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Huntingtin protein is essential for mitochondrial metabolism, bioenergetics and structure in murine embryonic stem cells

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ABSTRACT

Mutations in the Huntington locus (*htt*) have devastating consequences. Gain-of-poly-Q repeats in Htt protein causes Huntington's disease (HD), while $htt^{-/-}$ mutants display early embryonic lethality. Despite its importance, the function of Htt remains elusive. To address this, we compared more than 3700 compounds in three syngeneic mouse embryonic stem cell (mESC) lines: $htt^{-/-}$, extended poly-Q (Htt-Q140/7), and wild-type mESCs (Htt-Q7/7) using untargeted metabolite profiling. While Htt-Q140/7 cells did not show major differences in cellular bioenergetics, we find extensive metabolic aberrations in $htt^{-/-}$ mESCs, including (i) complete failure of ATP production despite preservation of the mitochondrial membrane potential; (ii) near-maximal glycolysis, with little or no glycolytic reserve; (iii) marked ketogenesis; (iv) depletion of intracellular NTPs; (v) accelerated purine biosynthesis and salvage; and (vi) loss of mitochondrial structural integrity. Together, our findings reveal that Htt is necessary for mitochondrial structure and function from the earliest stages of embryogenesis, providing a molecular explanation for $htt^{-/-}$ early embryonic lethality.

Introduction

The Huntington locus (*htt*) in the mammalian genome encodes a large protein called Huntingtin (Htt). Htt protein is maternally expressed from the first cell stage in the fertilized egg and subsequently is ubiquitously found in all cells of the embryo and adult. Mutations affecting the locus have dramatic consequences in both mouse and humans. Homozygote $htt^{-/-}$ embryos undergo developmental arrest, fail to undergo proper gastrulation, lack a proper node, display a shortened primitive streak, and exhibit an impaired patterning of embryonic germ layers – early embryonic lethality occurs by e7.0–7.5 (Duyao et al., 1995; Woda et al., 2005).

In contrast to the lethality of $htt^{-/-}$ embryos, mutation of a single *htt* allele that expands the poly-Q (CAG) repeat at the N-terminal domain of Htt protein results in the dominantly inherited Huntington's disease (HD) (Duyao et al., 1995; Jacobsen et al., 2011). HD is a devastating neurodegenerative disease typified by a progressive movement disorder, cognitive decline, and psychological impairment due to the death of medium spiny neurons in the striatum (Reiner et al., 1988), and other areas of the brain (Tabrizi et al., 2011). Some aspects of the disease can be recapitulated in rodent models by genetically increasing the length of the poly-Q repeat. As a platform to investigate Htt loss- and gain-of-function effects in the same genetic background, experiments were performed in syngeneic knock-in mouse embryonic stem cells (mESC) containing one copy of a humanized exon 1 (with an extended polyglutamine tract and adjacent proline-rich region; Htt-Q140/Q7), as well as knockout ($htt^{-/-}$) (Zheng et al., 2012; Nasir et al., 1995), in comparison with mESC expressing the mouse *htt* gene (Htt-Q7/7). Importantly, studies of $htt^{-/-}$ mESC afford an opportunity to assess Htt protein function because viability is maintained as pluripotent cells, despite the lethality that invariably ensues in $htt^{-/-}$ mice during embryogenesis.

The embryonic functions of the Htt protein remain essentially unknown. Notwithstanding, Htt has been implicated in diverse cell processes in multiple investigated cell types. These include, but are not limited to trafficking of growth factor complexes (del Toro

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et al., 2006; Liot et al., 2013), transcriptional regulation of a large variety of genes (Cha, 2007; Seong et al., 2010; Zuccato et al., 2001), mitotic spindle orientation (Godin et al., 2010), cell adhesion (Lo Sardo et al., 2012), endocytosis and vesicular transport (Metzler et al., 2001; Pardo et al., 2010; Qin et al., 2004; Smith et al., 2009), neuronal survival and neurogenesis (Reiner et al., 1988, 2003; White et al., 1997). Additionally, Htt protein is present in mitochondria of rat (Gutekunst et al., 1998), mouse, and human somatic cells(Choo et al., 2004) and therefore, may be important for mitochondrial activities. In human lymphoblasts, the ATP/ADP ratio, a key measure of cellular energy reserves has been correlated with the length of the poly-Q extension in Htt, even when this length is in the normal range (Seong et al., 2005). This apparent correlation raises the possibility that energy metabolism modulation may be a normal cellular role of Htt, not just a function acquired by the extension of the poly-Q region. Although Htt plays a crucial role in both embryonic development and disease initiation, the precise molecular and cellular functions of Htt in early embryonic cells and in the brain remain unknown (Woda et al., 2005). In particular, the connection between Htt and metabolism during early development is essentially unexplored to date.

In this study, we used global untargeted metabolite profiling to compare the small molecule metabolome (50–1000 Da) of mutant *htt* in mESC, both gain- and loss-of-function, in comparison with the metabolome of wild-type mESCs. Our findings demonstrate that *htt* mutation results in profound dysregulation of key metabolic pathways during pluripotency. These studies reveal that mouse $htt^{-/-}$ ESCs exhibit severe bioenergetic and metabolic defects, including an essentially complete failure of mitochondrial ATP generation, associated with defects in mitochondrial structure. Conversely, mutant mESCs with poly-Q (Q140/7) do not exhibit apparent mitochondrial structural defects, but do show metabolic alterations, including a higher respiratory capacity with wild-type levels of nucleotide mono-, di- and tri-phosphates. Our findings provide a molecular explanation for the early embryonic lethality phenotype of the $htt^{-/-}$ mouse, and reveal a previously unrecognized but essential role for Htt in supporting mitochondrial bioenergetics, metabolism and structural integrity during early embryogenesis.

Materials and methods

Cell culture

$Htt^{-/-}$, Htt-Q7/7, and Htt-Q140/7 mES cells were provided by Dr. Scott Zeitlin (University of Virginia) and propagated on 0.1% gelatin-coated plates, under serum- and feeder-free culture conditions in medium containing a 1:1 mix of DMEM-F12 (Invitrogen Cat# 12634) and neurobasal medium (Invitrogen Cat# 21103), supplemented with N2 (Invitrogen Cat# 17502), B27 supplement without Vitamin A (Invitrogen Cat# 12587), 2 mM GlutaMAX (Invitrogen Cat# 35050), 25 μg/ml of BSA (Invitrogen Cat# 15260), 10 μg/ml insulin (Sigma-Aldrich Cat# I9278), 0.1 mM β-mercaptoethanol (Invitrogen Cat# 21985), 10 ng/ml LIF (Millipore Cat# ESG1107), 0.5 μM PD0325901 (Axon Med Chem, Cat# Axon 1408) and 3 μM CHIR99021 (EMD Millipore, Cat# 361559). Cell culture medium was changed daily and the cells were passaged by trypsinization every 3 days.

Metabolite extraction for metabolomic analysis

Cells were grown in 6-well plates to 70% confluency. Culture medium and cells were collected separately for analysis by untargeted metabolite profiling. For media analysis, the medium was centrifuged at 1500 rpm for 10 min at 4 °C to remove dead cells and debris; supernatants were collected and frozen at −80 °C until the day of analysis. For analysis, media metabolites were directly extracted in 1:200 vol:vol 70% acetonitrile and aqueous 0.2% ammonium hydroxide and 3 μl of this extract was subjected to untargeted metabolite profiling by LC/MS. For analysis of cellular metabolites, cells were quickly washed twice with ice-cold PBS, followed by metabolic quenching and metabolite extraction using −70 °C 80:20 methanol:water (LC-MS grade methanol, Fisher Scientific). Cold-quenched cells were scrape-harvested using a teflon cell scraper and transferred with 80% cold MeOH to 2.0 ml Tissuelyzer tubes (Qiagen). The cell–MeOH mixtures were incubated on dry ice for 10 min then subjected to bead-beating for 45 sec using a *Tissuelyser* cell disrupter (Qiagen). Extracts were centrifuged for 5 min at 5000 rpm to pellet insoluble material and supernatants were tranfered to clean tubes. This extraction was repeated two additional times and all three supernatants were pooled, dried in a speed-vac (Savant) and stored at −80 °C until analysis. For normalization of sample analyses, post-extracted cell pellets were solubilized in 200 μl 0.2 M aqueous NaOH at 95 °C for 20 min and the pellet protein was quantified using the BioRad DC assay. On the day of metabolite analysis, dried cell extracts were reconstituted in 70% acetonitrile with 0.2% ammonium hydroxide at a relative protein concentration of 8 μg/μl and 3 μl of this reconsitued extract was injected for LC/MS-based untargted metabolite profiling.

Untargeted metabolite profiling

Both cell and culture medium extracts were analyzed by LC/MS essentially as described previously (Chen et al., 2012), using a platform comprised of an Agilent Model 1200 liquid chromatography system coupled to an Agilent Model 6230 time-of-flight MS analyzer. Metabolite separation was performed by using aqueous neutral phase gradient chromatography on a Diamond Hydride column (Microsolv) and mobile phases as follows: (A) 50% isopropanol, containing 0.025% acetic acid, and (B) 90% acetonitrile containing 5 mM ammonium acetate. Raw data were analyzed using Agilent MassHunter Qual software, and Mass Profiler Professional software. Briefly, Qual performs untargeted molecular feature extraction to generate compounds/metabolites based on the elution profile at identical mass and retention times within a specified mass accuracy (5 ppm). Aligned molecular features detected in all biological replicates from at least one cell group (i.e., $htt^{-/-}$, Htt-Q140/7 mutant and Htt-Q7/7 wild-type) were directly applied for statistical analysis across treatment groups by Mass Profiler Professional. The Bonferroni family-wise-error-rate correction was applied for multiple testing *correction* of *p*-values (corrected for $p < 0.05$).

Differentially expressed metabolite identification

Differentially expressed metabolites in *htt* mutant mESC, with fold-changes greater than 2.0 ($p < 0.05$), compared to *htt* wild-type mESC, were searched against an in-house annotated *METLIN* Personal Metabolite Database (Agilent Technologies), based on accurate monoisotopic neutral masses (< 5 ppm). A molecular formula generator (MFG) algorithm in MPP was used to generate and score empirical molecular formulae based on a weighted consideration of monoisotopic mass accuracy, isotope abundance ratios, and spacing between isotope peaks. A putative compound ID was tentatively assigned when METLIN and MFG scores concurred for a given candidate molecule. Tentatively assigned compound identities were assigned and verified based on a match of LC retention times and/or MS/MS fragmentation spectra to that of pure molecule standards in our progressively growing database.

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