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The Mechanism of Hepcidin-induced Degradation of Transmembrane Iron Transporters

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Abstract

Iron is an essential nutrient for most life because it is involved in fundamental processes such as oxygen transport, electron transfer, DNA replication, and a cofactor for enzymes. The expression of two transmembrane iron transporter proteins, DMT1 (an iron importer) and ferroportin (an iron exporter), is controlled by hepcidin, (a small protein signaling molecule made in the liver) [1,2]. Past research has shown that hepcidin decreases the expression of both DMT1 and ferroportin, but the biochemical mechanism for this is still unknown [3,4]. The specific aim of this research is to test whether hepcidin activates the Nedd4 system that mediates the ubiquitination of DMT1 and ferroportin using siRNA for Nedd4L knockdown.

Background

Iron homeostasis involves uptake, transport, storage, regulation, and metabolism of iron in the body. Alterations of iron transport can disrupt iron homeostasis and cause many iron-related diseases [9,10]. One way the body regulates the expression of these iron transporters is release of hepcidin, a 25-amino acid peptide secreted by the liver during inflammation or iron overload [1,2].

Ferroportin is the only known iron exporter in mammals, its expression is decreased by hepcidin. Hepcidin regulates the export of iron by binding to ferroportin which leads to its inactivation, internalization, and eventual degradation [3]. Hepcidin may activate a Nedd4 system to ubiquitinate ferroportin and decrease its expression [5,6]. The ubiquitin system marks a protein for degradation and Nedd4L belongs to the Nedd4 family of E3 HECT ubiquitin ligases [5,6].

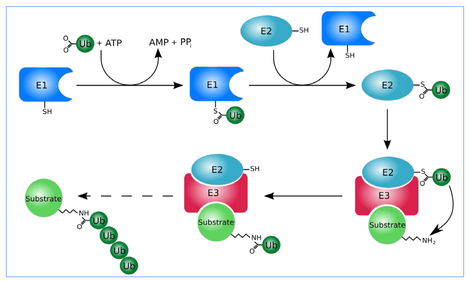

Fig 1. General mechanism of the ubiquitination system

DMT1 (divalent metal transporter 1) is a major transmembrane iron importer protein that has four isoforms (1A/+IRE, 1A/-IRE, 1B/+IRE, 1B/-IRE). In this project, two different DMT1 isoform expressing cell lines will be used (1A/+IRE and 2/-IRE). According to a previous student's research, hepcidin also decreases the expression of DMT1 but seemingly does not bind to it. Similarly, DMT1 may be regulated by the Nedd4 ubiquitination system [7,8]. This project will test for the presence of both hepcidin-induced ubiquitination systems of ferroportin and DMT1.

Results

HEK293 1A+: DMT1 Expression with varying incubations of Hepcidin, Doxycycline, and siRNA transfections

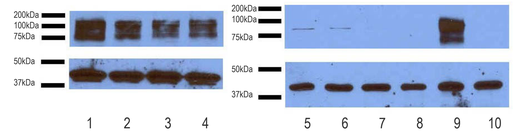

Fig 2. DMT1 is detected with 4EC primary antibody and secondary goat anti-mouse Alexa 568. Bands detected between 75-100kDa indicate DMT1 expression and there is a dramatic increase when doxycycline is added. There is also an increase in DMT1 expression when Nedd4L siRNA is transfected. Hepcidin only decreases DMT1 expression in non-specific siRNA transfected cells.

Protein loads:
(1) Nedd4L siRNA, Doxy, Hepc (2)Nedd4L siRNA, Doxy (3) NS siRNA, Doxy, Hepc (4) NS siRNA, Doxy (5) Nedd4L siRNA, Hepc (6) Nedd4L siRNA (7) NS siRNA, Hepc (8) NS siRNA (9) Doxy, Opti medium (10) Opti medium

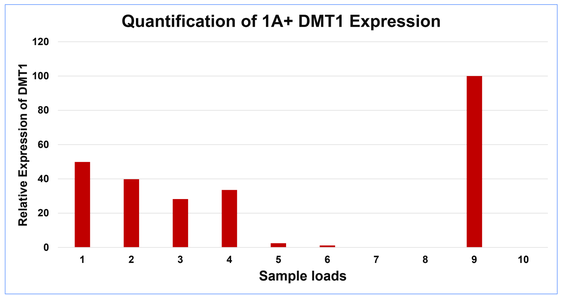

Fig 3. ImageJ is used to quantify the ratio of DMT1 protein to actin from the Western Blot in Fig 2. The relative expression of DMT1 compared to the Doxycycline control is shown. One can readily see the increase in DMT1 expression when Nedd4L siRNA is transfected.

HEK293 2-: DMT1 Expression with varying incubations of Hepcidin, Doxycycline, and siRNA transfections

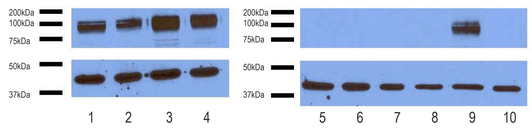

Fig 4. DMT1 is detected with Exon2 primary antibody and secondary goat anti-mouse Alexa 568. Bands detected between 75-100kDa indicate DMT1 expression and there is a dramatic increase when doxycycline is added. There is also a decrease in DMT1 expression when Nedd4L siRNA is transfected. Hepcidin does not seem to affect either transfected cells significantly.

Protein loads:
(1) Nedd4L siRNA, Doxy, Hepc (2)Nedd4L siRNA, Doxy (3) NS siRNA, Doxy, Hepc (4) NS siRNA, Doxy (5) Nedd4L siRNA, Hepc (6) Nedd4L siRNA (7) NS siRNA, Hepc (8) NS siRNA (9) Doxy, Opti medium (10) Opti medium

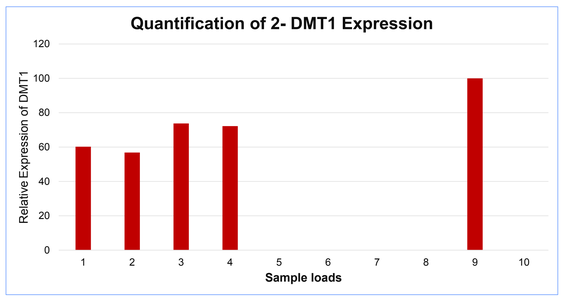

Fig 5. ImageJ is used to quantify the the ratio of DMT1 protein to actin from the Western Blot in Fig 4. The relative expression of DMT1 compared to the Doxycycline control is shown. One can readily see the decrease in DMT1 expression when Nedd4L is transfected is more clearly shown.

Results (continued)

HEK293 1A+: DMT1 Expression in Confocal Microscopy

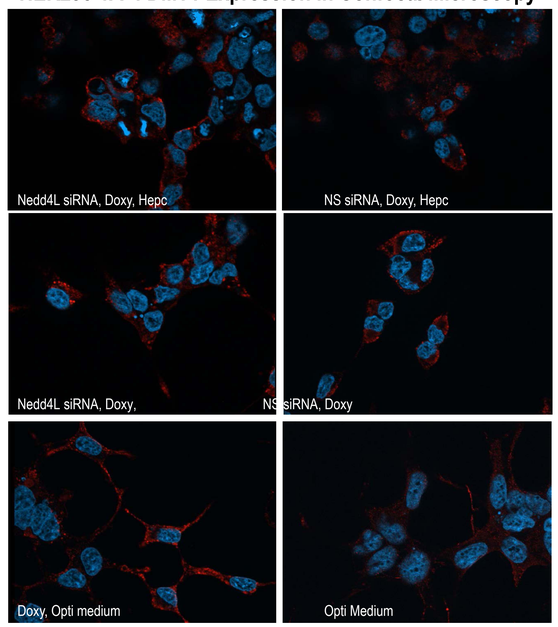

Fig 6. DMT1 is detected with Exon2 primary antibody and secondary goat anti-mouse Alexa 568 in red fluorescence. The nucleus is detected with DAPI in blue fluorescence. This data confirms the results of the previous Western blot data. Comparing from left to right, the increase in DMT1 expression when Nedd4L siRNA is transfected is shown in the increase in red fluorescence.

HEK293 2-: DMT1 Expression in Confocal Microscopy

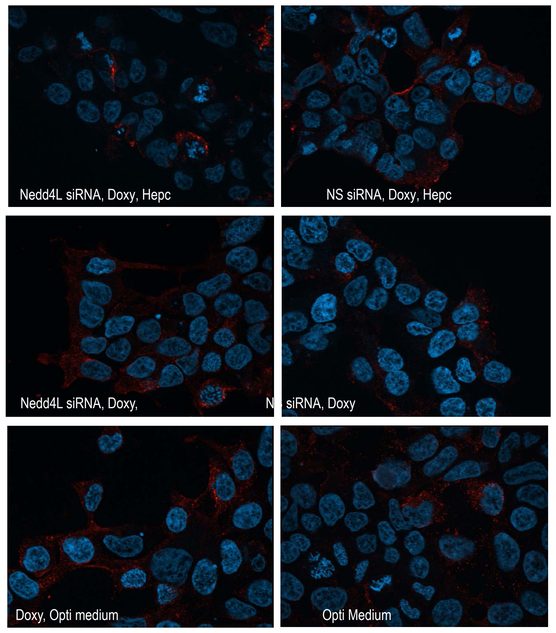

Fig 7. DMT1 is detected with Exon2 primary antibody and secondary goat anti-mouse Alexa 568 in red fluorescence. The nucleus is detected with DAPI in blue fluorescence. This does not replicate the Western Blot data as there is no apparent difference between the two transfected cells.

Methods

Cell lines and cell culture: HEK293 1A+IRE/1B-IRE cells were maintained at 37ºC with 5% CO_2 in DMEM and 10%(v/v) FBS containing 200 µg/ml of hygromycin, 100 µg/ml of streptomycin and 100 units/ml of penicillin.
Small Interfering RNA Transfection: Human Nedd4L and nonspecific siRNA oligonucleotide pools (catalog number M-007187-02) obtained from Dharmacon RNA Technologies (Lafayette, CO) were transfected at a final concentration of 25 nM with Dharmafect reagent (catalog number T-2001)
Confocal Microscopy: Cells were grown on coverslips coated in poly-L-lysine, washed with PBS, fixed with 4% PFA, permeabilized with 0.1% Triton and blocked with PBS/5% BSA before incubating with Exon2 primary antibody. The cells were stained with Alexa Anti Rabbit 568 secondary antibody, mounted on a glass slide with Prolong Gold with DAPI and imaged with Zeiss AxioImager Fluorescence Microscope with 63x oil lens.
Western Blot: Protein lysates were collected, separated by SDS/PAGE, blotted onto nitrocellulose membranes, and blocked in 5% nonfat milk. After incubation with 1:3000 dilution of Exon2 or 1:1000 dilution of 4EC, staining with a 1:10000 dilution of Alexa Anti Rabbit, Goat IgG 568 anti-rabbit secondary. Blots were developed on film with ECL substrate and stained with 1:10000 dilution of actin to confirm equal sample loading.

Conclusions

- DMT1 Expression is increased with Nedd4L siRNA transfection in 1A+ cells; Nedd4L protein knockdown interferes with Hepcidin-induced degradation of DMT1
- The effect of Nedd4L siRNA transfection on 2- cells is unclear; The hypothesis that there would be no effect because 2- cells use Parkin, a different E3 ubiquitin ligase, was met with mixed results

Further Directions

- Additional Western Blots and confocal microscopy to confirm preliminary data and provide clear results
- Perform same set of experiments on FPN-GFP cell line to test hypotheses on ferro portin

Acknowledgements

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References

[1] Garrick, M.D.; Garrick, L.M. Cellular iron transport. Biochim. Biophys. Acta Gen. Subj. 2009, 1790, 309–325. doi: 10.1016/j.bbagen.2009.03.018.
[2] Garrick MD. Human iron transporters. Genes & Nutrition. 2011;6(1):45-54. doi:10.1007/s12263-010-0184-8.
[3] Nemeth E, Tuttle M, Powelson J et al. Hepcidin Regulates Cellular Iron Efflux by Binding to Ferroportin and Inducing Its Internalization. Science. 2004;306(5704):2090-2093. doi:10.1126/science.1104742.
[4] 2. Mena N, Esparza A, Tapia V, Valdes P, Nunez M. Hepcidin inhibits apical iron uptake in intestinal cells. AJP: Gastrointestinal and Liver Physiology. 2007;294(1):G192-G198. doi:10.1152/ajpgi.00122.2007.
[5] Qiao B, Sugianto P, Fung E, et al. Hepcidin-induced endocytosis of ferroportin is dependent on ferroportin ubiquitination. Cell Metabolism. 2012;15(6):918-924. doi:10.1016/j.cmet.2012.03.018.
[6] De Domenico I, Lo E, Yang B, et al. The Role of Ubiquitination in Hepcidin-Independent and Hepcidin-Dependent Degradation of Ferroportin. Cell metabolism. 2011;14(5):635-646. doi:10.1016/j.cmet.2011.09.008. (Retracted but I will test what parts can be reproduced.)
[7] Brasse–Lagnel C, Karim Z, Letteron P, Bekri S, Bado A, Beaumont C. Intestinal DMT1 Cotransporter Is Down-regulated by Hepcidin via Proteasome Internalization and Degradation. Gastroenterology. 2011;140(4):1261-1271.e1. doi:10.1053/j.gastro.2010.12.037.
[8] Foot N, Dalton H, Shearwin-Whyatt L et al. Regulation of the divalent metal ion transporter DMT1 and iron homeostasis by a ubiquitin-dependent mechanism involving Ndfips and WWP2. Blood. 2008;112(10):4268-4275. doi:10.1182/blood-2008-04-150953.
[9] Roth, J.A., Singleton, S., Feng, J., Garrick, M. & Paradkar, P.N. 2010 Parkin regulates metal transport via proteasomal degradation of the 1B isoforms of divalent metal transporter 1 (DMT1). Journal of Neurochemistry 113:454-464, doi: 10.1111/j.1471-4159.2010.06607.x.
[10] Urrutia P, Aguirre P, Esparza A et al. Inflammation alters the expression of DMT1, FPN1 and hepcidin, and it causes iron accumulation in central nervous system cells. J Neurochem. 2013;126(4):541-549. doi:10.1111/jnc.12244.