Effect of the dysregulation of Cu-transport by ATP7A and ATP7B in the transcriptional regulation of myogenic genes in the skeletal muscle lineage

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BACKGROUND AND SIGNIFICANCE

Copper (Cu) is a critical micronutrient for the development of mammalian cells and tissues. It is a cofactor for several enzymes required for respiration, maintenance of redox homeostasis, neurotransmitter biogenesis, transcriptional regulation, among other essential cellular functions. However, Cu can also excerpt a toxic effect when the ion concentration exceeds the cellular needs. High Cu levels lead to the production of reactive oxygen species, disruption of Fe-S clusters, and oxidation of biomolecules. To prevent this, a complex network of transmembrane transport systems, soluble chaperones, chelating proteins, and TFs mediate Cu homeostasis (**Fig. 1**). Failure of this Cu network to maintain homeostasis leads to severe conditions, such as Wilson's (mutation of ATP7B) and Menkes (mutation of ATP7A) diseases. These are characterized by neurological deficiencies and defects in skeletal muscle development and function.

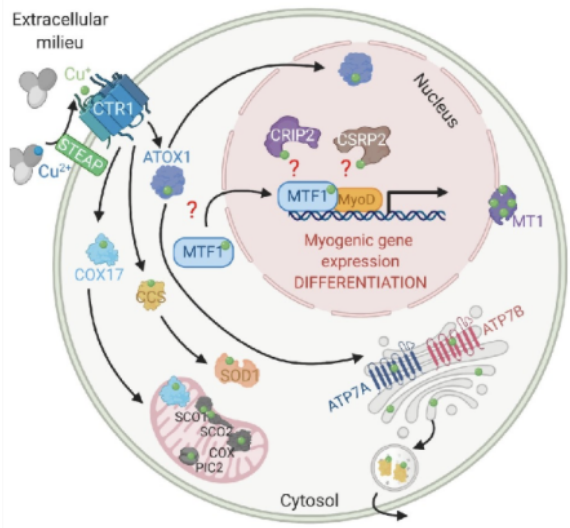

Fig. 1. Cu network and potential role of Cu-TFs in myogenesis. MTF1 as a CuTF and modulator of myogenic gene expression. CTR1 influx transporter. Cu-chaperone for SOD1, Cox17, ATOX1 cytosolic chaperones. ATP7A and ATP7B Cu-transporters.

Metabolic and morphological changes linked to Cu biology occur during myogenesis, such as energy production and redox homeostasis. During myoblast differentiation, increased production of mitochondria and cuproenzymes like cytochrome c oxidase (COX), and superoxide dismutase 1 (SOD1) will drive energy production, and redox homeostasis, respectively. Dysfunction or inhibition of mitochondrial protein synthesis impairs myogenesis. Our laboratory is interested in understanding the molecular mechanisms by which Cu promotes skeletal muscle growth and development. We provided the initial characterization of the biological contributions of Cu in skeletal muscle cell function, proliferation and differentiation using primary myoblasts derived from mouse satellite cells as a model (**Fig. 2**).[1] We demonstrated that Cu supplementation to cultured primary myoblasts promoted cell proliferation (**Fig. 2A**) and differentiation (**Fig. 2B**), while Cu depletion with the chelator tetraethylenepentamine (TEPA) prevented these processes (**Fig. 2A,C**). Cu contributions seems to be associated with regulation on the expression of myogenic genes (**Fig. 2D**). [1] In this regard, our lab recently showed that at least one Cu-binding transcription factor (Cu-TF), the metal responsive transcription factor MTF1[2], is required for the development of the skeletal muscle lineage. We reported that MTF1 binds and regulates the expression of genes required for skeletal muscle differentiation, in addition to genes that are related to maintenance of metal and redox homeostasis[2]. MTF1 is a highly conserved zinc-binding TF that recognizes and binds to the metal responsive elements (MREs) of genes that maintain metal homeostasis.[3] MTF1 binds to metallothionein (*Mt*) promoters, a family of small, cysteine-rich proteins that chelate excess metals and controls the expression of diverse

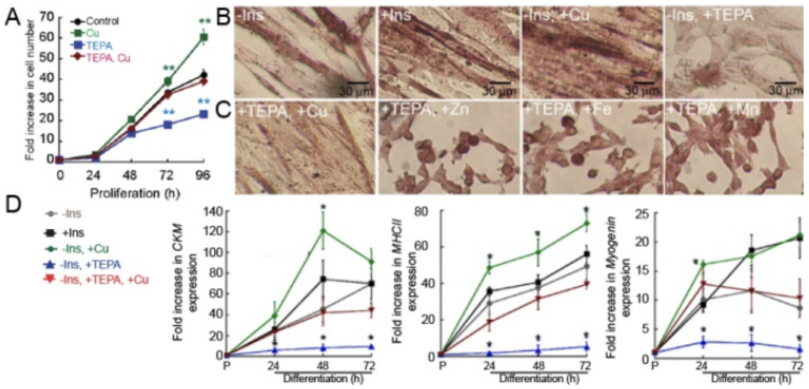

Fig. 2. Cu promotes primary myoblast proliferation and differentiation. **(A)** Cell counting assay of primary myoblasts grown in normal growth medium (control), or in the presence of $CuSO_4$, TEPA and Cu/TEPA. **(B)** Microscopy images of myotubes immunostained against Myosin Heavy Chain. **(C)** Effect of Cu in the expression on myogenic differentiation markers genes. Muscle-specific creatine kinase, Myosin Heavy Chain II and Myogenin. Data are the means±SD; n=4 *P<0.005.[1]

metal transporters to cope with metal imbalances.[3] MTF1 KO in mice is embryonic lethal at E14.5, which led to the hypothesis that MTF1 is important for the transcription of targets other than *Mts*. Using shRNA and CRISPR/Cas9 mediated targeting of MTF1 in primary myoblasts we demonstrated that partial depletion of MTF1 prevent myoblast differentiation.[2] Under standard myogenic conditions, MTF1 levels significantly increased after 24 h of differentiation, compared to proliferating myoblasts.[2] ChIP-Seq and ChIP-qPCR analyses show that MTF1 binds to the *Myogenin* promoter in addition to metal homeostasis gene promoters (*Mt1,* **Fig. 3**).[2] Cu supplementation to the culture media enhanced MTF1 expression and favored its binding to myogenic genes (**Fig. 3**).[2]

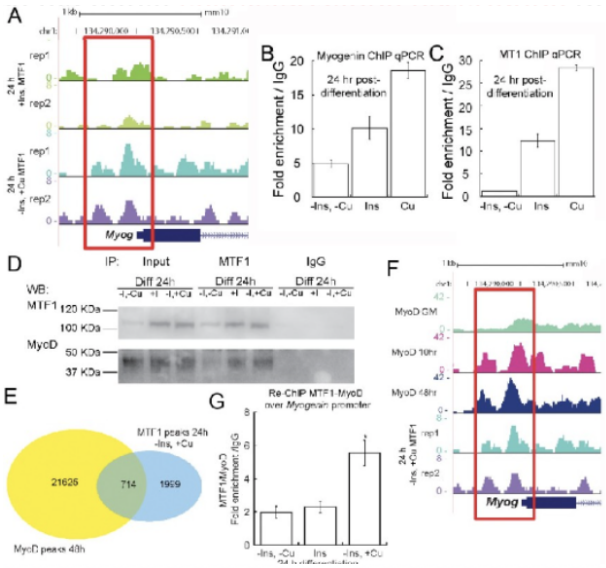

Fig. 3. MTF1 binds to promoters of myogenic and metal homeostasis genes, and interacts with MyoD in differentiating myoblasts.[2] ChIP-Seq **(A)** and -PCR **(B)** data shows MTF1 binding to the Myogenin and Metallothionein 1 **(C)** promoters. **(D)** IP of MTF1 and MyoD. **(E)** Venn diagram of the overlap between MTF1 51 and MyoD 70 ChIP-Seq datasets. **(F)** ChIP-Seq **(G)** and Re-ChIP analyses of MTF1 and MyoD peaks at Myogenin promoter. Data are the means ± SD, n=3. *P<0.005 over IgG.

Based on our preliminary and published studies,[1,2] we hypothesize that Cu is a regulator and component of a novel category of Cu-TFs that may act cooperatively with classic myogenic transcription factors (TFs) to facilitate the progression of skeletal muscle differentiation. Primary myoblasts are an excellent model to study Cu biology; however, we are aware of the limitations of cell culture systems. We began our studies with primary cells as the first step in a multi-step process, which eventually will utilize animal models. My proposal is focused to first mechanistically characterize Cu-TFs *in vitro* and cell culture, as there is little mechanistic information available about how these proteins contribute to lineage-specific gene expression in mammalian systems.

I aim to characterize the biological relevance of MTF1 transcriptional activity in the differentiation of primary myoblast cells knockdown for the Cu-exporters ATP7A and ATP7B. These two proteins are required for maintenance of systemic Cu homeostasis. Mutation of these transporters lead to Menkes and Wilson's diseases, which are associated with deficient Cu transport. Patients of both diseases present neuronal phenotypes which are largely characterized and are associated with metal and redox imbalance in the neurons and motor neurons. However, these patients also present phenotypes that are likely due to the direct effect of Cu dysregulation in the muscle, rather than a consequence of the neuronal effects. For instance, Menkes' patients present hypotonia, while Wilson's disease patients present ataxia and/or dystonia. In this regard, there is a significant gap in our understanding of the direct effect of Cu dysregulation on the muscle. We hypothesize that one of the multiple mechanisms by which aberrant cellular Cu levels may contribute to these pathological phenotypes is through a failure in the regulation of gene expression driven by Cu-TFs.

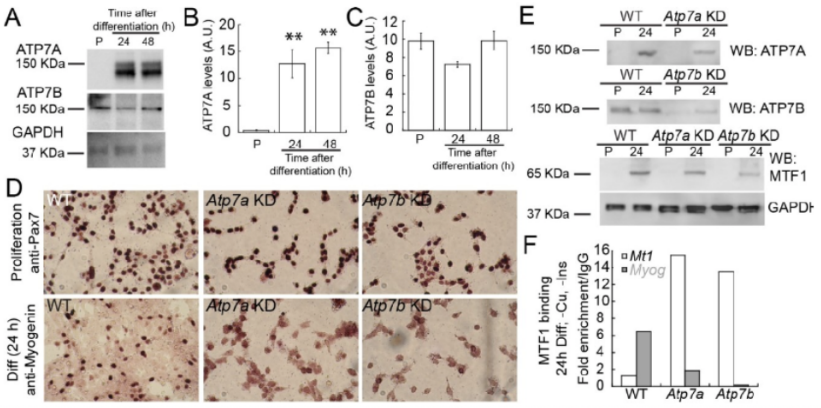

Fig. 4. ATP7A and ATP7B in the myogenic lineage. (A) Representative WB and quantification of ATP7A **(B)** and ATP7B **(C)** in primary myoblasts.[1] **(D)** *Atp7a* and *7b* deletion in proliferating (IHC: Pax7) and differentiating primary myoblasts (IHC: myogenin). **(E)** WB of ATP7A and ATP7B in KD myoblasts. GAPDH as loading control. **(F)** Representative ChIP of MTF1 binding to *Myogenin* and *metallothionein1* promoters in *Atp7a* and *Atp7b* KD myoblasts (n=2)

We will test our hypotheses using a multidisciplinary approach that is well-suited to determine the effect of Cu imbalance of primary myoblasts lacking the two Cu-transporters in the growth and differentiation associated with MTF1 transcriptional regulation. We have shown the differential

expression of ATP7A and ATP7B in the myogenic lineage.[1] ATP7A is expressed upon initiation of myogenesis (**Fig. 4A,B**), while ATP7B is present in proliferating and in differentiating primary myoblasts (**Fig. 4A,C**). Preliminary work shows that *Atp7a* or *7b* knockdown in myoblasts results in delayed growth, an inability to differentiate, and cell death (**Fig. 4D,E**). Initial screenings showed a 30-50% decrease in MTF1 levels in differentiating myoblasts (**Fig. 4E**). Preliminary ChIP-qPCR analysis of MTF1 in both *Atp7a* and *Atp7b* KD myoblasts showed a decrease in binding to the *Myogenin* promoter (**Fig. 4F**). Importantly, in both cases, MTF1 was bound to the *Metallothionein 1* (*Mt1*) promoter, consistent with its role in maintaining Cu homeostasis. This result provides evidence that MTF1 function is compromised when ATP7A and ATP7B expression is deficient and supports my hypothesis.

I will conduct a systematic, quantitative, and qualitative study of gene regulatory mechanisms prior to and during differentiation in cultured primary myoblasts knockdown for *Atp7a* and *Atp7b* (**Fig. 4**). Primary myoblasts can be easily isolated using standard techniques and cultured *ex vivo*. They recapitulate *in vivo* skeletal muscle differentiation and generate myotubes in culture. Additionally, the use of primary cell lines precludes potential complications associated with immortalized cell lines, like polyploidy. We have already established a lentiviral delivery system for shRNA and CRISPR/Cas9 to downregulate or express mutant Cu-TFs[2] and both Cu^{+}-ATPases (**Fig. 4**) in myoblasts. We will couple state of the art metalloproteomic and molecular approaches with established methodologies used for studies of skeletal muscle biology *in vitro*.

ATP7A and *ATP7B* knockdown myoblasts will be monitored for viability and gross defects to proliferate and differentiate in culture. The analyses will include:

a) Expression of proliferation and differentiation markers (Pax7, Desmin, Myogenin, MHC, and sarcomeric actin) by confocal microscopy, immunohistochemistry and western blot.
b) The effect of *Atp7a* and *Atp7b* knockdown in the establishment of the myogenic gene expression programs dependent on MTF1 to understand the transcriptional effect of Cu imbalance in the activity of these TFs in myogenesis. To understand changes in the expression of myogenic genes driven by Cu imbalance and MTF1, we will use quantitative RT-PCR analyses to measure endogenous mRNA synthesis of myogenic and metal homeostasis related genes. We will identify protein:chromatin interactions and changes in chromatin structure by chromatin immunoprecipitation qPCR (ChIP-PCR) with focus on myogenic target genes identified in our published studies (Fig. 2).[2]
c) We will evaluate changes in metal contend in myoblasts lacking both transporters. To this end, I will perform fine detection, quantification, and localization of Cu in cells using spectrophotometric techniques, such as atomic absorbance spectroscopy (AAS).
d) To understand whether the Cu-export defects in our *Atp7a* and *Atp7b* knockdown myoblasts can be rescued by Cu chelators, we will follow similar approaches for Cu chelation showed in Fig. 2 to deplete the ion from the media. Proliferation and differentiation markers as well as changes in gene expression, MTF1 chromatin binding capabilities and metal levels in cells will be assessed as described above.

Alternative outcomes and optional approaches: If there is an impact of *Atp7a* or *Atp7b* mutations in gene expression driven by MTF1, we expect to see a decrease in binding of MTF1 and transcription of myogenic genes. In this case we will explore the possibility of mislocalization or decreased expression of the Cu-TF.

Concluding remarks: The research proposed here will open up a much-needed, unexplored field by linking Cu to the regulation of gene expression in the skeletal muscle lineage. Our studies on MTF1 in the context of dysregulation of Cu homeostasis in skeletal muscle cells will build upon existing research in the characterization of novel mechanisms for lineage specific gene regulation. My research will provide a framework for understanding novel mechanistic details for Cu and Cu-TF in the regulation of gene expression during myogenesis. This work will contribute considerably to the very limited literature related to Cu biology in myogenesis, tissue differentiation, and in the mechanisms of transcriptional regulation.

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3 Gunther, V., Lindert, U. & Schaffner, W. The taste of heavy metals: gene regulation by MTF-1. *Bba-Bioenergetics* **1823**, 1416-1425, doi:10.1016/j.bbamcr.2012.01.005 (2012).

I became an undergraduate researcher in Professor Padilla-Benavides's lab this school year, and I am in-person in the lab roughly 12 hours per week. I have been doing general work such as preparing buffers and solutions, as well as work more specific to my research, including preparing and running SDS PAGE and agarose DNA gels, western blotting, and performing colony and mutagenesis PCR. I hope to solidify my current research project on copper transport and transcription factors in skeletal muscle cell differentiation over this upcoming summer.

Post-graduation from Wesleyan's undergraduate program in 2022, I plan to apply to and enroll in Wesleyan's BA/MA program for Molecular Biology and Biochemistry. Seeing as this program is primarily focused on research, a summer research experience would better prepare me for the BA/MA application process, and for the type of research I would conduct if admitted to the BA/MA program. I plan to continue as a researcher in Professor Padilla-Benavides's lab as long as possible, including throughout the duration of my time in the BA/MA program, if admitted. I also hope to eventually enroll in a PhD program in Molecular Biology and Biochemistry, at Wesleyan if possible, in which I would ideally use my undergraduate and graduate research as a foundation from which to continue with research projects of advanced complexity over which I have increased control in regards to proposals, technique, and procedure.

I plan to use the Molecular Biology and Biochemistry Bachelor's degree which I am currently pursuing, as well as any and all subsequent degrees that I pursue, to continue to conduct laboratory research. I am not currently Pre-Health, nor do I plan to pursue a career in medicine. As such, this summer research experience would directly inform my knowledge, preparedness, and skill for the professional research that I plan to participate in and conduct throughout my professional career as well as my academic path.

The content of the research that I would perform over the summer in Professor Padilla-Benavides's lab also informs my long-term academic and career goals as far as the types of research questions I wish to pose. My work this upcoming summer on the effects of the Cu-transporter Cu ATPase ATP7A deletion on the MTF1 Cu-binding transcription factor is in the same broader line of thought as the research I hope to eventually conduct on the effects of copper, iron and manganese transporters on oxidative injury that leads to Parkinson's Disease, a neuromuscular degenerative disease characterized by degeneration of brain stem neurons. Parkinson's disease relates to the research detailed in this proposal directly, as a link was drawn between CuTF MTF1 and muscle function in *Drosophila.* This summer research experience would allow me to gain experience and knowledge of the techniques and lines of question involved in identifying the effects of transition-metal binding transcription factors and transporters on transition metal transportation in and out of the cell, and how that altered transportation impacts specific pathogeneses.