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Silencing of the Menkes copper-transporting ATPase (Atp7a) gene increases cyclin D1 protein expression and impairs proliferation of rat intestinal epithelial (IEC-6) cells

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Abstract

The Menkes copper-transporting ATPase (Atp7a) has dual roles in mammalian enterocytes: pumping copper into the *trans*-Golgi network (to support cuproenzyme synthesis) and across the basolateral membrane (to deliver dietary copper to the blood). Atp7a is strongly induced in the rodent duodenum during iron deprivation, suggesting that copper influences iron homeostasis. To investigate this possibility, Atp7a was silenced in rat intestinal epithelial (IEC-6) cells. Irrespective of its influence on iron homeostasis, an unexpected observation was made in the Atp7a knockdown (KD) cells: the cells grew slower (~40% fewer cells at 96 h) and were larger than negative-control shRNA-transfected cells. Lack of Atp7a activity thus perturbed cell cycle control. To elucidate a possible molecular mechanism, expression of two important cell cycle control proteins was assessed. Cyclin D1 (CD1) protein expression increased in Atp7a KD cells whereas proliferating-cell nuclear antigen (PCNA) expression was unaltered. Increased CD1 expression is consistent with impaired cell cycle progression. Expression of additional cell proliferation marker genes (p21 and Ki67) was also investigated; p21 expression increased, whereas Ki67 decreased, both consistent with diminished cell growth. Further experiments were designed to determine whether increased cellular copper content was the trigger for the altered growth phenotype of the Atp7a KD cells. Copper loading, however, did not influence the expression patterns of CD1, p21 or Ki67. Overall, these findings demonstrate that Atp7a is required for normal proliferation of IEC-6 cells. How Atp7a influences cell growth is unclear, but the underlying mechanism could relate to its roles in intracellular copper distribution or cuproenzyme synthesis.

Keywords

Cell growth; Copper; Iron; Intestine

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Conflict of interest
The authors declare no conflict of interest.

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Introduction

Copper plays important roles in a variety of biological pathways and disease processes [1]. Although copper is important for normal cellular function, high intracellular copper is toxic since free copper mediates the production of reactive oxygen radicals [2]. Free copper may have similar affects in nuclei, as DNA instability, apoptosis and eventual cell death [3] were documented in hyper-proliferative cancer cells with elevated nuclear copper levels [4,5]. Cellular copper homeostasis must thus be tightly controlled. The Menkes copper-transporting ATPase (Atp7a) influences copper metabolism in intestinal epithelial cells (IECs), by pumping copper into the *trans*-Golgi network (TGN) to support the biosynthesis of cuproenzymes and by exporting copper from cells under conditions of excess [6]. Atp7a is highly expressed in the mammalian intestine and it was shown that copper stabilizes the protein in IECs [7]. Mutations in the *Atp7a* gene result in impaired intestinal copper transport, altered cuproenzyme synthesis and severe systemic copper deficiency [8]. Atp7a function in the intestine is thus critical for intestinal and whole-body copper homeostasis. Furthermore, iron deficiency induces Atp7a mRNA and protein expression in rodent enterocytes [9], suggesting a role for Atp7a and/or copper in intestinal iron transport.

Copper has been implicated as being important for angiogenesis, and cell growth and differentiation [10,11]. Copper deprivation impairs cell proliferation [5]. Similarly, lack of mitogenic agents, such as growth factors or other nutrients, impairs cell cycle control [12]. Proteins that regulate the cell cycle are another rate-limiting factor for normal cell growth and control of cell division. Two important proteins in this regard are cyclin D1 (CD1) and proliferating-cell nuclear antigen (PCNA). The eukaryotic cell cycle involves 4 phases: G1 (growth), S (DNA synthesis), G2 (growth, preparation for mitosis) and M (mitosis or cell division). CD1 is expressed at low levels during the S phase of the cell cycle, which is a signal for efficient DNA synthesis [13].CD1 levels then increase during the G2, M and G1 phases. CD1 expression subsequently decreases, allowing cells to enter the S phase. Low expression of CD1 during the S phase is coincident with increased PCNA expression, which is a signal for DNA replication [14]. PCNA levels remain high in replicating cells (during the G2 and S phases) [14].

Abnormally high CD1 expression during the G1/S transition and into the S phase impairs cell growth and division [15]. This cyclical pattern of CD1 expression during the cell cycle suggests that CD1 is an active player in the regulation of cell growth [16]. Moreover, CD1 overexpression has been linked to cell senescence [17,18]. Cell senescence is a normal physiological response to various homeostatic perturbations (e.g. nutrient deprivation, oxidative stress), and is characterized by a reduced rate of cell division or growth arrest. Furthermore, it was noted that high cytosolic copper levels induce senescence in human fibroblasts [19]. Senescence can lead to cell death via apoptosis [20], or alternatively, it can be reversible under certain cellular conditions [21].

A recent investigation showed that Atp7a KD in IEC-6 cells increased transepithelial iron flux, possibly via a mechanism involving transcriptional induction of the iron exporter ferroportin (Fpn) [22]. These observations were made in post-confluent, differentiated cells, but during the course of these experiments, it was noted that pre-confluent Atp7a KD cells

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grew slower and were larger in size than control cells. This led us to hypothesize that Atp7a influenced cell cycle control in IEC-6 cells. To test this hypothesis, experiments were performed to determine whether expression of proteins related to cell cycle regulation were altered in the KD cells. Notably, expression of cyclin D1 was elevated in Atp7a KD cells, perhaps providing a mechanistic explanation for the altered growth phenotype of the cells. Since CD1 overexpression was unaffected by copper loading of the cells, it is postulated that Atp7a influenced cell growth via its documented role in cuproenzyme synthesis.

Materials and methods

Cell culture

The rat intestinal epithelial (IEC-6) cell line was purchased from American Type Culture Collection. Cells were stably transfected with Atp7a-specific or non-specific shRNA-expressing plasmids [22]. Cells were maintained in DMEM medium with 10% fetal bovine serum, 1% penicillin/streptomycin and 0.1% insulin. Cells were grown in the presence of zeocin (25 μg/mL) to maintain expression of the shRNAs. In some experiments, cells were incubated with 100 μmol/L $CuCl_2$ for 48 h to induce copper overload. Intracellular copper levels were measured by atomic absorption spectroscopy.

Immunocytochemistry (ICC)

ICC experiments were performed essentially as described earlier [23]. Briefly, Atp7a KD and control IEC-6 cells were seeded on sterile coverslips that were coated with poly-D-lysine in 6-well cell culture dishes. Upon confluence, cells were fixed in methanol-free 4% formaldehyde (Pierce). Cells then were exposed to blocking buffer (Bethyl Laboratories) for 1 h followed by incubation with a 1:1000 dilution of Atp7a primary antibody (54–10) for 2 h. This antibody has been extensively characterized by us previously [23,24]. Alexa Fluor 647 goat anti-rabbit secondary antibody (Invitrogen) was subsequently used at a 1:2000 dilution for 30 min followed by rinsing, mounting and confocal microscopic imaging.

Cell counting and Western blotting

Atp7a KD and control cells (1×10^6) were seeded in 100 mm cell culture dishes and synchronized in serum-free medium for ~12 h followed by replacing with complete media and collecting cells at different time points thereafter. Cells were subsequently washed with PBS and trypsinized, and then counted using a hemocytometer. Total cellular proteins were extracted with RIPA buffer (50 mmol/L Tris-HCl, pH7.4; 150 mmol/L NaCl; 1% (v/v) NP-40; 0.1% (w/v) SDS; 0.5% (w/v) sodium deoxycholate plus protease inhibitors). Proteins were heat denatured, separated on 7.5% (for Atp7a) or 10% (for CD1 and PCNA) SDS-PAGE gels and then transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk in PBS for 1 h followed by reacting with the following primary antibodies: Atp7a (in-house [called 54–10]; 1:8000 for 1 h), CD1 (Santa Cruz, cat. # sc-717; 1:4000 overnight), PCNA (Santa Cruz, cat. # sc-25280; 1:5000 overnight). An α-tubulin antibody was used as a housekeeping control (Abcam, cat. #ab6046; 1:5000 for1 h). A secondary antibody from Bethyl Labs (cat. # A120-101P) was used for detection along with a homemade enhanced chemiluminescence buffer followed by autoradiography. The optical density of protein bands on film was calculated using the UN-SCAN-IT software (Silk

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Scientific), and average pixel numbers were used for comparison of data after normalization to the α-tubulin protein band.

Quantitative PCR

Total RNA was isolated from cells with TRIzol reagent (Invitrogen) according to manufacturer's protocol. 1 μg total RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad) and qPCR was performed as described previously [24] using gene-specific oligonucleotide primers (Supplemental Table 1). Rat cyclophilin was used to normalize expression of experimental genes. Mean fold changes in gene-specific mRNA expression were calculated by the $2^{-\Delta\Delta Ct}$ analysis method [24].

Supplementary Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/jjtemb.2014.07.010.

Statistical analyses

Results are expressed as mean ± SE. All statistical analyses were performed with GraphPad Prism. Student's *t*-test was used to compare groups.

Results

Atp7a KD IEC-6 cells grow slower and are larger than control cells

ICC experiments demonstrated that the Atp7a protein was located in an intracellular compartment (probably the *trans*-Golgi) and on the plasma membrane in IEC-6 cells (Fig. 1A). In KD cells, lesser amounts of protein were detected in a location consistent with the *trans*-Golgi, but the protein was not detected on the plasma membrane (Fig. 1B). The KD cells were also notably larger than control cells. Furthermore, Atp7a KD cells grew slower than cells transfected with a negative-control shRNA (Fig. 1C). At the 96 h time point, the number of Atp7a KD cells was ~40% lower.

CD1 protein is over-expressed inAtp7a KD cells

To elucidate the mechanism underlying the lower growth rate of Atp7a KD cells, CD1 and PCNA protein levels were quantified by immunoblot analysis. CD1 protein levels were increased in Atp7a KD cells at every time point analyzed, as compared to control cells (Fig. 2). PCNA protein levels, however, were not different between the 2 cell lines at any time point.

Copper accumulates in Atp7a KD cells upon copper treatment

Atp7a KD could alter intracellular copper levels or copper distribution, which could secondarily influence cell growth parameters. Atp7a KD cells, grown under basal conditions, accumulated more copper, but the differences did not reach statistical significance ($p = 0.31$; Fig. 3). Therefore, to ascertain whether the altered growth phenotype of Atp7a KD cells related to increases in intracellular copper levels, cells were loaded with copper prior to performing experiments. Copper accumulation occurred in both cell lines, but the increase in intracellular copper was more dramatic in the Atp7a KD cells (2.8-fold increase in Ctrl cells versus 5.3-fold in KD cells) (Fig. 3). This is consistent with defective

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Atp7a-mediated copper efflux. Mt1a mRNA levels also increased in both cell lines, and again, the increase was greater in the KD cells (1.8-fold in Ctrl cells versus 5.9-fold in KD cells). Furthermore, in control cells, copper treatment had no effect on Atp7a mRNA expression, but Atp7a protein levels increased (~50%; $p<0.05$) (Fig. 4). This observation is consistent with the postulate that copper stabilizes the protein [7]. Copper loading, however, did not alter Atp7a mRNA or protein expression in KD cells.

Copper loading does not alter CD1 protein expression

CD1 overexpression may alter cell cycle control in Atp7a KD cells and intracellular copper could be the trigger for enhanced CD1 expression. To test this possibility, CD1 expression was quantified in copper-loaded cells. CD1 mRNA expression did not change in control or KD cells with or without copper loading (Fig. 5). CD1 protein expression increased in KD cells (~4.2-fold, $p < 0.001$), but copper treatment did not further enhance CD1 protein levels. Furthermore, expression of 2 cell cycle control marker genes, cyclin-dependent kinase inhibitor 1 (p21) and proliferation-related Ki67 antigen (Ki67) was assessed in control and KD cells. P21 expression increased (~2.4-fold, $p< 0.05$) and Ki67 expression decreased (~10-fold, $p < 0.001$). Copper loading had no effect on the expression pattern of these marker genes.

Discussion

Rapidly growing cells increase in size and/or volume in preparation for cell division. Cell division, or mitosis, is regulated by key components of the cell cycle machinery [25]. Unfavorable environmental conditions, such as nutrient deficiency or excess, interfere with progression through the cell cycle. For example, increased copper levels in mice induced apoptosis and decreased cell count in spleen and thymus [26]. Also, iron depletion resulted in G1/S phase arrest and impaired cell growth [27].

Previously, an Atp7a KD IEC-6 cell line was developed to elucidate a possible role for Atp7a and/or copper in intestinal iron metabolism [22]. During the course of these experiments, two interesting observations were made: Atp7a KD IEC-6 cells grew significantly slower and were larger in size as compared to control cells. These findings suggested that normal progression through the cell cycle was perturbed by Atp7a silencing. Given that copper may influence cell proliferation [5] and Atp7a may influence copper and iron homeostasis [22,28], it is perhaps not surprising that inhibition of Atp7a activity altered cell growth parameters.

To elucidate the molecular mechanism responsible for this phenomenon, expression of key cell cycle markers, CD1 and PCNA, was assessed. CD1 levels are high in the G2 phase and gradually decrease into the G1/S phase transition. Decreased CD1 expression leads to release of the E2F transcription factor from its binding partner, the retinoblastoma (Rb) protein, allowing E2F nuclear translocation and initiation of DNA replication [29]. PCNA functions with DNA polymerase δ in DNA replication [14] and is considered a marker for the S phase of the cell cycle [30]. Expression is low in non-dividing cells, and it increases in the S phase [30]. CD1 and PCNA are thus critical regulators of the eukaryotic cell cycle.

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Consistent with the fact that CD1 expression decreases as cells enter the S phase, it was shown that CD1 overexpression reduced DNA synthesis and impaired cell proliferation [15]. CD1 protein levels were also elevated during induction of cell senescence [17,18]. Cell senescence, which may occur during unfavorable environmental conditions, results from impairment of cell cycle progression [31]. In the current investigation, CD1 protein levels were elevated in Atp7a KD cells, which correlated with the decreased proliferative rate. Conversely, PCNA levels were similar in Ctrl and KD cells, suggesting that PCNA does not alter the proliferative state of Atp7a KD cells. Furthermore, expression of the cell proliferation marker genes, p21 and Ki67, was assessed. p21 mRNA levels significantly increase during cell senescence and thus inversely correlate with cell growth rate [2]. Ki67 mRNA expression decreases when cell growth is arrested [32]. p21 mRNA levels increased and Ki67 mRNA levels were reduced in Atp7a KD cells, both being consistent with impaired growth.

High intracellular copper is toxic and might therefore interfere with cell proliferation. Studies have shown that metals such as iron, copper, and cadmium can produce reactive oxygen radicals, resulting in lipid peroxidation, DNA damage, and diminished cell proliferation [2]. Copper also interferes with cell proliferation by inducing senescence in human fibroblasts [19]. Relevant to the current study is the fact that Atp7a is only known intestinal copper exporter and decreased Atp7a levels may lead to elevated intracellular copper [33]. We thus sought to determine whether Atp7a KD in IEC-6 cells increased intracellular copper concentrations, perhaps providing a mechanistic explanation for altered growth parameters. Under basal culture conditions when cell growth was impaired, however, copper levels were not different in Atp7aKD as compared to control cells. Moreover, when cells were loaded with copper, expression of CD1, p21 and Ki67 was identical to that in control cells, suggesting that altered cell growth did not relate to changes in intracellular copper concentrations.

Mammalian intestinal epithelial cells, which are renewed every 2–3 days from primordial stem cells located in the crypts, are a unique and dynamic cell population [34]. Proper control of IEC proliferation and differentiation is necessary to maintain a functional intestinal barrier, which provides protection against infective organisms and foreign antigens, and promotes nutrient digestion and absorption. Moreover, perturbed regulation of IEC growth and maturation underlies important clinical entities in humans, for example in inflammatory bowel diseases [35]. A thorough understanding of the mechanisms which regulate IEC proliferation is thus important. Although copper has been implicated in control of cell proliferation [5], the Atp7a copper transporter has not been previously directly linked with cell growth. Since Atp7a has dual functions in IECs, impairment of either of these could potentially alter growth patterns. Atp7a influences intracellular copper levels (via its export function), copper distribution (since it pumps copper out of the cytosol into the TGN), and cuproenzyme synthesis (which occurs within the TGN). Since Atp7a KD did not change intracellular copper levels (under basal conditions) and copper loading did not alter CD1 protein expression, impaired cell growth could relate to defective cuproenzyme synthesis. Clearly, however, additional studies are required to elucidate additional mechanistic aspects of Atp7a and copper in the regulation of IEC proliferation and differentiation.

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Supplementary Material

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References

1. Wang Y, Hodgkinson V, Zhu S, Weisman GA, Petris MJ. Advances in the understanding of mammalian copper transporters. Adv Nutr. 2011; 2:129–37. [PubMed: 22332042]
2. Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem. 2005; 12:1161–208. [PubMed: 15892631]
3. Santon A, Albergoni V, Sturniolo GC, Irato P. Evaluation of MT expression and detection of apoptotic cells in LEC rat kidneys. Biochim Biophys Acta. 2004; 1688:223–31. [PubMed: 15062872]
4. Fuchs AG, de Lustig ES. Localization of tissue copper in mouse mammary tumors. Oncology. 1989; 46:183–7. [PubMed: 2470002]
5. Itoh S, Kim HW, Nakagawa O, Ozumi K, Lessner SM, Aoki H, et al. Novel role of antioxidant-1 (Atox1) as a copper-dependent transcription factor involved in cell proliferation. J Biol Chem. 2008; 283:9157–67. [PubMed: 18245776]
6. Kim BE, Petris MJ. Phenotypic diversity of Menkes disease in mottled mice is associated with defects in localisation and trafficking of the ATP7A protein. J Med Genet. 2007; 44:641–6. [PubMed: 17483305]
7. Xie L, Collins JF. Copper stabilizes the Menkes copper-transporting ATPase (Atp7a) protein expressed in rat intestinal epithelial cells. Am J Physiol Cell Physiol. 2013; 304:C257–62. [PubMed: 23174565]
8. Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J. Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. Nat Genet. 1993; 3:7–13. [PubMed: 8490659]
9. Collins JF, Franck CA, Kowdley KV, Ghishan FK. Identification of differentially expressed genes in response to dietary iron deprivation in rat duodenum. Am J Physiol Gastrointest Liver Physiol. 2005; 288:G964–71. [PubMed: 15637178]
10. Pena MM, Lee J, Thiele DJ. A delicate balance: homeostatic control of copper uptake and distribution. J Nutr. 1999; 129:1251–60. [PubMed: 10395584]
11. Harris ED. A requirement for copper in angiogenesis. Nutr Rev. 2004; 62:60–4. [PubMed: 15080367]
12. Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH, Thompson CB. Growth factors can influence cell growth and survival through effects on glucose metabolism. Mol Cell Biol. 2001; 21:5899–912. [PubMed: 11486029]
13. Yang K, Hitomi M, Stacey DW. Variations in cyclin D1 levels through the cell cycle determine the proliferative fate of a cell. Cell Div. 2006; 1:32. [PubMed: 17176475]
14. Xiong Y, Zhang H, Beach D. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell. 1992; 71:505–14. [PubMed: 1358458]
15. Guo Y, Yang K, Harwalkar J, Nye JM, Mason DR, Garrett MD, et al. Phosphorylation of cyclin D1 at Thr 286 during S phase leads to its proteasomal degradation and allows efficient DNA synthesis. Oncogene. 2005; 24:2599–612. [PubMed: 15735756]
16. Stacey DW. Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. Curr Opin Cell Biol. 2003; 15:158–63. [PubMed: 12648671]

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17. Atadja P, Wong H, Veillete C, Riabowol K. Overexpression of cyclin D1 blocks proliferation of normal diploid fibroblasts. Exp Cell Res. 1995; 217:205–16. [PubMed: 7698220]
18. Burton DG, Sheerin AN, Ostler EL, Smith K, Giles PJ, Lowe J, et al. Cyclin D1 over-expression permits the reproducible detection of senescent human vascular smooth muscle cells. Ann NY Acad Sci. 2007; 1119:20–31. [PubMed: 18056951]
19. Matos L, Gouveia A, Almeida H. Copper ability to induce premature senescence in human fibroblasts. Age. 2012; 34:783–94. [PubMed: 21695420]
20. Dimri GP. What has senescence got to do with cancer? Cancer Cell. 2005; 7:505–12. [PubMed: 15950900]
21. Rufini A, Tucci P, Celardo I, Melino G. Senescence and aging: the critical roles of p53. Oncogene. 2013; 32:5129–43. [PubMed: 23416979]
22. Gulec S, Collins JF. Silencing the Menkes copper-transporting ATPase (Atp7a) gene in rat intestinal epithelial (IEC-6) cells increases iron flux via transcriptional induction of ferroportin 1 (Fpn1). J Nutr. 2014; 144:12–9. [PubMed: 24174620]
23. Ravia JJ, Stephen RM, Ghishan FK, Collins JF. Menkes copper ATPase (Atp7a) is a novel metal-responsive gene in rat duodenum, and immunoreactive protein is present on brush-border and basolateral membrane domains. J Biol Chem. 2005; 280:36221–7. [PubMed: 16081413]
24. Collins JF, Hua P, Lu Y, Ranganathan PN. Alternative splicing of the Menkes copper Atpase (Atp7a) transcript in the rat intestinal epithelium. Am J Physiol Gastrointest Liver Physiol. 2009; 297:G695–707. [PubMed: 19679821]
25. Lim S, Kaldis P. Cdks, cyclins and CKIs: roles beyond cell cycle regulation. Development. 2013; 140:3079–93. [PubMed: 23861057]
26. Mitra S, Keswani T, Dey M, Bhattacharya S, Sarkar S, Goswami S, et al. Copper-induced immunotoxicity involves cell cycle arrest and cell death in the spleen and thymus. Toxicology. 2012; 293:78–88. [PubMed: 22239859]
27. Nurtjahja-Tjendraputra E, Fu D, Phang JM, Richardson DR. Iron chelation regulates cyclin D1 expression via the proteasome: a link to iron deficiency-mediated growth suppression. Blood. 2007; 109:4045–54. [PubMed: 17197429]
28. Gulec S, Collins JF. Investigation of iron metabolism in mice expressing a mutant Menke's copper transporting ATPase (Atp7a) protein with diminished activity (Brindled; Mo (Br) (/y)). PLoS ONE. 2013; 8:e66010. [PubMed: 23776592]
29. DeGregori J. The Rb network. J Cell Sci. 2004; 117:3411–3. [PubMed: 15252123]
30. Morris GF, Mathews MB. Regulation of proliferating cell nuclear antigen during the cell cycle. J Biol Chem. 1989; 264:13856–64. [PubMed: 2569465]
31. Faragher RG, Kipling D. How might replicative senescence contribute to human ageing? Bioessays. 1998; 20:985–91. [PubMed: 10048298]
32. Narla G, DiFeo A, Yao S, Banno A, Hod E, Reeves HL, et al. Targeted inhibition of the KLF6 splice variant, KLF6 SV1, suppresses prostate cancer cell growth and spread. Cancer Res. 2005; 65:5761–8. [PubMed: 15994951]
33. Mann JR, Camakaris J, Danks DM, Walliczek EG. Copper metabolism in mottled mouse mutants: copper therapy of brindled (Mobr) mice. Biochem J. 1979; 180:605–12. [PubMed: 573618]
34. van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol. 2009; 71:241–60. [PubMed: 18808327]
35. Henderson P, van Limbergen JE, Schwarze J, Wilson DC. Function of the intestinal epithelium and its dysregulation in inflammatory bowel disease. Inflamm Bowel Dis. 2011; 17:382–95. [PubMed: 20645321]

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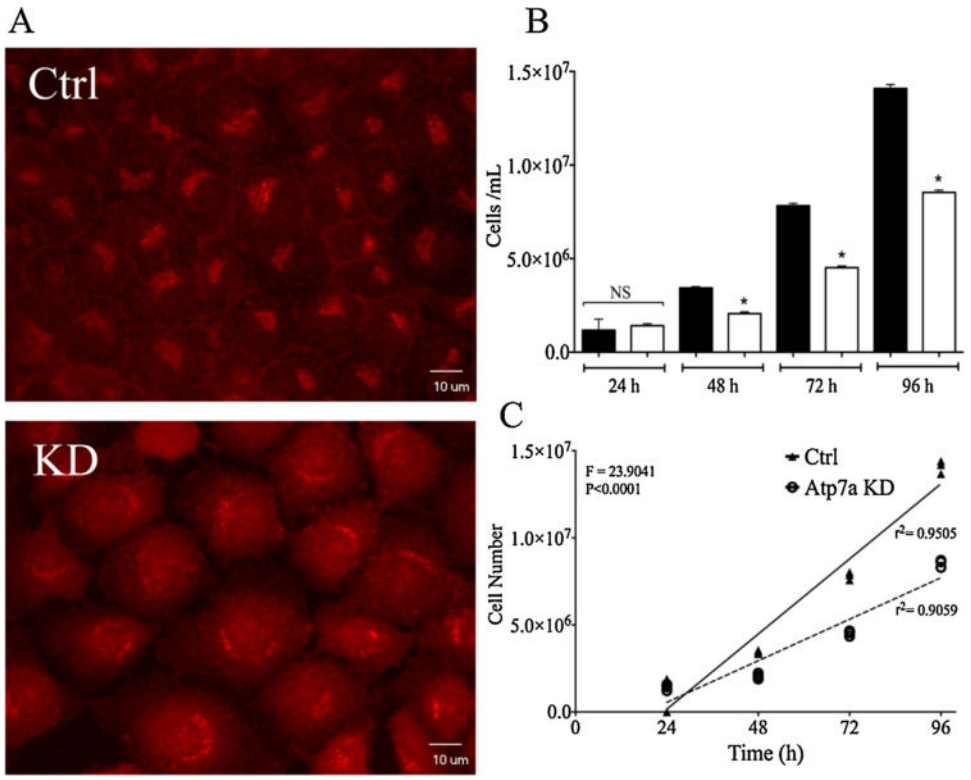

Fig. 1.

Effect of Atp7a KD on IEC-6 cell growth; control (Ctrl) and Atp7a KD IEC-6 cells were incubated with an Atp7a-specific antibody followed by a fluorescent-tagged secondary antibody and imaged by confocal microscopy (A). In separate experiments, control (black bars) or KD (open bars) cells were plated at low density and then synchronized for ~12 h in serum-free medium. Regular medium was subsequently used to replace the serum-free medium, and cells were grown for various time periods and counted (B); *different from Ctrl, $p < 0.001$. To better exemplify differences ingrowth rates, cell growth data were fitted with lines and slopes were calculated (C). Slopes from Ctrl and KD cells were significantly different. Values are means ± SE; $n = 3$ independent experiments. NS, not significant.

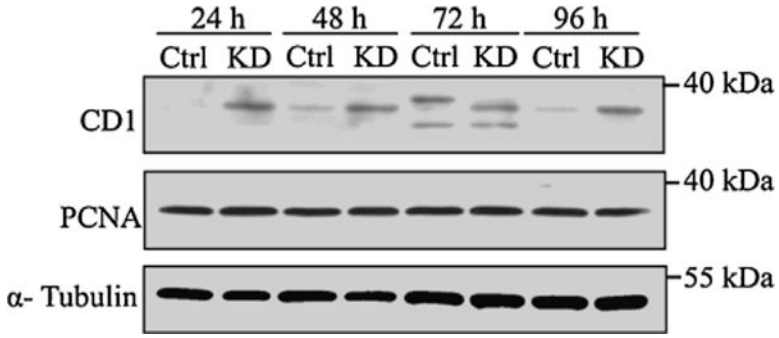

Fig. 2.

CD1 and PCNA protein expression in control and Atp7a KD IEC-6 cells; cells were synchronized in serum-free medium for ~12 h, and then grown in regular medium (with serum) for various time periods. Total cell lysates were then prepared and immunoblots were performed for CD1 (A) and PCNA (B) protein expression. α-Tubulin was used as a constitutively expressed housekeeping protein. Each group represents combined samples from 3–8 experimental repetitions. The position of the closest molecular weight marker is shown on the right hand side. kDa, kilodaltons.

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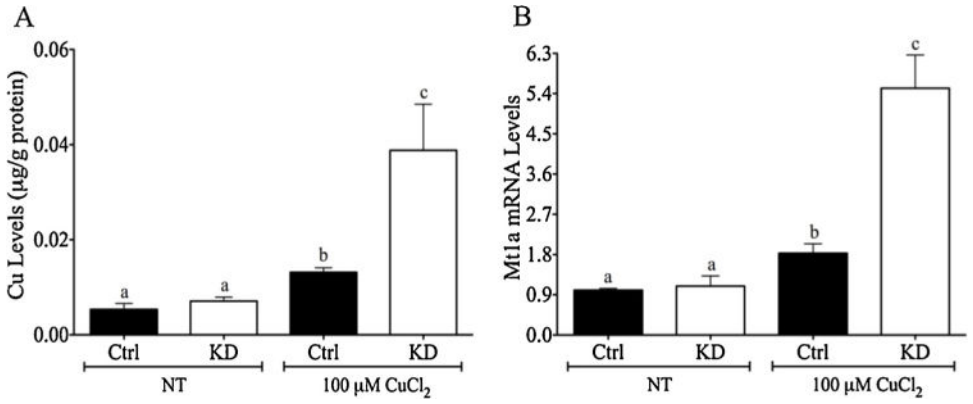

Fig. 3.

Copper loading of control and Atp7a KD IEC-6 cells; cells were synchronized in serum-free medium for ~ 12 h, and then grown in regular medium (plus serum) with or without 100 µM/L $CuCl_2$ for 48 h. Intracellular copper levels (by AAS) and Mt1a mRNA expression (by qRT-PCR) were subsequently determined. Different letters indicate statistical significance between groups within each panel ($p < 0.05$). Values are means ± SE; $n = 3$ independent experiments. NT, no treatment.

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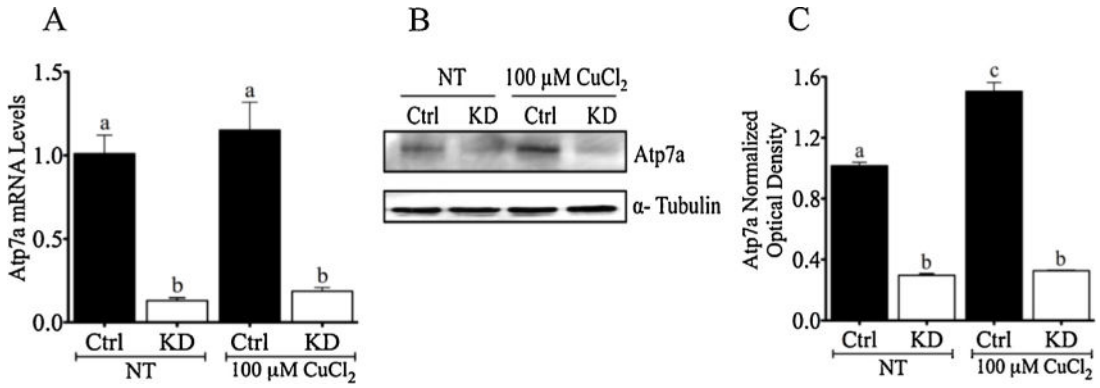

Fig. 4.

Effect of copper loading on Atp7a expression in control and Atp7a KD IEC-6 cells; cells were synchronized in serum-free medium for ~12 h, and then grown in regular medium (plus serum) with or without 100 μM/L $CuCl_2$ for 48 h. Relative Atp7a mRNA (A) and protein (B, C) expression was subsequently determined. Atp7a protein expression was normalized to α-tubulin. Different letters indicate statistical significance between groups within each panel ($p < 0.05$). Values are means ± SE; $n = 3$ independent experiments. NT, no treatment.

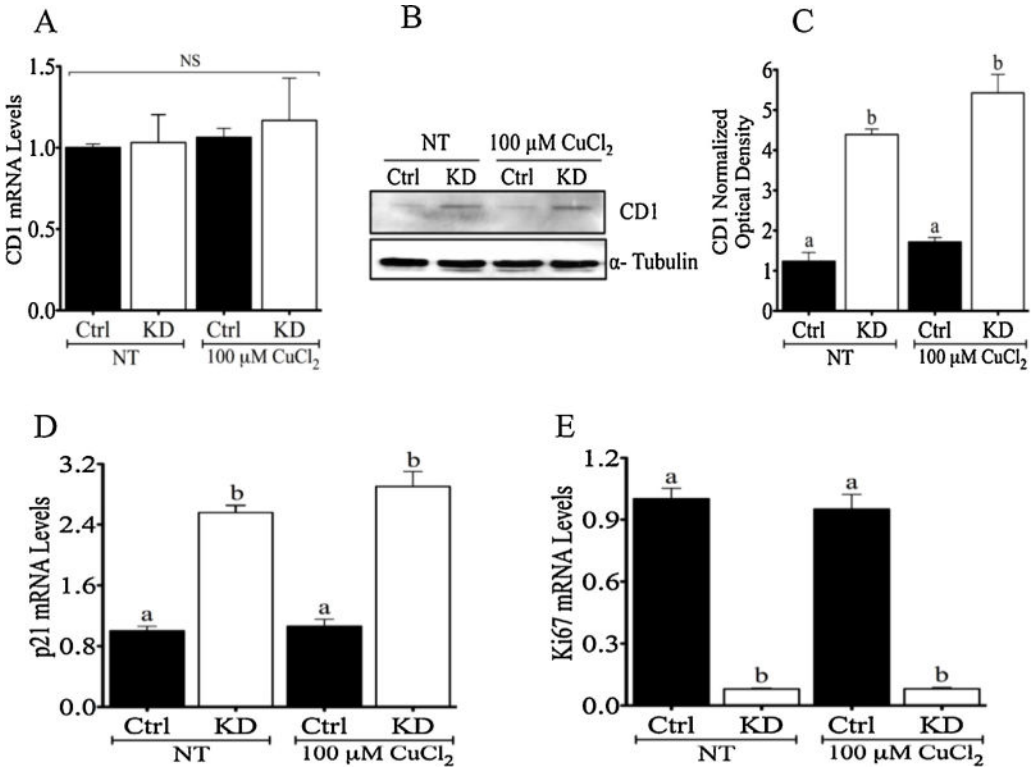

Fig. 5.

Effect of copper loading on the expression of cell cycle marker genes/proteins; cells were synchronized in serum-free medium for ~12 h, and then grown in regular medium (plus serum) with or without 100 μM/L $CuCl_2$ for 48 h. Subsequently, CD1 mRNA(A) and protein (B, C) expression was determined, as well as p21 (D) and Ki67 (E) mRNA expression. CD1 protein expression was normalized to the expression of α-tubulin. Different letters indicate statistical significance between groups within each panel ($p < 0.05$). Values are means ± SE; $n = 3$ independent experiments. NT, no treatment; NS, not significant.

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