CIRC*HTT*, A CIRCULAR RNA FROM THE HUNTINGTON'S DISEASE GENE LOCUS: FUNCTIONAL CHARACTERIZATION AND POSSIBLE IMPLICATIONS FOR DISEASE MODULATION

Jessica Döring[1], Simone Ferrari[1], Alan Monziani[1], Claudio Oss Pegorar[1], Takshashila Tripathi[1], Francesca Di Leva[1], Emanuela Kerschbamer[1], Virginia B. Mattis[2], Christoph Dieterich[3], Erik Dassi[4], Vanessa C. Wheeler[5], Hana Hansíková[6], Zdenka Ellederová[7], Jeremy E. Wilusz[8], Marta Biagioli[1]

[1] NeuroEpigenetics laboratory, Department of Cellular, Computational and Integrative Biology - CIBIO, University of Trento (ITALY)

[2] The Board of Governor's Regenerative Medicine Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA; Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA 90048, (U.S.A.)

[3] Section of Bioinformatics and Systems Cardiology, University Hospital Heidelberg (GERMANY)

[4] Computational biology laboratory, Department of Cellular, Computational and Integrative Biology – CIBIO, University of Trento (ITALY)

[5] Molecular Neurogenetics Unit, Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA02114, USA; Department of Neurology, Harvard Medical School, Boston, MA 02115, USA, Neurogenetics Unit, Center for Genomic Medicine, Massachusetts General Hospital, Boston (U.S.A.)

[6] Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague (CZECH REPUBLIC)

[7] Laboratory of Cell Regeneration and Plasticity, Research Center PIGMOD, Institute of Animal Physiology and Genetics, Czech Academy of Science, Libechov (CZECH REPUBLIC)

[8] Department of Biochemistry & Biophysics, University of Pennsylvania Perelman School of Medicine, Philadelphia (U.S.A.)

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This work was supported by:

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Introduction: identification of a circular RNA from the *HTT* locus

We **identified the first ever known circular RNA (circRNA) originating from the *HTT* locus** (Circ*HTT*: 484 nt, Exons 2-6, chr4:3088665-3109150) (circBASE). Circ*HTT* orthologues were found in mouse (circ*Htt*) and minipig, suggesting a possible conserved function. However, circ*HTT*'s role and implication for HD pathogenesis are not known yet. Here, we present its expression pattern in human and mouse body districts and in iPS-derived neuronal cell lines with different CAG repeats. Furthermore, we show that overexpression of the circle might modulate wild-type and mutant huntingtin expression. Our observations unveil **a new piece of HD biology/pathology** and might pave the way for future studies of innovative avenues for therapeutic intervention.

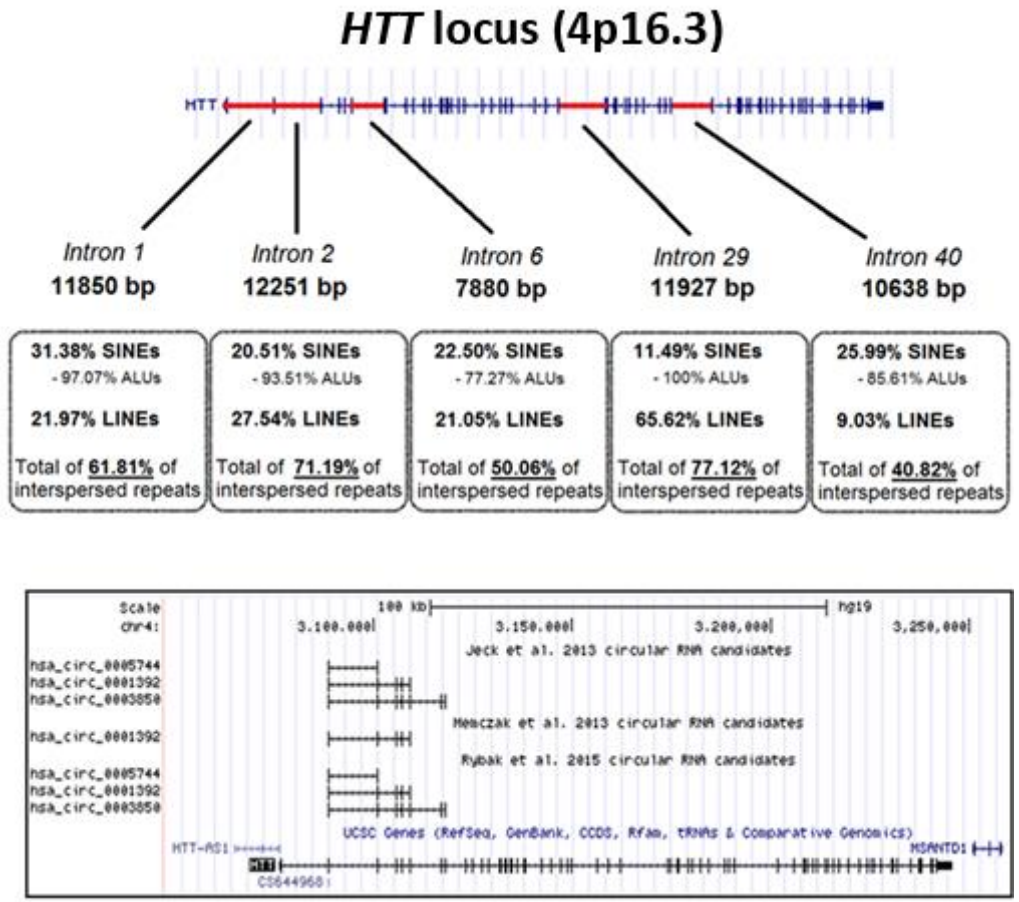

Figure 1. circ*HTT* identification from human circRNA-seq database. Long introns (> 7500 bp), here in red, and the presence of interspersed repeats – calculated with RepeatMasker tool – are known hallmarks for circular RNAs biogenesis. CircBASE tracks depict the annotated circular RNAs stemming from the *HTT* locus.

Circ*HTT* detection and *proof-of-circularity*

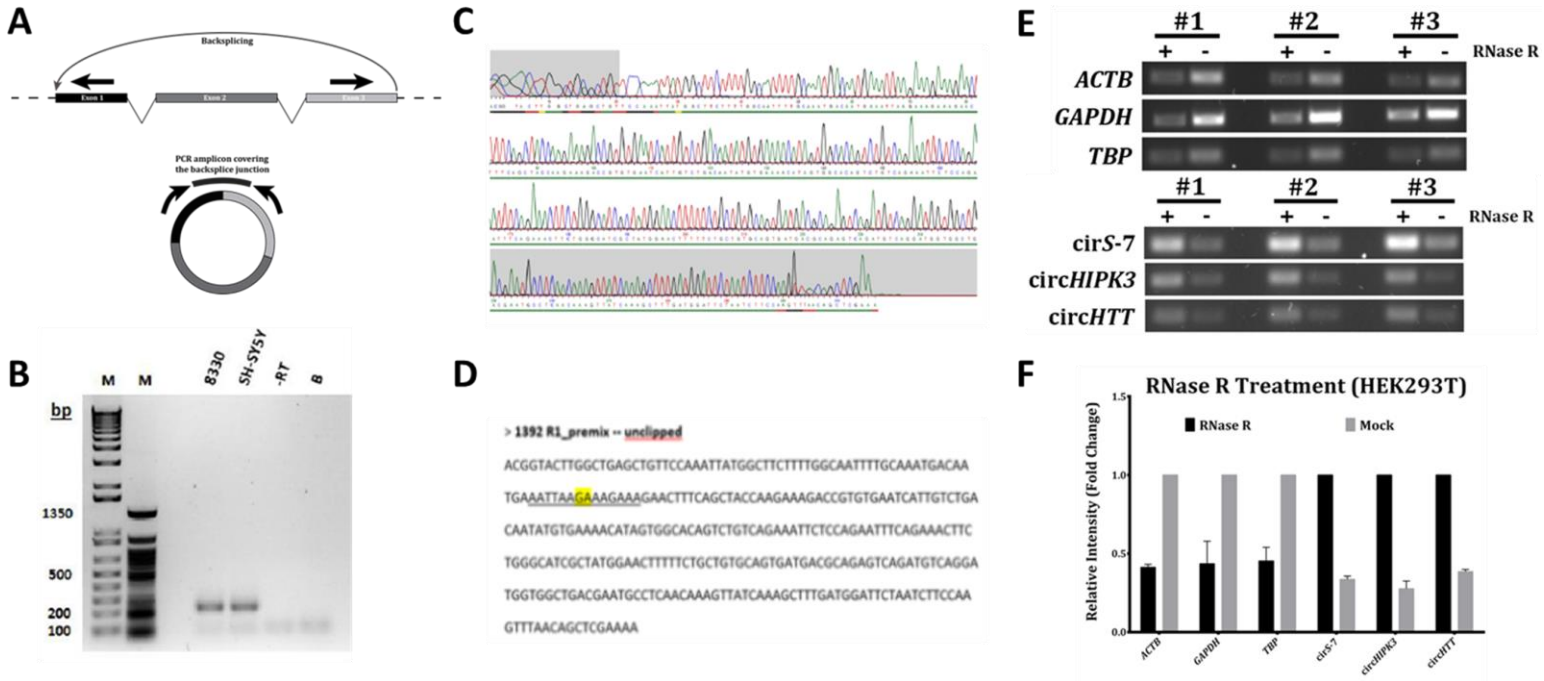

Figure 2. circ*HTT* detection in human cell lines and *proof-of-circularity.* **A.** The schematic drawing is showing divergent primers, producing an amplicon, spanning the unique backsplice junction (bottom). **B.** End-Point RT-PCR experiments show amplification of circ*HTT* in human iPS-derived NPC (8330-8) and human neuroblastoma cell lines (SH-SY5Y). **C-D.** All circ*HTT* amplicons are specific, correctly spanning the backsplicing junction as confirmed by Sanger sequencing. Backsplice junction is marked in yellow and underlined]. **E-F.** Following RNase R treatment, equal amounts of treated and untreated RNA samples were subjected to RT-PCR. Images report PCR amplified bands of linear RNAs (*ACTB, GAPDH, TBP*) and circular RNAs (*cirS-7* and *circHIPK3*), as well as circ*HTT* (E, bottom). While linear RNA are depleted in RNase R treated (+) conditions (Fig. E, top panel), circRNAs show an enrichment. (F) The bar graph reports amplicon RT-PCR bands quantification by Image Lab, and the conditions presenting the highest intensities were used as normalizers (F).

Results 1. circ*HTT/Htt* is mainly expressed in CNS in both human and mouse

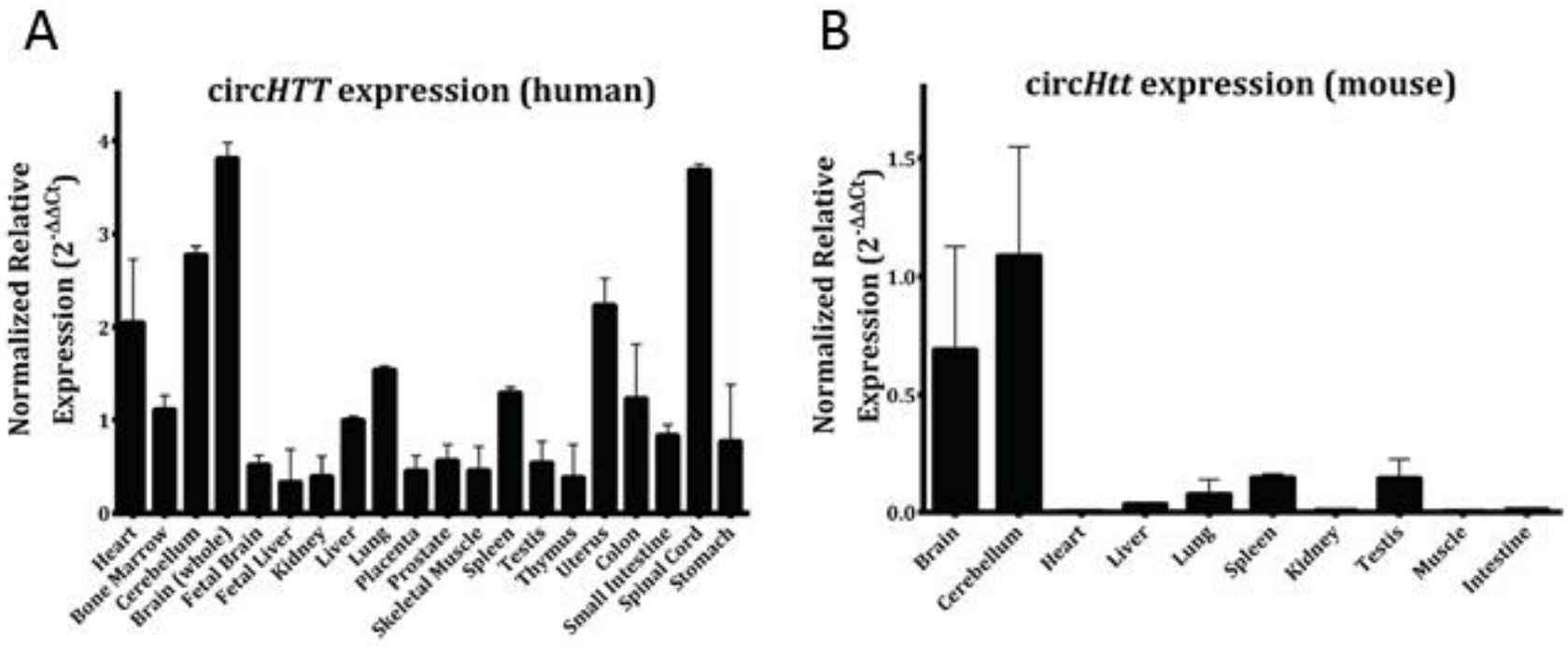

Figure 3. Circ*HTT/Htt* expression in different human and mouse tissues. To explore circ*HTT*/*Htt* expression profile in different human and mouse tissues, human RNA tissues panel were purchased from Clontech and RNA extracted from a panel of tissues were used. Here, we report that circ*HTT*/*Htt* is expressed within all analyzed body districts, with higher levels within CNS (whole brain, cerebellum and spinal cord) and heart. **A.** Interestingly, the levels dramatically drop in fetal compared to adult brain in human. **B.** In agreement with human data, circ*Htt* is predominantly expressed in brain and cerebellum, while it is lowly expressed in the rest of the analyzed body district.

Results 2. circ*HTT* expression increases with increasing CAG repeats in human terminally differentiated neurons

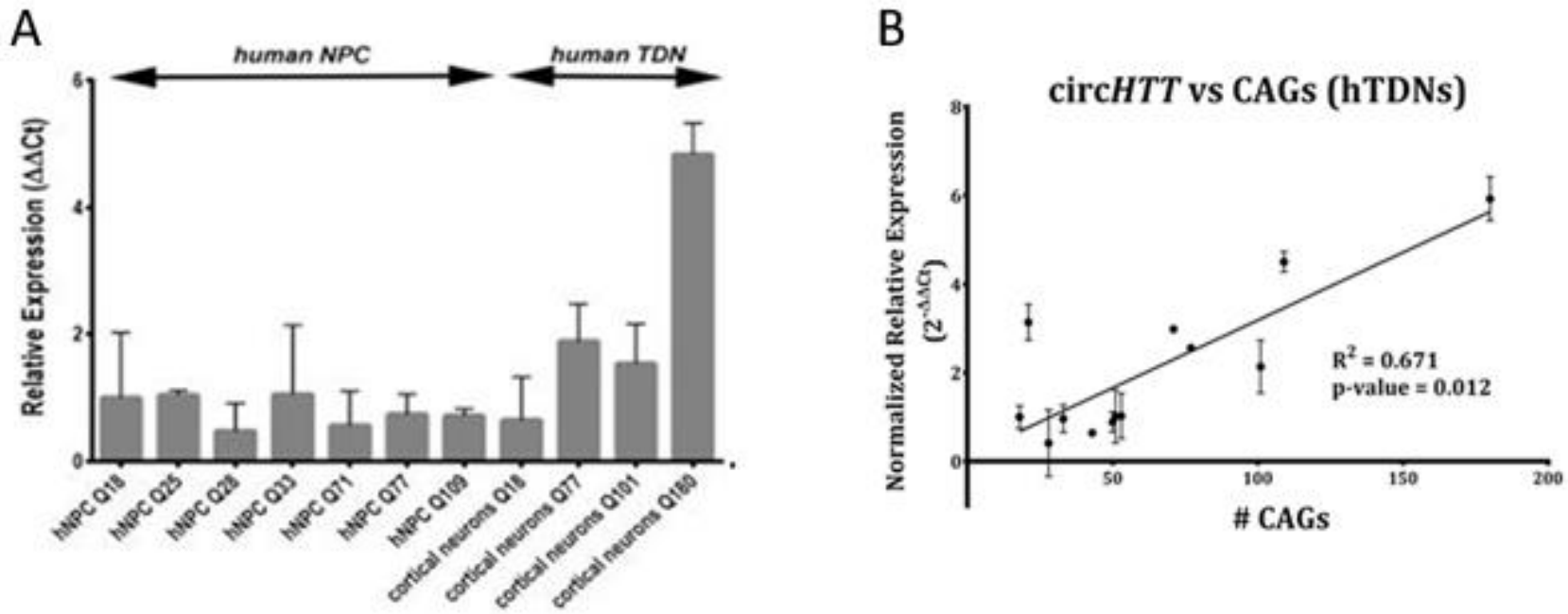

Figure 4. circ*HTT* expression in iPS-derived neuronal cell lines with elongated CAG sizes. To determine whether circ*HTT*'s expression occurs in a CAG repeat dependent manner in iPS-derived neuronal cell lines, thanks to a collaboration with Dr. Virginia B. Mattis (Department of Biomedical Sciences, Cedars-Sinai Hospital, Los Angeles, USA), we obtained two sets of hiPSCs-derived neural progenitor cells (hNPCs) as well as three independent collections of terminally-differentiated neurons (hTDNs) carrying different polyglutamine tracts in one single *HTT* allele, ranging from wild type (CAGs < 36) to very juvenile forms of the disease (CAGs > 100). **A.** We demonstrate that circ*HTT* is expressed in iPS-derived neuronal cell lines [neural progenitors (NPC) and terminal differentiated cortical neurons (hTDN)]. **The levels of circ*HTT* expression in hTDN are elevated compared to NPC**. **B. circ*HTT* expression increases with the number of CAG repeats in HD patients iPSCs-derived terminally-differentiated neurons**. A significant correlation between circ*HTT* expression and CAG repeats size is detected within hTDNs is shown.

Results 3. circ*Htt* is significantly more expressed in brain districts of Q111 and zQ175 knock-in mice

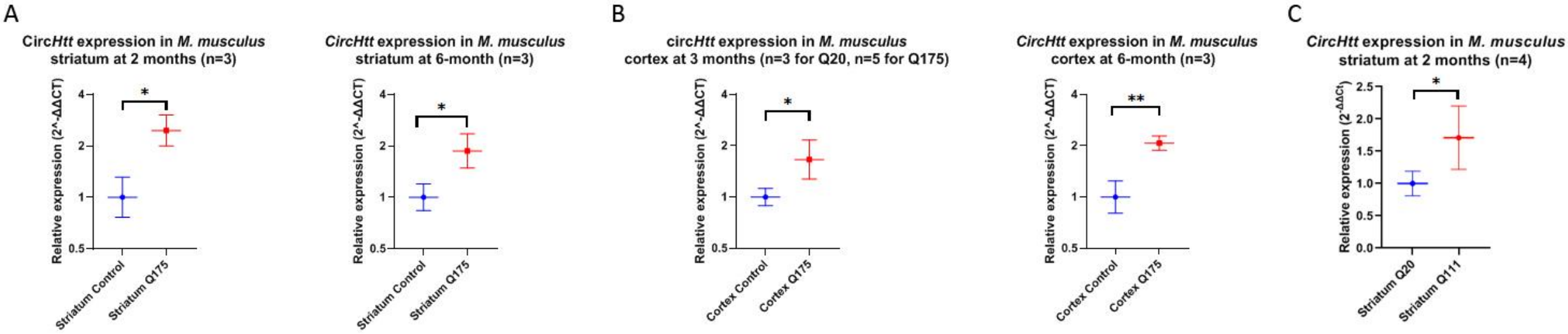

Figure 5. Circ*Htt* expression in wild-type and mutant mouse brains. To investigate the levels of expression of circ*Htt* in brain districts of different HD-mimicking animals, wild-type, knock-in C57BL/6J Q20, Q111, and zQ175 mice were used as ideal model systems. Striatal tissues of Q20 and Q111 mice were kindly provided by Dr. Wheeler Vanessa C. (Massachusetts General Hospital, Boston, USA). **A.** C*ircHtt* expression levels are significantly higher in mutant striatum (Q175) than in wild-type striatum (Q7) at both 2 and 6 months old stages. **B.** Similarly, circ*Htt* cortical levels were higher in mutant than in wildtype mice at 6 months. **C.** Additional experiments comparing Q20 and Q111 mouse striatum at 2 months indicated a significantly higher expression of circ*Htt* in mutant mice.

Results 4. circ*HTT*/*Htt* overexpression affects huntingtin protein levels, with no alteration of the *HTT*/*Htt* mRNA

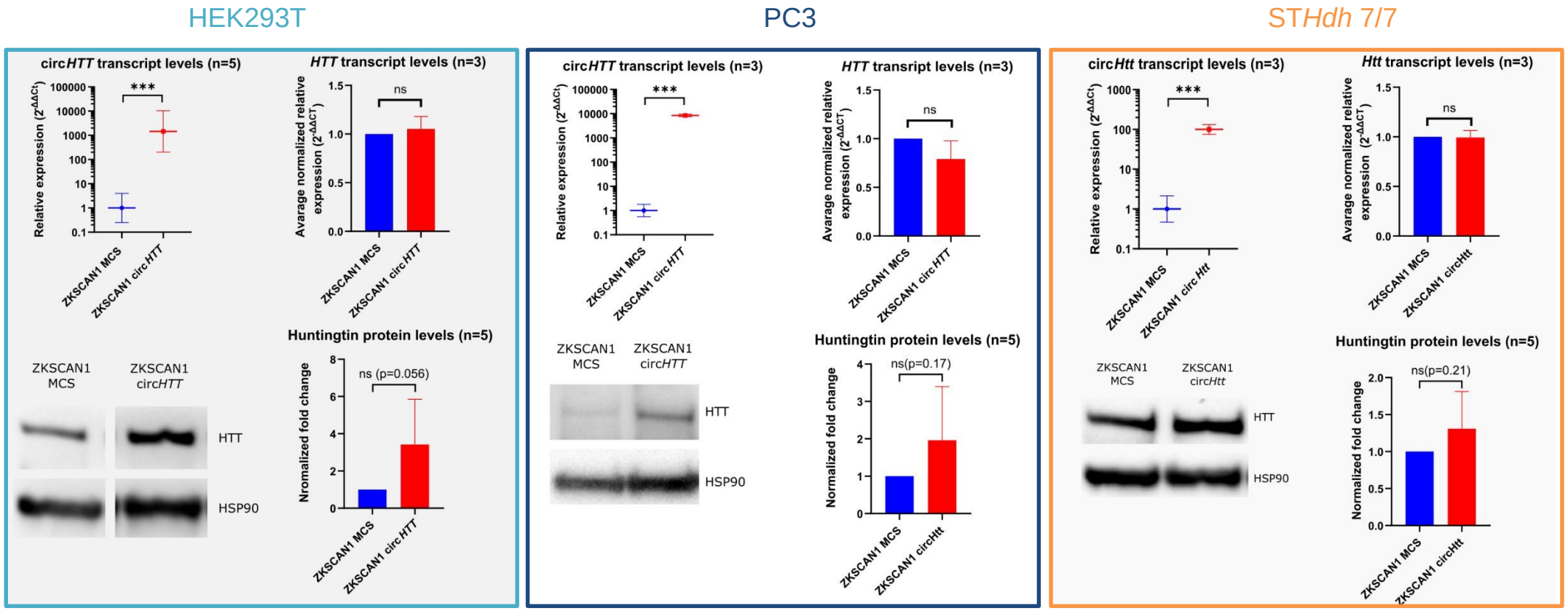

Figure 6. Effects of circ*HTT*/circ*Htt* overexpression in HEK293T, PC3, and ST*Hdh* Q7/7 cells. To characterize the function of circ*HTT*/circ*Htt*, the circular RNA was overexpressed in human and mouse cell lines by cloning its sequence into the ZKSCAN1 MCS Exon vector (Addgene #69901), kindly provided by Prof. Jeremy E. Wilusz. Cells were transfected with the ZKSCAN1-circ*HTT*/*Htt* or the ZKSCAN1 MCS vector, as control. HEK293T (left panel), PC3 (middle panel), and mouse striatal cell line mST*Hdh* Q7/7 (right panel) were used. We show that **circ*HTT*/circ*Htt* overexpression in human and mouse cells increases wild-type huntingtin protein levels with no alteration of the endogenous *HTT*/*Htt* linear transcript.**

Results 5. circ*Htt* overexpression affects huntingtin protein levels, with no alteration of the *Htt* mRNA

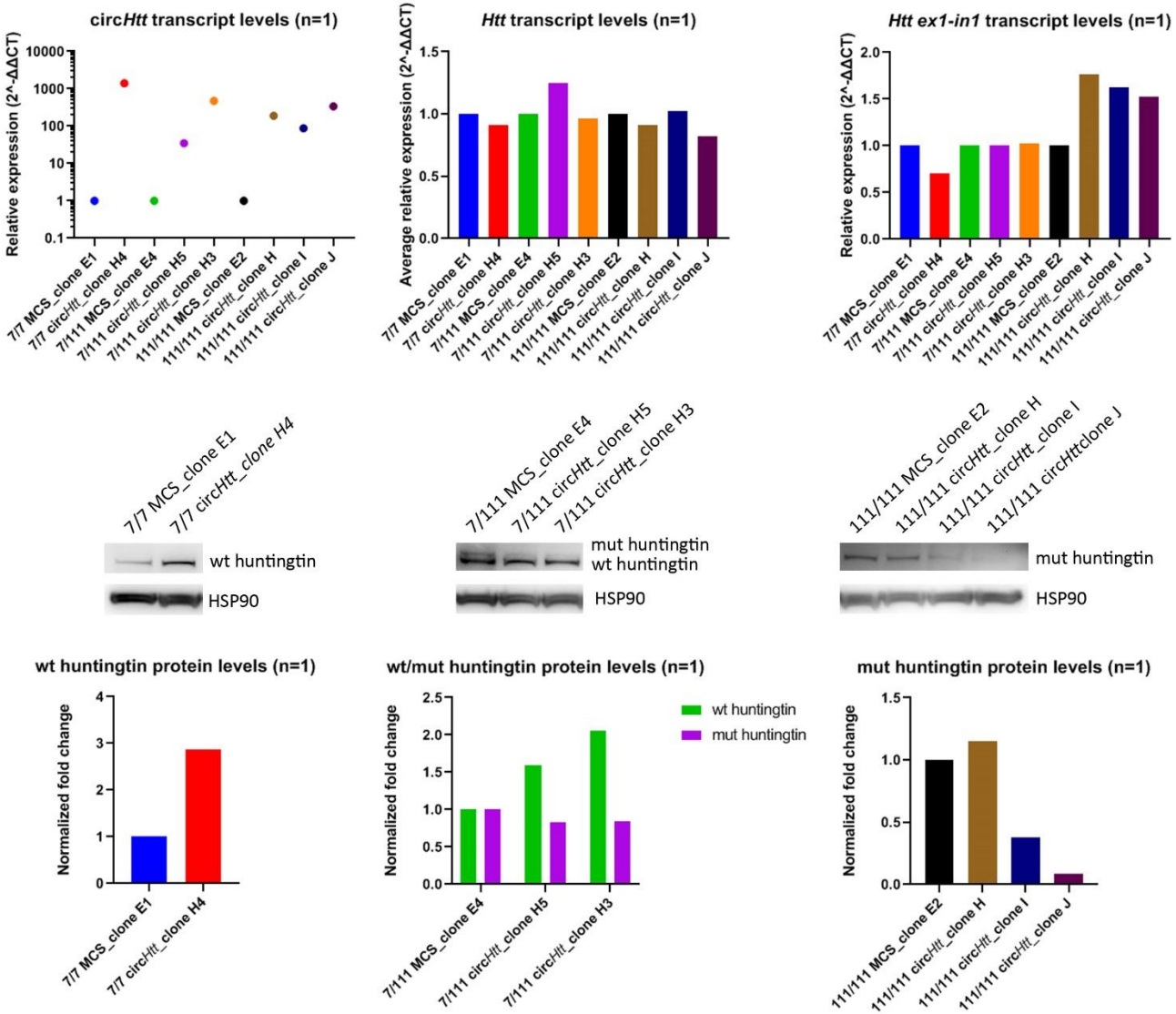

Figure 7. Effects of circ*Htt* overexpression in monoclonal ST*Hdh* Q7/7, Q7/111, and Q111/111 cell lines. To characterize the function of circ*Htt*, the circular RNA was overexpressed in mouse striatal cell lines (mST*Hdh* Q7/7, Q7/111, and Q111/111) expressing wild type (Q7/7), heterozygous (Q7/111), and homozygous mutant (Q111/111) by cloning its sequence into the ZKSCAN1 MCS Exon vector (Addgene #69901), kindly provided by Prof. Jeremy E. Wilusz. Cells were transfected with the ZKSCAN1-circ*HTT*/*Htt* or the ZKSCAN1 MCS vector, as control. In order to produce a more controlled and homogenous system in mST*Hdh*, we plated single-cells in 96-wells to obtain monoclonal cell lines. **Apparently, overexpression of the circular RNA might increase wild-type huntingtin, while decreasing mutant huntingtin protein, with no alteration of the *Htt* transcript level.**

Conclusion

We identified a **circular RNA** stemming from the *HTT* locus that is expressed in a **CAG repeat dependent manner in brain districts and neurons,** typical hallmark of HD pathology. We suggest that circ*HTT*/*Htt* might modulate wild-type and mutant huntingtin translation without affecting the transcription. These observations will pave the way for future studies of innovative avenues for therapeutic intervention to treat Huntington's Disease.

Acknowledgements

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