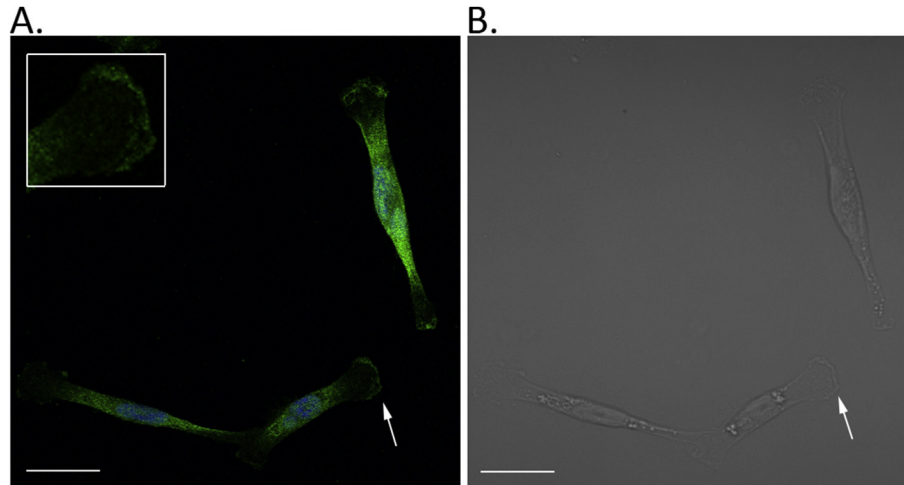
## 2.2. Western blot

Proteins were extracted by lysis buffer containing 50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton and 100  $\mu$ L/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, California). Membranes were first probed with primary antibodies (rabbit monoclonal anti-Atox1 antibody (Abcam); rabbit polyclonal anti-beta actin antibody

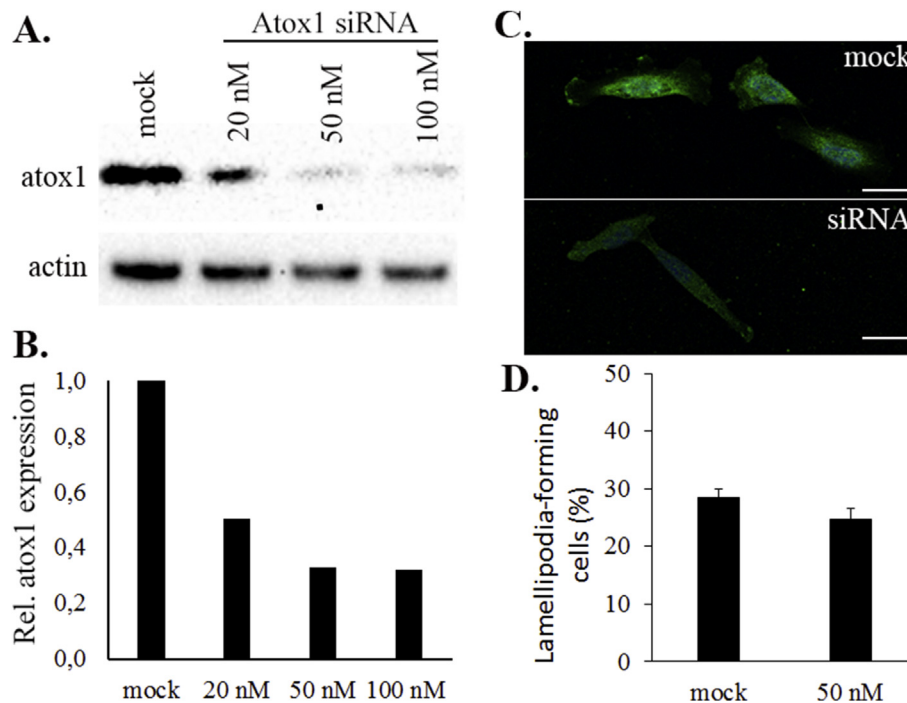
(Abcam)) and subsequently with HRP-conjugated goat anti-rabbit secondary antibody (Sigma). Protein bands were detected using the ECL plus Western blotting detection system (GE Healthcare).

## 2.3. Immunofluorescence staining

Cells plated on poly-L-lysine coated coverslips were fixed during 20 min incubation with 4% paraformaldehyde, washed with PBS, and blocked plus permeabilized during 45 min incubation with 10% fetal bovine serum plus 0.1% Triton-X-100 in PBS. Samples were then incubated with mouse monoclonal anti-Atox1 primary antibody (Abcam) diluted in 1% blocking buffer overnight at 4°. After washing, samples were incubated with Alexa Fluor 488-conjugated



**Fig. 1.** Atox1 accumulates in the lamellipodium border of MDA-MB-231 cells. (A) Confocal fluorescent image (green = Atox1; blue = DAPI), and (B) bright field image. (Scale bar = 30  $\mu$ m).



**Fig. 2.** Atox1 silencing in MDA-MB-231 cells results in a uniformly-distributed Atox1 downregulation, but no effect on lamellipodia-forming potential of the cells. (A) Immunoblot and (B) quantification of total Atox1 expression in cells transfected with increasing concentrations of siRNA towards Atox1. (C) Atox1 immunofluorescence staining of mock versus 50 nM Atox1 siRNA transfected cells. (D) Percentage of lamellipodia-forming cells in mock and Atox1 siRNA transfected cells 1) (error bar; standard error of the mean); 2) ( $n_{\text{micrographs}}=5$ ;  $n_{\text{cells}}=350$ ).

goat anti-mouse secondary antibody (Thermo Fisher) diluted in 1% blocking buffer for 1 h. Samples were washed with PBS, stained with DAPI, mounted and analyzed with a LSM700 inverted microscope (Carl Zeiss Jena GmbH, Germany) using a plan-apochromat 40×/1.3 oil DIC objective.

#### 2.4. Atox1 silencing

Cells were transfected with FlexiTube siRNAs targeting four different Atox1 sequences (Qiagen; see Cat.No. 1027416 for sequence information) using Lipofectamine RNAiMAX transfection reagent (Invitrogen).

#### 2.5. Wound healing assay

Artificial wounds were created 24 h after cell seeding in tissue culture-treated 6-well plate using a P-200 pipette tip to scratch on the cell monolayer. Wounds were measured on bright field micrographs.

### 3. Results

#### 3.1. Atox1 accumulates in the lamellipodium border of breast cancer cells

We first confirmed expression of Atox1 in MCF7 and MDA-MB-231 breast cancer cell lines. Atox1 immunofluorescence staining demonstrated that the Atox1 is not only located in the nucleus and cytoplasm of the cells, but also, surprisingly, at lamellipodia borders

of both cell lines (Fig. 1).

#### 3.2. Atox1 silencing does not affect the lamellipodia formation by MDA-MB-231 cells

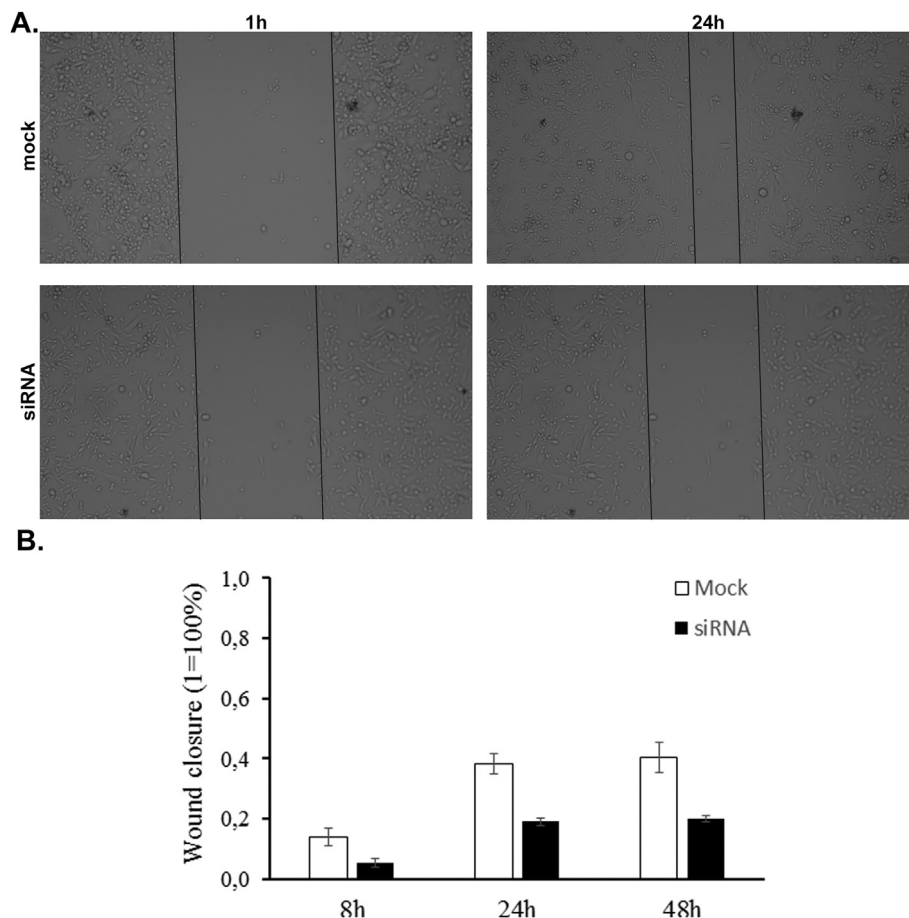
Because lamellipodia are membrane protrusions that drive cell migration, we investigated the role of Atox1 in the migration of MDA-MB-231 cells by evaluation of its morphology (lamellipodia-formation) and functionality (cell migration) upon Atox1 silencing. We reached 60% downregulation of Atox1 protein expression in cells transfected with 100 nM siRNAs against Atox1 (Fig. 2A). Reduction in Atox1 protein appeared uniformly-distributed in the cell (Fig. 2B) and did not affect the lamellipodium-formation potential of the cells (Fig. 2C).

#### 3.3. Atox1 downregulated expression reduces the migration of MDA-MB-231 cells

To probe the role of Atox1 in the motility of the MDA-MB-231 cells we applied a wound healing assay. We detected a 50% reduction in wound closure for cells silenced to 60% with respect to Atox1 expression (Fig. 3). This result suggests that presence of Atox1 is required for MDA-MB-231 cell migration *in vitro*.

### 4. Discussion

Cu and Cu-binding proteins are essential in hallmarks of cancer, and there is a growing evidence that Cu directly influences the



**Fig. 3. Atox1 downregulation reduces migration of MDA-MB-231 cells.** (A) Bright field micrographs of wounds at 0 h and 24 h after wounding of mock and 100 nM siRNA-treated cell monolayers (lines along the border of the wounds). (B) Wound closure measured at 0 h, 24 h and 48 h after wounding (error bar; standard error of the mean) ( $n_{\text{micrographs}}=10$ ).

ability of cancerous cells to invade and metastasize [15,16,18]. Cancer cell migration is mediated by lamellipodial protrusions and is a key phenomenon during cancer metastasis. Here, we discovered that the Cu chaperone Atox1 plays an essential role in breast cancer cell migration *in vitro*, and it localizes to the borders of cellular lamellipodia of migrating cells. In accord with our findings, a recent study reported that Atox1 was essential for platelet-derived growth factor-induced vascular smooth muscle cell migration. It was demonstrated that the process involved relocation of Atox1 (and ATP7A) to the leading edge of lamellipodia structures [21]. Thus it appears that the discovered role of Atox1 in breast cancer cell migration may be extended to other migrating cell types.

In conclusion, in addition to cytoplasmic Cu transport and nuclear transcription factor roles, Atox1 appears to have a new role in cell migration that entails positioning at the cell border. Lamellipodia are powered by actin polymerization, which depends on the activity of many proteins [22]. Atox1 may provide Cu to Cu-binding proteins (e.g., MEMO, LOX) activated at the leading edge of migratory cells. MEMO is a novel Cu-dependent protein found to be important for cancer cell migration and regulation of actin dynamics [18,23] that may be loaded with Cu via Atox1 at the lamellipodia border. On the other hand, Atox1 may deliver Cu to LOX via ATP7A at the lamellipodia border, thereby activating LOX extra-cellular matrix remodeling [21]. Further molecular studies are needed to elucidate Atox1 interaction partners at the lamellipodia border and involved signaling pathways.

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## Transparency document

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## References

- [1] D.L. Huffman, T.V. O'Halloran, Function, structure, and mechanism of intracellular copper trafficking proteins, *Annu. Rev. Biochem.* 70 (2001) 677–701.
- [2] S. Puig, D.J. Thiele, Molecular mechanisms of copper uptake and distribution, *Curr. Opin. Chem. Biol.* 6 (2) (2002) 171–180.
- [3] E.D. Harris, Basic and clinical aspects of copper, *Crit. Rev. Clin. Lab. Sci.* 40 (5) (2003) 547–586.
- [4] T.V. O'Halloran, V.C. Culotta, Metallochaperones, an intracellular shuttle service for metal ions, *J. Biol. Chem.* 275 (33) (2000) 25057–25060.
- [5] Festa, R.A. and Thiele D.J., Copper: an essential metal in biology. *Curr. Biol.* 21(21): p. R877–R883.
- [6] N.J. Robinson, D.R. Winge, Copper metallochaperones, *Annu. Rev. Biochem.* 79 (2010) 537–562.
- [7] I. Hamza, M. Schaefer, L.W. Klomp, J.D. Gitlin, Interaction of the copper chaperone HAH1 with the Wilson disease protein is essential for copper homeostasis, *Proc. Natl. Acad. Sci. U. S. A.* 96 (23) (1999) 13363–13368.
- [8] S. Itoh, H.W. Kim, O. Nakagawa, K. Ozumi, S.M. Lessner, H. Aoki, K. Akram, R.D. McKinney, M. Ushio-Fukai, T. Fukai, Novel role of antioxidant-1 (Atox1) as a copper-dependent transcription factor involved in cell proliferation, *J. Biol. Chem.* 283 (14) (2008) 9157–9167.
- [9] S. Itoh, K. Ozumi, H.W. Kim, O. Nakagawa, R.D. McKinney, R.J. Folz, I.N. Zelko, M. Ushio-Fukai, T. Fukai, Novel mechanism for regulation of extracellular SOD transcription and activity by copper: role of antioxidant-1, *Free Radic. Biol. Med.* 46 (1) (2009) 95–104.
- [10] K. Ozumi, V. Sudhakar, H.W. Kim, G.F. Chen, T. Kohno, L. Finney, S. Vogt, R.D. McKinney, M. Ushio-Fukai, T. Fukai, Role of copper transport protein antioxidant 1 in angiotensin II-induced hypertension: a key regulator of extracellular superoxide dismutase, *Hypertension* 60 (2) (2012) 476–486.
- [11] G.F. Chen, V. Sudhakar, S.W. Youn, A. Das, J. Cho, T. Kamiya, N. Urao, R.D. McKinney, B. Surenkhuu, T. Hamakubo, H. Iwanari, S. Li, J.W. Christman, S. Shantikumar, G.D. Angelini, C. Emanueli, M. Ushio-Fukai, T. Fukai, Copper transport protein Antioxidant-1 promotes inflammatory neovascularization via chaperone and transcription factor function, *Sci. Rep.* 5 (2015) 14780.
- [12] M. Matson Dzebo, C. Ario, P. Wittung-Stafshede, Extended functional repertoire for human copper chaperones, *Biomol. Concepts* 7 (1) (2016) 29–39.
- [13] A. Grubman, A.R. White, Copper as a key regulator of cell signalling pathways, *Expert Rev. Mol. Med.* 16 (2014) e11.
- [14] M.L. Turski, D.J. Thiele, New roles for copper metabolism in cell proliferation, signaling, and disease, *J. Biol. Chem.* 284 (2) (2009) 717–721.
- [15] J.T. Erler, K.L. Bennewith, T.R. Cox, G. Lang, D. Bird, A. Koong, Q.T. Le, A.J. Giaccia, Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche, *Cancer Cell* 15 (1) (2009) 35–44.
- [16] M.A. Morrissey, R. Jayadev, G.R. Miley, C.A. Blebea, Q. Chi, S. Ihara, D.R. Sherwood, SPARC promotes cell invasion *in vivo* by decreasing type IV collagen levels in the basement membrane, *PLoS Genet.* 12 (2) (2016) e1005905.
- [17] M. Yang, C.Z. Huang, Mitogen-activated protein kinase signaling pathway and invasion and metastasis of gastric cancer, *World J. Gastroenterol.* 21 (41) (2015) 11673–11679.
- [18] G. MacDonald, I. Nalvarte, T. Smirnova, M. Vecchi, N. Aceto, A. Dolemeyer, A. Frei, S. Lienhard, J. Wyckoff, D. Hess, J. Seebacher, J.J. Keusch, H. Gut, D. Salaun, G. Mazzarol, D. Disalvatore, M. Bentires-Alj, P.P. Di Fiore, A. Badache, N.E. Hynes, Memo is a copper-dependent redox protein with an essential role in migration and metastasis, *Sci. Signal* 7 (329) (2014) ra56.
- [19] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, *Cell* 144 (5) (2011) 646–674.
- [20] L. Finney, S. Mandava, L. Ursos, W. Zhang, D. Rodi, S. Vogt, D. Legnini, J. Maser, F. Ikpat, O.I. Olopade, D. Glesne, X-ray fluorescence microscopy reveals large-scale relocalization and extracellular translocation of cellular copper during angiogenesis, *Proc. Natl. Acad. Sci. U. S. A.* 104 (7) (2007) 2247–2252.
- [21] T. Kohno, N. Urao, T. Ashino, V. Sudhakar, R.D. McKinney, T. Hamakubo, H. Iwanari, M. Ushio-Fukai, T. Fukai, Novel role of copper transport protein antioxidant-1 in neointimal formation after vascular injury, *Arterioscler. Thromb. Vasc. Biol.* 33 (4) (2013) 805–813.
- [22] M. Krause, A. Gautreau, Steering cell migration: lamellipodium dynamics and the regulation of directional persistence, *Nat. Rev. Mol. Cell Biol.* 15 (9) (2014) 577–590.
- [23] M. Meira, R. Masson, I. Stagljar, S. Lienhard, F. Maurer, A. Boulay, N.E. Hynes, Memo is a cofilin-interacting protein that influences PLCgamma1 and cofilin activities, and is essential for maintaining directionality during ErbB2-induced tumor-cell migration, *J. Cell Sci.* 122 (Pt 6) (2009) 787–797.