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Analysis of white adipose tissue gene expression reveals CREB1 pathway altered in Huntington´s disease

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Gene expression changes in HD white adipose tissue

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Abstract

BACKGROUND: In addition to classical neurological symptoms, Huntington´s disease (HD) is complicated by peripheral pathology and both the mutant gene and the protein are found in cells and tissues throughout the body. Despite the adipose tissue gene expression alterations described in HD mouse models, adipose tissue and its gene expression signature have not been previously explored in human HD.

OBJECTIVE: We investigated gene expression signatures in subcutaneous adipose tissue obtained from control subjects, premanifest HD gene carriers and manifest HD subjects with the aim to identify gene expression changes and signalling pathway alterations in adipose tissue relevant to HD.

METHODS: Gene expression was assessed using Affymetrix GeneChip® Human Gene 1.0 ST Array. Target genes were technically validated using real-time quantitative PCR and the expression signature was validated in an independent subject cohort.

RESULTS: In subcutaneous adipose tissue, more than 500 genes were significantly different in premanifest HD subjects as compared to healthy controls. Pathway analysis suggests that the differentially expressed genes found here in HD adipose tissue are involved in fatty acid metabolism pathways, angiotensin signalling pathways and immune pathways. Transcription factor analysis highlights CREB1. Using RT-qPCR, we found that *MAL2*, *AGTR2*, *COBL* and the transcription factor *CREB1* were significantly upregulated, with *CREB1* and *AGT* also being significantly upregulated in a separate cohort.

CONCLUSIONS: Distinct gene expression profiles can be seen in HD subcutaneous adipose tissue, with CREB1 highlighted as a key transcription factor.

Keywords:

Huntington's disease, adipose tissue, gene expression, CREB1

Introduction

Traditionally, Huntington´s disease (HD) has been defined as a condition caused by selective neurodegeneration of the basal ganglia and cerebral cortex in the brain [1]. However, in addition to classical neurological symptoms, HD is complicated by peripheral pathology, including weight loss, altered body composition and skeletal muscle wasting [2], and both the mutant gene and protein are found in cells and tissues throughout the body [2-4].

In several HD mouse models there is, despite weight loss, an enhanced accumulation of body fat in midlife [5-7]. Both the R6/2 and CAG140 strains exhibit age-dependent alterations in white adipose tissue mass [5-7] and white adipose tissue abnormalities are progressive [7], accompanied by impaired expression of mature adipocyte genes in both HD mouse models [7]. Importantly, some of these changes occur prior to weight loss and development of some of the characteristic neurological symptoms [5, 7]. Interestingly, a direct effect of mutant huntingtin in adipocytes has also been demonstrated. Gene expression alterations in mouse adipocytes can be replicated in an adipocyte cell line expressing mutant huntingtin with 103 CAG repeats, mimicking the gene expression changes that have been observed in HD mice [7].

White adipose tissue not only influences whole body energy metabolism by being a source of energy, but also through being a large endocrine organ [8]. Interestingly, several peripheral features of HD, such as endocrine abnormalities, muscle wasting, weight loss and low-grade inflammation [2, 9] could all potentially be influenced by changes in adipose tissue function.

Changes in gene expression in brain, blood and peripheral tissues, such as skeletal muscle, have been shown in human HD as well as in HD mice [10]. However, human HD adipose tissue has not previously been studied and we therefore set out to analyse white adipose tissue samples from control subjects, premanifest HD subjects, and manifest HD subjects, in order to identify HD-related gene expression signatures. Using affymetrix and subsequent qPCR validation we could identify early gene expression changes in human HD adipose tissue.

Materials and Methods

Patient demographics, and biopsies

The study was conducted at the Royal Free London NHS Foundation Trust. Participants were recruited through the HD Multidisciplinary Clinic at the National Hospital for Neurology and Neurosurgery, London, UK. Written informed consent was obtained from all subjects. Each participant signed a consent form with specific sections for every procedure, including the adipose tissue biopsy. The study protocol (including consent procedure) was approved by the joint University College London (UCL) / University College London Hospitals (UCLH) ethics committee and was conducted in accordance with the Declaration of Helsinki. Patients were eligible for enrolment if they were 18 years of age or older, had completed either a predictive test for premanifest subjects, or had a confirmed genotype consistent with HD (CAG repeat ≥40). Controls were recruited principally from the partners, spouses, or carers of the HD group and exclusion criteria were the same as for the HD group.

Cohort 1: Subcutaneous adipose tissue biopsies (38 in total, Table 1) were obtained from 13 premanifest HD gene carriers (5 male, 8 female), 11 stage II/III HD patients (7 male, 4

female) and 14 control subjects (8 male, 6 female) by needle aspiration from the buttock under local, topical anaesthetic. The patients were fasted overnight prior to the procedure. The samples were snap frozen in liquid nitrogen and stored at -80°C prior to analysis.
For circulating Angiotensin II levels, non-fasting plasma samples were collected in the morning from the same subjects as cohort 1 with one to two additional participants per group: 14 premanifest HD gene carriers (5 male, 9 female), 13 stage II/III HD patients (8 male, 5 female) and 15 controls (9 male, 6 female) (see [11] for more information on cohort 1).

Cohort 2: Adipose samples (28 in total, Table 2) were taken from an open biopsy of the subcutaneous adipose overlying the vastus lateralis in the left thigh under an injected local anaesthetic from 9 premanifest HD gene carriers (5 male, 4 female), 9 stage I/II HD patients (5 male, 4 female) and 10 control subjects (5 male, 5 female). The patients were fasted overnight prior to the procedure. The samples were snap frozen in liquid nitrogen and stored at -80°C prior to analysis.

RNA extraction

RNA was extracted using either the RNeasy Lipid Tissue Mini Kit (Qiagen) (cohort 1) or E.Z.N.A. Total RNA Kit II (VWR) (cohort 2) following manufacturer's protocol. Briefly, snap frozen tissues were homogenized in lysis buffer using a Fastprep-24 homogeniser (MP Biomedicals) at 4 m/s for 40 sec prior to RNA extraction via spin column methods. RNA concentration and quality were measured using a Nanodrop spectrophotometer (Thermo Scientific). RNA integrity was analysed by Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA), and only samples with RIN values greater than or equal to 5 were utilised for

affymetrix analysis. For RT-qPCR validations, RNA was then reverse transcribed to cDNA using iScript™ cDNA Synthesis Kit (BioRad) and stored at -20°C. For further validations, RNA was reverse transcribed to cDNA using QuantiTect Whole Transcriptome Kit (Qiagen) and stored at -20°C.

Affymetrix

Gene expression analysis was performed on samples with RIN values greater than or equal to 5.0, using Affymetrix GeneChip® Human Gene 1.0 ST Array and RT-qPCR. This group included 8 premanifest (2 male, 6 female), 5 stage II/III HD patients (1 male, 4 females) and 7 control subjects (3 males, 4 females) (Table 3). The affyemtrix data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [12] and are accessible through GEO Series accession number GSE73655 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73655).

Microarray Data Analysis

Microarray data were initially pre-processed and normalized using Robust Multi-array Analysis (RMA) method [13]. These analyses were performed using Affymetrix Expression Console Software v1.1.2. Non-annotated probe sets and probe sets that did not have a signal intensity above the negative control intensity signal median in each group were excluded. Replicate probe sets were merged by the median of signal intensity values.

To identify significantly differentially expressed genes between groups, we used Significance Analysis of Microarrays (SAM) method [14]. SAM analysis was performed using TMEV v4.0 software.

We selected differentially expressed genes having a q-value < 10% for the Pathway analysis, which was performed using MetaCore™ pathway analysis software [15].

Validation

Significantly altered genes from premanifest HD versus controls were selected from the SAM analysis to confirm affymetrix findings by RT-qPCR. Validations were initially carried out using RT-qPCR on samples from cohort 1; both the samples with RIN ≥ 5 used for above affymetrix and on all remaining samples: 5 premanifest (3 male, 2 female); 6 stage II/III HD patients (6 male, 0 female); and 7 control subjects (5 male, 2 female). Further validations were then performed on a separate cohort (cohort 2): 9 premanifest (5 male, 4 female); 9 stage II/III HD patients (5 male, 4 female); and 10 control subjects (5 male, 5 female) (See Tables 1 - 3). For RT-qPCR experiments, all samples were run in triplicate for each target gene and housekeeping gene, and relevant negative and positive controls were run on each plate. Melt curves were inspected for all assays, with the Tm checked to be within known specifications for each assay. Sample assay data points were included in data analysis only if detected with Ct < 37 and at least 3 Ct values lower than the corresponding negative control [16]. Any data that did not pass these criteria were omitted from all further analyses. Primers utilised for RT-qPCR validations (see Table 4) were designed using either QuantPrime [17], Primer3 [18, 19] or PrimerQuest from Integrated DNA Technologies (http://eu.idtdna.com/PrimerQuest).

Circulating Angiotensin II levels

Angiotensin II was measured in duplicate by an Angiotensin II Enzyme Immunoassay (EIA)

Kit (Sigma-Aldrich) according to the manufacturer's instructions using non-fasting plasma samples taken in the morning (see [11]).

Statistics

Validations: RT-qPCR analysis was performed using Bio-Rad CFX Manager 3.1 software (BioRad). Data were analyzed using the ΔΔCt method [20] and normalized to the housekeeping genes Peptidylprolyl Isomerase A (Cyclophilin A) (*PPIA*), *18S* ribosomal RNA (cohort 2) and also hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) (cohort 1). Student's t-test was used for comparisons in gene expression levels and a critical value of $P < 0.05$ was used as a significance threshold.

The non-parametric Kruskal-Wallis test was used to assess group differences in plasma angiotensin II levels.

Results

Gene expression data analysis

In order to investigate possible gene expression alterations in HD subcutaneous adipose tissue, gene expression analysis was performed on RNA extracted from subcutaneous adipose tissue from control subjects and HD gene carriers (premanifest HD and HD stage II/III) (see Table 1-3) using the Affymetrix platform.

SAM analysis was performed to identify significantly differentially expressed genes between groups. Based on the selected criteria, more than 500 genes were found to be significantly altered in premanifest HD patients compared to healthy controls (See Table 5 for top 10, Supplementary Table 1 for complete data list). Of these significantly altered genes, a total of

227 top significant genes having a q-value <10% from premanifest HD vs. controls in the SAM analysis (see Supplementary Table 1) were selected for further data analysis. Principal components analysis of this dataset revealed distinct grouping of the control and premanifest HD patients (Fig. 1). We next performed hierarchical clustering, using Pearson correlation as distance matrix and average linkage, on our premanifest HD versus control data (Fig. 2). This yielded two major clusters; one representing the control group and the other representing premanifest HD patients, suggesting that the molecular signature of subcutaneous adipose tissue from HD gene carriers can be readily distinguished from that of healthy controls.

In this study we had a limited n number for the stage II/III group in the affymetrix experiment. Therefore, we are careful with the interpretation of the data in the SAM analysis for stage II/III HD vs. controls, which did not result in any significantly differentially expressed genes (Supplementary Table 2).

Pathway and Transcription factor analyses

MetaCore™ pathway analysis results from control vs. premanifest HD suggest that the differentially expressed genes are involved in angiotensin system maturation, fatty acid synthesis and metabolic pathways (see Table 6).

To gain further insight into which factors might facilitate regulation of gene expression in HD subcutaneous adipose tissue we performed transcription factor analysis using MetaCore™. MetaCore™ analysis showed cAMP responsive element binding protein 1 (CREB1) to be a highly significant transcription factor and also showed it as central node for 35 of the 100 top differentially expressed genes between healthy controls and premanifest HD gene carriers (Table 7, Fig. 3, the 35 transcripts are summarized in Supplementary Table 3). We

further ran our data through KEGG and REACTOME databases, however, these databases yielded no more information as compared to the commercial MetaCore™ software (data not shown).

RT-qPCR

Among the most significantly changed genes, based on the affymetrix data, fifteen genes were selected for validation using RT-qPCR. Since CREB1 was identified in the above described transcription factor analysis and angiotensin signalling pathway was highlighted, we included *CREB1*, Angiotensin II Receptor, Type 1 (*AGTR1*) and Angiotensin II Receptor, Type 2 (*AGTR2*) in our validation. The RT-qPCR was conducted on samples from 2 different cohorts, cohort 1: 13 premanifest HD gene carriers, 11 stage II/III HD patients and 14 control subjects (Table 1); cohort 2: 9 premanifest HD gene carriers, 9 stage I/II HD patients and 10 control subjects (Table 2). Since the samples obtained were taken from different adipose depots (buttock and thigh, respectively), we analysed data as two separate cohorts. Previous studies have indicated that adipose tissue displays depot specific gene/protein expression in both mice [21] and humans [22, 23].

Using this approach, we could show a significant upregulation of the *CREB1* gene in both cohorts (see Fig. 4 and 5), supporting our transcription factor analysis, which suggests altered CREB1 in premanifest HD versus controls.

First, we performed validation RT-qPCR on all samples (controls, premanifest HD and stage II/III HD patients selected for affymetrix in addition to the remaining samples) from cohort 1 (Fig. 4). Here, we were able to confirm the affymetrix findings that showed significantly upregulated gene expression of cordon-bleu WH2 repeat protein (*COBL*), corticotropin

releasing hormone binding protein (*CHRBP*) and Mal, T-cell differentiation protein 2 (*MAL2*) in the premanifest HD group. In line with our transcription factor analysis, *CREB1* is significantly altered in both premanifest HD and stage II/III HD patients. We also observed significant upregulation of *AGTR2* in both the premanifest HD and stage II/III HD patients.

Next, we repeated the validation RT-qPCR on a second sample cohort, cohort 2, comprising controls, premanifest HD and stage I/II HD patients (Fig. 5). Here, we could confirm our affymetrix data showing a significant downregulation of WNT1 inducible signalling pathway protein 2 (*WISP2*) and upregulation of angiotensinogen (*AGT*) in HD gene carriers. In line with both our transcription factor analysis and cohort 1 validation RT-qPCR, we also observed significant upregulation of *CREB1* in our stage II/III HD patients versus controls. A summary of our qPCR results, with a comparison to the affymetrix results can be found in Table 8.

Circulating angiotensin II levels

Adipose tissue exerts the largest source of extra hepatic circulating angiotensin [24], and here we found angiotensin pathways to be altered along with significant upregulation of *AGT* alongside, *AGTR2* gene expression. We therefore investigated whether circulating levels of angiotensin II were altered. Plasma samples collected from cohort 1 [11] were analyzed for possible alterations in angiotensin II levels between the three groups. In this cohort, we could detect no significant differences between the healthy controls, premanifest HD gene carriers and stage II/III HD patients (p 0.144, Kruskal-Wallis) (Fig. 6).

Discussion

Adipose tissue is a key determinant of whole body metabolism and energy homeostasis. Evidence of abnormal energy metabolism in the early stages of HD has accumulated for both HD patients and mouse models [25]. Increased understanding of adipose tissue gene expression in human HD could potentially shed light on important physiological pathways for further investigation.

In HD mice, altered body composition with increased fat deposits, alongside reduced lean mass has been shown [5]. White adipose tissue gene expression changes have been shown to be progressive in two different HD mouse models [7]. Our affymetrix data illustrates the possibility of distinguishing between control adipose tissue and premanifest HD adipose tissue by principal components analysis (Fig. 1) and hierarchical cluster analysis (Fig. 2). This indicates that human HD subcutaneous adipose tissue has a distinct molecular profile from that of control tissue.

In R6/2 mice, altered functional adipocyte capacity with impaired noradrenaline-stimulated lipolysis has been shown [5]. In line with this, pathway analyses here highlight fatty acid metabolism to be altered in human HD subcutaneous adipose tissue. Whilst there was no detectable alteration in plasma levels of free fatty acids, triglycerides or lipoproteins in HD patients from this subject cohort [26], it is possible that a challenge, for example noradrenaline-stimulation of lipolysis, is required in order to elicit a phenotype [27].

We were able to identify several transcription factors that regulate a substantial proportion of the differentially expressed transcripts as determined by affymetrix analysis of premanifest HD versus control subcutaneous white adipose tissue. Of the 100 most significantly altered transcripts, *CREB1* was found to regulate 35 transcripts, while c-Myc and ESR1 were found to regulate 27 and 21 transcripts, respectively. *CREB1* has been shown to

be activated in adipocytes under obese conditions where it can lead to insulin resistance and type II diabetes through its modulation of lipid metabolism [28]. Further, CREB has been demonstrated as a primary regulator of adipogenesis and induces adipogenesis in the 3T3-L1 preadipocyte cell line [29]. Interestingly, metabolic disturbances have been shown in HD [30-32] and HD patients have been shown to display a higher incidence of impaired glucose tolerance the general population [33]. c-Myc has been shown to play a role in adipogenesis, in that expression of myc in 3T3-L1 cells prevented adipogenesis by inhibiting C/EBPα [34], while ESR1 knockout mice display significant increases in epididymal, perirenal and inguinal white adipose tissue depots in both males and females, with no changes observed in brown adipose tissue [35]. Since altered fat distribution and adipocyte function is observed in HD mice [5, 7], the importance of these transcription factors in HD adipose tissue warrants further study. Due to small sample sizes of adipose tissue collected, this study focussed on gene expression data from white adipose tissue only. Further studies into human HD adipose tissue are therefore warranted, such as investigation of protein levels.

Adipose tissue angiotensin has recently become recognized as a contributor to metabolic regulation [24]. The local adipose renin-angiotensin-system exerts important functions in modulating lipogenesis, lipolysis, adipogenesis as well as systemic and adipose tissue inflammation [36, 37]. Here, we show a significant increase in *AGTR2* expression in early HD from our RT-qPCR validations in subcutaneous adipose tissue samples from cohort 1. *Agtr2* has been shown to promote differentiation of subcutaneous preadipocytes [38] and *AGTR2* expression is elevated following the induction of adipocyte differentiation [39]. Further, *Agtr2* has been indicated as playing a key role in angiotensin II-induced development of fat

mass in mice and adipose tissue inflammation [40]. Interestingly, an altered inflammatory response is seen both centrally and peripherally in HD patients and mouse models of disease [41]. We were unable to replicate this alteration in cohort 2, however, this may be due to the different adipose depot samples for this cohort, since different depots have been shown previously to display altered gene expression profiles in humans [22, 23]. As such, further studies into HD adipose tissue are warranted to further examine possible alterations.

Adipose tissue exerts the largest source of extra hepatic circulating angiotensin, and adipose tissue–derived angiotensin has been suggested to be a factor contributing to the association between obesity and hypertension [24], with plasma levels of both angiotensinogen and angiotensin II showing positive correlations with BMI [42]. In our study, however, plasma angiotensin II levels are not altered with disease state. Whether altered adipose tissue angiotensin signalling alters adipose tissue function in HD warrants further studies.

Using tissue biopsies from subject cohorts is a challenging source of research material. Many factors, such as for example gender, age, diet, alcohol consumption, smoking and medication are likely to contribute to biological variance of any features investigated within the biopsy material. Importantly, previous studies have indicated that adipose tissue displays depot specific gene/protein expression in both mice [21] and humans [22, 23], which could here contribute to the gene expression differences obtained in the 2 different subject cohorts.

The study presented here has the limitation of a small subject number within the cohorts investigated and there is a great need of further studies using larger subject cohorts. Key gene expression changes and their role to mutant huntingtin, as well as possible functional consequences, need to be investigated further in for example primary cell cultures of HD

adipocytes. Of the genes identified as significantly altered in HD by affymetrix, we were unable to validate all of those chosen for RT-qPCR. Similar discrepancies between microarray and RT-qPCR have been previously reported along with possible explanations for such discrepancies, including different sensitivity and specificity between the methods as well as differing normalization strategies employed for each [43-45]. This could also in our study provide an explanation for why we are unable to validate all findings (as well as for some targets, finding expression alterations in different directions).

To our knowledge, this is the first study to examine adipose tissue from human HD patients for molecular alterations. Our data suggest gene expression alterations linked to fatty acid metabolism, differentiation, angiotensin signalling and immune pathways in human HD adipose tissue, and highlight a key role of the transcription factor CREB1 in HD adipose tissue. These findings contribute to the growing body of evidence that peripheral tissues are valuable resources for studying molecular mechanisms of HD.

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Conflict of Interest

The authors have no conflict of interest to report.

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Tables

Table 1. Subject demographics: cohort 1

Group	N (M/F)	Mean Age/years	Mean CAG	Mean BMI
Control	14 (8/6)	52.4 (3.17)	-	25.9 (1.20)
Premanifest HD	13 (5/8)	45.8 (1.72)	42 (0.39)	29.1 (1.34)
Stage II/III HD	11 (7/4)	55.9 (2.76)	43 (0.60)	26.3 (1.01)

Subject demographics for all samples collected from cohort 1. N: number; M: male; F: female. Data for age, CAG and BMI are presented as mean (SEM).

Table 2. Subject demographics: cohort 2

Group	N (M/F)	Mean Age/years	Mean CAG	Mean BMI
Control	10 (5/5)	42.0 (2.27)	-	24.2 (1.28)
Premanifest HD	9 (5/4)	41.1 (2.38)	44 (0.75)	24.2 (0.95)
Stage I/II HD	9 (5/4)	46.6 (1.90)	44 (0.40)	24.6 (1.01)

Subject demographics for samples collected from cohort 2. N: number; M: male; F: female.

Data for age, CAG and BMI are presented as mean (SEM).

Table 3. Subject demographics: affymetrix

Group	N (M/F)	Mean Age/years	Mean CAG	Mean BMI
Control	7 (3/4)	54.9 (3.56)	-	26.2 (2.23)
Premanifest HD	8 (2/6)	45.4 (3.15)	42 (0.49)	28.8 (1.58)
Stage II/III HD	5 (1/4)	56.0 (3.83)	42 (0.00)	27.3 (0.51)

Subject demographics for samples selected, based on RNA integrity (RIN ≥ 5), for affymetrix analysis from cohort 1. N: number; M: male; F: female. Data for age, CAG and BMI are presented as mean (SEM).

Table 4. Primer sequences

Target	Forward primer	Reverse primer
ADIPOR2	GGGTCTCTCGGCTCTTCTCTAAAC	TGCCCAGCACACAGATGACAATC
ADRA2A	GTCAGCACATGTTGCTAATGACAG	ACAGACAAGAGGACCACTCCATTC
AGT	GATGTTGCTGCTGAGAAGATTG	AGTGGACGTAGGTGTTGAAAG
AGTR1	AGAAATGCAATCTCCCTAGCC	CATCTCCTGTTGCTCCTCTAAC
AGTR2	CACCAACAGCTGCGTTAATCCG	ACACACTGCGGAGCTTCTGTTG
CASQ2	AATCACAGCACCCACTACCA	GAATGATGCTGCTCCTGACG
COBL	TTGATTGGGACCCTGAATGTGC	ACGACCAAACGCACAGATTTCTC
CREB1	GGCAGCCTATCCCATCAGAT	CCACATGTCACCCCAAAAGG
CRHBP	AAGAAATCCTCAGCAGGTTGCG	TGGAAGGGTCCAATCCAGTTCC
FAT2	GTTCTGTGCCCCTCTCTTCT	GGAGGGGTAGAACACAGCTT
GATA3	GCGGGCTCTATCACAAAATG	TCCCCATTGGCATTCCTC
*HPRT1**	TGCAGACTTTGCTTTCCTTGGTC	CAAGCTTGCGACCTTGACCATC
MAL2	TCAGGCAGAGTCCTGGATATAG	TGAGGATCTTGCTCAGTTGTTAG
*PPIA**	ACGCCACCGCCGAGGAAAAC	CAAAGGAGACGCGGCCCAAG
TMEM93	TGGTGCACGTCTACTGAAATGGG	TGTTAATTGCTGGCCACAGTCC
TSPAN18	TGGGGATCTGAGGTTTGGTC	AGGGGAGAGAAGAGAGCAGA
WISP2	GTCTGGATCCCGAGGTATGG	GCCCGTGTGCATGTTTGATA
*18S**	ACTCAACACGGGAAACCTCACC	ACCCACGGAATCGAGAAAGAGC

Primer sequences used for validation RT-qPCR of affymetrix results.

* denotes housekeeping genes.

Table 5. Top 10 altered genes

Rank	Gene	Fold change	T-test (p-value)	q-value (%)
1	WNT1 Inducible Signaling Pathway Protein 2 (***WISP2***)	-1.55	2.23E-05	0
2	Corticotropin Releasing Hormone Binding Protein (***CRHBP***)	1.82	0.00172	0
3	Calsequestrin 2 (Cardiac Muscle) (***CASQ2***)	1.91	0.00109	0
4	Tetraspanin 18 (***TSPAN18***)	1.41	4.01E-04	0
5	Adrenoceptor Alpha 2A (***ADRA2A***)	1.48	8.36E-04	0
6	FAT Tumor Suppressor Homolog 2 (Drosophila) (***FAT2***)	1.57	0.00124	0
7	ER Membrane Protin Complex Subunit 6 (***TMEM93***)	1.26	1.12E-04	0
8	Protocadherin 7 (***PCDH7***)	1.47	7.79E-04	0
9	Leucine Rich Repeat Neuronal 3 (***LRRN3***)	1.78	2.68E-04	0
10	Cordon-Bleu WH2 Repeat Protein (***COBL***)	1.70	2.08E-04	0

Top 10 significantly altered gene targets following SAM analysis based on fold change and p-value, comparing 8 premanifest HD and 7 control subjects.

Table 6. Top 10 Pathway maps

Rank	Maps	Total nodes	Nodes in data	pValue	Min FDR
1	Protein folding and maturation_Angiotensin system maturation \ Human version	43	8	3,445E-12	2,791E-10
2	Protein folding and maturation_Angiotensin system maturation \ Rodent version	48	8	8,845E-12	3,582E-10
3	n-6 Polyunsaturated fatty acid biosynthesis	64	3	2,040E-03	4,131E-02
4	n-3 Polyunsaturated fatty acid biosynthesis	64	3	2,040E-03	4,131E-02
5	Regulation of lipid metabolism_PPAR regulation of lipid metabolism	42	2	1,201E-02	1,946E-01
6	Galactose metabolism	59	2	2,288E-02	2,905E-01
7	Unsaturated fatty acid biosynthesis	62	2	2,510E-02	2,905E-01
8	Vitamin E (alfa-tocopherol) metabolism	81	2	4,105E-02	3,199E-01
9	Sphingolipid metabolism	91	2	5,062E-02	3,199E-01
10	Sphingolipid metabolism / Human version	92	2	5,162E-02	3,199E-01

Top 10 significant pathway maps based on top 100 significantly altered transcripts between premanifest HD and control affymetrix analysis.

Table 7. Top 10 Transcription factor networks

Rank	Network	Total nodes	p-value	zScore
1	CREB1	35	7.240E-96	139.49
2	c-Myc	27	9.950E-73	121.44
3	ESR1 (nuclear)	21	1.280E-55	105.92
4	GCR-alpha	12	2.380E-30	77.05
5	SP1	12	2.380E-30	77.05
6	Oct-3/4	10	8.260E-25	69.06
7	c-Jun	9	4.730E-22	64.70
8	Androgen receptor	9	4.730E-22	64.70
9	P53	9	4.730E-22	64.70
10	RelA (p65 NF-kB subunit)	8	2.650E-19	60.05

Top 10 transcription factor networks involving the 100 most significantly altered genes from affymetrix analysis of 8 premanifest HD versus 7 control subjects.

Table 8. Summary of genes selected for validation by RT-qPCR

	Cohort 1		Cohort 1				Cohort 2			
Gene symbol	Affymetrix – Pre-HD		Pre-HD		HD		Pre-HD		HD	
ADIPOR2	↑	**	↑		↑		↓		↓	†
ADRA2A	↑	***	↓	†	↓	††	↓		↓	
AGT	↑	**	↑		↑		↑	†	↑	†
AGTR1	-		↓	†	↓		↓	†††	↓	
AGTR2	§		↑	††	↑	††	↓		↓	†
CASQ2	↑	**	↓		-		↓	†	↓	†
COBL	↑	***	↑	††	↓		↓		↓	
CREB1	-		↑	†	↑	††	↓		↑	†
CRHBP	↑	**	↑	†	↓		↓		↑	
FAT2	↑	*	-		↓	††	↓	††††	↓	
GATA3	↑	**	↓	†	↓		↓		↓	
MAL2	↑	**	↑	†	↑	†	↓		↓	†††
TMEM93	↑	***	↑		↓		↓		↓	†
TSPAN18	↑	***	↑		↑		↑		↓	†
WISP2	↓	****	↓		↓		↓	†	-	

Table of genes selected for RT-qPCR validations. Arrows depict up- or down-regulation as compared to controls, while dashes depict no change. § not included on affymetrix platform.

Affymetrix: Significance Analysis of Microarrays, * $p < 0.05$, ** $p < 0.01$, *** $p > 0.001$, **** $p < 0.0001$

RT-qPCR: Student's t-test, † $p < 0.05$, †† $p < 0.01$, ††† $p > 0.001$, †††† $p < 0.0001$

Figure Legends

Fig. 1. 3D Principal components analysis of affymetrix gene expression data shows separation of control and premanifest HD groups. Control samples (green circles); premanifest HD (blue).

Fig. 2. Heat map and hierarchical clustering of 227 genes from affymetrix data yields distinct clustering of control and premanifest HD groups. Hierarchical clustering analysis performed on control versus premanifest HD for the 227 top significant genes following SAM analysis of the Affymetrix data, using Pearson correlation as distance matrix and average linkage. The heat map diagram shows the result of the two-way hierarchical clustering of genes and samples. Each row represents one target gene, and each column represents one sample. The gene clustering tree is shown on the left and gene names are depicted on the right side of the image. The colour scale shown at the top illustrates the relative expression level of a gene across all samples: red colour represents an expression level above mean, green colour represents expression lower than the mean. Control samples are denoted in green while premanifest HD samples are denoted in blue.

Fig. 3. **Network analysis highlights CREB1 as a significant transcription factor in early HD white adipose tissue.** Network analysis based on the top 100 significant results from control vs. premanifest HD affymetrix analysis. CREB1 network is the highest scored network. Red

circles indicate upregulation in premanifest HD carriers relative to control subjects. Green lined arrows indicate positive interaction/activation; red lines, negative interaction/inhibition; grey lines, unspecified interaction.

Fig. 4. RT-qPCR confirms upregulation of *COBL*, *CRHBP* and *MAL2* in HD gene carriers and highlights upregulation of *CREB1* in cohort 1. Cohort 1: validated RT-qPCR results for 15 of the most significantly altered targets based on premanifest HD vs. control affymetrix data. Notably, significant results for the premanifest group (*COBL*, *CRHBP* and *MAL2*) confirm the findings of the affymetrix analysis. In line with our transcription factor analysis, *CREB1* is significantly altered in both premanifest HD and stage II/III HD patients. In line with angiotensin signalling pathway alterations, *AGTR2* was significantly altered. Group sizes ranged from n = 11-14 (control), n = 9-13 (premanifest) and n = 9-11 (stage II/III HD). * $P < 0.05$, ** $P < 0.01$, student's t-test.

Fig. 5. RT-qPCR confirms downregulation of *WISP2* and upregulation of AGT in HD gene carriers and highlights upgregulation of *CREB1* in cohort 2. Cohort 2: validated RT-qPCR results for 15 of the most significantly altered targets based on premanifest HD vs. control affymetrix data. Validations were performed on all samples from cohort 2. Notably, significant results for the premanifest HD group confirm the downregulation of *WISP2* and upregulation of *AGT* in HD gene carriers as determined by affymetrix analysis. In line with our transcription factor analysis, *CREB1* is significantly upregulated in stage I/II HD patients.

Similar to cohort 1, *AGTR2* was significantly upregulated in manifest HD. Group sizes ranged from n = 5-10 (control), n = 6-9 (premanifest) and n = 6-9 (stage I/II HD). * P < 0.05, *** P < 0.001, **** P < 0.0001, student´s t-test.

Fig. 6. Circulating plasma angiotensin II levels are not altered in HD. Circulating angiotensin II levels from plasma of 15 controls, 14 premanifest and 13 stage II/III HD patients (cohort 1).

Supplementary Data

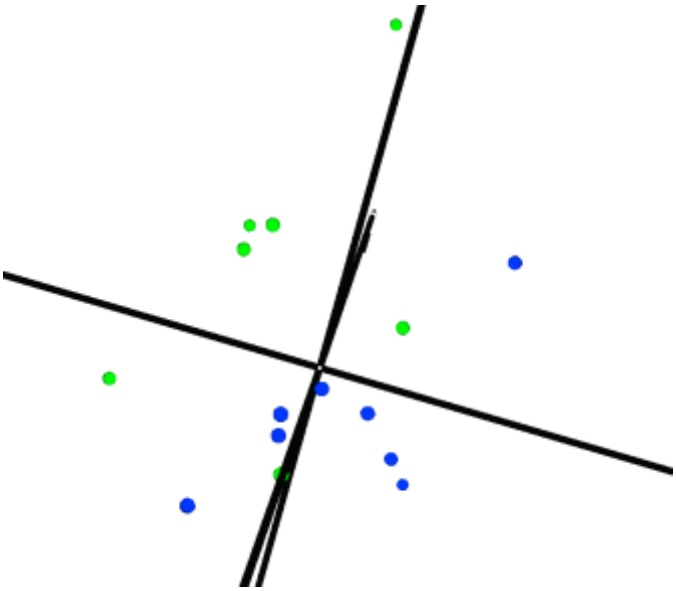
S1 Table. Premanifest HD vs. Control Affymetrix Data. Complete affymetrix dataset for premanifest HD vs. controls following SAM analysis.

S2 Table. Stage II/III HD vs. Control Affymetrix Data. Complete affymetrix dataset for stage II/III HD vs. controls following SAM analysis.

S3 Table. Significantly altered CREB1 related targets. Significantly upregulated genes from control vs. premanifest HD affymetrix analysis involved in the CREB1 network.

Figures

Fig. 1.



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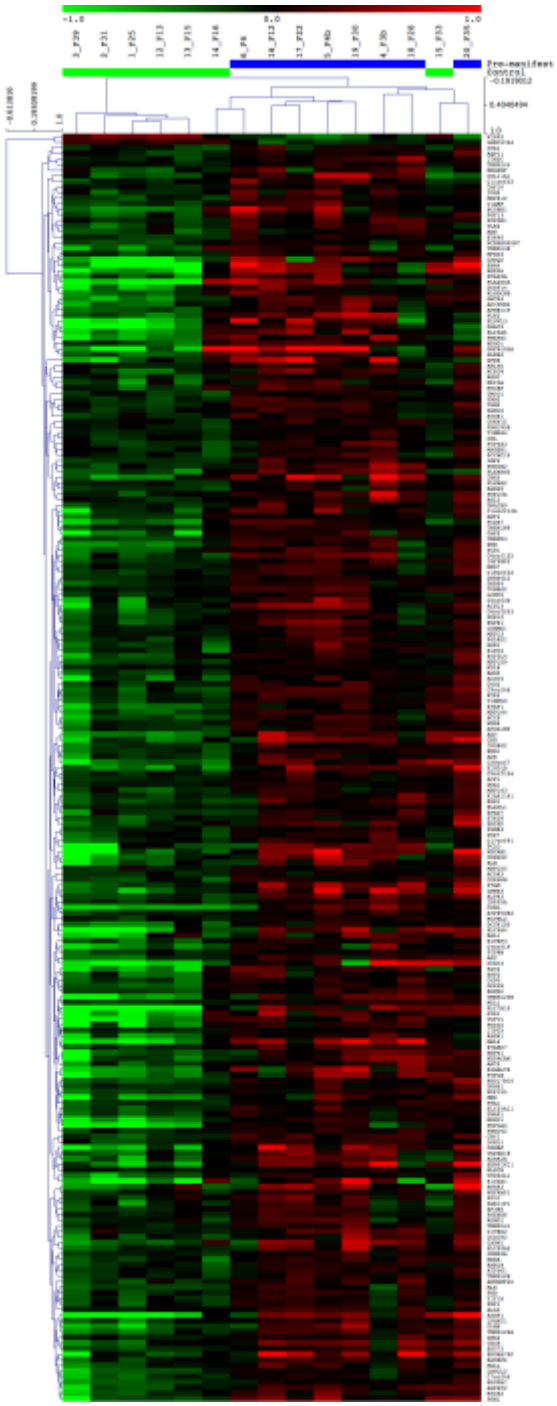

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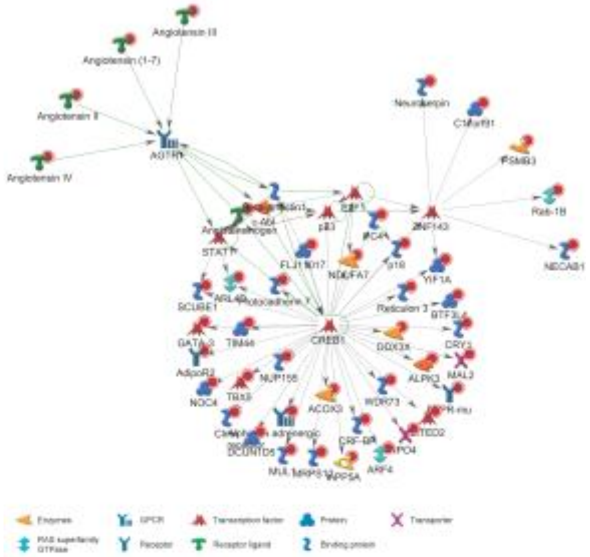

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GATA-2
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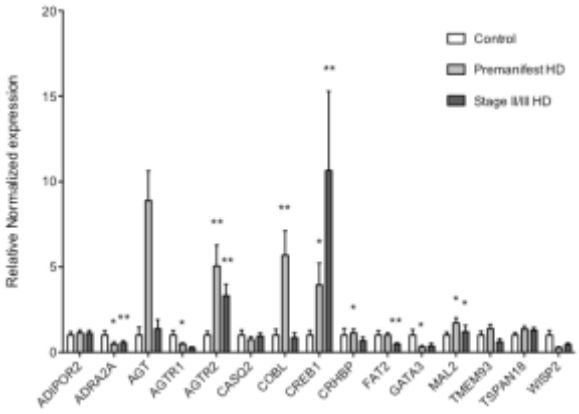
Fig. 4.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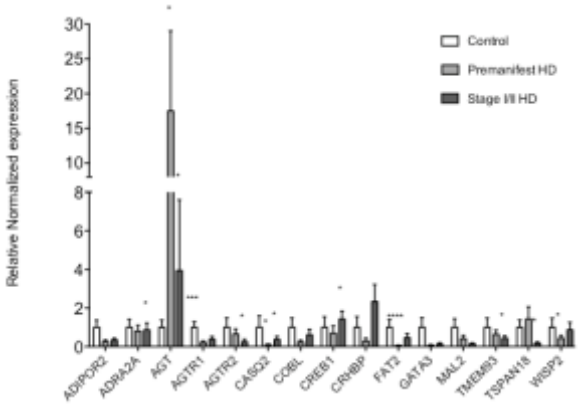
WISP2

Fig. 5.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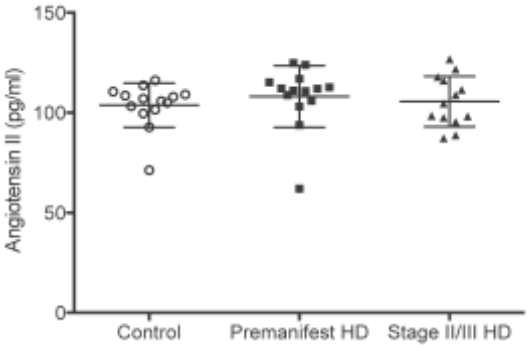
TMEM93
TSPAN18
WISP2

Fig. 6.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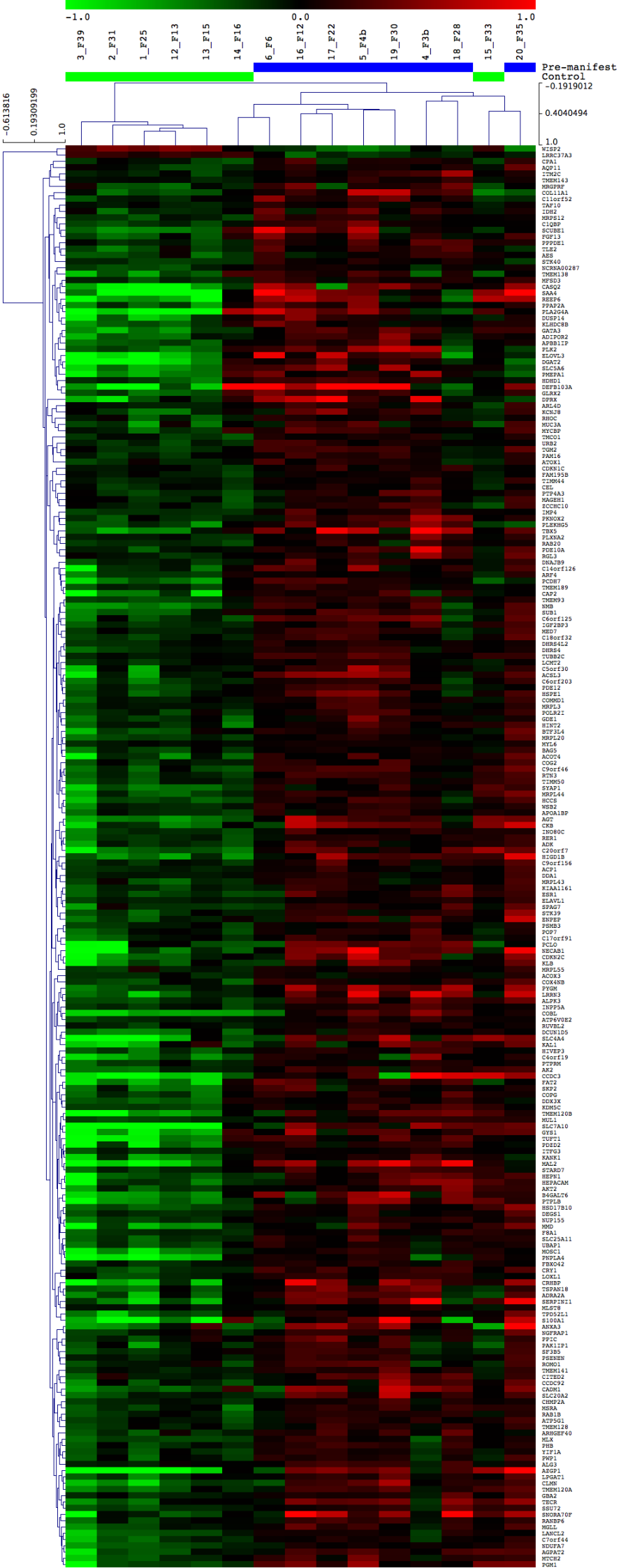
Control
Premanifest HD
Stage II/III HD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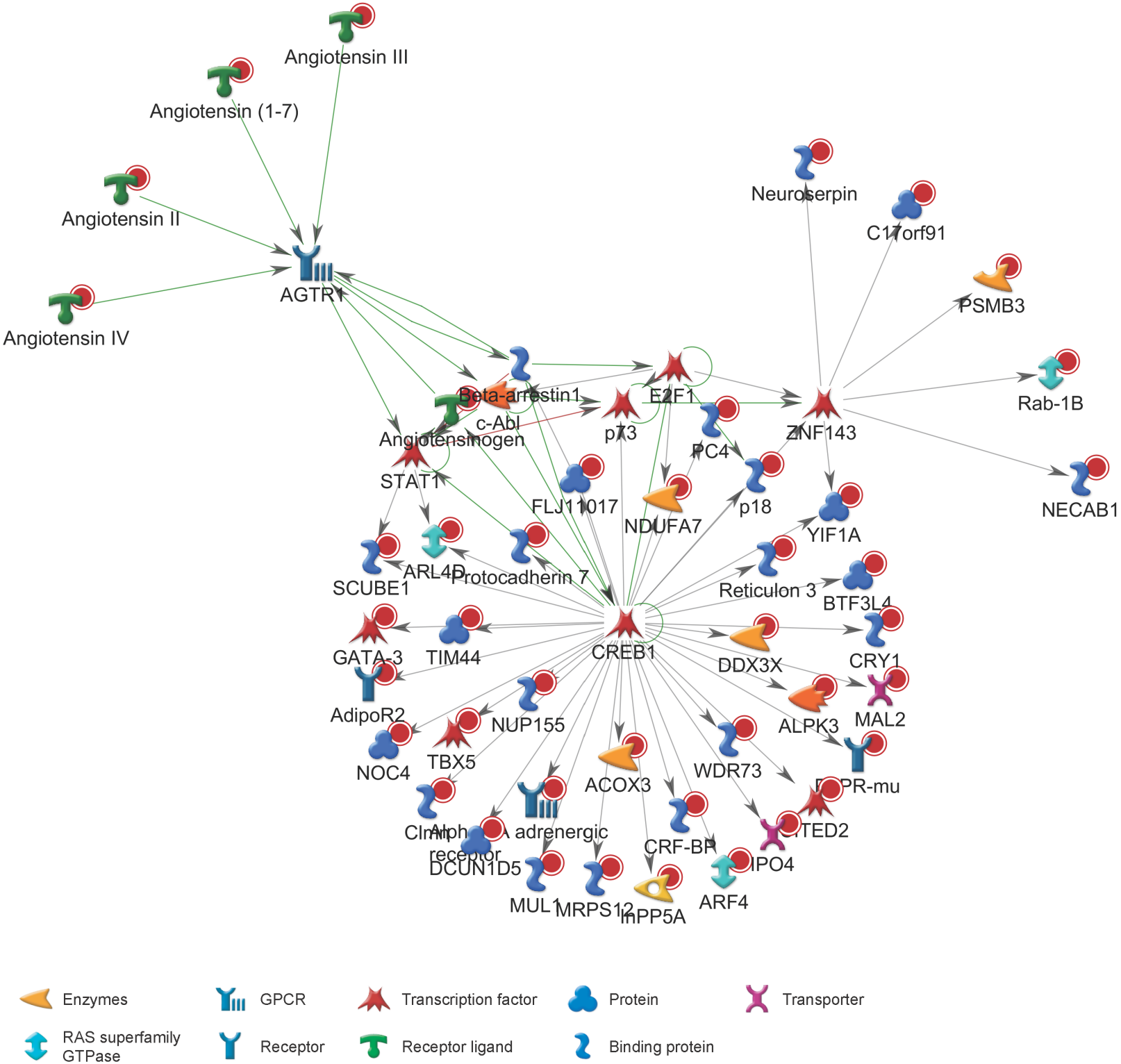
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Beta-arrestin1
c-Abl
Angiotensinogen
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FLJ11017
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YIF1A
SCUBE1
ARL4D
Protocadherin 7
Reticulon 3
BTF3L4
GATA-3
TIM44
CREB1
DDX3X
CRY1
AdipoR2
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MUL1
MRPS12
InPP5A
DCUN1D5
Enzymes
GPCR
Transcription factor
Protein
Transporter
RAS superfamily GTPase
Receptor
Receptor ligand
Binding protein

Supplementary Data

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S2 Table. Stage II/III HD vs. Control Affymetrix Data. Complete affymetrix dataset for stage II/III HD vs. controls following SAM analysis.

S3 Table. Significantly altered CREB1 related targets. Significantly upregulated genes from control vs. premanifest HD affymetrix analysis involved in the CREB1 network.