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Molekularne mechanizmy regulacji ekspresji genów w komórkowych i zwierzęcych modelach choroby Huntingtona

Molecular mechanisms regulating gene expression in cellular and animal models of Huntington's disease

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Każda relacja jest bodźcem biologicznym: może wzmacniać, osłabiać lub przebudowywać sieci neuronalne, za pośrednictwem których interpretujemy świat. Dziękuję wszystkim osobom, które pokazały mi, że to od nas zależy, jakie sieci tworzymy, i w jaki sposób możemy dzięki nim współtworzyć naukę oraz otaczający nas świat.

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Streszczenie rozprawy doktorskiej

Choroby neurodegeneracyjne stanowią heterogenną grupę schorzeń charakteryzującą się postępującym uszkodzeniem i utratą neuronów w różnych obszarach ośrodkowego i obwodowego układu nerwowego. Towarzyszą im zmiany histologiczne i hematologiczne, przekładające się na kliniczne objawy patologiczne. Mechanizm neurodegeneracji obejmuje utratę neuronów oraz współwystępujące zaburzenia wynikające z defektów na poziomie molekularnym, takich jak patologiczna agregacja białek, dysfunkcja synaps i sieci neuronalnych, zaburzona proteostaza, nieprawidłowości cytoszkieletu, zmieniona homeostaza energetyczna, uszkodzenia DNA i RNA, stan zapalny oraz śmierć komórek nerwowych.

Flawonoidy stanowią dużą podgrupę polifenoli, obejmującą ponad 6000 związków występujących w produktach pochodzenia naturalnego. Ze względu na liczne właściwości, takie jak działanie antyoksydacyjne, przeciwzapalne oraz zdolność do przenikania przez barierę krew–mózg, są one coraz częściej brane pod uwagę w poszukiwaniu terapii dla wielu chorób. Przykładowe mechanizmy chorób neurodegeneracyjnych, takich jak choroba Alzheimera (AD), choroba Parkinsona (PD), stwardnienie zanikowe boczne (ALS) oraz choroba Huntingtona (HD), a także główne grupy flawonoidów, ich właściwości i najczęściej opisywane mechanizmy działania neuroprotekcyjnego, zostały omówione w następującym artykule przeglądowym, wchodzącym w skład tej rozprawy doktorskiej: Szulc, A., Wiśniewska, K., Żabińska, M., Gaffke, L., Szota, M., Olendzka, Z., Węgrzyn, G., & Pierzynowska, K. (2024). Effectiveness of flavonoid-rich diet in alleviating symptoms of neurodegenerative diseases. *Foods*, *13*, 1931.

W niniejszej rozprawie doktorskiej położono nacisk na HD, jedną z najczęstszych chorób neurodegeneracyjnych związanych z agregacją białek. Należy ona do grupy monogenowych chorób poliglutaminowych (PolyQ), czyli dziedzicznych zaburzeń neurologicznych spowodowanych zwiększoną liczbą powtórzeń trinukleotydów CAG (cytozyna, adenina, guanina). W HD zwiększona liczba powtórzeń CAG występuje w genie *HTT*, kodującym białko huntingtynę (HTT). Patogenny wariant *HTT* prowadzi do powstania nieprawidłowo sfałdowanego białka z wydłużonym odcinkiem reszt glutaminowych, tworzącego toksyczne, trudne do usunięcia agregaty. Agregaty te, wraz z nieprawidłowymi strukturami drugorzędowymi zmutowanego mRNA *HTT*, wpływają na proces splicingu poprzez sekwestrowanie składników spliceosomu. Te dwa elementy są uznawane za kluczowe defekty inicjujące kaskadę zaburzeń funkcjonowania komórki, prowadzącą do rozwoju objawów patogennych u pacjentów. Liczba powtórzeń CAG niższa niż 27 uznawana jest za prawidłową. Zakres 27–35 powtórzeń wiąże się z ryzykiem pojawienia się objawów choroby w kolejnych pokoleniach, natomiast ponad 36 powtórzeń prowadzi do manifestacji choroby. Pomimo obecności zwiększonej liczby powtórzeń CAG już na etapie zygoty, pierwsze objawy choroby obserwuje się w trzeciej lub czwartej dekadzie życia. Najnowsze doniesienia sugerują, że za patogenność oraz

opóźniony początek choroby może odpowiadać somatyczna ekspansja powtórzeń CAG do ponad 150 kopii w trakcie życia chorego. Niemniej jednak nadal nie jest jasne, dlaczego taka ekspansja występuje wyłącznie w komórkach posiadających allele *HTT*, w których pierwotna liczba powtórzeń CAG przekracza 36. Ponieważ HD jest obecnie chorobą nieuleczalną, oczywistym jest poszukiwanie strategii terapeutycznych umożliwiających skuteczne leczenie tej choroby. Opracowanie nowych leków wymaga jednak dogłębnego zrozumienia molekularnych mechanizmów choroby będącej celem terapii.

W moich badaniach zastosowałam komórkowe modele choroby Huntingtona (HD) oparte na linii HEK293. Linie te zostały wcześniej skonstruowane z zastosowaniem mutagenezy CRISPR-Cas9 i obejmowały linię kontrolną zawierającą 16/17 powtórzeń CAG w eksonie 1 genu *HTT* (HEK) oraz linie HD zawierające 41, 53 lub 84 powtórzenia CAG (odpowiednio HEK 41, HEK 53 i HEK 84). W badaniach wykorzystywano również mysi, genetycznie modyfikowany model HD R6/1 (116 powtórzeń CAG) oraz myszy kontrolne C57BL/6J.

W pracy Szulc, A., Walter, B., Gaffke, L., Wiśniewska, K., Żabińska, M., Rintz, E., Cyske, Z., Grabski, M., Pankiv, O., Podlacha, M., Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the HTT gene. *Biochemical and Biophysical Research Communications, 816*, 153711 z zastosowaniem AlphaFold 3 przewidziane zostały struktury białkowe ludzkich wariantów HTT z 16, 41, 53 i 84 powtórzeniami CAG. Ujawniły się istotne zmiany w formach patogennych w porównaniu z formą prawidłową. W dalszej części pracy analizowane były globalne zmiany w ekspresji genów w komórkowym i zwierzęcym modelu HD względem kontroli oraz w obrębie modelu komórkowego, w liniach zawierających różną liczbę powtórzeń CAG (41, 53, 84).

Zaobserwowałam, że patologiczne wydłużenie ciągu glutamin może prowadzić do przewidywanej reorientacji N-końcowego regionu polyQ. Wyniki te sugerują, że patomechanizm HD może wykraczać poza tradycyjny pogląd o agregacji białek indukowanej przez polyQ, wskazując na możliwość zmian konformacyjnych wynikających z konkretnej długości polyQ, wpływających na przebieg choroby, co jest zgodne z niedawnymi propozycjami dotyczącymi somatycznej ekspansji powtórzeń CAG i jej dalszych konsekwencji. Przedstawione modele AlphaFold 3, które opisują monomeryczną HTT, należy interpretować jednak jako hipotezy dotyczące stanu „apo", który do tej pory nie został bezpośrednio stwierdzony w badaniach strukturalnych.

Analizy transkryptomiczne wykazały obecność tysięcy genów o zaburzonej ekspresji w komórkach HEK41 (2892 geny), HEK53 (4193 geny) oraz HEK84 (6091 genów) w porównaniu z linią kontrolną HEK. Ponadto w komórkach mózgowych myszy R6/1 zidentyfikowałam 4185 genów o zaburzonej ekspresji w porównaniu z komórkami myszy typu dzikiego. Zarówno w komórkowym, jak i zwierzęcym modelu choroby zmiany te dotyczyły funkcji mitochondriów, apoptozy, autofagii, odpowiedzi zapalnej, replikacji i naprawy DNA, ale także odpowiedzi na uszkodzenia DNA i stres. Ponadto zaburzenia dotyczyły ekspresji genów zaangażowanych w transport białek i neurotransmiterów na różnych etapach funkcjonowania komórek. Oczywiście zmiany transkryptomiczne

są konsekwencją obecności patogennych form HTT, a nie pierwotną przyczyną choroby, jednak podwyższony poziom reaktywnych form tlenu (ROS) w połączeniu z zaburzeniami ekspresji dziesiątek genów odpowiedzialnych za odpowiedź na stres, odpowiedź na uszkodzenia DNA i zaburzenia odpowiadające specyficznym procesom mogą sprzyjać somatycznej ekspansji trójek CAG w komórkach zawierających patogenne allele *HTT*.

Spośród wspomnianych procesów, w obrębie których obserwowałam najliczniejsze zaburzenia globalnej regulacji ekspresji genów, kluczowa w kontekście degradacji toksycznych form mHTT jest autofagia. Proces ten jest ściśle regulowany w komórce a jego podstawową funkcją jest utrzymywanie homeostazy. Odpowiada on za usuwanie uszkodzonych organelli, długo żyjących białek, agregatów białkowych, kompleksów makrocząsteczkowych oraz innych nieprawidłowych struktur wewnątrzkomórkowych. Makroautofagia rozpoczyna się od utworzenia fagoforu czyli błony, która stopniowo wydłuża się i otacza elementy przeznaczone do degradacji. Tak powstały autofagosom dojrzewa i łączy się z lizosomem tworząc autofagolizosom, wewnątrz którego dzięki niskiemu pH i obecności enzymów hydrolitycznych dochodzi do degradacji jego zawartości. Powstałe produkty mogą ponownie zostać wykorzystane przez komórkę.

Wykorzystywanie modulatorów autofagii przynosi obiecujące rezultaty w komórkowych i zwierzęcych modelach HD. Jednak silne stymulatory autofagii, takie jak rapamycyna i inne związki, mimo że są bardzo użyteczne w badaniach laboratoryjnych, nie mogą być stosowane w praktyce klinicznej u pacjentów z chorobami wymagającymi leczenia przez całe życie, ze względu na działania niepożądane wywoływane podczas długotrwałego podawania. Dlatego w kontekście mojej pracy szczególnie obiecujące było wykorzystanie genisteiny (4',5,7-trihydroksyizoflawonu lub 5,7-dihydroksy-3-(4-hydroksyfenylo)-4H-1-benzopiran-4-onu). Związek ten może do pewnego stopnia stymulować autofagię przy jednoczesnym korzystnym profilu bezpieczeństwa.

Przy zastosowaniu specyficznych przeciwciał anty-HTT, we wszystkich trzech badanych liniach komórkowych zaobserwowałam zwiększoną intensywność fluorescencji pochodzącej od HTT w porównaniu z linią kontrolną. Po zastosowaniu genisteiny intensywność fluorescencji uległa istotnemu obniżeniu, co może wskazywać na zmniejszenie ilości zmutowanej huntingtyny (mHTT). Redukcja liczby agregatów może zachodzić za pośrednictwem modulacji autofagii.

W modelach HD stwierdziłam zaburzenia ekspresji licznych genów związanych z autofagią, obejmujące zarówno ich indukcję, jak i represję względem komórek kontrolnych. Liczba genów o zmienionej ekspresji zwiększała się wraz ze wzrostem liczby powtórzeń CAG w genie *HTT*. Szczególnie często podwyższoną ekspresję wykazywały geny kodujące negatywne regulatory autofagii, natomiast geny niezbędne do jej prawidłowego przebiegu charakteryzowały się zróżnicowanym profilem ekspresji. W obecności genisteiny nasiliły się obserwowane zmiany, przy czym dominowała indukcja genów zaangażowanych w pozytywną regulację autofagii. Podobne zależności obserwowałam również dla genów związanych z poszczególnymi typami autofagii.

W celu określenia interakcji pomiędzy białkami będącymi produktami genów o zmienionej ekspresji wykonałam modelowanie sieci interakcji białkowych w oparciu o bazę STRING. Określiłam białka kodowane przez geny, których ekspresja uległa zmianie, a wśród nich te, których ekspresja powróciła do poziomu kontrolnego po zastosowaniu genisteiny. Pozwoliło to wytypować potencjalne punkty działania terapeutyku. W kolejnym etapie, metodą Western blot określiłam poziomy białek zaangażowanych w inicjację autofagii zależną od ULK1 oraz w elongację autofagosomu związaną z Atg12 i LC3-I/II. Wykazałam, że zaburzenie autofagii w HD zachodzi głównie na etapie inicjacji tego procesu i obejmuje obniżenie poziomów pULK1(Ser555) oraz Atg13. Genisteina może natomiast przywracać prawidłowy przebieg autofagii poprzez mechanizmy obejmujące zwiększenie wydajności fosforylacji ULK1(Ser555) oraz podwyższenie poziomu Atg13, jednak nie ograniczając się wyłącznie do nich. Jednoczesne zwiększenie poziomów pULK1(Ser757) i białka Rubicon, które są znanymi negatywnymi regulatorami autofagii, zapobiega nadmiernej stymulacji tego procesu przez badany izoflawon. Taka nadmierna aktywacja autofagii byłaby problematyczna w przypadku długotrwałego jego stosowania, natomiast łagodna lecz skuteczna stymulacja może z jednej strony pozwalać na efektywną degradację mHTT, a z drugiej być bezpieczna dla organizmu. Bardziej szczegółowa analiza zaburzeń procesów autofagii oraz wpływu genisteiny przedstawiona została w artykule Szulc, A., Wiśniewska, K., Szota, M., Żabińska, M., Pierzynowska, K., Gaffke, L., & Węgrzyn, G. (2026). Autophagy disorders and their improvement by 4′,5,7-trihydroxyisoflavone in cellular models of Huntington's disease. [Manuskrypt złożony do czasopisma *Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease* BBADIS-26-4684].

Podsumowując, przeprowadzone przeze mnie badania dotyczyły molekularnych mechanizmów HD, ze szczególnym uwzględnieniem wpływu długości ekspansji powtórzeń CAG w genie *HTT* na zmiany strukturalne HTT, regulacje ekspresji genów i przebieg autofagii. Przewidywania strukturalne sugerują, że wydłużenie odcinka poliglutaminowego może zmieniać konformację huntingtyny. Analizy transkryptomiczne wykazały liczne zaburzenia ekspresji genów, nasilające się wraz ze wzrostem liczby powtórzeń CAG i obejmujące m.in. funkcje mitochondriów, odpowiedź na stres i uszkodzenia DNA oraz autofagię - proces kluczowy dla prawidłowego usuwania mHTT. Szczegółowe badania wskazały na zaburzenie inicjacji autofagii w modelach HD, związane z obniżeniem poziomów pULK1(Ser555) i Atg13. Genisteina zwiększała poziomy tych białek oraz zmniejszała intensywność fluorescencji sygnału HTT w badaniach immunocytochemicznych, co może wskazywać na redukcję ilości zmutowanej huntingtyny. Wyniki te sugerują, że genisteina może modulować autofagię na etapie inicjacji tego procesu, a przez to stanowić potencjalny kierunek dalszych badań nad terapią HD.

Lista prac wchodzących w skład rozprawy doktorskiej

1. **Szulc, A.,** Wiśniewska, K., Żabińska, M., Gaffke, L., Szota, M., Olendzka, Z., Węgrzyn, G., & Pierzynowska, K. (2024). Effectiveness of flavonoid-rich diet in alleviating symptoms of neurodegenerative diseases. *Foods*, *13*, 1931. https://doi.org/10.3390/foods13121931
   [IF= 6] [MNisW=100]

2. **Szulc, A.,** Walter, B., Gaffke, L., Wiśniewska, K., Żabińska, M., Rintz, E., Cyske, Z., Grabski, M., Pankiv, O., Podlacha, M., Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the HTT gene. *Biochemical and Biophysical Research Communications*, *816*, 153711. https://doi.org/10.1016/j.bbrc.2026.153711
   [IF= 2.5] [MNisW=100]

3. **Szulc, A.,** Wiśniewska, K., Szota M., Żabińska, M., Pierzynowska, K., Gaffke, L., & Węgrzyn, G. (2026). Autophagy disorders and their improvement by 4′,5,7-trihydroxyisoflavone in cellular models of Huntington's disease. [Manuskrypt złożony do czasopisma *Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease* BBADIS-26-4684].

Abstract of doctoral dissertation

Neurodegenerative diseases constitute a heterogeneous group of disorders characterized by progressive damage and loss of neurons in various areas of the central and peripheral nervous systems. They are accompanied by histological and hematological changes that manifest as clinical pathological symptoms. Neurodegeneration involves neuronal loss and co-occurring disorders resulting from molecular-level defects. These defects include pathological protein aggregation, synaptic and neural network dysfunction, impaired proteostasis, cytoskeletal abnormalities, altered energy homeostasis, DNA and RNA damage, inflammation, and neuronal cell death.

Flavonoids constitute a large subgroup of polyphenols, comprising over 6,000 compounds found in natural products. They are of high interest in the search for treatments for various diseases because of their numerous properties, such as antioxidant and anti-inflammatory activity, and their ability to cross the blood-brain barrier. The main groups of flavonoids, their properties, and the most commonly described mechanisms of neuroprotective action are discussed in the following review article, which is part of this doctoral dissertation: Szulc, A., Wiśniewska, K., Żabińska, M., Gaffke, L., Szota, M., Olendzka, Z., Węgrzyn, G., & Pierzynowska, K. (2024). Effectiveness of flavonoid-rich diet in alleviating symptoms of neurodegenerative diseases. *Foods*, 13, 1931. Moreover, this article also discusses mechanisms underlying neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD).

This doctoral dissertation focuses on HD, one of the most common neurodegenerative diseases associated with protein aggregation. It belongs to the group of monogenic polyglutamine (PolyQ) diseases, which are inherited neurological disorders caused by an increased number of CAG (cytosine, adenine, guanine) trinucleotide repeats. In HD, an increased number of CAG repeats occurs in the *HTT* gene, which encodes the huntingtin (HTT) protein. The pathogenic *HTT* variant codes for an abnormally folded protein with an elongated glutamine residue sequence, resulting in toxic aggregates that are difficult to remove. These aggregates, along with abnormal secondary structures of the mutated *HTT* mRNA, affect the splicing process by sequestering spliceosome components. These two elements are considered key defects that initiate a cascade of cellular dysfunction, leading to the development of pathogenic symptoms in patients. In a healthy individual, up to 27 CAG repeats are considered normal. A range of 27–35 repeats is associated with a risk of disease symptoms appearing in subsequent generations, while more than 36 repeats lead to disease manifestation. An increased number of CAG repeats appears as early as at the zygote stage; however, the first symptoms of the disease are observed much later, in the third or fourth decade of life. Recent reports suggested that somatic expansion of CAG repeats during the patient's lifetime to more than 150 copies may drive pathogenicity and delayed disease onset. Nevertheless, it remains unclear why such expansion occurs exclusively in cells carrying the *HTT* allele, in which the original number

of CAG repeats exceeds 36. HD is currently an incurable disease; thus, seeking therapeutic strategies that enable effective treatment of this disease is needed. However, developing new drugs requires a thorough understanding of the molecular mechanisms of the targeted disease.

In my research, I used cellular models of Huntington's disease (HD) based on the HEK293 cell line. These cell lines were previously generated using CRISPR-Cas9 mutagenesis and included a control line containing 16/17 CAG repeats in exon 1 of the *HTT* gene (HEK) and HD lines containing 41, 53, or 84 CAG repeats, respectively (HEK 41, HEK 53, and HEK 84, respectively). The study also utilized a genetically modified R6/1 mouse model of HD (116 CAG repeats) and C57BL/6J control mice.

In the study: Szulc, A., Walter, B., Gaffke, L., Wiśniewska, K., Żabińska, M., Rintz, E., Cyske, Z., Grabski, M., Pankiv, O., Podlacha, M., Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the HTT gene. *Biochemical and Biophysical Research Communications*, 816, 153711., protein structures were predicted for human HTT variants with 16, 41, 53, and 84 CAG repeats using AlphaFold3. Significant changes were observed in the pathogenic forms compared to the wild-type form. The study also analyzed global changes in gene expression in cellular and animal models of HD relative to controls, as well as within the cellular model across cell lines with different CAG repeat numbers (41, 53, 84).

I have observed that pathological elongation of the glutamine chain can lead to the predicted reorientation of the N-terminal polyQ region. These results suggest that the pathomechanism of HD may extend beyond the traditional view of polyQ-induced protein aggregation, pointing to the possibility that specific polyQ lengths drive conformational changes that influence disease course, consistent with recent proposals on somatic CAG repeat expansion and its downstream consequences. However, the AlphaFold3 models presented here describe monomeric HTT and should be interpreted as hypotheses about the "apo" state, which structural studies have not yet directly confirmed.

Transcriptomic analyses revealed thousands of genes with altered expression in HEK41 cells (2,892 genes), HEK53 cells (4,193 genes), and HEK84 cells (6,091 genes) compared to the HEK control line. Furthermore, in R6/1 mouse brain cells, I identified 4,185 genes with altered expression compared to wild-type mouse cells. In both cellular and animal models of the disease, these changes were related to mitochondrial function, apoptosis, autophagy, the inflammatory response, DNA replication and repair, and responses to DNA damage and stress. Furthermore, the alterations included the expression of genes involved in the transport of proteins and neurotransmitters at various stages of cellular function. These transcriptomic changes are a consequence of the presence of pathogenic forms of HTT, not the primary cause of the disease; however, elevated levels of reactive oxygen species (ROS), combined with alterations in the expression of dozens of genes responsible for the stress response, the response to DNA damage, and processes specific

to certain pathways, may promote the somatic expansion of CAG trinucleotide repeats in cells containing pathogenic *HTT* alleles.

Among the processes most frequently disrupted in global gene expression regulation, autophagy plays a key role in degrading toxic forms of mHTT. This process is tightly regulated within the cell, and its primary function is to maintain homeostasis. It removes damaged organelles, long-lived proteins, protein aggregates, macromolecular complexes, and other abnormal intracellular structures. Macroautophagy begins with the formation of a phagophore - a membrane that gradually elongates and encloses the components destined for degradation. The resulting autophagosome matures and fuses with a lysosome to form an autophagolysosome, where its contents are degraded at low pH by hydrolytic enzymes. The cell can reuse the resulting products.

Autophagy modulators have shown promising results in cellular and animal models of HD. However, potent autophagy stimulators, such as rapamycin and other compounds, although useful in laboratory studies, cannot be used in clinical practice for patients with diseases requiring lifelong treatment because their long-term administration causes adverse effects. Therefore, in the context of my work, the use of genistein (4',5,7-trihydroxyisoflavone or 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), was particularly promising. This compound can stimulate autophagy to a certain extent while maintaining a favorable safety profile.

Using specific anti-HTT antibody, I observed increased HTT fluorescence intensity in all three cell lines compared with the control line. After genistein treatment, fluorescence intensity decreased significantly, which may indicate reduced mutant huntingtin (mHTT). The reduction in the number of aggregates may occur through the modulation of autophagy.

In the HD models, I observed altered expression of numerous autophagy-associated genes, including both induction and repression compared with control cells. The number of genes with altered expression increased with CAG repeat length in the HTT gene. Genes encoding negative regulators of autophagy were particularly frequently upregulated, whereas genes essential for its proper progression exhibited a varied expression profile. In the presence of genistein, the observed changes were exacerbated, with the induction of genes involved in the positive regulation of autophagy predominating. I also observed similar relationships for genes associated with specific types of autophagy.

To identify interactions among proteins encoded by genes with altered expression, I modeled protein interaction networks using the STRING database. I identified proteins encoded by genes with altered expression, including those whose expression returned to control levels after genistein treatment. This allowed me to identify potential therapeutic targets. Next, using Western blot analysis, I measured the levels of proteins involved in ULK1-dependent autophagy initiation and autophagosome elongation and flux, associated with ATG12 and LC3-I/II, respectively. I showed that autophagy disruption in HD occurs primarily during the initiation phase of the process and involves

a decrease in the levels of pULK1(Ser555) and Atg13. Genistein, by contrast, can restore normal autophagy through mechanisms that include, but are not limited to, increasing the efficiency of ULK1(Ser555) phosphorylation and raising Atg13 levels. The simultaneous increase in levels of pULK1(Ser757) and Rubicon protein - which are known negative regulators of autophagy - prevents excessive stimulation of this process by the studied isoflavone. Such excessive activation of autophagy would be problematic with long-term use, whereas mild yet effective stimulation may, on the one hand, allow effective degradation of mHTT and, on the other hand, be safe for the body. A more detailed analysis of autophagy disorders and the effects of genistein are presented in the article Szulc, A., Wiśniewska, K., Szota, M., Żabińska, M., Pierzynowska, K., Gaffke, L., & Węgrzyn, G. (2026). Autophagy disorders and their improvement by 4′,5,7-trihydroxyisoflavone in cellular models of Huntington's disease. [Manuscript submitted to *Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease* BBADIS-26-4684].

In summary, my research focused on the molecular mechanisms of HD, with particular emphasis on how CAG repeat length in the *HTT* gene affects HTT structure, gene expression regulation, and autophagy. Structural predictions suggest that the lengthening of the polyglutamine track may alter the conformation of huntingtin. Transcriptomic analyses revealed numerous gene expression abnormalities that become more pronounced as the number of CAG repeats increases and involve, among other things, mitochondrial function, the response to stress and DNA damage, and autophagy - a process critical for proper clearance of mHTT. Detailed studies have shown disrupted autophagy initiation in HD models, associated with reduced levels of pULK1(Ser555) and Atg13. Genistein increased levels of these proteins and reduced the fluorescence intensity of the HTT signal in immunohistochemical studies, which may indicate reduced mutant huntingtin levels. These results suggest that genistein may modulate autophagy at the initiation stage and thus represent a potential new direction for further research into HD therapy.

List of publications included in the doctoral dissertation

1. **Szulc, A.,** Wiśniewska, K., Żabińska, M., Gaffke, L., Szota, M., Olendzka, Z., Węgrzyn, G., & Pierzynowska, K. (2024). Effectiveness of flavonoid-rich diet in alleviating symptoms of neurodegenerative diseases. *Foods*, *13*(12), Artykuł 1931. https://doi.org/10.3390/foods13121931
   [IF= 6] [MNisW=100]

2. **Szulc, A.,** Walter, B., Gaffke, L., Wiśniewska, K., Żabińska, M., Rintz, E., Cyske, Z., Grabski, M., Pankiv, O., Podlacha, M., Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the HTT gene. *Biochemical and Biophysical Research Communications*, *816*, Artykuł 153711. https://doi.org/10.1016/j.bbrc.2026.153711
   [IF= 2.5] [MNisW=100]

3. **Szulc, A.,** Wiśniewska, K., Szota M., Żabińska, M., Pierzynowska, K., Gaffke, L., & Węgrzyn, G. (2026). Autophagy disorders and their improvement by 4′,5,7-trihydroxyisoflavone in cellular models of Huntington's disease. [Manuskrypt złożony do czasopisma *Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease* BBADIS-26-4684].

Prace wchodzące w skład rozprawy doktorskiej

Effectiveness of Flavonoid-Rich Diet in Alleviating Symptoms of Neurodegenerative Diseases

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Review

Effectiveness of Flavonoid-Rich Diet in Alleviating Symptoms of Neurodegenerative Diseases

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Abstract: Over the past decades, there has been a significant increase in the burden of neurological diseases, including neurodegenerative disorders, on a global scale. This is linked to a widespread demographic trend in which developed societies are aging, leading to an increased proportion of elderly individuals and, concurrently, an increase in the number of those afflicted, posing one of the main public health challenges for the coming decades. The complex pathomechanisms of neurodegenerative diseases and resulting varied symptoms, which differ depending on the disease, environment, and lifestyle of the patients, make searching for therapies for this group of disorders a formidable challenge. Currently, most neurodegenerative diseases are considered incurable. An important aspect in the fight against and prevention of neurodegenerative diseases may be broadly understood lifestyle choices, and more specifically, what we will focus on in this review, a diet. One proposal that may help in the fight against the spread of neurodegenerative diseases is a diet rich in flavonoids. Flavonoids are compounds widely found in products considered healthy, such as fruits, vegetables, and herbs. Many studies indicated not only the neuroprotective effects of these compounds but also their ability to reverse changes occurring during the progression of diseases such as Alzheimer's, Parkinson's and amyotrophic lateral sclerosis. Here, we present the main groups of flavonoids, discussing their characteristics and mechanisms of action. The most widely described mechanisms point to neuroprotective functions due to strong antioxidant and anti-inflammatory effects, accompanied with their ability to penetrate the blood-brain barrier, as well as the ability to inhibit the formation of protein aggregates. The latter feature, together with promoting removal of the aggregates is especially important in neurodegenerative diseases. We discuss a therapeutic potential of selected flavonoids in the fight against neurodegenerative diseases, based on in vitro studies, and their impact when included in the diet of animals (laboratory research) and humans (population studies). Thus, this review summarizes flavonoids' actions and impacts on neurodegenerative diseases. Therapeutic use of these compounds in the future is potentially possible but depends on overcoming key challenges such as low bioavailability, determining the therapeutic dose, and defining what a flavonoid-rich diet is and determining its potential negative effects. This review also suggests further research directions to address these challenges.

Keywords: soy-rich diet; neurodegenerative diseases; flavonoids; animal models; prevention; treatment

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1. Introduction

Neurodegenerative diseases are a heterogeneous group of disorders characterized by progressive damage and loss of neurons in different areas of the central or peripheral nervous system (CNS and PNS, respectively) [1]. They are accompanied by pathological, histological, and hematological changes [2,3]. The mechanism of neurodegeneration itself

(neuronal loss and accompanying disorders) is due to defects at the molecular level, i.e., pathological protein aggregation, synaptic and neuronal network dysfunction, aberrant proteostasis, cytoskeletal abnormalities, altered energy homeostasis, DNA and RNA defects, inflammation, and neuronal cell death [3] (Figure 1). Moreover, every individual has a risk factor leading to the development of changes that favor the onset of neurodegeneration, and that is age. In the ageing brain, there are various processes facilitating neuronal dysfunctions, like increased oxidative stress, abnormalities in cell metabolism (mitochondria-related energy disturbances), alterations in protein processing leading to the accumulation of protein aggregates, and immune system activation [4]. Abnormalities at the molecular and cellular levels translate into a specific clinical picture, i.e., signs and symptoms characteristic for a given disease, in which cognitive impairment, memory impairment, motor dysfunctions, and speaking disability predominate [3,5]. Hundreds of neurodegenerative diseases are now known, but the highest attention is given to the most common ones: Alzheimer disease (AD), Parkinson disease (PD), prion disease, Amyotrophic lateral sclerosis (ALS), Huntington disease (HD), and spinal muscular atrophy (SMA) [6,7].

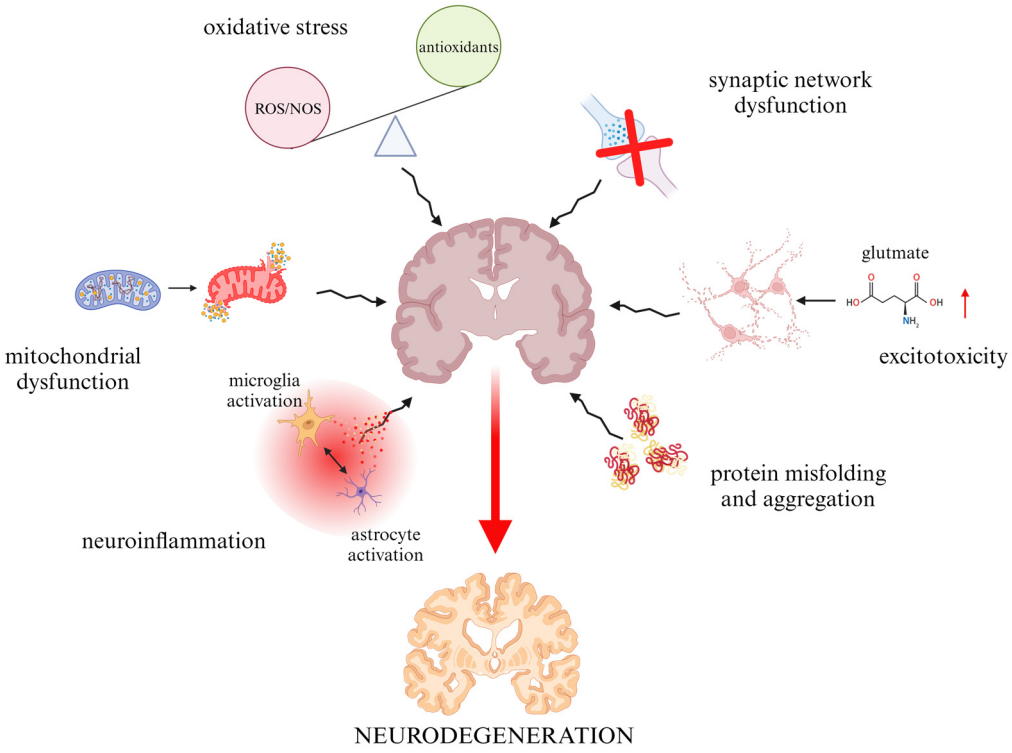


Figure 1. The causes of neurodegeneration. The neurodegeneration process may be caused by various types of abnormalities; the most common of them are (i) increased levels of oxidative stress, (ii) synaptic network dysfunction, (iii) excitotoxity resulting from high glutathione levels, (iv) incorrect folding of proteins and their accumulation in the form of aggregates, (v) neuroinflammation resulting from excessive stimulation of microglia, and (vi) mitochondrial dysfunction causing, among others, increasing concentrations of reactive oxygen species (ROS) and intensifying apoptosis. This figure was made with Biorender.com (accessed on 15 May 2024; Agreement no. YP26TQSS3W).

No effective treatment is currently available for any of these diseases, and only some drugs are registered to help reduce or relieve the symptoms [6]. The main difficulty in developing new therapeutics is not only the inability to inhibit neurodegeneration at different levels, but also the need to cross the blood-brain barrier [6]. An advantage would be the possibility of using compounds able to prevent the development of changes facilitating the initiation of neurodegeneration, which appear with age [4]. All the above-mentioned features are possessed by flavonoids [4,5]. These compounds are being extensively studied

for their use in the treatment and prevention of neurodegenerative diseases, some of which are proving to be effective not only in alleviating disorders at the cellular level, but also in the clinical picture.

In this review, we will focus on discussing the effectiveness of the most intensively studied flavonoids. These compounds can be divided into six main groups, namely flavones, flavonols, flavanones, flavanonols, anthocyanidins and isoflavones [4]. Flavonoids are being studied for their potential benefits in conditions such as chronic inflammation, arthritis, cardiovascular disease, diabetes, cerebral ischemia, hemorrhagic conditions including stroke, and even cancer [8–12]. Because of their neuroprotective functions, they are also considered for therapy or prevention in neurodegenerative diseases [13–18].

Despite many promising therapeutic effects of flavonoids, low bioavailability remains a significant challenge. These compounds are abundant in common food, such as potatoes, cucumbers, onions, strawberries, seeds, nuts and grapes. However, achieving a therapeutic effect requires consuming these foods in large quantities. For example, an isoflavone-rich diet requires approximately 100 mg of isoflavones per day, which means consuming 250–500 g of tofu, drinking 1–1.7 L of soy milk, or taking 200–500 g of edamame (green soybeans) daily [19]. This is difficult to achieve through diet alone.

Another problem is that the most common source of flavonoids in food is soy and related soy-derived products, while potential adverse effects of their consumption, especially at relatively high amounts, cannot be ignored [20,21]. Especially, isoflavones included in soy act as phytoestrogens, potentially disrupting hormone levels and affecting endocrine function. High soy intake can also interfere with thyroid function due to goitrogens [22]. Moreover, the high fiber content in soy may cause digestive problems, like bloating and gas [23]. Additionally, soy contains phytates that can hinder the absorption of essential minerals, possibly leading to their deficiencies [24]. For those with soy allergies, consumption of soy-derived products can trigger allergic reactions. It was also suggested that excessive soy intake might impact reproductive health and even influence cancer risk, though the latter effect appears controversial [20,21].

Therefore, it is important to investigate whether consuming a combination of polyphenols can provide a synergistic effect, improving bioavailability and reducing the need to consume large amounts of specific foods, taking into consideration also possible adverse effects. It is crucial to address the information gap regarding the combination of products containing various flavonoids to increase their bioavailability. For instance, fisetin, which exerts neuroprotective effects through the gut-brain axis [25], may benefit from being studied in combination with other gut-brain axis compounds or those with high bioavailability, potentially enhancing its absorption and neuroprotective effects. Solving the bioavailability problem could bring us closer to treating or preventing neurodegenerative diseases. Another research goal should be identifying the optimal forms of consuming these compounds through food. Food processing can enhance the therapeutic value and acceptance of these foods, as seen with fermented soy products showing better therapeutic effects than steamed soybean [26,27]. Although flavonoids have relatively low toxicity, excessive consumption may lead to digestive side effects. Proper processing methods, such as heat treatment, can help mitigate these effects. Additionally, it is important to disseminate knowledge about flavonoid consumption among doctors and patients. There are recurring myths about the impact of isoflavones in soy on male feminization [28] and risk of potential interactions with medications, similar to how grapefruit juice can affect drug efficacy [29,30]. For instance, hesperidin, found in large amounts in citrus fruits, including grapefruits, may interact with medications, which is also worth investigating in the case of this flavonoid and the others.

In this review, we present the latest knowledge on neurodegenerative diseases and the most commonly researched flavonoids with an emphasis on therapeutic effects they may cause, with the limitations observed today.

2. Overview of the Use of Flavonoids in Neurodegenerative Disorders

2.1. Alzheimer Disease (AD)

AD is a neurodegenerative disorder with a complex etiology. Neurotransmitter abnormalities, accumulation of short β-amyloid segments and aggregation of the hyperphosphorylated form of the tau protein (pTau) into neurofibrillary tangles (NFTs) underlie the disease [31]. In familial forms of the disease (FAD), the occurrence of the aforementioned disorders is associated with mutations in the gene encoding the β-amyloid precursor, APP, or in genes of the prenisilin (*PSEN1* and *PSEN2* genes), which cut the β-amyloid precursor protein APP. Mutations in the *APOE4* gene, which encodes an apolipoprotein responsible for cholesterol transport within the nervous system, are also considered a genetic risk factor for AD [32]. Protein aggregation is accompanied by increased levels of reactive oxygen species (ROS), increased oxidative stress, decreased neurotransmission, and microglial cell-activated neuroinflammation [33]. The first symptom, usually appearing significantly earlier than other disorders, is dementia. Over time, cognitive decline and memory impairment occur [34].

Studies conducted on the use of flavonoids have shown that their regular consumption reduced the risk of dementia and AD by lowering the aggregation of pathological proteins [34,35]. However, the efficacy depended on the type of flavonoid used for the therapy. One of the tested compounds was quercetin showing efficacy in improving cognitive function, learning ability and memory in AD [2]. The effect of quercetin was to inhibit the aggregation of both β-amyloid and NFTs and to decrease the level of phosphorylation of the tau protein [36,37]. Improvements in cognitive functions are associated with the inhibition of acetylcholinesterase, an enzyme that degrades acetylcholine [38]. In addition, this compound stimulates antioxidant enzymes and in itself has the ability to reduce ROS levels, thus lowering the oxidative stress [39]. Quercetin also has an inhibitory effect on excessive activation of the immune system by reducing ROS abundance, decreasing the production of pro-inflammatory cytokines in macrophages, and down-regulating the expression of genes encoding them [36].

Another compound with proven efficacy in alleviating AD symptoms is rutin, which is a quercetin glycoside. It was demonstrated that rutin improved both behavioral and cognitive impairment [40]. The action of rutin is to alleviate inflammation by inhibiting FβB (nuclear factor kappa light chain enhancer of activated B cells), and NFβB-dependent pro-inflammatory cytokines, and down-regulating the expression of genes encoding pro-inflammatory cytokines [40]. In addition, this compound lowers ROS levels and reduces lipid peroxidation [41]. Administration of rutin also promotes the elevation of levels of markers of neurotrophic factor activity [42].

Genistein is another flavonoid with a therapeutic potential in AD [43]. Like all flavonoids, genistein has antioxidant and anti-inflammatory properties and stimulates autophagy [44]. However, the most important aspect of its action is the ability to lower the levels of the pTau protein, to reduce Aβ deposits and to inhibit the production of this protein [44,45]. In addition, this compound inhibits the activity of acetylcholinesterase, thus demonstrating neuroprotective effects [46]. All of the above-mentioned properties of genistein contribute to its therapeutic effects. Studies using animal models demonstrated that genistein normalized the behavior of animals and improved memory, learning ability and cognitive functions [44–47]. Moreover, genistein turned out to be effective in delaying the development of dementia in patients with prodromal AD [48].

More flavonoid compounds with their effects and mechanisms of action in AD are shown in Table 1.

2.2. Parkinson Disease (PD)

PD is the second most common progressive neurodegenerative disease [49]. Underlying the disease is the accumulation of so-called Lewy bodies composed of α-synuclein and ubiquitin, and Lewy neurites (LN) [50]. A characteristic feature of PD is degeneration of dopaminergic neurons (50–70% degeneration occurs even before the first symptoms)

leading to patient-specific motor slowing, rigidity and resting tremor, associated with a decrease in dopamine (DA) level [51,52]. The pathogenesis of PD also includes oxidative stress, high levels of ROS, mitochondrial dysfunction and excessive activation of inflammatory processes [53]. As with other neurodegenerative disorders, there is also no effective treatment of PD, and only partial control of the symptoms and the course of the disease is currently available [54].

Myricitrin is being investigated for its potential use not only in the treatment of neurodegenerative diseases, but also in their prevention [55,56]. Research into the mechanism of action of this flavonoid has shown that it effectively reduces oxidative stress, inhibits apoptosis, and reduces the neuroinflammatory response. It is also effective in inhibiting mutant α-synuclein. In addition, after stimulating the activation of inhibitory neurons of the paraventricular nuclei of the hypothalamus, myricitrin exhibits antiepileptic activity. The aim of myricitrin-based therapy of PD is not only to stabilize the abnormalities characteristic for neurodegenerative diseases, but primarily to raise dopamine by increasing efficiency of its synthesis, using agonists for the DA receptor or inhibiting DA degradation [55,56]. Myricitrin has the ability to slow down DA degradation by inhibiting monoamine oxidases (MAO). MAO is a family of oxidoreductases that deaminate catecholamines. In dopaminergic axons, DA from the extracellular space is re-transported for the reuse or degraded by MAO [57]. Myricetin-treated mice showed a reduction in MAO activity and an increase in DA levels in the striatum. Perhaps this compound could be used in the early stages of PD to inhibit the disease progression or to serve as an adjuvant to L-DOPA in more advanced stages [55].

There are promising results from studies on the use of baicalein in the treatment of PD. This compound is known, among other things, for its antioxidant, anti-inflammatory and neuroprotective properties [58]. Studies using animal models demonstrated that administration of baicalein increases the levels of neurotransmitters, i.e., DA, DOPAC (3,4-dihydroxyphenylacetic acid), and HVA (homovanillic acid) [59]. In addition, baicalein protects nervous system cells from 6-hydroxydopamine (6-OHDA)-, 1-methyl-4-phenylpyridinium (MPP+)-, glutamate-, Aβ-, hydrogen peroxide (H_2O_2)-, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-, and methamphetamine-induced neurotoxicity [16]. Since one of the main causes of PD is α-synuclein accumulation, baicalein has also been tested for inhibition/degradation of toxic aggregates. It was found that this compound is effective in both inhibiting the aggregation of abnormal proteins and degrading already formed deposits [60]. Importantly, this flavonoid prevented the loss of dopaminergic neurons, and improved the behavioral disturbances that are part of the clinical picture of PD [61,62].

More flavonoid compounds with their effects and mechanisms of action in PD are shown in Table 1.

2.3. Amyotrophic Lateral Sclerosis (ALS)

ALS is a neurodegenerative disease of a progressive nature. The main cause of ALS is the loss of motor neurons (responsible for voluntary muscle movements) resulting in progressive paralysis leading to death around 5 years after diagnosis. Protein aggregation is also observed during the course of the disease [4]. Despite intensive research, to date only two therapies have been approved with limited efficacy on both motor function improvement and patient survival [63,64].

Several studies have been dedicated to the use of flavonoids in the treatment of ALS. One of the compounds tested was fisetin [2]. This compound is a natural antioxidant with anti-inflammatory and neuroprotective properties. The ability to reduce ROS levels translated into a reduction in oxidative stress and neurotoxicity, and preservation of normal mitochondrial functions [65]. Studies in a mouse model of ALS have shown that oral fisetin supplementation improved motor fusions and increased the number of spinal cord motor neurons. It was also observed that fisetin could reduce disease progression and increase life expectancy [66].

Another compound whose efficacy is being investigated in the context of ALS therapy is kaempferol. This compound has the ability to inhibit the aggregation of pathogenic proteins, such as Aβ, tau, and α-synuclein, preventing the activation of microglia, reducing pro-inflammatory factors, and lowering ROS levels, possibly through inhibition of the actions of certain enzymes [67]. Kaempferol appears to be a good candidate for ALS therapy due to its ability to counteract neurotoxicity caused by mutations in the *SOD* gene. Aggregation of toxic proteins in cells leads to mitochondrial damage and excessive ROS production. The action of kaempferol is to inhibit the aggregation of the mutant SOD1 protein, to inhibit ROS production and to stimulate autophagy. In addition, it binds to abnormal proteins, preventing the mutant forms from interacting with each other and stabilizing their structure [68,69].

More flavonoid compounds with their effects and mechanisms of action in ALS are shown in Table 1.

2.4. Huntington Disease (HD)

HD, a genetic, progressive, neurodegenerative disease, is caused by a mutation resulting in the expansion of the CAG nucleotide triplet in exon 1 of the *IT15* (*HTT*) gene [70]. This leads to creation of a long stretch of glutamine residues in the amino acid sequence of the huntingtin (HTT) protein, which impairs its proper folding. As a result, mutant huntingtin (mHTT) accumulates in cells, including neurons, as insoluble, difficult-to-remove aggregates that impair cell function [71]. Pathological changes in the nervous system are accompanied by neuroinflammation, neurotoxicity, microglial activation, elevated ROS levels, and oxidative stress, leading to mitochondrial dysfunction and impaired neurotransmission [71].

In recent years, genistein has been of great interest to researchers investigating treatments for diseases with neurodegeneration. Similar to the previously mentioned flavonoids, genistein crosses the blood-brain barrier and exhibits neuroprotective effects at multiple levels [72]. Like other flavonoids, genistein has an oxidative effect, reducing oxidative stress by lowering ROS levels in cells and restoring normal mitochondrial function [73,74]. In addition, this compound prevents neuro-inflammatory effects by inhibiting the acetylcholinesterase (AChE) factor-activated B cells' (NF-κB) activity [73,75]. In the context of the treatment of HD (and other neurodegenerative diseases), an important aspect is the ability of genistein to stimulate autophagy [76]. Autophagy is a process by which, among other things, compounds that are harmful to the cell (like abnormally folded proteins) are removed. This process is often impaired in diseases with accumulation of various compounds, and thus also in neurodegenerative diseases. Genistein improves the efficiency of autophagy, so that mutated huntingtin aggregates are efficiently removed from the cells [76]. Such multi-level effects of genistein lead to improvements in motor, cognitive, behavioral and memory functions [70,73,76].

Another compound from the flavonoid group with a therapeutic potential in HD is luteolin, a substance belonging to the flavones [77]. In traditional Chinese medicine, herbs, vegetables and fruits rich in lutein were used to treat hypertension, inflammation, and cancer [78]. The use of luteolin in the treatment of neurodegenerative diseases is due, among other things, to its anti-inflammatory properties. This compound reduces levels of pro-inflammatory cytokines, interleukins and inflammatory mediators [79]. In addition, luteolin reduces ROS levels and oxidative stress (in HD generated by, among others, the aggregation of mutant huntingtin) by increasing the levels of enzymes and compounds with antioxidant properties, like superoxide dismutase (SOD), glutathione peroxidase and catalase (CAT) [80]. Additionally, in a mouse model of HD, luteolin was shown to stimulate the Nrf2-HO-1 antioxidant pathway [81]. Studies with a cellular model of HD confirmed that luteolin effectively reduced levels of mHTT aggregates and protected cells from the cytotoxic effects of the mutation in *HTT* [81]. Experiments with the *Drosophila* model confirmed the results obtained in studies with cell lines, additionally indicating a delay in the loss of motile functions in flies treated with luteolin [82].

More flavonoid compounds with their effects and mechanisms of action in HD are shown in Table 1.

Table 1. Selected flavonoids and their actions in neurodegenerative diseases.

Neurodegenerative Disease	Flavonoid	Study Model	Therapeutic Effects	References
Alzheimer's disease	7,8-DHF	animal	• rescues memory deficits • neuroprotective effect by ameliorating oxidative stress and mitochondrial dysfunction • promoted dendrite branching and synaptogenesis • inhibited the loss of hippocampal synapses • restored synapse number and synaptic plasticity	[83–85]
	Catechin	animal	• reduce oxidative stress in peripheral and brain tissue • decrease alfa-synuclein levels • reduce amyloid-β plaque aggregation and tau phosphorylation	[86]
		human	• improvement of cognitive function	[87]
	Genistein	animal	• reduction of the production of Aβ • reduce the accumulation of Aβ1-25 and Aβ3342 fragments. • reduce the neurotoxicity induced by Aβ42 and Aβ accumulation • significantly improve the cognition • activation of autophagy • improve cognitive function • protective effect of on cholinergic neurons • decrease the hyperphosphorylation of tau protein	[45,46]
		human	• reduction of the production of Aβ • improve cognitive function • reduction of inflammation markers	[48]
	Luteolin	animal	• exhibits anti-inflammatory neuroprotective properties • ameliorated memory and recognitive impairment • reduction amyloid-beta (Aβ) production and promotion Aβ degradation • amelioration of spatial learning and memory impairment • protective effects against amyloid beta-induced oxidative stress	[88–90]
		cellular	• restored mitochondrial function, decreased mitochondrial superoxide, and preserved mitochondrial morphology	[91]
	Nobiletin	animal	• improved cognitive deficits • memory improvement • protection against cholinergic innervation of the hippocampus • reduction of the amount of Aβ and inhibited formation of Aβ plaques • ameliorates learning and memory impairment • reduced ROS levels in the hippocampus • promoting the survival of nerve cells, and promoting axon growth	[92–95]

Table 1. *Cont.*

Neurodegenerative Disease	Flavonoid	Study Model	Therapeutic Effects	References
Amyotrophic Lateral Sclerosis	7,8-DHF	animal	• improved motor deficits • preserved spinal MNs and dendritic spines • enhanced spatial memory • slowed the advance of ALS • improve the motor ability • promote mitochondrial biogenesis • attenuated motor neurons loss	[96–98]
	Fisetin	animal	• decreases free radical levels to protect against oxidative stress • upregulates the expression of antioxidant factors by activation • detergent-insoluble mutant hSOD1 proteins • improvement of motor ability	[66]
	Genistein	animal	• suppressed the production of pro-inflammatory cytokines • improved the viability of motor neurons • induced autophagy • neuroprotective effects by protection against oxygen singlet-induced cerebral damage	[99,100]
	Kaempferide and kaempferol	animal	• reduced SOD1 aggregation • antioxidant effects • prevent neurotoxicity by pumping autophagy	[101,102]
	Quercetin	cellular	• inhibit misfolding and aggregation	[103]
		animal	• reduced oxidative stress prevents mitochondria	[104]
Parkinson's disease	Genistein	animal	• reduced oxidative stress • improvement of spatial learning and memory • protection of dopaminergic neurons • improved rotational behavior • protection against protein aggregation toxicity • preserved motor dysfunction	[58,105–107]
	Baicalein	cellular	• inhibited the formation of α-synuclein oligomers • protected cells against cytotoxicity • restore mitochondrial function	[58,108,109]
		animal	• prevented the progression of α-synuclein accumulation • protected DA neurons against α-synuclein oligomer-induced toxicity • inhibited the formation of α-synuclein oligomers • prevented abnormal behavior by increasing DA neurons, as well as dopamine and serotonin levels in the striatum • inhibited oxidative stress and the astroglial response • induces mitochondrial autophagy	[110–113]

Table 1. *Cont.*

Neurodegenerative Disease	Flavonoid	Study Model	Therapeutic Effects	References
Quercetin	Hesperetin	animal	• improvement in learning and cognitive impairment • promotes neuronal survival • reduced memory loss • decrease astrocyte and microglia activation • reduced motor dysfunction	[114–117]
		cellular	• suppression of glial activations in primary microglia and astrocytes • protection against ROS	[118–120]
	Morin	animal	• attenuated behavioral deficits and DA deprivation • prevented motor dysfunction • reduce neuroinflammation • ameliorated motor dysfunction • alleviated MPTP-induced astrocyte activation	[118,119,121]
	Nobiletin	animal	• improved motor and cognitive deficits • inhibition of neuroinflammation • partial protection of dopaminergic neurons against neurotoxicity	[92]
Huntington's disease	7,8-DHF	animal	• delay motor deficits associated with HD • reverse cognitive deficits • provide neuronal protection by improving enkephalin levels and preventing volume loss in the striatum normalize levels of induced and neuronal nitric oxide synthase	[122]
	Anthocyanins	animal	• delaying motor dysfunction • improvement in motor function • reduced cholesterol oxidation products in the cortex • gender differences in motor function improvement highlight the significance of considering gender disparities in treatment approaches • reduction in CAG repeat instability	[123,124]
	Genistein	cellular	• correction of HTT levels • leads to the disappearance of aggregates formed by mHTT in HD fibroblasts • stimulation of autophagy • improvement of HD phenotype • reduction of mutated huntingtin levels increased cell viability	[76,125]
		animal	• antioxidant and anti-inflammatory properties correction of behavioral abnormalities	[126]
	Hesperidin	animal	• improved motor function • protective effects on the oxidative system, reducing oxidative damage levels and increasing catalase (CAT) enzyme activity • inhibiting microglial activation • improved behavioral and cognitive function • decreasing lipid peroxidation • decreasing nitrite concentration • increasing superoxide dismutase • increasing glutathione levels • attenuates neuroinflammation	[127,128]

Table 1. *Cont.*

Neurodegenerative Disease	Flavonoid	Study Model	Therapeutic Effects	References
Huntington's disease	Naringin	animal	• preventive effects against 3-nitropropionic acid • memory function improvement • restoration of glutathione system functioning • increased intracellular glutathione concentrations • reduction of free radicals • modulates histological changes • reduces the expression of inflammatory mediators	[129,130]

3. General Characteristics and Targets of Flavonoids

Flavonoids are a large subgroup of polyphenols which include over 6000 compounds that can be found in products of natural origin, including fruits, vegetables, barks and herbs (tea, berry fruits, apples, red wine, and orange or orange juice). In this review, we will focus on investigating the effectiveness of the most common flavonoids researched. These compounds can be divided into six main groups called: flavones (e.g., luteolin, apigenin, quercetin), flavonols (e.g., quercetin, fisetin, kaempferol), flavanones (e.g., naringenin, hesperidin), flavanonols (e.g., taxifolin, dihydroxyflavonol), anthocyanidins (e.g., cyanidin, pelargonidin, delphinidin) and isoflavones (e.g., genistein, daidzein, glycitein, biochanin A) (Figure 2). This classification is based on the oxidation state of the central C ring, the ring hydroxylation pattern, and the 3-position substitution. Within each group, a diversity of compounds results from the arrangement of hydroxyl groups in combination with glycosylation or alkylation [4]. Due to their numerous properties, for instance antioxidant function, anti-inflammatory activity, and availability to penetrate the blood-brain barrier, flavonoids are increasingly being noticed in the search for therapy for many diseases. In vitro and in vivo studies have demonstrated that flavonoids can penetrate the blood-brain barrier (BBB) and exert effects within the brain. Studies conducted with rats and pigs have specifically shown that one of the exemplary flavonoids discussed in more detail later in this work, anthocyanin, is capable of crossing the blood-brain-barrier [131]. It has also been shown that quercetin, kaempferol, and isorhamnetin are detected in the brain of rats after ingestion of ginkgo biloba extract [132]. Due to their functions, such as anti-apoptotic effects along the Nrf2, NF-κB and p53 pathways, flavonoids play a crucial role in coordinating cellular responses to various stimuli, ultimately determining the survival or death of neurons. Flavonoids are being explored for the treatment of numerous conditions including inflammations, diabetes, and cancer. Moreover, many flavonoids are currently under investigation for their neuroprotective properties, particularly in the context of neurodegenerative diseases [13–15]. The strong anti-inflammatory properties of flavonoids in the brain are a promising aspect of therapy for both the most frequent neuroinflammatory diseases, such as AD or PD [16–18], but also other disorders, like cerebral ischemia or hemorrhagic conditions, including stroke.

Because flavonoids belong to a diverse family of plant polyphenolic molecules, it remains unclear whether consuming individual polyphenols or a combination of them (synergistic effects) provides the greatest health benefits to humans. Moreover, studies have revealed the low bioavailability of flavonoid compounds in humans, presenting a significant challenge in establishing recommended intake doses and, consequently, determining their therapeutic value. This challenge is compounded by the declining quality and nutrient density of modern food sources. Despite their possible toxicity, flavonoids are relatively safe and non-invasive drugs of a natural origin. Excessive consumption of flavonoids leading to adverse effects often seems to be related to the overuse of supplements without consulting a doctor or knowing the proper dosage. This is especially a risk for people taking other medications because flavonoids may interact with them [133]. These seem to be extreme cases out of ignorance because flavonoids offer cognitive health benefits in

humans, as long-term consumption through food has been shown to reduce the risk of cognitive decline. Cohort studies indicated that flavonoids also lower the risk of AD, PD, and related dementia. Interestingly, the average daily intake of flavonoids from food varies across different regions of the world, for example 132 mg in the United States, 250–900 mg in Europe, 200–650 mg in Asia, and 1650 mg in the Middle East [134].

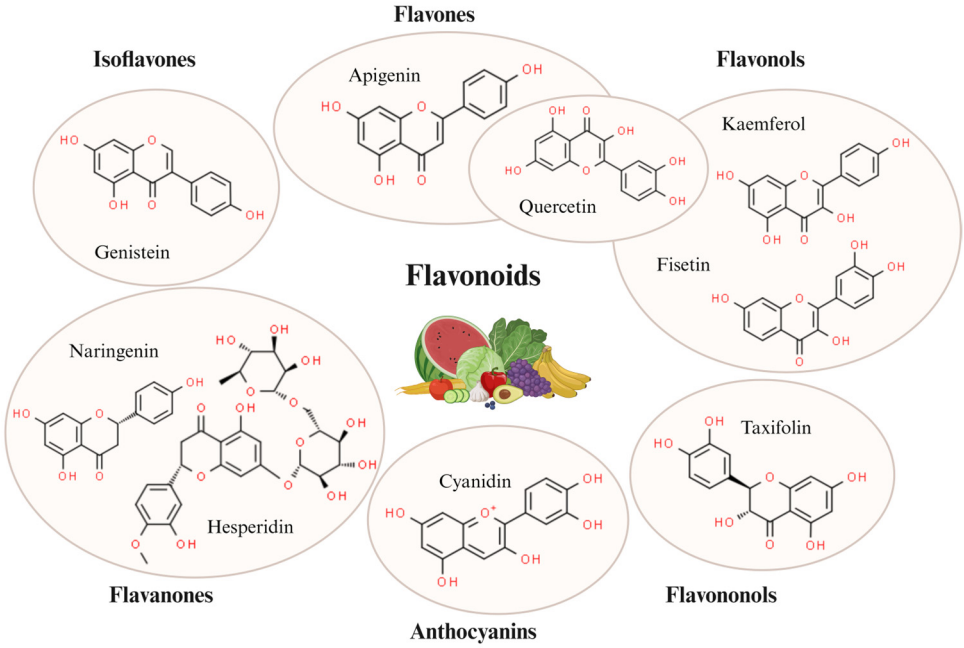


Figure 2. Classification of flavonoids and representatives of each group, described in the text. This figure was made with Biorender.com (accessed on 15 May 2024; Agreement no. GD26TLR8VA).

Greater efficacy is observed with the use of plant extracts in supplementation than with the use of synthetic compounds. The challenge with the bioavailability of flavonoids has led to many pharmacokinetic studies in animals and humans and numerous clinical trials. For example, in the case of genistein, nano- and micro-formulations for encapsulation were reported, including lipid nanoparticles, liposomes, tocotrienol-rich nano-emulsions, polymeric nanoparticles, dextran complexes, chitosan complexes [135] or nanoencapsulation of Cyanidin 3-O-Glucoside [136]. Research is ongoing to improve the pharmacokinetics of compounds synthesized ex planta [137].

The presence of hydroxyl and carbonyl groups in flavonoids enables chelation with biological metal ions and interaction with biomolecules, affecting their pharmacological properties. This structural feature allows for designing metal complexes with enhanced biological properties. Flavonoids, weak acids that deprotonate, strongly favor metal ion coordination, influencing reaction kinetics and thermodynamics [138,139]. While metal complexes may enhance antioxidant properties, it is not a universal rule, and each case requires individual examination. This method of modifying flavonoids has proven successful in the case of quercetin, where it has been shown that this treatment supports radical scavenging activity [140]. Increasing the bioavailability of flavonoids and intensifying their action in the human body is certainly an important issue and worth taking action due to their therapeutic potential and pleiotropic functions. Although the exact mechanisms have not yet been fully elucidated, accumulating data increasingly indicate the ability of flavonoids to interact with neuronal receptors, transcription factors and modulate signaling pathways. These pathways include actions of kinases crucial for neuronal activation and synaptic strengthening. As a result, flavonoids are recognized as promising bioactive compounds capable of influencing various aspects of brain functioning, such as synaptic plasticity, cognitive functions improvement, learning and memory in mammalian species [18,141].

It has been suggested that the neuroprotective effects of natural compounds are related to the potential prevention of cellular damage by reducing cellular oxidative stress and inflammation, but also potentially reversing pathological changes already present in the brain [18,142,143]. However, it appears that the molecular mechanisms of actions of flavonoids are significantly more complex. Mechanisms of biological activities of flavonoids vary from one another; however, there are also some common features, presented schematically in Figure 3. In following subsections, we present discussions on several selected flavonoids whose regulatory properties have been investigated most extensively.

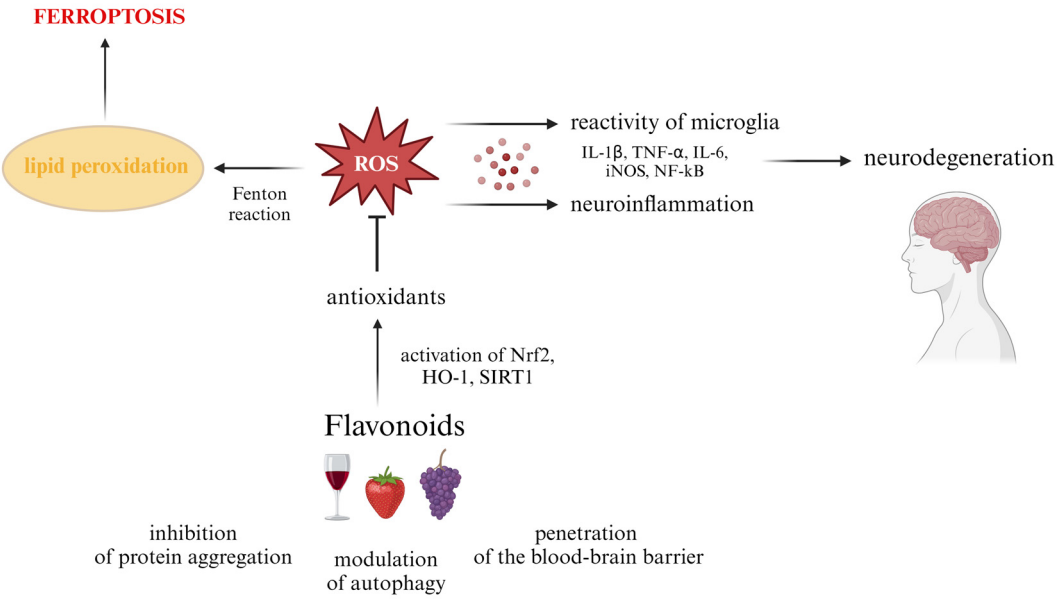


Figure 3. Examples of some of the neuroinflammatory and neuroprotective effects of flavonoids. Flavonoids, due to their ability to modulate autophagy, prevent the formation of protein aggregates and the ability to cross the blood-brain barrier, have important neuroprotective functions, particularly important in neurodegenerative diseases. By activating the Nrf1/HO-1 pathway, as well as improving mitochondrial function by targeting SIRT1 activity, these compounds alleviate the oxidative stress, and effectively reduce ROS formation. When occurring at high levels, ROS can act in various ways, including (i) stimulation of the Fenton reaction, which by increasing lipid peroxidation may contribute to ferroptosis that seems to be important in neurodegenerative diseases, (ii) induction of neuroinflammation which is one of the causes of the initiation and progression of neurodegeneration. Flavonoids inhibit ferroptosis by reduction of ROS levels, and cause a decrease in levels of inflammatory markers (TNF-α, IL-6, IL-1β, iNOS, and NF-κB), positively influencing the survival of neurons and silencing neuroinflammation. This figure was made with Biorender.com (accessed on 15 May 2024; Agreement no. SC26TLT68T).

3.1. Flavones

3.1.1. Apigenin

As one of the most frequently consumed flavonoids from the flavones subgroup, apigenin (4′,5,7-trihydroxyflavone) is present in the highest amounts in parsley, celery, mint, chamomile, lavender, apples, onions, oranges, lemons, broccoli, and spinach. It has a positive effect on learning, memory, and locomotor activity, as well as revealing anxiolytic properties, weaking depressive behaviors, and improving sensorimotor and motor coordination in animals with cognitive disease and neurobehavioral deficits [144–147]. It has been found that apigenin reduces the reactivity of microglia in the hippocampus, which is important in the initiation and progression of neurodegenerative diseases, such as AD [148]. Apigenin inhibits the loss of NAD^+ and activation of SIRT3, thus, appropriate homeostasis and mitochondrial functions are maintained in the event of neurotoxicity [149]. Apigenin also inhibits the release of pro-inflammatory cytokines TNF-α, IL-6 and the

pro-inflammatory enzyme iNOS-1 in induced PD. Additionally, it prevents the reduction of mRNA expression of neurotrophic factors BDNF and GDNF, reduces the aggregation of α-synuclein and increases the level of the TH protein, and also enhances the action of the dopamine D2 receptor in rats with ROT damage [150]. Moreover, apigenin reversed changes in the expression of genes encoding TNF-α, IL-1β, IL-6, IL-10 and TGF-β [151]. It also has a neuroprotective effect by increasing the number of living neurons and reducing the release of cytochrome c, which may suggest inhibition of the apoptosis pathway [152]. This flavone affects ERK/CREB/BDNF signaling, which translates into long-term enhancement and memory functions. It also alleviates cholinergic and acetylcholine deficits in the hippocampus, which are important for cholinergic neurotransmission and required for memory functioning. Additionally, it has antidepressant and anxiolytic effects, which may be related to the inhibition of inflammatory markers (TNF-α, IL-6, IL-1β, iNOS and COX-2) and the activation of NF-κB [144]. One should note that most of the results of experiments with apigenin come from studies on mouse and rat disease models [153]. Apigenin, like most other flavonoids, has the ability to penetrate the blood-brain barrier, while, as in the case of other flavonoids, the greatest challenge is to increase the bioavailability of these compounds [154].

3.1.2. Nobiletin

Nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone) is the main component of polymethoxylated flavones found in citrus fruit peels [16]. Specifically, nobiletin was demonstrated to be a promising compound in ameliorating cognitive deficits, memory impairment, and pathological features associated with AD, such as excessive accumulation of Aβ, hyperphosphorylation of tau and oxidative stress in animal models [92]. Nobiletin at a dose of 10–50 mg/kg was administered daily to rats for 7 days before and after the disease-inducing surgery. The impairment of reference and working memory in Aβ-treated rats was improved by nobiletin treatment, demonstrating its effectiveness. Furthermore, nobiletin treatment improved motor and cognitive deficits in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model [92]. The research results should encourage researchers to continue studies on this compound, which could potentially become a drug for the treatment and prevention of neurodegenerative diseases. It is worth continuing to examine the effects of this compound alone and in combination with other currently researched therapies for neurological diseases.

3.2. Flavonols

3.2.1. Quercetine

Quercetin (3,3,4,5,7-pentahydroxyflavone) is one of the most common secondary metabolites in the plant kingdom and one of the most frequently studied compounds. The Latin term for this flavonoid means oak forest. It belongs to the class of flavonols and is not synthesized in the human body. Quercetin is commonly found in the form of glycosides, i.e., it is conjugated to sugar residues. The largest amounts of this compound are found in onions, but it is also present in apples, berries, herbs (such as dill), asparagus, seeds and nuts, as well as in some varieties of tea and wine. It is the dominant flavonoid in the diet [155,156].

Quercetin has antioxidant, anti-inflammatory, antiproliferative, anticancer, antidiabetic and antiviral properties. This molecule is lipophilic and can easily cross the blood-brain-barrier. It is a powerful antioxidant that has been studied both in vivo and in vitro and is currently used in various pharmaceuticals [156,157]. Quercetin is known to be used to treat cancer, allergic reactions, inflammation, arthritis, and cardiovascular diseases. This flavonol also plays an important role in platelet aggregation and lipid peroxidation and enhances mitochondrial biogenesis [158]. The mechanism of its action in AD may be related to the direct suppression of Aβ formation and the stability of Aβ fibrils [159].

The neuroinflammatory process is suppressed by quercetin, which down-regulates pro-inflammatory cytokines, which include iNOS and NF-κB, thus influencing neuronal

regeneration. Quercetin reduces lipid peroxidation, and thus, reduces the risk of oxidative damage to neurons, as seen in studies involving a mouse model of AD, in which cognitive functions improved after 3 months of intraperitoneal quercetin administration [160]. The neuroprotective role of quercetin included in an energy drink was demonstrated. Consumption of quercetic included in the Red Bull drink increased mRNA expression levels and protein levels of Nrf2 and heme oxygenase-1 (HO-1), which appeared to protect the brain against neurotoxicity. This flavonoid protected the rat cerebral cortex against oxidative damage by acting as an anti-inflammatory agent by reducing the levels of the pro-inflammatory marker interleukin 1 beta and DNA damage markers [161].

In the case of dementia, where there is a disruption in iron homeostasis in the brain, quercetin has been shown to inhibit protein aggregation and prevent cell overload with iron. It regulated genes related to iron metabolism, removed free radicals and silenced Fenton reactions leading to ferroptosis [162]. As a result, quercetin may also be used to remove oxidative damage to the brain caused by iron oxide nanoparticle therapy (IONP), which may cause adverse effects [163]. Reports have shown that quercetin improved the antioxidant enzymes and anti-inflammatory cytokines, and also improved mitochondrial function by targeting SIRT1 activity through the SIRT1/AMPK/NF-κB, SIRT1/Keap1/Nrf2/HO-1, and SIRT1/PI3K/Akt pathways [164]. It has been observed that quercetin exerted neuroprotective effects by modulating microRNA expression in microglial cells. This demonstrated its ability to regulate inflammation, differentiation and apoptosis in neurological diseases [165]. Such treatment was proven to protect primary neurons from microglial toxicity and neurodegeneration induced by LPS in mouse models of PD [166].

3.2.2. Fisetin

Fisetin (3,3′,4′,7-tetrahydroxyflavone) is found in grapes, apples, onions, strawberries, and cucumbers [65]. Like other flavonoids, it is taken up by brain cells and crosses the blood-brain barrier and supports synaptic functions in the hippocampus [4,65]. It has shown effectiveness in neurodegenerative diseases such as AD, PD, HD and ALS [167]. Fisetin acts to regulate neuroinflammation, mediated by SIRT1, Nrf2/HO-1 and p-JNK/NF-κB, abolishes oxidative stress and neuroinflammation by regulating inflammatory mediators (IL-1β and TNF-α) and related markers with autophagy (Atg-3 and Beclin-1), which was confirmed by biochemical and behavioral studies in a mouse model of neuroinflammation and memory impairment [168], and supports synaptic functions in the hippocampus [167]. The effect of fisetin on the physiology of mitochondria in hippocampal HT22 neuronal cells under oxytotic/ferroptotic stress was examined and it has been shown that it is possible to restore mitochondrial homeostasis at the level of redox regulation, calcium uptake, biogenesis, and respiration modulation [169]. Fisetin significantly abolished LPS-induced increased ROS/oxidative stress and activated phosphorylated N-c-JUN terminal kinase (p-JNK) in the adult mouse hippocampus. The treatment inhibited LPS-induced activation of inflammatory Toll-like receptors (TLR4)/cluster of differentiation 14 (CD14)/phosphonuclear factor kappa (NF-κB) and attenuated other inflammatory mediators (tumor necrosis factor-α (TNF)-α), interleukin-1β (IL1-β) and cyclooxygenase (COX-2). Additionally, fisetin improved hippocampus-dependent synaptic and memory functions in adult mice after LPS treatment [170]. It also reduced the accumulation of p-tau and Aβ and additionally increased the expression of genes responsible for the formation of neprilysin, which additionally removed Aβ NEP in the brains of mice [171]. Interestingly, fisetin may play a role in the function of the gut-brain axis, positively influencing the intestinal microbiome, thereby providing neuroprotection to the brain [25].

3.2.3. Kaempferol

Kaempferol (3,4′,5,7-tetrahydroxyflavone) occurs widely in apples, strawberries, grapes, tomatoes, beans, and potatoes, *Gingko biloba* leaves, propolis and oregano [172]. It has antioxidant, antimicrobial, anticancer, neuroprotective and hepatoprotective properties and additionally reduces lipid oxidation [101]. In a mouse model, kaempferol improved

learning performance and memory deterioration in streptozotocin-treated rats. At the molecular level, streptozotocin-induced neurodegeneration resulted in decreased expression of GAD67, reelin, and phosphorylated NMDAR. However, kaempferol treatment alleviated these changes, increasing their levels up to the values measured in the control group [173]. It was determined that the NLRP3 (NLR family, pyrin domain cleavage 3) inflammasome is involved in the progression of PD in patients and various mouse models, however, kaempferol protected mice against LPS-induced neurodegeneration by inhibiting the treatment of the NLRP3 inflammasome. The reason for this was noted to be that kaempferol decreased the expression of CASP1-related genes and interfered with the assembly of NLRP3-PYCARD-CASP1 complexes. In parallel, the ingredient also reduced the secretion of IL1B, which means that it is one of the causes of neurodegenerative pathologies. Mechanistically, kaempferol promoted autophagy in microglia, causing decreased expression of the gene encoding NLRP3, which in turn inactivated the NLRP3 inflammasome. Interestingly, ubiquitination was involved in kaempferol-induced autophagic degradation of NLRP3, suggesting the connection of ubiquitination and autophagy by kaempferol, which was confirmed by in vivo studies [25].

3.3. Flavanones

3.3.1. Hesperetin

Hesperetin is a hesperidin ((2*S*)-3′,5-dihydroxy-4′-methoxy-7-[α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyloxy]flavan-4-one) aglycone, naturally occurring in large amounts in oranges, tangerines, lemons, and traditional Chinese medicinal herbs; in fact, this compound is heavily used in Chinese medicine [117,174]. Studies with humans have shown that the maximum concentration of hesperetin in plasma improves after oral consumption of citrus juices, such as grapefruit and orange juice [175]. It has various pharmacological properties, including antioxidant and anti-inflammatory properties. The latter properties enhance neuroprotection by supporting neuronal survival via phosphatidylinositol 3-kinase and protein kinase Akt B (PI3K-Akt), by inhibiting the pro-inflammatory cytokines (IL-1β, TNF-α and IL-6), and by controlling the p38 mitogen-activated protein kinase (MAPK) signaling pathway. Moreover, it can act by increasing levels of antioxidant proteins such as Nrf2, as shown in an LPS-stimulated mouse model [176,177]. Modulation of the signaling pathway, as well as antioxidant and anti-inflammatory effects of flavanones may contribute to the observed improvement in cognitive and motor disorders, which was reported in animal models of AD treated with hesperetin [117].

The neuroprotective effect of hesperetin was tested in rat models, where it was found that doses of 10 and 20 mg/kg administered for three weeks showed significant improvement in learning skills and cognitive functions and a reduction in the level of oxidative stress. A similar study was conducted to evaluate effects of this molecule in Aβ-stimulated AD, where it was observed to reduce neuroinflammation, apoptosis and neurodegeneration induced by oxidative stress [18,178]. Other studies have shown that hesperetin significantly protected cells against increased oxidative stress by inhibiting apoptotic cell death, ROS production and improving the expression of SOD and GSH, which probably caused the activation of Keap-1, Nrf2 and HO-1 [179]. These results strongly indicated that hesperetin ameliorated LPS-induced pathological features of neurodegenerative diseases in vivo (in adult mouse brains) and in vitro (in BV2 and HT-22 cells). Hesperetin also reduced LPS-induced neuronal apoptosis by inhibiting the action of phosphorylated N-terminal c-Jun kinases (p-JNK), B-cell lymphoma-associated protein 2 (Bcl-2) X (Bax), and caspase-3, and promoting Bcl-2 protein levels. Additionally, hesperetin enhanced synaptic integrity, cognitive function, and memory processes by enhancing phosphorylated cAMP response element binding protein (p-CREB), PSD-95 protein, and syntaxin. It was suggested that hesperetin provides neuroprotection by regulating the TLR4/NF-κB signaling pathway that protects against the harmful effects of LPS [176].

3.3.2. Naringenin

Naringenin ((2*S*)-4′,5,7-trihydroxyflavan-4-one) is abundant in various citrus fruits, tomatoes, bergamots and other fruits. Some studies have highlighted its neuroprotective properties. In male rats, naringenin was found to prevent anxiety-like behavior, orofacial dyskinesia and neurodegeneration induced by respine. Moreover, this inductor increased oxidative stress markers and neuronal degeneration in the striatum, both of which were alleviated by naringenin [180]. In neonatal mice, propofol-induced anesthesia led to neurodegeneration via apoptosis, while naringenin administration prevented neuronal loss. After exposure to propofol in adulthood, the mice showed long-term neuronal deficits, impaired neurocognitive functions and problems with memory and learning, while naringenin helped to mitigate these effects in the treated group [181]. The neuroprotective effect of naringenin was additionally exhibited in cellular and animal models of PD, when paraquat was used as an inducer of oxidative stress. Naringenin treatment in SH-SY5Y cells resulted in increased cell viability, reduced oxidative stress, and increased mitochondrial membrane potential. In a rat model, naringenin treatment showed significant neuroprotection against paraquat-induced behavioral deficits. This flavanone seems to modulate levels of mRNAs derived from genes coding for DRD2, DAT, LRRK2, SNCA, β-catenin, caspase-3, and BDNF genes. It also increased TH protein abundance and modulated its immunoreactivity in the striatum of rats and zebrafish, where oxidative stress was induced by 6-OHDA and the levels of oxidative stress biomarkers such as CAT, GSH, SOD and ROS decreased [182–184].

3.4. Isoflavones

Genistein

Genistein (4′,5,7-trihydroxyisoflavone), a naturally occurring isoflavone found in legumes, like soybeans, green peas, chickpeas and peanuts, shares the ability of other flavonoids to cross the blood-brain barrier, making it a subject of intense study in the aspect of neurodegenerative diseases [65,66,72,185]. In studies involving fibroblast from patients suffering from HD, it was showed that the addition of genistein led to notable degradation of mHTT within cells [125]. Furthermore, research has unveiled its capacity to mitigate oxidative stress and neuroinflammation in neonatal mice modeling cerebral hypoxia and ischemia. Genistein, by increasing cell viability, weakened the effect of neuronal damage and apoptosis, increasing the prospects of long-term recovery in mice. The mechanisms involve the activation of the Nrf1/HO-1 pathway while simultaneously inhibiting the NF-κB pathway [186]. Notably, genistein has shown promising properties in reducing cognitive deficits and neuronal loss in rats administered with ketamine to induce neurodegeneration. These effects were achieved through the regulation of apoptosis-related genes such as those encoding Bax, Bcl-2, caspace 3, and phosphorylated GSK-3b and Akt [187]. Genistein can inhibit the activity of NF-κB signaling pathway, which can be activated via TLR4 and which is one of the important elements influencing the development of neurodegenerative disease. This is consistent with the findings demonstrating ability of genistein to disrupt the binding of NF-κB to target DNA, thereby counteracting neuroinflammatory effects in AD [188]. Moreover, genistein reduced the production of NO in rat microglia induced by LPS and lowered the level of IRF-1 and STAT1. This isoflavone has antioxidant properties that can eliminate ROS and prevent neuronal necrosis and apoptosis induced by Aβ [46].

Genistein structurally resembles estradiol, thus, it acts through estrogen receptors, imitating the action of estrogen and influencing both the endocrine and nervous systems. This activity extends to the regulation of cognitive processes such as learning, concentration and memory. Genistein has demonstrated efficacy in restoring or enhancing memory functions across various animal models and human studies [189]. It was found that genistein exerts anti-inflammatory effects in microglial cells by activating the GPER receptor (one of estrogen receptors). These findings were supported by data from both the BV2 microglial cell line and primary microglial cultures, which sheds light on the contribution of GPER to genistein-mediated anti-inflammatory effects on microglial after LPS treatment [190].

There are more and more reports confirming that the action of isoflavones is at least partially exerted through the GPER signal transduction pathway [191–194]. Moreover, by acting through this receptor, which are increasingly recognized for their significance in neurodegenerative diseases [195], genistein also proved its neuroprotective effects. Studies also indicated that genistein induces dendritogenesis via Erα and GPER which is a newly described mechanism in neuronal protection and synaptic plasticity [196,197].

3.5. Anthocyanins

Cyanidin

Cyanidin (3,3′,4′,5,7-pentahydroxyflavylium), a natural flavonoid compound abundant in various plants like cherries, blueberries, blackcurrants, mulberries, black soybeans and honeyberries, exhibits neuroprotective properties against degeneration caused by oxidative damage. Both in vitro experiments and mouse model studies have demonstrated the ability of cyanidin-3-O-glucoside to reduce the mRNA expression of proinflammatory cytokines such as IL-1β, TNF-α and IL-6 within the cerebral cortex. Additionally, the treatment has been associated with increased levels of PPARγ and TREM2, leading to the elimination of accumulated Aβ through increased phagocytosis [198]. In a mouse model, oral administration of cyanidin-3-O-glucoside has been shown to protect the brain and improve cognitive behavior by reducing the level of the amyloid precursor presenilin-1, and β-secretase in the cortical and hippocampal regions. Such treatment has been associated with an up-regulation of autophagy-related markers such as LC3-II, LAMP-1, TFEB, and PPAR-α, along with a down-regulation of SQSTM1/p62, improving the autophagy of Aβ plaques and neurofibrillary tangles [199]. Intragastric administration effectively mitigated the decline in brain glucose uptake, alleviated neuronal damage in the hippocampus and cortex, and decreased the Aβ level in the brain [200]. Thus, this anthocyanin derivative may be a promising neuroprotective agent acting by inhibition of glutamate-induced oxidative stress signal and endoplasmic reticulum stress, while activating ERK/Nrf2 pathways [201].

Cyanidin treatment has shown significant attenuation of Aβ-induced PC12 cell death and morphological changes by restoring mitochondrial membrane potential through the increased Bcl-2 protein level. Cyanidin also protected cells against DNA damage by blocking ROS and by inhibiting mitochondrial apoptosis [202]. Cyanidin chloride inhibited the upregulation of proinflammatory cytokines TNF-α and IL-6, and suppressed hyperactivity of microglia, induced by LPS, indicating the anti-inflammatory action in the hippocampus. Moreover, this treatment restored the control of expression of genes encoding glial fibrillary acidic protein (GFAP), brain-derived neurotrophic factor (BDNF), glutamate-aspartate transporter (GLAST), and excitatory amino acid transporter 2 (EAAT2), which are members of the excitatory amino acid transporter family [203]. Finally, cyanidin has shown promising features in mitigating bisphenol A-induced oxidative stress and improving impaired neurogenesis in hippocampal and cortical regions of the brain [204].

3.6. Flavononols

Taxifolin

Taxifolin (3,5,7,3,4-pentahydroxy flavanone or dihydroquercetin), occurs in many plants, like Siberian pine, Siberian spruce, oak, cedar, walnut but also apples, onions, capers, tea and coffee. It has been shown to be able to limit the formation of Aβ fibrils, protect neurons, maintain normal dopamine levels, improve cerebral blood flow and cognitive abilities. Currently, the health supplement is used in clinical practice. Taxifolin improves cognitive and motor impairment in neurodegenerative diseases such as PD, AD, and HD [205]. The mechanisms underlying taxifolin-mediated neuroprotection against neurodegeneration are presented in Table 1.

It was demonstrated that oral administration of taxifolin to a mouse model of cerebral amyloid angiopathy (CAA) effectively inhibited the intracerebral production of Aβ by suppressing ApoE-ERK1/2-amyloid-β precursor protein axis, despite its limited permeability through the blood-brain barrier. Taxifolin reduced inflammation by alleviating the

accumulation of TREM2-expressing cells in the brain, which are associated with heightened inflammation. I also moderated glutamate levels and attenuated oxidative tissue damage, as well as decreased active caspases, indicating a decrease in apoptotic cell death [206].

4. Effects of a Flavonoid-Rich Diet on Neuroprotection

4.1. Animal Studies

Experiments employing animal models are especially useful in preclinical studies, when the efficacy of tested compounds can be extensively tested. This is also the case with studies on the effects of a flavonoid-rich diet on neuroprotection.

Animal studies conducted with adult Sprague-Dawley rat females after ovariectomy showed that a soy-rich diet reduced programmed cell death following a 90 min transient middle cerebral artery occlusion (tMCAO). Before tMCAO, rats were fed for two weeks with a diet low in isoflavonoids or a soy-rich diet. The soy-rich diet significantly reduced the extent of infarction and apoptosis in the ischemic brain cortex. A decrease in the translocation of the apoptosis-inducing factor was observed, along with a significant reduction in the number of active caspase-3 positive cells and caspase-mediated α-spectrin cleavage. Additionally, unlike estradiol, the soy diet affects the expression of *bcl-xL* in the ischemic cortex, enhancing its neuroprotective action, suggesting that a soy diet may have a positive impact on protecting the brain from stroke-induced damage [207]. Moreover, a soy diet in male N-Marry albino rats could prevent disturbances in the blood-brain barrier, alleviate increased intracranial pressure and vestibular-motor, and alleviate neurological disorders in traumatic brain injury [208].

Other studies conducted with ovariectomized Sprague-Dawley rats fed for 12 weeks with phytoestrogens from soy sprouts or estradiol demonstrated that both estradiol and phytoestrogens can significantly increase the expression of the brain-derived neurotrophic factor (BDNF)-encoding gene and a gene of its primary receptor TrkB (tyrosine kinase receptors B) in the hippocampus. The increased expression of genes coding for proteins involved in the postsynaptic BDNF-TrkB pathway and presynaptic vesicle proteins (synaptophysin, synaptotagmin 1, and synapsin 1) in the hippocampal tissue had a beneficial effect on the development of synaptic plasticity, synaptic transmission, and overall cognitive function development, which might be responsible for the observed effect of improved spatial learning and memory performance in rats [209]. Similar results of improved spatial memory performance and faster learning of rates were observed in young animals subjected to a 7-week supplementation with a flavonoid-rich berry diet. The cognitive function results correlated with elevated levels of pro- and mature brain-derived neurotrophic factor (BDNF) in the hippocampus [210]. Furthermore, the use of phytoestrogens, unlike the use of estradiol, did not cause negative effects on the reproductive system of animals, suggesting that phytoestrogens with potentially neuroprotective action in the brain may be a safer alternative to estrogen replacement therapy, used among others in postmenopausal women to improve cognitive functions [209,211].

It was demonstrated that genistein has a protective effect on neuron degeneration in a mouse model with ApoE-encoding gene deletion, that was fed a high-fat diet. The absence of apolipoprotein E in the brain leads to increased susceptibility to neurodegeneration. A high-fat diet in mice induces oxidative stress, inflammation of neurons, accumulation of Aβ, hyperphosphorylation of tau, and neuronal cell death. Mice whose diet was supplemented with genistein showed alleviated inflammation of the nervous system by reducing the level of oxidative stress, decreasing Aβ levels, and reducing hyperphosphorylation of the tau protein [212]. It was further observed that enriching the high-fat diet of mice with steamed soy and doenjang, a traditional Korean fermented soybean paste, mitigated inflammation and slowed down neurodegenerative processes. In both diets, as in the case of the use of genistein, a reduction in oxidative stress in the hippocampus and cerebral cortex was observed, as well as altered accumulation of Aβ and hyperphosphorylation of tau. Moreover, it was observed that increased levels of isoflavone aglycone may play a role in improving synaptic plasticity and defending against neurodegenerative diseases.

Additionally, it was noted that fermented soy products had greater neuroprotective efficacy than steamed soy, which may be related to the presence of bioactive compounds produced during fermentation [26,27].

Numerous studies indicated the effectiveness of both oral and injectable administration of flavonoids in preventing or alleviating symptoms of neurodegenerative diseases [213]. In further analyses, we will focus on the potential of oral application of flavonoids for neuroprotective action, as this way of drug administration is more likely to be used in clinical practice if the treatment is approved.

4.1.1. Animal Models of Alzheimer's Disease (AD)

In aging mouse models, particularly in SAMP8 mice, there is an early decline in learning and memory along with a series of pathophysiological changes in the brain, including increased oxidative stress, inflammation, vascular disorders, accumulation of Aβ, and hyperphosphorylation of tau [214].

It has been demonstrated that administering the flavonol fisetin (at the dose of 25 mg/kg) can regulate the levels of proteins involved in synaptic functions and inflammation, thereby affecting key processes correlated with improved cognitive functions [215]. The impact of flavonoids was also tested with the transgenic AD model 5 × FAD (multiple AD-linked mutations in the genes encoding amyloid precursor protein (APP) and presenilin 1 (PS1)). Chronic administration of the flavone 7,8-dihydroxyflavone (7,8-DHF)-activated TrkB signaling pathways in the brain mitigated synaptic loss, reversed synaptic plasticity, reduced Aβ deposition, and slowed spatial memory deficits by mimicking the action of BDNF, whose main functions are to enhance synaptic transmission, facilitate synaptic plasticity, and promote synaptic growth [83]. The flavone apigenin (at the dose of 40 mg/kg) administered in the 2 × FAD mouse model (AD-linked mutations in genes coding for APP and PS1) for 12 weeks alleviated learning and memory disorders associated with AD similarly to how 7,8-DHF alleviated Aβ burdens, inhibited the amyloidogenic process, oxidative stress, and restored the ERK/CREB/BDNF pathway in the cerebral cortex of mice [216].

A rat model of sporadic AD, induced by streptozotocin (STZ), was used to test effects of genistein [21]. The AD rats were treated orally with genistein at a dose of 150 mg/kg/day, followed by a series of behavioral, biochemical, and histological tests. In biochemical studies, the genistein-supplemented group showed normalization of APP and β-amyloid levels, including Aβ40 and Aβ42 forms, as well as Tau and pTau levels. An increase in the levels of LC3-II protein, an autophagy marker, and TFEB, a transcription factor responsible for stimulating lysosome biogenesis, was also observed. Histological analyses revealed a reduction in Aβ aggregates. Administering genistein at a dose of 150 mg/kg/day normalized the defects in the rat brain, induced by STZ, and corrected all behavioral parameters, otherwise deteriorated by STZ injection [45].

4.1.2. Animal Models of Parkinson's Disease (PD)

The most commonly used models for Parkinson's disease include the use of toxic compounds 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and 1-methyl-4-phenylpyridinium (MPP(+)). Transgenic models of PD have not been widely used in research due to inconsistent phenotypes of mice with the same mutation [217], however, the MitoPark model appears to be promising [218].

The flavone baicalein (at the dose of 200 mg/kg), administered to C57BL/6 mice before exposing them to MPTP, mitigated the loss of dopaminergic neurons, reduced behavioral disturbances, and lowered markers of the oxidative stress and astroglia response [219]. Rats subjected to 6-OHDA and treated with 7,8-DHF (at the doses of 12–16 mg/kg) in their drinking water before and after exposure showed significant behavioral improvement and a reduction in the degradation of dopaminergic neurons in the substantia nigra [220]. In a primate model of PD, intracerebroventricular injections of MPP++ were used, and oral administration of 7,8-DHF at a dose of 30 mg/kg was applied. The flavonoid demonstrated

neurotrophic action, protecting dopaminergic neurons from apoptosis in the face of induced neurotoxicity. Moreover, long-term use of 7,8-DHF in monkeys at doses significantly higher than previously used in mice (5 mg/kg) and rats did not show toxicity and did not cause abnormal hematological functions, nor did it impair liver, heart, muscle, kidney, and pancreatic functions [221]. Additionally, other flavonoids like chrysin [222] and nobiletin [223] also demonstrated neuroprotective actions in neurodegenerative diseases, including mouse and rat models of both 6-OHDA- and MPTP-induced PD.

4.1.3. Animal Models of Amyotrophic Lateral Sclerosis (ALS)

Despite promising results in the use of flavonoids for other neurodegenerative diseases, very few flavonoids have been tested on animal models of ALS. The transgenic mouse model SOD1-G93A exhibits protein aggregation, loss of motor neurons, axonal denervation, progressive paralysis, and shortened lifespan [224]. Treatment with fisetin provided neuroprotective action, mitigated motor impairment, reduced ROS-mediated damage, regulated redox homeostasis, and increased survival rates compared to the control group [66].

4.1.4. Animal Models of Huntington's Disease (HD)

For assessing the neuroprotective nature of flavonoids in HD, both chemical induction of striatal neuron degeneration using 3-Nitropropionic acid (3-NP) and genetic mouse models were employed [225].

3-NP is an irreversible inhibitor of mitochondrial complex-II that causes transcriptional dysregulation, bioenergetic failure, protein aggregation, and oxidative damage similar to HD pathogenesis. Chrysin, an active flavonoid, demonstrated neuroprotective efficacy against 3-NP-induced oxidative stress, mitochondrial dysfunction, and neurodegeneration. When administered orally at a dose of 50 mg/kg for 14 days, it improved all behavioral functions, markers of oxidative stress and cell death, and increased the survival of striatal neurons in Wistar rats [226]. Similar neuroprotective effects have been observed with two other flavonoids, quercetin (at the dose of 25 mg/kg) [227] and hesperidin (at 100 mg/kg) [213]. Additionally, neuroprotective effects were observed in the transgenic mouse model R6/1, which exhibits an aggressive phenotype and shortened lifespan. Administration of 7,8-dihydroxyflavone (at 5 mg/kg) delayed the development of motor deficits and reversed deficits in the Novel Object Recognition Test. Furthermore, morphological and biochemical analyses demonstrated prevention of loss in the striatal neurons [122]. In another study with the R6/2 mouse model, phenotypically similar to R6/1, fisetin and resveratrol were used. It was found that both compounds had neuroprotective abilities that helped in preserving normal brain functions in HD through activation and maintenance of the Ras-extracellular signal-regulated kinase (ERK) pathway [228].

Recent studies with the R6/1 mouse model of HD also indicated that treatment with genistein at a dose of 150 mg/kg/day, initiated after the first HD symptoms appeared (at 16 weeks of age), has a positive impact on the health of the animals [126]. Genistein treatment corrected emerging abnormalities in both cognitive functions and anxiety levels, as observed in numerous behavioral tests. Additionally, motor functions, which are severely impaired in HD, also improved. There was a reduction in mHTT aggregates and levels of the mutant protein in the brain, likely due to their effective degradation through the autophagy process stimulated by genistein. Oxidative stress levels, as well as pro-inflammatory and anti-inflammatory interleukins, normalized, thereby reducing the inflammatory process, which is a secondary pathomechanism of neurodegeneration in HD. Improvements were observed in biochemical parameters such as GPT/ALAT (glutamic pyruvic transaminase, soluble) and GOT1/ASPAT (glutamic-oxaloacetic transaminase 1, soluble) activities, and levels of bilirubin, CK (creatine kinase), TNN (troponin I), and ROS, which deteriorate with the progressive dysfunctions of organs such as the liver, kidneys, and heart in the course of HD. In summary, genistein administration corrected not only behavioral abnormalities but

also most of the selected hematological and biochemical parameters in the blood, skeletal muscles, and heart muscle, whose deterioration was observed in diseased mice [126].

Moreover, animal studies reported positive aspects of a soy-enriched diet on other matters. Eliminating soy from the diet of BALB/c mice for two generations resulted in a stronger anaphylactic reaction and degranulation of mast cells after oral sensitization with peanut extract. Introducing soy or isoflavones into the diet significantly suppressed allergic reactions [229]. Introducing soy milk into the diet of Wistar rats for almost the entire lifespan of the animals caused reduction of the loss of neurons in the hippocampus and extended the lifespan of the animals relative to the control group [230].

4.2. Population Studies

Studies on the use of flavonoid-rich diet by humans might be more informative than those conducted with animal models from the clinical point of view. However, the limitations are the restrictions in the use of human subjects in experiments, and the inability to conduct all the test which can be performed with animals. Therefore, interpretation of the results of studies with human subjects are usually more difficult and conclusions are less clear and more indirect, as compared to experiments with animals.

Diets rich in flavonoids include those of the Mediterranean and Asian countries, which are abundant in fruits, vegetables, and legumes. In Asian countries, soy products rich in flavonoids are widely consumed. Studies on the consumption of soy protein and isoflavones among adults from Japan, China, Singapore, and Hong Kong indicated that, on average, in Japan, about 6–11 g of soy protein and 25–50 mg of isoflavones are consumed daily. In Singapore and Hong Kong, consumption is lower than in Japan, while in China, there is considerable regional variation. Moreover, $\leq$10% of the Asian population consumes as much as 25 g of soy protein or 100 mg of isoflavones (expressed as aglycone equivalents) daily [231]. Prospective population studies conducted by the Japan Public Health Center (JPHC) over a period of 9.4 years found no association between total soy product consumption and the risk of dementia-causing disability in Japanese women and men [232]. The Singapore Chinese Health Study found no link between soy or isoflavone consumption and the risk of cognitive impairment [233]. Similar results were obtained among the elderly population of Taiwan, where soy product consumption was not associated with worsening cognitive functions [234].

A comparison of consuming a soy-rich diet (100 mg of isoflavones daily) versus a low-soy diet (0.5 mg of isoflavones daily) for 10 weeks among young healthy adults of both sexes indicated a beneficial effect of the isoflavone-rich diet on the improvement of short-term and long-term memory and a significant improvement in tests of mental flexibility involving the frontal cortex. Additionally, among women, improvements were observed in tests of letter fluency and planning involving the frontal lobes [235]. Furthermore, prospective cohort studies demonstrated that the consumption of citrus fruits, strawberries, and grapes is associated with a lower risk of stroke in Japanese women [236]. A meta-analysis of preclinical and observational studies, which included both post- and pre-menopausal women, as well as men, concluded that soy isoflavones may improve cognitive functions in adults, especially in the memory domain, and did not indicate the occurrence of serious adverse effects [237].

A randomized, controlled clinical trial concerning the impact of soy isoflavones on cognitive functions in older adults with AD was performed. Individuals diagnosed with early-stage AD underwent a six-month therapy with isoflavones at a dose of 100 mg/day. The patients were evaluated for cognitive functions, including visual spatial memory, visual-motor skills, verbal fluency, and speeded dexterity. The study did not reveal an overall improvement in cognitive functions in the isoflavone-treated group compared to the placebo, and global cognitive functions in both groups deteriorated at a similar rate. During the study, researchers observed significant individual variability in plasma concentrations of genistein, daidzein, and equol in the treatment group, despite adherence to therapeutic recommendations. In the subgroup able to efficiently metabolize the soy

isoflavone daidzein to equol, correlations were observed between circulating plasma equol levels and improvement in two cognitive speed-dependent parameters, verbal fluency and speeded manual dexterity. However, due to the small number of participants effectively metabolizing isoflavones among the study population, there is a risk of false interpretation of the results. On the other hand, these studies suggested that soy isoflavones might exert a positive, albeit moderate, impact on cognitive functions among individuals capable of metabolizing isoflavones [238]. It was demonstrated that the ability to metabolize isoflavones was significantly influenced by the intestinal microbiome. This was confirmed by the presence of anaerobic, Gram-positive bacteria, capable of transforming daidzein into S-equol, isolated from the feces of humans and animals capable of metabolizing flavonoids [239]. Studies conducted with 152 older adults from Japan, among whom 40% were capable of effectively converting isoflavones to equol in the intestine, showed that among those able to produce equol, a clearly beneficial impact on cognitive functions occurred [240].

In 2022, results of another clinical trial (ClinicalTrials.gov NCT01982578) were published, in which 24 subjects, aged 54–75, with prodromal AD (defined as amyloid-positive minimal cognitive impairment) were given daily oral supplementation of genistein at a dose of 120 mg for 12 months [48]. The results of this trial indicated that patients with prodromal AD treated with genistein for one year showed less amyloid-β deposition in the anterior cingulate cortex (which plays a crucial role in some functional features such as cognitive processes and emotions), as determined by flutemetamol uptake in the specified brain region, compared to patients receiving placebo. Furthermore, the impact of genistein supplementation on the progressive loss of cognitive functions was assessed at two time points, 6 and 12 months of supplementation. At the second time point, a statistically significant improvement was observed in the total dichotomized TAVEC and Centil REY Delayed Copy tests among subjects receiving genistein supplementation. In other tests performed, such as the Mini-Mental State Exam (MMSE), Memory Alteration Test (M@T), Clock-Drawing Test, and Barcelona Test-Revised (TBR), a trend towards improvement was observed. However, making solid conclusions on efficacy of genistein in AD treatment would require further clinical studies on a larger sample size over a longer period than 12 months.

In 2024, results of a meta-analysis focused on the impact of dietary supplementation, food, and dietary patterns on PD were published [241]. The analysis included 24 randomized and crossover clinical trials from 1989 to 2022. It was concluded that diet and dietary supplements had a modest but statistically significant impact on QUICK (quantitative insulin sensitivity check index). The results of the systematic review indicated that Mediterranean, low-fat, and ketogenic diets significantly reduced the total Unified Parkinson Disease Rating Scale (UPDRS) score, while low-protein diets significantly alleviated motor symptoms.

In a clinical trial conducted in Iran, 70 idiopathic PD patients with an average age of approximately 60 years were investigated (Iranian Clinical Trials Registry IRCT20141108019853N4) [242]. The effect of a Mediterranean diet on PD patients was examined and compared with a control group consuming a traditional Iranian diet. The total antioxidant capacity (TAC) in serum, as well as motor and non-motor aspects of the disease were assessed using the UPDRS scale. The Mediterranean diet significantly increased TAC and lowered the UPDRS score. It was suggested that the Mediterranean diet provides some benefits, but further research is necessary.

Prospective studies with the goal of examining whether higher consumption of all flavonoids and their subclasses (flavanones, anthocyanins, flavan-3-ols, flavonols, flavones, and polymers) was associated with a lower risk of developing PD were conducted. The experiment included 120,617 individuals of both sexes, among whom, over 20–22 years of observation, 805 participants developed PD. The study identified five main dietary sources of high flavonoid content (tea, berries, apples, red wine, and orange juice/oranges). Indi-

viduals consuming a high amount of flavonoids in their diet had a 40% lower probability of developing PD, particularly among men [243].

Although various cellular and animal studies have evaluated the effect of flavonoids on HD models, a lack of human trial has limited their therapeutic application. In recent years, two significant randomized clinical trials investigated the impact of flavonoids on HD. The first one focused on the effects of consuming 1200 mg of epigallocatechin gallate (EGCG) for 12 months (ClinicalTrials.gov identifier: NCT01357681), assessing its potential neuroprotective and antioxidant benefits in slowing disease progression. The second study examined the impact of administration of 80 mg daily resveratrol on the caudate nucleus volume in HD patients (ClinicalTrials.gov identifier: NCT02336633), exploring its anti-inflammatory and antioxidant properties. Both studies aimed to develop therapeutic strategies for HD, utilizing natural substances to potentially alleviate symptoms or slow progression, providing valuable insights for clinical applications. According to the information available on the ClinicalTrials.gov website (last accessed on 16 May 2024), the data from these studies have not yet been published yet.

A diet rich in flavonoids contained in soy has many positive effects while the negative aspects of an excess of flavonoids in the diet cannot be overlooked. Soy in its raw form contains anti-nutritional factors that alter the amount of amino acids, mineral salts and vitamins absorbed [244]. Soy flour contains a trypsin inhibitor, which can induce hypertrophy and pancreatic hyperplasia. Prolonged exposure to elevated levels of the trypsin inhibitor present in soy resulted in pancreatic nodular hyperplasia and germ cell adenoma in rats subjected to low levels of pancreatic carcinogens [244]. To ensure optimal nutritional properties, soy must undergo a heating process to eliminate internal compounds that adversely affect the gastrointestinal tract [245]. To inactivate the trypsin inhibitors, it is necessary to cleave the two disulfide bonds present within them. This can be accomplished through a variety of processing methods, including physical, chemical, and enzymatic treatments, each conducted under specific conditions of temperature and duration [244]. Dietary supplements containing soy extracts are considered possibly safe for use up to six months. However, soy may cause mild gastrointestinal side effects, including constipation, bloating, nausea and diarrhea. The antinutrients present in soy may compromise the gut barrier function, potentially leading to inflammation and digestive issues [23]. Additionally, it can trigger allergic reactions in some individuals, manifesting as respiratory difficulties, itching and rash [109]. Moreover, concerns exist that soy may negatively affect thyroid function and alter thyroid hormone levels [246]. Dietary soy intake has been associated with an increased risk of bladder cancer and dementia. Due to the activity of estrogen-like substances, it is reasonable for women with ER tumors to be able to safely consume soy and possibly other phytoestrogens. Despite studies suggesting a protective role for phytoestrogens, there is some evidence that genistein, the most abundant isoflavone in soy, may stimulate the growth of estrogen receptor (ER+) breast cancer and interfere with the anti-tumor effect of tamoxifen at low levels [247]. By the same token, soy may also be dangerous when consumed in large quantities (usually in medications) during pregnancy and early childhood development (use of modified soy milk), adversely affecting fetal development and infants during the steroid hormone-sensitive period when estrogen exposure may alter the normal arrangement and function of reproductive tissues [248]. Additionally, soy consumption during pregnancy has been shown to alter the epigenome in offspring, potentially leading to health implications [20]. On the other hand, insufficient soy intake may increase the risk of gestational diabetes mellitus, suggesting that adequate soy consumption could play a beneficial role in preventing this condition [249]. All the above-described effects of an increased uptake of flavonoids present in soy indicate that a soy-rich diet should be used carefully with understanding potential benefits and adverse effects.

The impact of diet and its components on the body is an interesting and important research topic. Diets characterized by high consumption of fruits and vegetables, legumes, grains, dried fruits, and nuts, with low consumption of red meat, low to moderate consumption of dairy, and moderate to high consumption of fish, have a broad beneficial

impact on health. The trend of shifting towards a Western diet, which is characterized by high consumption of saturated fats, highly processed foods, regular consumption of red meat, high content of products with added sugars, and low content of vegetables, fruits, and fiber, is concerning and makes it even more important to pay attention to the beneficial effects of a healthy, balanced diet on the body [250,251].

5. Concluding Remarks

There are numerous reports indicating that a flavonoid-rich diet might be effective in alleviating symptoms of neurodegenerative diseases. Indeed, flavonoids reveal biological activities which can be particularly helpful in both preventing the symptoms of such diseases and improving functions of the brain in already affected organisms. Studies with animal models confirmed that some soy-derived flavonoids can be effective in treatments of various neurodegenerative diseases, like AD, PD, and HD. Experiments with animal and cellular models indicated specific molecular mechanisms of actions of flavonoids which might explain their efficacy in stimulation of autophagy, inhibition of neuroinflammation, and reduction of the oxidative stress, which lead to correcting neurological defects. On the other hand, studies on the effects of these compounds, including in a diet, in human diseases gave less clear and not so straightforward results. A specific problem is the low bioavailability of flavonoids, especially when used as diet supplements, which has been suggested as one of the major limitations in their potential use as drugs for neurodegenerative diseases. In summary, there is some potential of flavonoids included in foods for the treatment of neurodegenerative diseases; however, further investigations are mandatory to precisely estimate their therapeutic potential and to develop optimal conditions for their potential use in the clinical practice.

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- przygotowaniu figur i tabeli
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Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the HTT gene

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Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the *HTT* gene

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ABSTRACT

Although Huntington's disease – a severe, inherited, neurodegenerative disorder – is primarily caused by a pathological variant of the *HTT* gene (encoding huntingtin protein) which is characterized by the extension of CAG repeats in the 1st exon exceeding 36 triplets, the biochemical mechanisms underlying the disease remain poorly understood. Mutant huntingtin forms toxic aggregates in cells; however, the clinical symptoms usually appear in adulthood. Recent reports suggested that somatic expansion of CAG repeats to more than 150 copies may be responsible for the pathogenicity and could explain the delayed onset of symptoms in this inherited disease. Nevertheless, it remains unclear why such an expansion occurs only in cells bearing *HTT* alleles with the original number of CAG repeats over 36. Here, we used cellular models of Huntington disease, consisting of human HEK293 cell lines with either normal (16/17) or pathogenic (41, 53, 84) numbers of CAG repeats in exon 1 of *HTT*, as well as the R6/1 mouse model. Using AlphaFold3, protein structures were predicted for the human huntingtin variants, revealing significant changes in pathogenic forms compared to the normal form. Transcriptomic analyses indicated that expression of a few thousand genes is significantly dysregulated in cells with increased CAG repeat numbers. Products of these genes are involved in various processes, such as apoptosis, autophagy, and DNA repair and recombination. Thus, we suggest that stress conditions, caused by dysregulation of cellular processes, might facilitate abnormal somatic expansion of CAG repeats, contrary to cells bearing normal *HTT* alleles.

1. Introduction

Huntington's disease (HD) is a severe, inherited, neurodegenerative disease. The genetic defect causing this disease is an increased number (over 36) of CAG triplets in exon 1 of the *HTT* gene, which encodes huntingtin (HTT) [1]. The pathogenic HTT variant produces an improperly folded protein with an elongated tract of glutamine residues, which forms toxic aggregates. Moreover, it was proposed that *HTT* transcripts with a pathological CAG expansion form secondary structures that disrupt RNA splicing [2].

One of the most intriguing features of HD is the late onset of symptoms, despite its dominant mode of inheritance. In most cases, the first symptoms appear during the third or fourth decade of life, despite the presence of the pathogenic variant from the zygote stage [3]. Recent studies have proposed that appearance of HD symptoms requires the number of CAG repeats exceeding 150, and that somatic expansion of

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CAG repeats may be responsible for the late onset of symptoms [4,5]. According to this hypothesis, the appearance of such long CAG tracks in patients' genomes takes years, as one would expect a gradual process that adds shorter stretches of DNA during each expansion event. This is an attractive proposal, potentially explaining the delayed onset of HD symptoms. However, it remains unclear why such a large somatic expansion occurs only in cells of patients with over 36 repeats in the *HTT* gene, and not in healthy individuals, despite the corresponding DNA fragment being shorter by only several base pairs.

One possibility which might explain the above uncertainty is that either mRNA transcript derived from the mutant *HTT* allele or the protein product of such an allele, or both, although unable to cause severe cell damage, might nevertheless affect the cell physiology resulting in stress conditions which facilitate abnormalities in DNA transactions, leading to huge expansion of CAG repeats, contrary to healthy cells, devoid of toxic mRNA and/or protein variant(s). To test this hypothesis, we investigated gene expression profiles, using transcriptomic approaches, in cellular models of HD, consisting of human HEK293 cell lines bearing different numbers of CAG repeats in the *HTT* gene: 16/17 (control), 41, 53 or 84, as well as the R6/1 mouse model of HD (with recombinant human *HTT* exon 1 containing 116-120 CAG repeats) and control mice. Although transcriptomic studies were performed previously with different HD models (either cellular models or tissues), they employed single *HTT* pathogenic alleles and relevant controls (summarized in a recent analysis [6]). Here, using models with different numbers of CAG repeats, it was possible to test the effects of extended repeat lengths on transcriptomes and to predict changes in the structures of the protein products of mutant *HTT* alleles.

2. Materials and methods

2.1. Structure prediction of huntingtin variants

The huntingtin structures of the reference variant with 16 glutamine (Q) residues (HTT-16Q) and the mutant variants with 41, 53, and 84 Q residues (HTT-41Q, HTT-53Q, and HTT-84Q, respectively) were predicted using AlphaFold3. The predictions were based on the amino acid sequence from the NCBI database under accession no. NM_001388492.1, which was appropriately modified to include the specified number of glutamine residues. The predicted structures were visualised using ChimeraX 1.10. The predicted variants were aligned using Matchmaker with the best-aligning pair of chains between the reference and the matched structures. Atom-atom contacts were calculated using ChimeraX with specific interaction parameters. The van der Waals (VDW) overlap threshold was set to ≥ -0.40 Å, and the centre-to-centre distance cutoff was set to ≤ 4.00 Å. Interactions between atoms separated by four or fewer bonds were ignored. Residues separated by fewer than 5 positions in sequence were excluded from interactions.

2.2. Cells and cell cultures

Cell lines derived from HEK293, carrying either normal (16/17) number of CAG repeats in the *HTT* gene (used as a control, and called HEK further in the text) or otherwise isogenic HD cellular models, genetically modified using the CRISPR-Cas9 system to introduce expanded numbers of CAG repeats (41, 53 or 84; called HEK41, HEK53 or HEK83, respectively) [7], were employed. The presence of the specific alleles was confirmed by DNA sequencing, as described [7], and by PCR (Fig. S1) with primers described in Table S1. Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium; Gibco™ Thermo Fisher Scientific, #11995-065) supplemented with 10% FBS (Fetal Bovine Serum; Gibco ™ Thermo Fisher Scientific, #A5256701) and 1:100 Anti-Anti (Antibiotic-Antimycotic 100x; Gibco ™ Thermo Fisher Scientific, #15240062), under standard conditions (37 °C, 95% humidity, and atmosphere saturated with 5% CO_2).

2.3. The mouse model of HD

The R6/1 transgenic mouse model of HD and wild-type C57BL/6J control mice (WT), purchased from Jackson Laboratories, were used. Both males and females were investigated (merged into one group). The detailed methodology for animal experimentation has been described previously by this research group [8]. Brains were collected at the age of 6 months, after euthanasia with 2 ml/kg of morbital, as described elsewhere [9], and used for RNA extraction. All procedures were approved by the Local Ethics Committee for Animal Experiments in Bydgoszcz (Poland) (resolution no. 48/2021).

2.4. RNA isolation

In all analyses, four samples (independent biological repeats or individual animals) per group were investigated ($n = 4$). For RNA isolation from brains, 300 mg of the brain tissue per sample was used. For RNA isolation from cultured cells, 3×10^4 cells were passaged on plates (3 cm diameter) and allowed to attach overnight. Following 48 h incubation of cells or homogenization of the brain tissue (previously frozen at -80^oC), cells were lysed employing the QIAshredder column (Qiagen, #79656), with the use of a solution containing guanidine isothiocyanate and β-mercaptoethanol. RNA extraction was performed with the use of RNeasy Mini kit (Qiagen, #74106) and treatment with Turbo DNase (Invitrogen ™ Thermo Fisher Scientific, #AM1907), according to the manufacturers' instructions. The quality of isolated RNA samples was assessed by RNA Nano Chips (Agilent Technologies) in the Agilent 2100 Bioanalyzer System.

2.5. Transcriptomic analyses

RNA sequencing, using Illumina NovaSeq X Plus platforms, based on the sequencing-by-synthesis mechanism, was performed as outsourcing by the external company (Novogen). The RNA-seq data were deposited in the NCBI database, with BioProject ID: PRJNA1345724.

2.5.1. Library construction, quality control and sequencing

Isolated mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers, followed by the second strand cDNA synthesis using either dUTP for a directional library or dTTP for a non-directional library. The non-directional library was obtained after end repair, A-tailing, adapter ligation, size selection, amplification, and purification. The directional library was ready after end repair, A-tailing, adapter ligation, size selection, USER enzyme digestion, amplification, and purification.

The library was checked by Qubit and real-time PCR for quantification, and by a bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms, according to effective library concentration and data amount.

2.5.2. Bioinformatic analysis pipeline

Raw readings were mapped to the Human Ensemble Homo Sapiens GRCh38.p12 (GCA_000001405.27) or Mouse Ensembl GRCm38.p6 (GCA_000001635.8) reference genome; gene model annotation files were downloaded from the genome website directly. Index of the reference genome was built using Hisat2 v2.0.5, and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. Prediction of novel transcripts was conducted using StringTie(v1.3.3b) in a reference-based approach. FeatureCounts v1.5.0-p3 was used to count the reads' numbers mapped to each gene. Then FPKM of each gene was calculated based on the length of the gene and reads' count mapped to this gene.

2.5.3. Differential expression analysis

For DESeq2 with biological replicates, differential expression

analysis of two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package (1.20.0). The resulting *p*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *p*-value <0.05 found by DESeq2 were assigned as differentially expressed. For edgeR without biological replicates, prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR program package through one scaling normalized factor. Differential expression analysis of two conditions was performed using the edgeR R package (3.22.5). The *p*-values were adjusted using the Benjamini-Hochberg method. Corrected *p*-value of 0.05 and absolute foldchange of 2 were set as the threshold for significantly differential expression.

2.6. Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was reverse transcribed using the NG dART RT Kit (EURx, #E0801-02) according to the manufacturer's instructions. RT-qPCR analyses were conducted using a LightCycler 480 II system (Roche) with SYBR Green Master Mix (Roche). The primers used are listed in Table S2. Gene expression efficiency was calculated using the $2^{-\Delta\Delta CT}$ method, with *GAPDH* serving as the reference gene.

2.7. Western-blotting

Western-blotting was performed using the WES system (WES - Automated Western Blots with Simple Western; ProteinSimple, San Jose, California, USA), exactly as described previously [8] with primary antibodies listed in Table S3.

2.8. Determination of reactive oxygen species (ROS) levels

Levels of ROS in blood samples were measured using OxiSelect™ In Vitro ROS/RNS Assay Kit (Green Fluorescence) (CELL BIOLABS, #STA-347-5).

3. Results and discussion

3.1. Predicted structures of huntingtin with different lengths of polyglutamine (polyQ) stretch

AlphaFold3 predicts protein structures from sequences and provides two confidence metrics: a pTM score (overall fold quality for monomers) and a per-atom pLDDT score (local reliability). The four HTT models (HTT-Q16, HTT-Q41, HTT-Q53, and HTT-Q84) scored very similarly, with high pTM values (0.82, 0.82, 0.82, and 0.81, respectively) and pLDDT values in the mid-70s (76.85, 77.33, 76.84, and 76.18, respectively), indicating a moderately confident global fold. Thus, these models could be used as hypothesis-generating representations of HTT structure (Fig. 1A, Fig. S2A).

The disease-associated polyQ stretch lies within the N-terminus (Fig. 1B). The predicted pLDDT for this fragment averages about 53 across the four polyQ lengths (52.57, 53.74, 53.03, 54.24 for HTT-Q16, HTT-Q41, HTT-Q53 and HTT-Q84, respectively), which falls within the low-confidence range (pLDDT 50-59). Low pLDDT can reflect either poor modelling of a rigid conformation or, more plausibly here, the presence of an intrinsically disordered region (IDR). Consequently, the predicted low-confidence zones flag portions of HTT that are likely to be flexible, dynamic and potentially involved in transient interactions. This aligns with experimental observations as exon 1 remains unresolved in all available high-resolution cryoEM maps of the HTT-HAP40 complex, despite near-atomic resolution for other domains [10]. Interestingly, we observed that the pathological increase in glutamine residues may lead to a predicted reorientation of the N-terminal polyQ region (Fig. 1B). As the polyQ lengthens, the predicted contacts of the adjacent Lys135 and Lys136 residues drop from 25 in HTT-Q16 to 19, 18 and 16 in HTT-Q41, HTT-Q53 and HTT-Q84, respectively, suggesting a polyQ-dependent reorientation of the N-terminal region. This flexibility could contribute to the observed aggregation propensity of expanded polyQ alleles, linking possible structural changes to the pathogenic outcome.

We also observed a second putative IDR spanning residues Gly1360 to His1460 in HTT-Q16 (Fig. 1C). We compared the corresponding fragments in other HTT variants and noticed that the HTT-Q53 prediction may vary markedly, unlike those for shorter or longer polyQ variants. All high-resolution structures of HTT to date have been obtained in complex with HAP40, and these structures show that polyQ expansion has little to no effect on the global architecture or stability of the HTT-HAP40 complex [10]. At the same time, it is not established that HTT is always in the HAP40-bound state in cells. Moreover, biophysical work on apo HTT (without HAP40) indicated that the monomeric protein is substantially more flexible and prone to self-association, with polyQ expansion promoting more extended conformations and enhanced oligomerisation [10]. In this context, the fact that this fragment is predicted to be disordered in all variants but adopts a distinct conformation specifically in HTT-Q53 may suggest that intermediate polyQ lengths place particular stress, potentially leading to non-linear changes in flexibility that might be reflected in accessibility to post-translational modifications or partner binding.

Our results suggest that the pathomechanism of HD may extend beyond the traditional view of HTT-HAP40 heterodimer and polyQ-induced protein aggregation, highlighting the possibility of conformational changes implied by specific polyQ elongation affecting the outcome of the disease, which is also compatible with the recent proposals about the somatic expansion of CAG repeats and further consequences [4,5]. Our AlphaFold3 models, which describe monomeric HTT, should be interpreted as hypothesis-generating for the apo state, which to date has not been directly captured by structural studies.

3.2. Transcriptomic changes in cells bearing extended CAG repeats in the HTT gene

Our transcriptomic analyses indicated that there are thousands of dysregulated genes in HEK41 (2892 genes), HEK53 (4193 genes) and HEK84 (6091 genes), relative to the control HEK cell line (Fig. 1D). Moreover, 4185 genes were dysregulated in R6/1 mouse brain cells compared with wild-type cells (Fig. 1D). These results demonstrated global dysregulation of gene expression in both cellular and animal models of HD (specifically in the brains of the latter), even without drastic somatic expansion (over 150 repeats) of CAG triplets. The effect of the length of the CAG repeat region was also evident: the longer the region, the greater the changes in gene expression patterns in cellular HD models. Moreover, expression of some genes was severely dysregulated, with $\log_2$ fold change ($\log_2$FC) values ranging from 7 to 9 (indicating about 100-500-fold differences in transcript levels relative to control cells) (Fig. S2B). Interestingly, the differences between HEK84 and HEK53 were higher than those between HEK84 and HEK41 (Fig. 1D), corroborating the results of the modeling of huntingtin structure, which indicated that HTT-Q53 differs significantly not only from HTT-Q16, but also from HTT-Q41 and HTT-Q83, while HTT-Q41 and HTT-Q83 are more similar to each other, though considerably different from HTT-Q16 (Fig. 1C).

Genes with the highest levels of dysregulation relative to control (HEK) cells in all three variants of the HD cellular model, as well as in the brains of the R6/1 mouse model relative to controls, are presented in the form of volcano plots in Fig. 2. The threshold for the "highest level" was set at $\log_2$FC values of 6.5 for cellular models and 4.5 for the mouse model (these thresholds allowed us to select 10-20 genes in each comparison). Using this approach, the identified genes represented those with mRNA levels over 90-fold up- or down-regulated in HEK293-derived HD cells and over 23-fold dysregulated in the brains of the mouse HD model. Among them are genes coding for transcription factors (e.g., BEX1, TWIST2), an activator of the ubiquitin ligase (MAGEA6), an

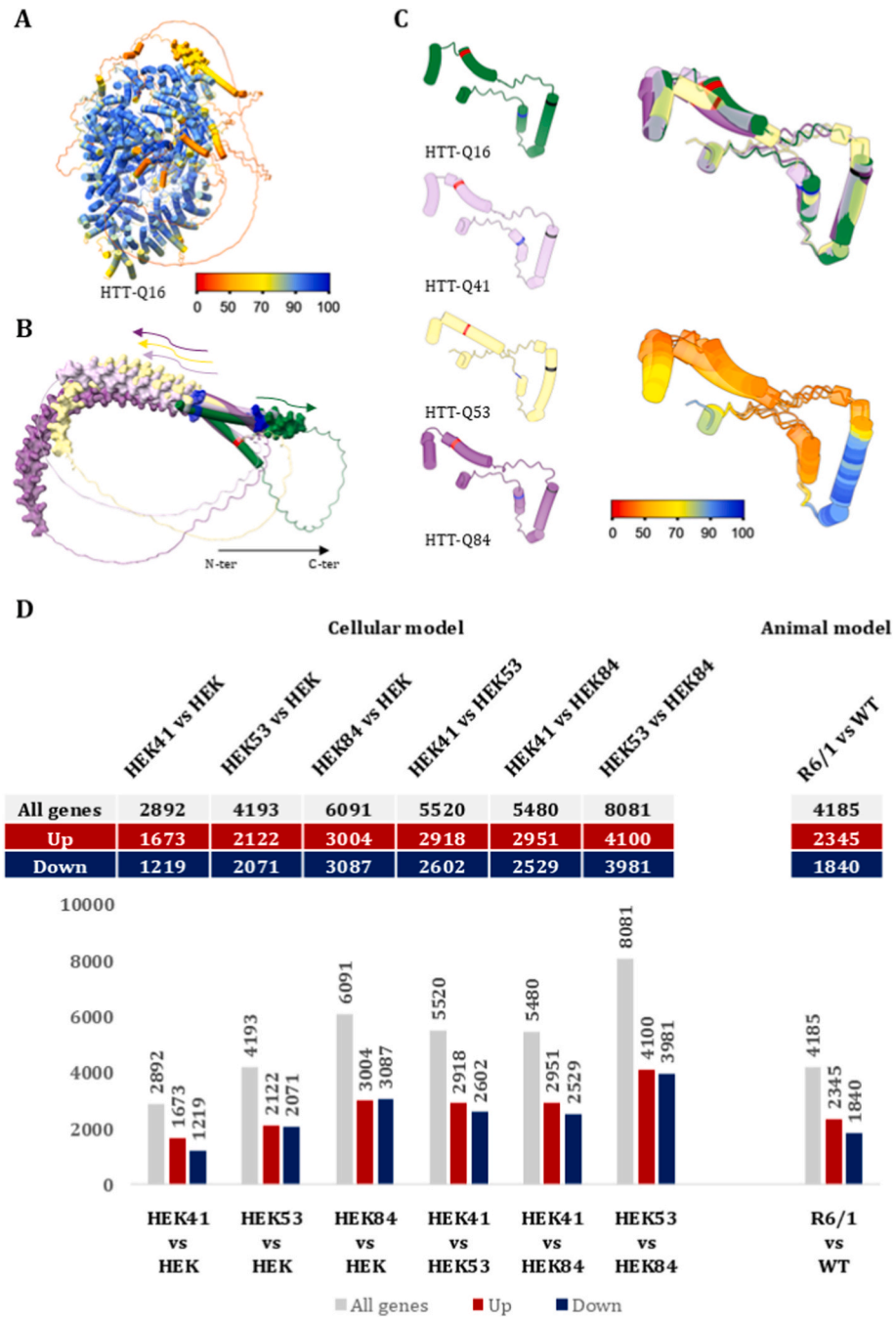


Fig. 1. AlphaFold3 modelling of HTT variants (A-C) and summary of transcriptomic changes in cellular and animal models of HD (D). (A) An AlphaFold prediction of HTT-16Q reference with the interface predicted template modelling (ipTM) score of 0.82 and pLDTT score of 76.85, reflecting a highly confident prediction. The standard pLDDT score was used to visualise predicted confidence (very high confidence (pLDDT ≥90): blue; high confidence (pLDDT 80-89): light blue; medium confidence (pLDDT 70-79): green; low confidence (pLDDT 50-59): yellow; very low confidence (pLDDT<50): orange/red). (B) Polyglutamine (PolyQ) elongation induces an *in silico* reorientation of the N-terminal polyQ region. The region shown spans residues Asp36 to Val150 in HTT-Q16 reference (with Val175, Val187 and Val218 corresponding to Q41, Q53 and Q84, respectively). The polyQ region is displayed as a surface. The arrows indicate the N-ter/C-ter direction. Lysine residues K135 and K136, shown in red for the HTT-Q16 variant, exhibit fewer contacts with increasing polyQ length in the mutants, indicating greater regional flexibility that could enable the structural reorientation of the region. (C) Distinct structural features in the intrinsically disordered region in HTT-Q53 compared to other polyQ variants. Modelling of the second IDT, spanning residues Gly1360 to His1460 in HTT-Q16 (corresponding to Gly1385, Gly1395 and Gly1428 in Q41, Q53 and Q84, respectively), showed noticeable variations. The most divergent conformation was observed in the HTT-Q53 mutant, with the prediction differing markedly from that for shorter or longer polyQ variants. Lysine K1441 (in HTT-Q53), Serine S1413, and Glycine G1379 – and their corresponding residues in the other mutants – are highlighted in red, black and blue, respectively, to emphasise structural differences. The standard pLDDT score visualises predicted confidence of the fragments as described in (A), suggesting an intrinsically disordered region (IDR). (D) Number of up- and down-regulated transcripts (at $p < 0.05$) in cellular (HEK) and mouse (R6/1) models of HD relative to controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

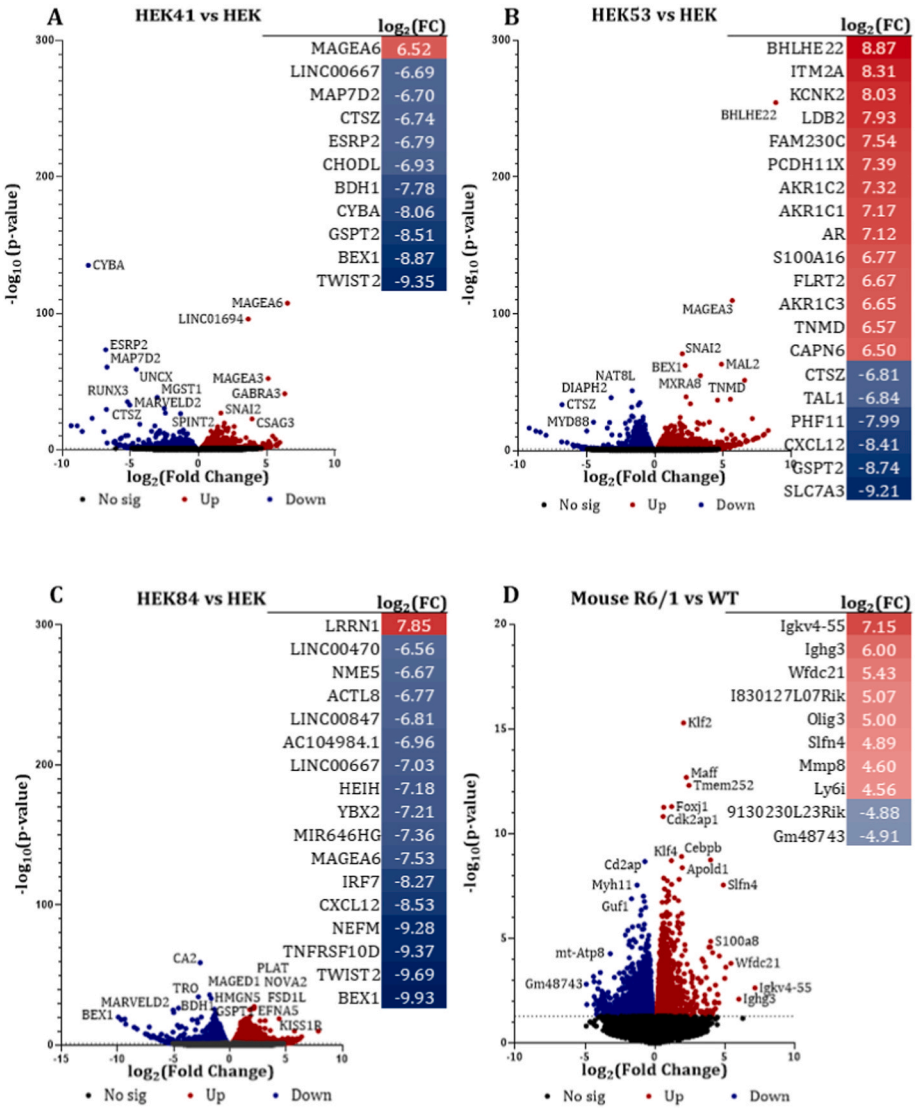


Fig. 2. Genes with especially severely changed expression in cellular (A-C) and animal (D) models of HD relative to controls. The thresholds for this category of genes were assumed to be $\log_2FC > 6.5$ for the cellular (HEK41, HEK53, and HEK84) models and >4.5 for the R6/1 mouse model.

amino acid transporter (SLC7A3), a receptor protein (TNFRSF10D), and others involved in crucial cell regulatory processes. Therefore, one might predict that severe dysregulation of the expression of such genes may cause serious stress conditions and metabolic perturbations in cells.

Comparisons of HEK41, HEK53 and HEK84 cell lines indicated again that when analyzing the highly dysregulated genes, differences in gene expression patterns are more pronounced between HEK53 and other HD cellular models than between HEK41 and HEK84 (Fig. S3). This confirms the previous analyses and predictions of the dissimilarity between the structure of the HTT-Q53 variant and other forms of mutant huntingtin.

When assessing the up- and down-regulated genes between control and HD cells, expression of 488 genes was commonly dysregulated in all three disease cellular models (Fig. 3A). These include genes with the most severely changed transcript levels. The full list of 488 genes commonly dysregulated in HEK41, HEK53 and HEK84 lines (with fold change values) is shown in Fig. S4. Comparisons between particular HD lines are shown in Fig. S5, and dysregulation in the expression of the 488 genes commonly changed in the HEK41, HEK53, and HEK84 lines relative to HEK cells is shown as a volcano plot in Fig. S6A.

Reliability of the presented transcriptomic analyses was confirmed using RT-qPCR with selected genes from the above mentioned group (Fig. S6B) and Western-blotting with antibodies against relevant proteins (Fig. 3C). The transcriptomic analyses, supported by the above mentioned results of additional experiments, demonstrated that there are common features of changes in gene expression patterns among tested HD cellular models, including genes especially affected and involved in crucial cellular processes (like *BEX1*, *MAGEA6*, *SLC7A3*, and others). Such changes may lead to significant disturbances in cell physiology, thereby facilitating further genetic rearrangements, such as the somatic expansion of CAG triplets.

To determine which cellular processes might be the most severely affected in HD cells due to dysregulation of a battery of genes, the Gene Ontology database QuickGO was employed (https://www.ebi.ac.uk/QuickGO/; last assessed on 11 November 2025). Such analysis demonstrated that some cellular processes were severely disturbed in HD cells, irrespective of the length of extended CAG repeats over the threshold of the pathogenicity (Fig. 4, Fig. S7–S22). They include transport (GO:0006810) (with subcategories shown in Fig. S16–S22), mitochondrial functions (GO:0005739), apoptotic process (GO:0006915), inflammatory response (GO:0006954), autophagy (GO:0006914), DNA repair (GO:0006281), and DNA replication (GO:0006260). Importantly, expression of genes related to responses to stress and DNA damage was affected (Fig. S13–S15) and elevated levels of ROS were detected (Fig. 3B) in all cellular HD models. Interestingly,

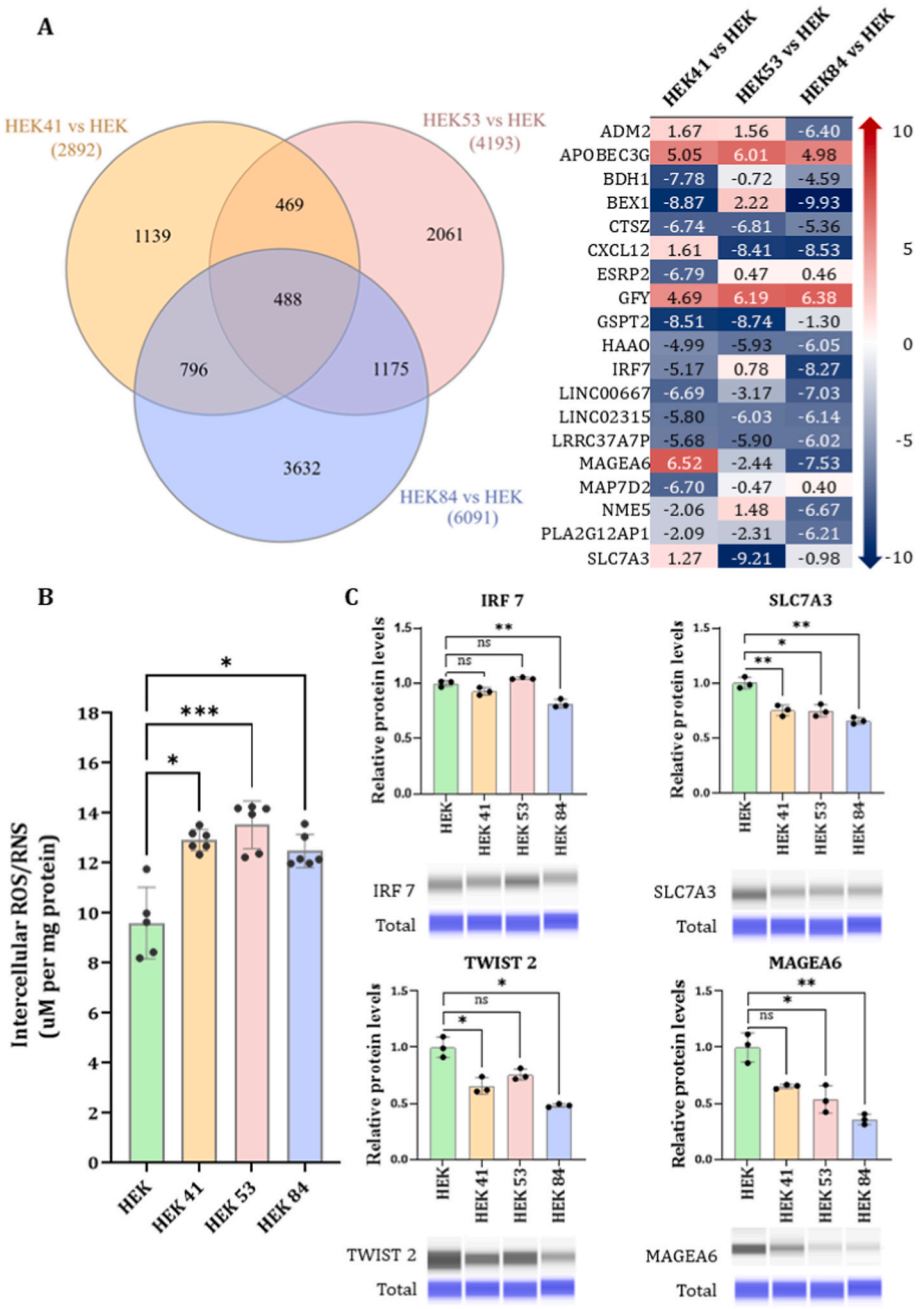


Fig. 3. Numbers of commonly dysregulated genes (A), ROS levels (B), and protein abundances (estimated by Western-blotting) (C) in various cellular models of HD relative to control cells and between particular HD models. In panel A, genes with especially changed levels of transcripts in HD models relative to control cells are indicated in the right side.

comparison of results reported here with those published previously [6] revealed that expression of genes from the same families (e.g. *DNAJB1*, *DNAJC30* and *DNAJC9* – coding for heat shock proteins; *HDAC1* and *HDAC5* – coding for histone deacetylases; *NFKB1* and *NFKB2*, and *SP1*, *SP4*, and *SP110* – coding for transcription factors acting in various stress reactions) was dysregulated in both experimental systems. These analyses corroborate the hypothesis that crucial cellular processes can be dysfunctional in HD cells, causing different stress conditions (including DNA damage stress) and resulting in various defects which, between other consequences, might facilitate somatic CAG repeat expansion in cells bearing over 36 CAG triplets in *HTT*, contrary to healthy cells, where such dysregulations do not occur.

4. Conclusions

These are significant changes in the regulation of expression of thousands of genes in cellular and animal models of HD relative to controls, occurring in cells bearing different numbers of pathogenic extensions of CAG repeats in exon 1 of the *HTT* gene (from 41 through 53 to 84 in cellular models, and 116-120 in the mouse R6/1 model). These changes roughly correlate with the length of the CAG stretch in cellular HD models. Obviously, transcriptomic changes are consequences of the occurrence of pathogenic huntingtin forms rather than the primary cause of the disease, however, they may significantly contribute to the somatic expansion of CAG triplets. The most severe dysregulation involves genes encoding proteins that perform crucial cellular functions, such as transport, mitochondrial functions, apoptosis, the inflammatory

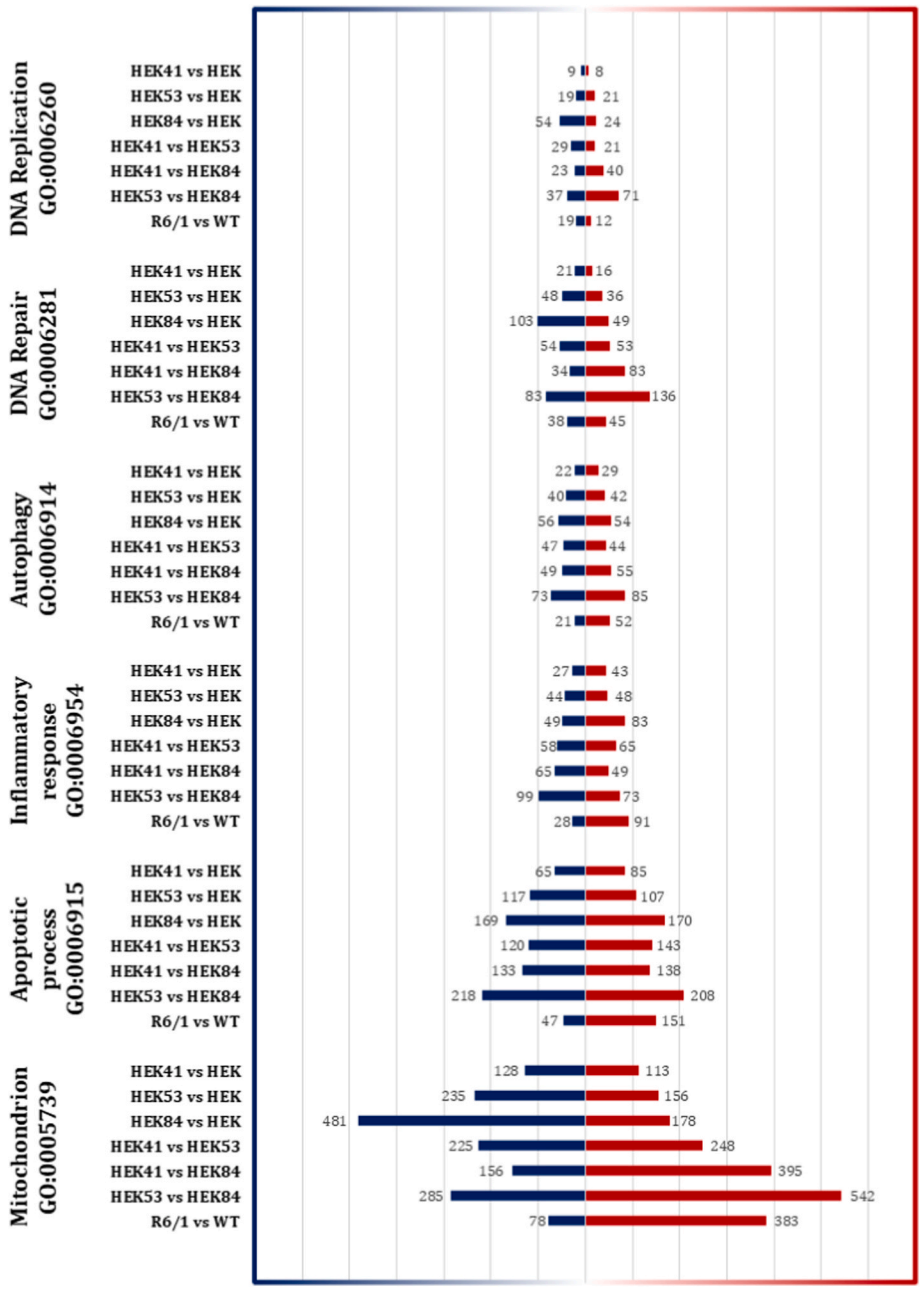


Fig. 4. Numbers of genes with significantly changed ($p < 0.05$) levels of transcripts in HD models relative to controls, grouped according to processes (from the QuickGO database) they are involved in.

response, autophagy, DNA repair, and DNA replication. Importantly, significant changes were detected in expression of genes related to responses to stress and DNA damage. We propose that corresponding changes in these processes may cause stress conditions, facilitating somatic expansion of CAG triplets in cells bearing pathogenic *HTT* alleles, contrary to normal cells. If this hypothesis is true, it might explain the selectivity of the mechanism of pathological genetic process (somatic CAG triplet expansion restricted only to cells with 36 or more repeats in *HTT*), proposed recently by others [4,5], as the major cause of neurodegeneration in HD and the reason for the latency of the disease symptoms.

Data availability statement

The RNA-seq raw results are deposited in the NCBI Sequence Read Archive (SRA) under the accession no. PRJNA1345724.

Ethical approval

The experiments with the animal model were approved by the Local Ethics Committee for the Care and Use of Laboratory Animals in Bydgoszcz, Poland (resolution no. 48/2021).

The use of generative AI statement

Generative AI or AI-assisted tools were not used to write this paper.

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CRediT authorship contribution statement

Aneta Szulc: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Visualization, Writing – review & editing. **Beata M. Walter:** Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Lidia Gaffke:** Conceptualization, Investigation, Methodology, Validation, Writing – review & editing. **Karolina Wiśniewska:** Investigation, Writing – review & editing. **Magdalena Żabińska:** Investigation, Writing – review & editing. **Estera Rintz:** Investigation, Writing – review & editing. **Zuzanna Cyske:** Investigation, Writing – review & editing. **Michał Grabski:** Formal analysis, Methodology, Writing – review & editing. **Oleksandr Pankiv:** Methodology, Writing – review & editing. **Magdalena Podlacha:** Investigation, Methodology, Writing – review & editing. **Karolina Pierzynowska:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing. **Grzegorz Węgrzyn:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2026.153711.

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SUPPLEMENTARY MATERIAL

Table S1. Primers used for PCR

Gene name	Forward 5'... 3'	Reverse 5'... 3'
htt	CCGCTCAGGTTCTGCTTTTA	GGCTGAGGCAGCAGCGGCTG

Table S2. Primers used for RT- qPCR

	Gene name	Forward 5'... 3'	Reverse 5'... 3'
Human	***GAPDH***	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
	MAGEA6	AGGGGAGGGAAGACAGTATCT	AAAGCCCACTCATGCAGGAG
	ENPP2	ACTTTTGCCGTTGGAGTCAAT	GGAGTCTGATAGCACTGTAGGA
	IRF7	GCTGGACGTGACCATCATGTA	GGGCCGTATAGGAACGTGC
	HTT	GCTCTTAGGCTTACTCGTTCCT	TTCCTTGTCACTCCGAAGCTG
Mouse	***gapdh***	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
	magea6	CCCAAGGGCTCTTGCAGAAA	AATGGTCAGAGAAATTGGAGCAT
	enpp2	ATGGCAAGACAAGGCTGTTTC	TTGACGCCGATGGCAAAAGT
	irf7	GAGACTGGCTATTGGGGGAG	GACCGAAATGCTTCCAGGG
	htt	CTCCATGCTGTTACGACTCATC	AGGGAGGAAGGAGCCAAAATC

Table S3. Antibody used for Wester Blot

Protein name	Producer	Cat no.
IRF 7	Proteintech	11752-1-AP
SLC7A3	Proteintech	27183-1-AP
TWIST2	Proteintech	11752-1-AP
MAGEA6	Proteintech	14602-1-AP

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Verification of CAG repeat number in cellular models using PCR

Figure S2. Models of HTT variants prepared using AlphaFold3 (A) and transcriptomic changes in cellular and animal models of HD revealing numbers of genes with up- or down-regulated levels of transcripts (relative to controls) with groups accrding to log_2FC range (B).

Figure S3. Genes with significantly changed *(p*<0.05) levels of transcripts between the HEK41, HEK53, and HEK83 cellular models of HD. In each pair of cell lines, genes with the highest levels of change are indicated, assuming the log_2FC threshold >7.

Figure S4. The list of 488 genes commonly dysregulated in HEK41, HEK53 and HEK84 lines relative to control HEK line (with log_2FC values).

Figure S5. Numbers of commonly dysregulated genes in various cellular models of HD relative to control cells and between pairs of particular HD models. Genes with especially changed levels of transcripts are indicated next to corresponding Venn diagrams.

Figure S6 A. Expression dysregulation of 488 genes commonly changed in the HEK41, HEK53, HEK84 models of HD relative to control cells. For selected genes, changes in expression levels relative to controls are marked.
B. RT-qPCR analysis of selected transcripts, confirming reliability of transcriptomic results.

Figure S7. Numbers of genes involved in DNA replication (GO:0006260) with significantly changed (*p*<0.05) levels of transcripts in HD models relative to controls

Figure S8. Numbers of genes involved in DNA repair (GO:0006281) with significantly changed (*p*<0.05) levels of transcripts in HD models relative to controls

Figure S9. Numbers of genes involved in autophagy (GO:0006914) with significantly changed (*p*<0.05) levels of transcripts in HD models relative to controls.

SUPPLEMENTARY FIGURE LEGENDS

Figure S10. Numbers of genes involved in inflammatory response (GO:0006954) with significantly changed (p<0.05) levels of transcripts in HD models relative to controls.

Figure S11. Numbers of genes involved in apoptotic process (GO:0006915) with significantly changed (p<0.05) levels of transcripts in HD models relative to controls.

Figure S12. Numbers of genes involved in mitochondrion functions (GO:0005739) with significantly changed (p<0.05) levels of transcripts in HD models relative to controls.

Figure S13. Numbers of genes with significantly changed (p<0.05) levels of transcripts in HD models relative to controls, grouped according to processes (from the QuickGO database) they are involved in.

Figure S14. Numbers of genes involved in response to stress (GO:0006950) with significantly changed (p<0.05) levels of transcripts in HD models relative to controls

Figure S15. Numbers of genes involved in DNA damage response (GO:0006974) with significantly changed (p<0.05) levels of transcripts in HD models relative to controls

Figure S16. Numbers of genes with significantly changed (p<0.05) levels of transcripts in HD models relative to controls, grouped according to processes (from the QuickGO database) they are involved in.

Figure S17. Numbers of genes involved in transport (GO:0006810) with significantly changed (p<0.05) levels of transcripts in HD models relative to controls.

Figure S18. Numbers of genes involved in vesicle-mediated transport (**GO:0016192**) with significantly changed (p<0.05) levels of transcripts in HD models relative to controls.

Figure S19. Numbers of genes involved in intercellular transport (GO:0010496) with significantly changed (p<0.05) levels of transcripts in HD models relative to controls.

SUPPLEMENTARY FIGURE LEGENDS

Figure S20. Numbers of genes involved in protein transport (GO:0015031) with significantly changed (p<0.05) levels of transcripts in HD models relative to controls.

Figure S21. Numbers of genes involved in nuclear transport (GO:0051169) with significantly changed (p<0.05) levels of transcripts in HD models relative to controls.

Figure S22. Numbers of genes involved in neurotransmitter transport (GO:0006836) with significantly changed (p<0.05) levels of transcripts in HD models relative to controls.

Figure S1

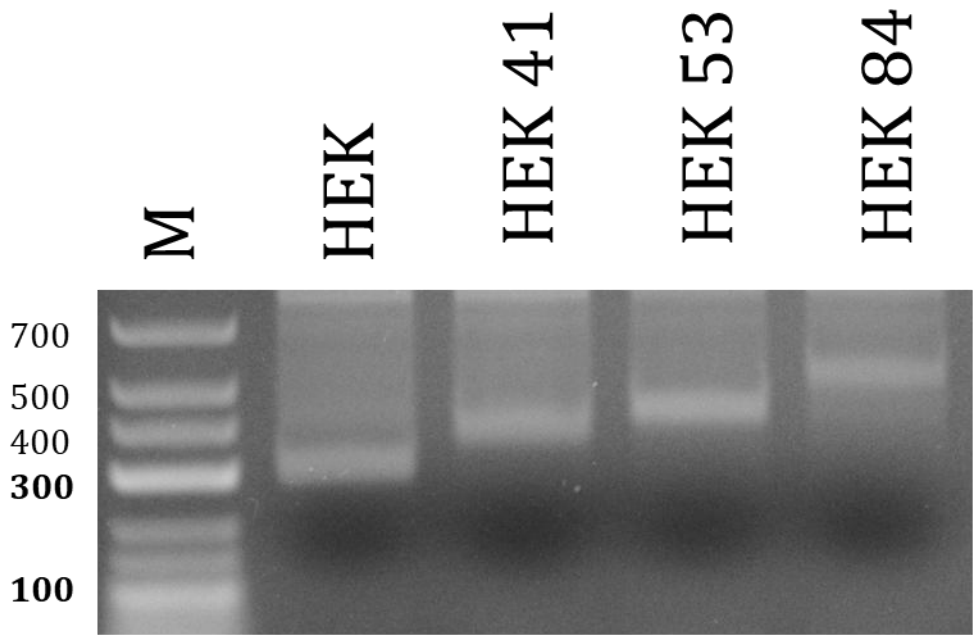
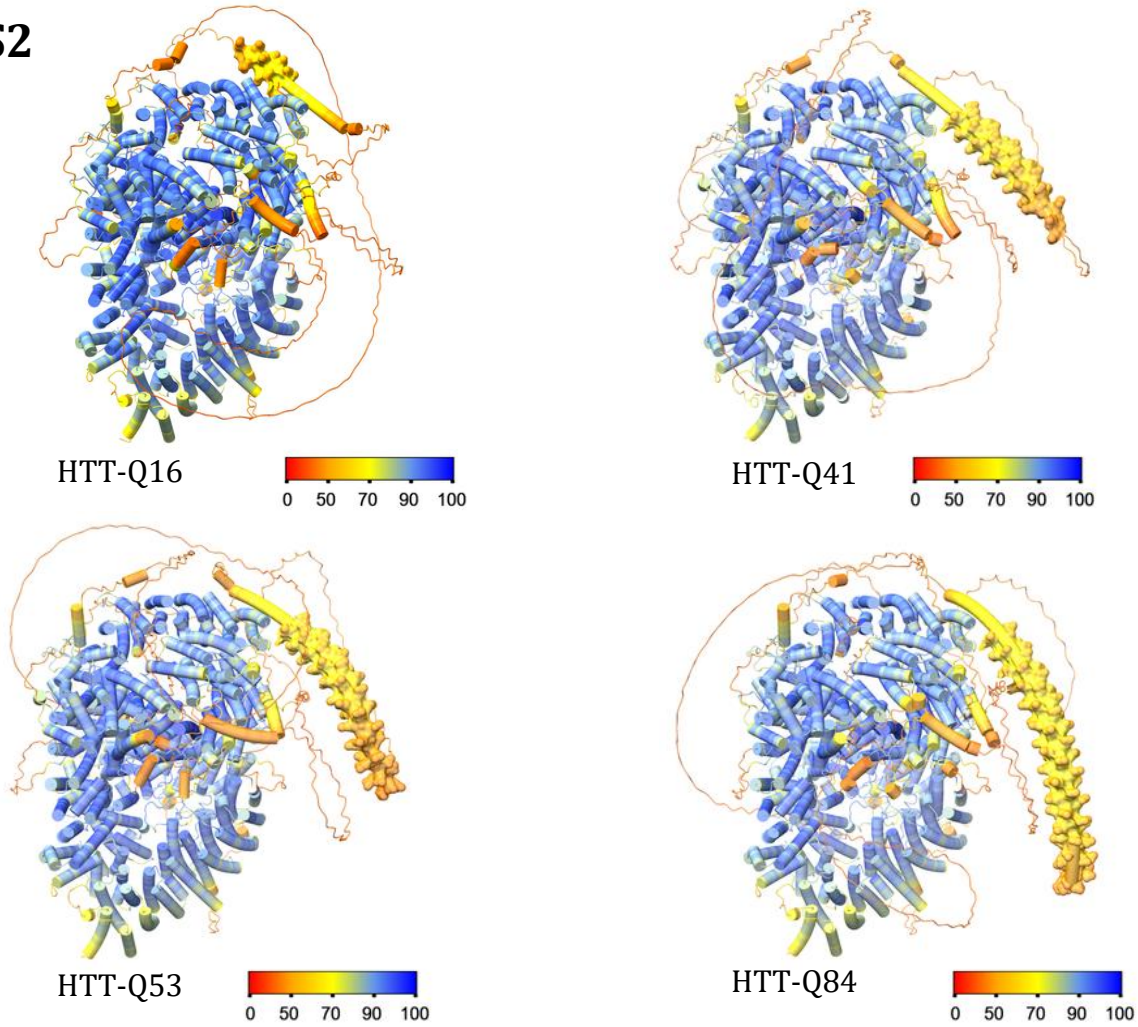


Figure S2

A



B

Level log_2(FC)	Cellular model: HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	Animal model: R6/1 vs WT	log_2(FC)
> 10	0	0	0	2	4	5	0	> 10
9 -10	0	0	0	0	2	4	0	10
8 - 9	0	3	0	2	2	4	0	9
7 - 8	0	6	1	3	8	12	1	8
6 - 7	2	11	2	2	12	16	1	7
5 - 6	16	40	46	5	27	64	2	6
4 - 5	52	102	86	53	70	135	14	5
3 - 4	68	100	90	72	61	84	31	4
2 - 3	85	137	158	102	102	147	49	3
1 - 2	250	255	628	527	430	492	240	2
0 - 1	1200	1468	1993	2150	2233	3137	2007	1
0 - (-1)	794	1645	2431	1756	1519	2720	1289	-1
-1 - (-2)	217	268	385	384	602	856	351	-2
-2 - (-3)	89	68	87	166	179	221	106	-3
-3 - (-4)	50	33	49	120	101	81	79	-4
-4 - (-5)	37	35	47	95	84	73	15	-5
-5 - (-6)	19	14	58	38	25	21	0	-6
-6 - (-7)	8	4	19	17	13	5	0	-7
-7 - (-8)	1	1	5	16	6	3	0	-8
-8 - (-9)	3	2	2	4	0	1	0	-9
-9 - (-10)	1	1	4	3	0	0	0	-10
< (-10)	0	0	0	3	0	0	0	< (-10)

Figure S3

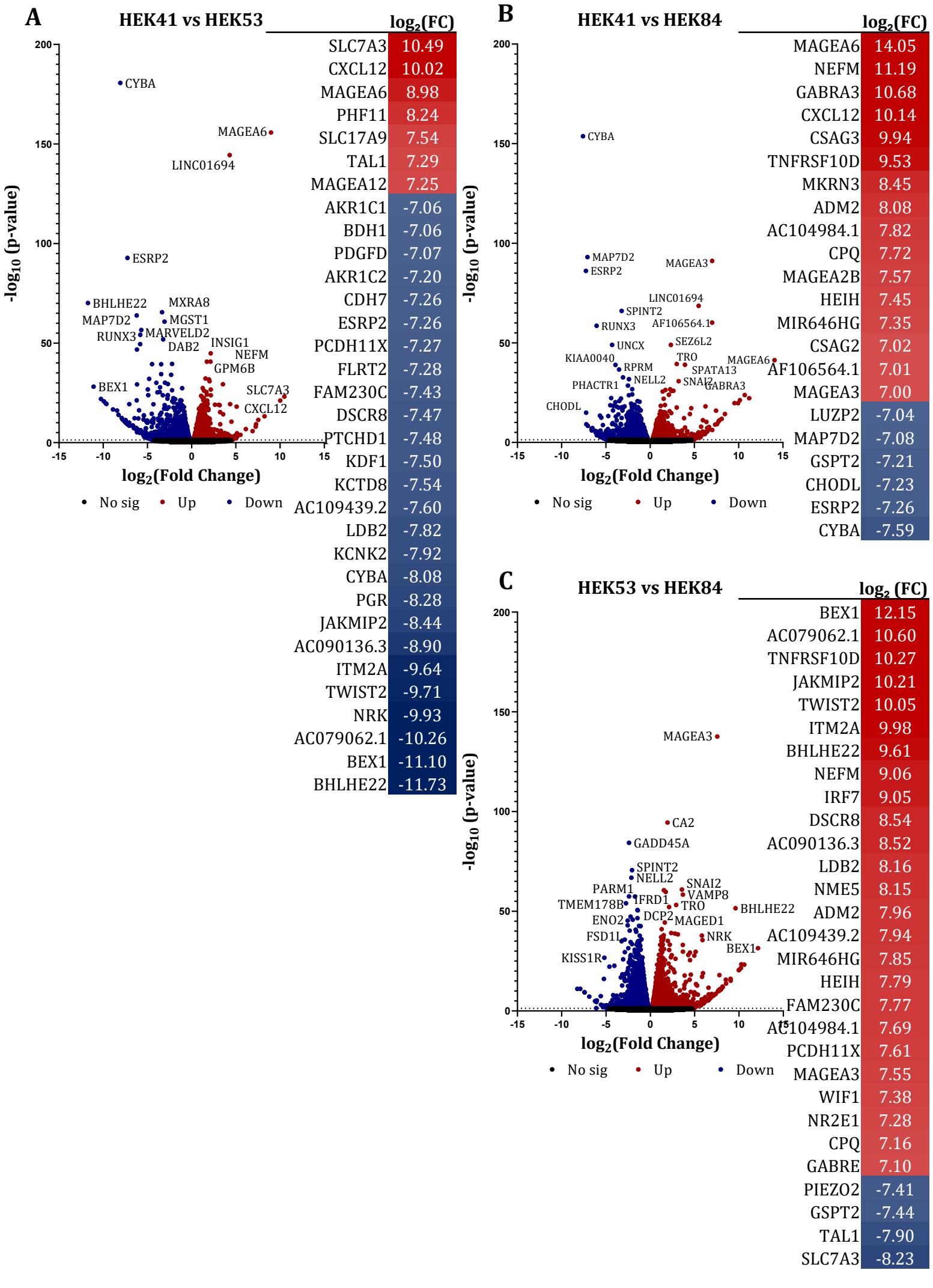

A
HEK41 vs HEK53
log2(FC)
SLC7A3 10.49
CXCL12 10.02
MAGEA6 8.98
PHF11 8.24
SLC17A9 7.54
TAL1 7.29
MAGEA12 7.25
AKR1C1 -7.06
BDH1 -7.06
PDGFD -7.07
AKR1C2 -7.20
CDH7 -7.26
ESRP2 -7.26
PCDH11X -7.27
FLRT2 -7.28
FAM230C -7.43
DSCR8 -7.47
PTCHD1 -7.48
KDF1 -7.50
KCTD8 -7.54
AC109439.2 -7.60
LDB2 -7.82
KCNK2 -7.92
CYBA -8.08
PGR -8.28
JAKMIP2 -8.44
AC090136.3 -8.90
ITM2A -9.64
TWIST2 -9.71
NRK -9.93
AC079062.1 -10.26
BEX1 -11.10
BHLHE22 -11.73
-log10 (p-value)
log2(Fold Change)
No sig
Up
Down
B
HEK41 vs HEK84
log2(FC)
MAGEA6 14.05
NEFM 11.19
GABRA3 10.68
CXCL12 10.14
CSAG3 9.94
TNFRSF10D 9.53
MKRN3 8.45
ADM2 8.08
AC104984.1 7.82
CPQ 7.72
MAGEA2B 7.57
HEIH 7.45
MIR646HG 7.35
CSAG2 7.02
AF106564.1 7.01
MAGEA3 7.00
LUZP2 -7.04
MAP7D2 -7.08
GSPT2 -7.21
CHODL -7.23
ESRP2 -7.26
CYBA -7.59
C
HEK53 vs HEK84
log2 (FC)
BEX1 12.15
AC079062.1 10.60
TNFRSF10D 10.27
JAKMIP2 10.21
TWIST2 10.05
ITM2A 9.98
BHLHE22 9.61
NEFM 9.06
IRF7 9.05
DSCR8 8.54
AC090136.3 8.52
LDB2 8.16
NME5 8.15
ADM2 7.96
AC109439.2 7.94
MIR646HG 7.85
HEIH 7.79
FAM230C 7.77
AC104984.1 7.69
PCDH11X 7.61
MAGEA3 7.55
WIF1 7.38
NR2E1 7.28
CPQ 7.16
GABRE 7.10
PIEZO2 -7.41
GSPT2 -7.44
TAL1 -7.90
SLC7A3 -8.23

Figure S4

	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK
ABCA11P ENSG00000251595	-1.37	-0.94	-1.70
ABCA5 ENSG00000154265	0.55	0.31	-0.53
ABCA8 ENSG00000141338	2.52	2.44	3.40
ABLIM2 ENSG00000163995	-0.75	-0.98	0.91
AC000099.1 ENSG00000236340	-4.96	-5.19	-5.30
AC004233.3 ENSG00000272079	-0.88	-1.39	-2.44
AC019069.1 ENSG00000272711	-0.43	-0.46	-0.85
AC023946.1 ENSG00000255351	1.65	1.82	2.36
AC027031.2 ENSG00000254615	0.48	0.46	0.64
AC027307.3 ENSG00000268798	-1.08	-1.09	-1.59
AC040977.1 ENSG00000262089	-1.09	-1.44	-1.02
AC072062.1 ENSG00000227769	-4.71	-4.21	-5.05
AC079062.1 ENSG00000263711	-5.45	4.82	-5.79
AC087477.2 ENSG00000259275	-1.08	-1.88	-1.65
AC110491.1 ENSG00000261292	-1.75	-2.25	-2.22
AC123768.3 ENSG00000262728	1.97	2.26	2.24
AC125257.1 ENSG00000259623	-0.28	0.46	-0.30
AC156455.1 ENSG00000256546	0.87	0.63	-1.34
ACADL ENSG00000115361	-1.13	0.57	-0.79
ACSS3 ENSG00000111058	-4.31	0.48	-4.52
ACTB ENSG00000075624	-0.35	-0.42	-0.56
ADAMTS5 ENSG00000154736	-4.40	1.02	-4.14
ADGRB2 ENSG00000121753	0.65	-1.32	0.64
ADGRL1 ENSG00000072071	0.69	0.53	0.78
ADM2 ENSG00000128165	1.67	1.56	-6.40
AFAP1 ENSG00000196526	0.64	0.49	0.98
AGAP2 ENSG00000135439	0.44	0.54	1.66
AHCYL1 ENSG00000168710	0.52	0.33	0.46
AIFM2 ENSG00000042286	-2.52	-3.33	-2.92
AIMP2 ENSG00000106305	-0.52	-0.37	-0.86
AL034346.1 ENSG00000261730	-1.56	-1.03	-1.32
AL353807.2 ENSG00000232519	-2.23	-1.63	-2.57
ALDOC ENSG00000109107	0.75	1.05	0.97
ALPK1 ENSG00000073331	0.69	1.08	1.05
ANP32E ENSG00000143401	-0.37	0.34	0.25
AP003068.4 ENSG00000278952	-2.02	-2.04	-1.84
APOBEC3F ENSG00000128394	0.65	1.03	1.33
APOBEC3G ENSG00000239713	5.05	6.01	4.98
APOL2 ENSG00000128335	-0.41	0.74	-0.48
ARGLU1 ENSG00000134884	0.37	-0.70	-0.72
ARHGAP22 ENSG00000128805	-0.39	-0.32	-0.93
ARHGAP23 ENSG00000275832	1.22	1.65	1.85
ARHGAP29 ENSG00000137962	0.58	-0.41	0.54
ARL2BP ENSG00000102931	-0.35	-0.35	-0.95
ARRDC1 ENSG00000197070	-0.74	-0.63	-0.51
ARRDC4 ENSG00000140450	0.52	-0.31	0.84
ASIC1 ENSG00000110881	0.77	0.47	0.54
ATP1B1 ENSG00000143153	-0.29	-0.36	0.38
ATXN7L3 ENSG00000087152	0.27	0.37	0.32



Figure S4, continued

	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK
AXL ENSG00000167601	3.55	3.84	4.20
B4GALNT4 ENSG00000182272	0.57	-0.50	0.79
BACE1 ENSG00000186318	-0.55	-1.37	-0.78
BCAR3 ENSG00000137936	0.66	-0.77	0.59
BCDIN3D-AS1 ENSG00000258057	0.99	1.25	1.45
BCHE ENSG00000114200	1.84	1.90	2.02
BDH1 ENSG00000161267	-7.78	-0.72	-4.59
BEX1 ENSG00000133169	-8.87	2.22	-9.93
BLVRA ENSG00000106605	-0.29	-0.40	-0.66
BMP2 ENSG00000125845	0.57	1.45	-0.60
BMP5 ENSG00000112175	2.45	3.13	3.52
BMP6 ENSG00000153162	0.65	-0.54	1.52
C1orf122 ENSG00000197982	-0.58	-0.35	-0.44
C1QL4 ENSG00000186897	1.09	1.08	1.64
C22orf39 ENSG00000242259	0.29	0.62	0.50
C5orf63 ENSG00000164241	0.64	0.48	0.78
CALCB ENSG00000175868	2.53	-1.99	0.57
CAMK2D ENSG00000145349	0.53	0.42	0.83
CASP10 ENSG00000003400	1.34	1.37	0.60
CBWD1 ENSG00000172785	-0.39	-0.24	-0.46
CBWD6 ENSG00000215126	-0.80	-0.81	-0.50
CCDC113 ENSG00000103021	1.17	0.49	1.03
CCDC68 ENSG00000166510	-3.40	2.06	-3.20
CCDC80 ENSG00000091986	0.62	0.87	1.39
CCDC85B ENSG00000175602	-0.46	-0.39	-0.93
CCDC86 ENSG00000110104	-0.26	0.27	-0.78
CD109 ENSG00000156535	-0.61	1.26	0.95
CDA ENSG00000158825	2.81	4.93	4.02
CDK18 ENSG00000117266	0.52	0.86	0.91
CDK2AP2 ENSG00000167797	-0.44	-0.36	-0.41
CECR7 ENSG00000237438	-4.53	-4.75	-4.87
CENPV ENSG00000166582	-0.54	-0.80	-0.29
CEP120 ENSG00000168944	0.45	0.27	1.60
CERS2 ENSG00000143418	-0.26	0.28	0.57
CHMP4B ENSG00000101421	-0.28	-0.21	-0.38
CHRM3 ENSG00000133019	-2.56	2.44	1.09
CKB ENSG00000166165	-0.33	-0.20	-0.70
CLCNKB ENSG00000184908	3.77	2.76	3.48
CLDN1 ENSG00000163347	-0.93	-1.21	0.74
CLU ENSG00000120885	-0.62	0.63	1.19
CLYBL ENSG00000125246	0.53	-0.78	0.60
CNNM2 ENSG00000148842	0.49	0.34	0.67
CNST ENSG00000162852	-0.66	0.35	0.45
CNTNAP1 ENSG00000108797	0.72	0.61	0.67
CNTNAP2 ENSG00000174469	-0.54	0.34	0.84
COL1A1 ENSG00000108821	0.66	1.20	0.54
COL3A1 ENSG00000168542	1.51	3.24	2.60
COL6A3 ENSG00000163359	3.07	3.67	3.27
CORO2A ENSG00000106789	0.69	0.64	1.09

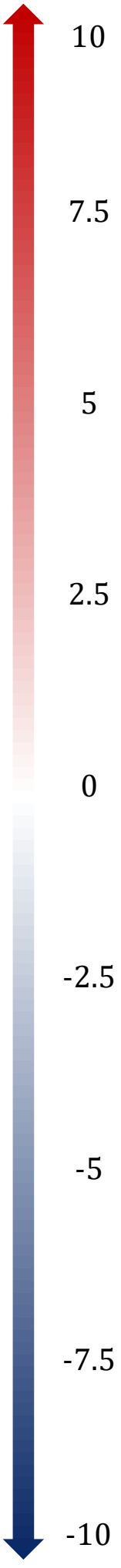


Figure S4, continued

	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK
CPED1 ENSG00000106034	3.45	2.70	3.50
CPQ ENSG00000104324	2.09	1.53	-5.62
CRABP2 ENSG00000143320	1.20	1.28	2.43
CREBRF ENSG00000164463	0.70	0.70	1.07
CSDC2 ENSG00000172346	0.99	2.19	1.98
CSRNP1 ENSG00000144655	0.85	0.97	1.37
CSRNP3 ENSG00000178662	0.91	0.66	0.74
CTSZ ENSG00000101160	-6.74	-6.81	-5.36
CXCL12 ENSG00000107562	1.61	-8.41	-8.53
CXCL16 ENSG00000161921	-0.88	-0.36	0.78
CXCR4 ENSG00000121966	1.07	-0.66	0.79
CYB5R3 ENSG00000100243	0.49	0.63	0.48
CYP1B1 ENSG00000138061	-0.64	-0.71	0.52
D2HGDH ENSG00000180902	-0.35	-0.36	-0.87
DAB2 ENSG00000153071	-1.28	1.94	-1.15
DCAF6 ENSG00000143164	-0.28	0.35	0.52
DCHS1 ENSG00000166341	1.31	1.17	1.05
DDN ENSG00000181418	-0.84	1.39	-0.97
DDR1 ENSG00000204580	0.51	0.53	0.92
DDX56 ENSG00000136271	-0.35	-0.33	-0.68
DEGS2 ENSG00000168350	-0.87	-1.06	0.79
DHRS13 ENSG00000167536	0.76	0.43	0.74
DHRS3 ENSG00000162496	0.80	-0.71	0.57
KFZP434K028 ENSG00000124915	-2.22	-3.60	-3.60
DLX2 ENSG00000115844	-0.42	-0.38	-0.84
DMBX1 ENSG00000197587	1.00	0.75	1.00
DMPK ENSG00000104936	-0.44	-0.61	-0.43
DMTN ENSG00000158856	-1.25	-0.71	0.79
DNAJC30 ENSG00000176410	-0.48	-0.38	-0.66
DNAJC9 ENSG00000213551	-0.40	-0.25	-1.00
DOC2A ENSG00000149927	0.45	0.39	-0.50
DOK6 ENSG00000206052	-2.50	1.49	1.44
DPYSL3 ENSG00000113657	-1.45	2.00	-0.97
EBF4 ENSG00000088881	1.22	-0.94	0.83
EDNRA ENSG00000151617	-0.79	0.57	0.80
EHD2 ENSG00000024422	0.78	0.67	0.91
EIF4E3 ENSG00000163412	0.40	-0.30	0.43
ELK3 ENSG00000111145	0.60	0.64	0.43
ENDOD1 ENSG00000149218	0.44	0.36	0.37
ENO2 ENSG00000111674	-0.65	-0.80	1.77
ENOX1 ENSG00000120658	0.52	-0.53	-0.54
ENPP2 ENSG00000136960	1.46	1.80	2.02
ERMARD ENSG00000130023	0.48	0.36	0.60
ESRP2 ENSG00000103067	-6.79	0.47	0.46
ESRRB ENSG00000119715	-0.56	-1.07	0.71
ETNK2 ENSG00000143845	0.29	0.30	0.58
ETV1 ENSG00000006468	-0.94	-1.14	1.02
FADS3 ENSG00000221968	0.60	-0.74	0.59
FAM131C ENSG00000185519	1.34	0.96	2.03

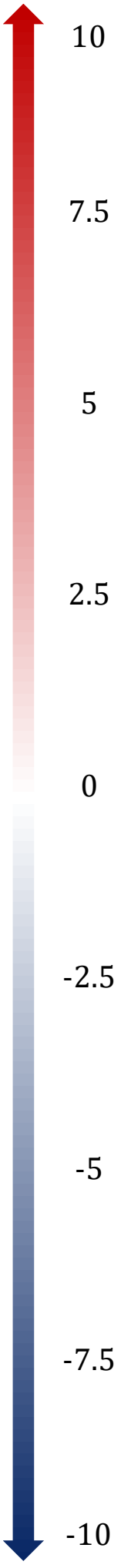


Figure S4, continued

	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK
FAM133B ENSG00000234545	-0.38	-0.63	-0.36
FAM173A ENSG00000103254	-0.64	-0.95	-1.36
FAM189A1 ENSG00000104059	0.78	-0.63	0.95
FAM20B ENSG00000116199	-0.44	0.26	0.43
FAM43B ENSG00000183114	0.89	0.95	1.25
FAM57A ENSG00000167695	-0.33	-0.50	0.42
FBLIM1 ENSG00000162458	0.80	0.57	1.39
FBN2 ENSG00000138829	-0.85	-0.35	0.36
FBXL16 ENSG00000127585	-0.98	-2.17	-1.12
FERMT1 ENSG00000101311	-2.85	-1.01	-2.45
FGF13 ENSG00000129682	1.83	2.44	1.48
FGFR2 ENSG00000066468	0.41	-0.52	0.55
FGFR4 ENSG00000160867	0.50	0.59	0.66
FH ENSG00000091483	-1.33	-0.30	-0.67
FIGNL2 ENSG00000261308	4.96	3.50	4.98
FMR1-IT1 ENSG00000236337	-2.33	-1.48	-2.49
FNDC3B ENSG00000075420	0.55	0.55	0.81
FRMD4B ENSG00000114541	0.86	0.56	0.95
FRRS1 ENSG00000156869	0.79	0.79	0.57
FUS ENSG00000089280	-0.54	-0.36	-0.92
FUT1 ENSG00000174951	2.61	3.50	5.74
GAD1 ENSG00000128683	-0.46	-0.69	-0.88
GADD45A ENSG00000116717	0.49	-1.08	1.33
GAL ENSG00000069482	-0.54	-0.35	-0.84
GAL3ST1 ENSG00000128242	1.13	3.09	1.34
GAR1 ENSG00000109534	-0.39	0.29	-0.37
GFI1 ENSG00000162676	0.79	-0.70	0.99
GFY ENSG00000261949	4.69	6.19	6.38
GGT1 ENSG00000100031	0.89	1.93	1.00
GIPC3 ENSG00000179855	0.86	1.30	1.59
GLMP ENSG00000198715	-0.34	0.20	0.38
GNAZ ENSG00000128266	0.47	-0.53	0.83
GPAM ENSG00000119927	-0.39	-0.29	0.40
GPAT3 ENSG00000138678	0.90	0.45	0.92
GPATCH2 ENSG00000092978	-0.36	0.34	0.37
GPATCH4 ENSG00000160818	-0.73	0.26	-0.52
GPC1 ENSG00000063660	-0.40	-0.59	-0.78
GPC6 ENSG00000183098	0.64	-0.64	0.83
GPR176 ENSG00000166073	0.67	0.58	0.66
GRAMD1B ENSG00000023171	0.77	0.61	0.63
GRAMD1C ENSG00000178075	0.66	0.75	1.06
GRIN2D ENSG00000105464	0.54	0.51	0.88
GRTP1 ENSG00000139835	0.46	-0.62	-0.55
GSPT2 ENSG00000189369	-8.51	-8.74	-1.30
GTPBP6 ENSG00000178605	-0.47	-0.33	-0.52
GUCY1B1 ENSG00000061918	0.52	0.59	0.44
HAAO ENSG00000162882	-4.99	-5.93	-6.05
HBQ1 ENSG00000086506	1.00	-0.69	0.79
HDAC5 ENSG00000108840	0.63	0.48	0.42

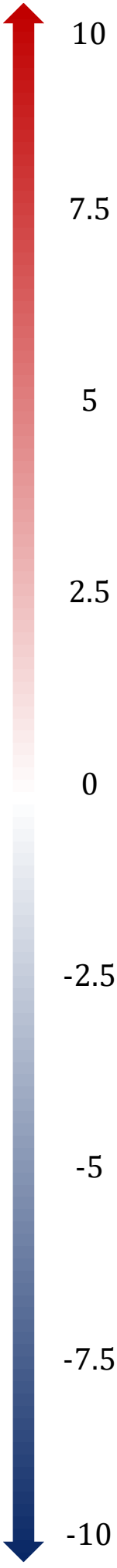


Figure S4, continued

	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK
HERC6 ENSG00000138642	-1.14	0.50	0.98
HHIP-AS1 ENSG00000248890	1.29	1.16	1.18
HIP1 ENSG00000127946	0.62	-0.64	0.76
HMGXB4 ENSG00000100281	0.34	0.46	0.29
HOXA11-AS ENSG00000240990	-0.60	0.61	-0.84
HOXC8 ENSG00000037965	-0.56	-0.62	-0.74
HOXD11 ENSG00000128713	-0.59	0.42	-0.80
HPCAL4 ENSG00000116983	0.89	0.85	1.44
HSD17B8 ENSG00000204228	0.58	0.66	1.18
HTR3A ENSG00000166736	5.68	5.07	5.93
ICOSLG ENSG00000160223	0.83	0.84	1.27
IER5 ENSG00000162783	-0.37	-0.28	0.36
IFNAR2 ENSG00000159110	-0.45	-0.44	-0.56
IFRD1 ENSG00000006652	0.42	-0.85	0.60
IGF2BP2 ENSG00000073792	0.57	0.30	0.48
IGSF3 ENSG00000143061	0.98	0.41	0.54
IL12RB2 ENSG00000081985	-3.13	1.44	-5.13
IL6R ENSG00000160712	1.24	0.64	1.77
IMPACT ENSG00000154059	0.27	-0.34	0.50
INHBB ENSG00000163083	1.16	1.03	0.98
INSIG1 ENSG00000186480	1.41	-0.75	1.05
INTS6L ENSG00000165359	0.74	0.72	-0.93
IRF7 ENSG00000185507	-5.17	0.78	-8.27
IRF8 ENSG00000140968	0.60	0.61	-0.95
ISM2 ENSG00000100593	1.37	1.24	2.26
ITGA5 ENSG00000161638	0.59	0.71	0.53
ITGA9 ENSG00000144668	1.47	1.57	1.35
JAKMIP2 ENSG00000176049	-3.87	4.58	-5.66
JDP2 ENSG00000140044	1.12	0.87	1.67
KIF3C ENSG00000084731	0.67	0.54	1.00
KIFAP3 ENSG00000075945	-0.32	0.24	0.31
KLF12 ENSG00000118922	0.46	-0.41	0.37
KLF9 ENSG00000119138	0.90	0.39	0.66
KLHDC9 ENSG00000162755	-5.21	-5.44	-5.55
KRT8 ENSG00000170421	0.84	2.28	1.23
KYAT3 ENSG00000137944	0.55	-0.36	-0.40
LANCL2 ENSG00000132434	-0.62	-0.43	-0.45
LARGE2 ENSG00000165905	0.64	0.75	1.26
LATS2 ENSG00000150457	0.59	0.50	-0.81
LDLR ENSG00000130164	0.61	0.36	0.85
LDOC1 ENSG00000182195	-0.26	0.52	-1.21
LGI1 ENSG00000108231	4.34	3.38	5.10
LINC00632 ENSG00000203930	0.49	0.58	-1.61
LINC00667 ENSG00000263753	-6.69	-3.17	-7.03
LINC00852 ENSG00000231177	-1.18	-1.10	-1.03
LINC01694 ENSG00000233922	3.65	-0.65	-1.82
LINC02315 ENSG00000251363	-5.80	-6.03	-6.14
LIPH ENSG00000163898	2.91	4.07	4.54
LMO4 ENSG00000143013	0.41	-0.42	0.33

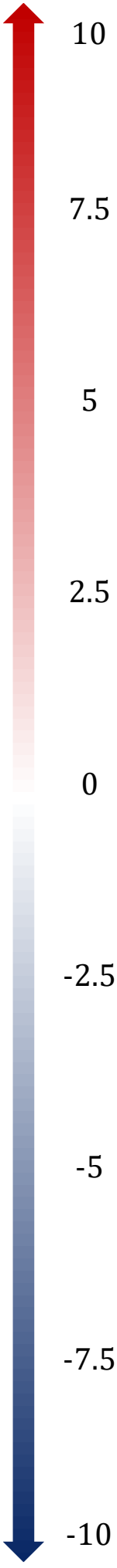


Figure S4, continued

	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK
LONRF2 ENSG00000170500	-1.09	-1.63	-0.55
LRP1 ENSG00000123384	0.51	0.50	1.19
LRP12 ENSG00000147650	0.50	0.92	0.83
LRP2 ENSG00000081479	0.59	-0.93	0.53
LRP4 ENSG00000134569	0.61	0.68	0.47
LRRC37A7P ENSG00000265158	-5.68	-5.90	-6.02
LRRC8D ENSG00000171492	0.36	-0.27	1.05
LTBP2 ENSG00000119681	1.18	1.07	2.71
LUC7L2 ENSG00000146963	-0.30	-0.51	-0.31
LYPD1 ENSG00000150551	-1.09	-1.35	0.47
LYST ENSG00000143669	0.47	0.40	0.54
MAGEA3 ENSG00000221867	5.10	5.66	-1.94
MAGEA6 ENSG00000197172	6.52	-2.44	-7.53
MAGEE1 ENSG00000198934	-0.45	-0.41	-1.27
MAGI2-AS3 ENSG00000234456	-0.46	-0.58	-0.45
MALAT1 ENSG00000251562	0.90	0.38	1.18
MAP4K2 ENSG00000168067	0.88	0.67	1.50
MAP7D2 ENSG00000184368	-6.70	-0.47	0.40
MAPT ENSG00000186868	0.63	-0.41	0.97
MARCKSL1 ENSG00000175130	-0.29	-0.76	-0.90
MARVELD2 ENSG00000152939	-5.03	0.77	-5.07
MCRIP2 ENSG00000172366	-0.49	-0.74	-0.77
MED13L ENSG00000123066	0.56	0.43	0.62
MEGF10 ENSG00000145794	1.70	1.65	1.48
MET ENSG00000105976	0.49	-0.66	0.80
METTL13 ENSG00000010165	-0.52	0.26	-0.47
MFAP2 ENSG00000117122	0.56	0.99	0.87
MGME1 ENSG00000125871	-0.32	0.46	0.50
MID1 ENSG00000101871	1.11	0.92	0.57
MIDN ENSG00000167470	-0.63	-0.26	-0.34
MMP24OS ENSG00000126005	-0.57	0.30	-0.47
MROH6 ENSG00000204839	-1.38	-0.73	-1.08
MRPS2 ENSG00000122140	-0.27	-0.23	-0.52
MSANTD2 ENSG00000120458	-0.43	-0.39	-0.67
MSI1 ENSG00000135097	0.79	-0.30	0.36
MTMR11 ENSG00000014914	0.72	1.18	0.93
MT-TV ENSG00000210077	-2.45	-2.48	-2.64
MYC ENSG00000136997	0.48	0.60	1.11
MYD88 ENSG00000172936	-2.43	-4.51	-3.09
MYO18A ENSG00000196535	0.55	0.56	0.47
MYOF ENSG00000138119	1.84	1.77	2.37
MYRF ENSG00000124920	0.87	1.07	1.70
N4BP2L2-IT2 ENSG00000281026	-1.07	-0.90	-1.18
NAALAD2 ENSG00000077616	-0.80	1.25	-1.35
NAB2 ENSG00000166886	0.82	0.40	1.09
NAP1L3 ENSG00000186310	-0.47	0.46	-1.52
NAT8L ENSG00000185818	-0.44	-1.67	-0.56
NDRG1 ENSG00000104419	0.44	0.72	1.25
NELL2 ENSG00000184613	-1.35	-1.09	1.05

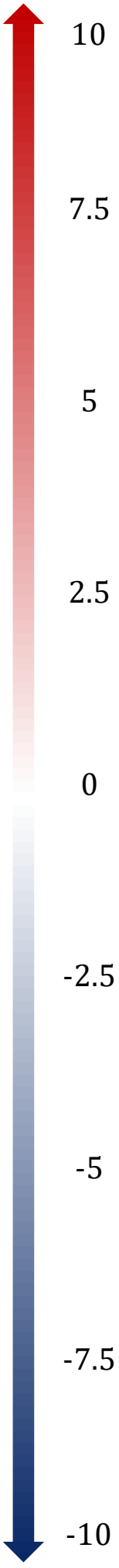


Figure S4, continued

	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK
NETO2 ENSG00000171208	-0.36	0.29	-0.84
NEURL1B ENSG00000214357	1.45	0.98	1.86
NFATC4 ENSG00000100968	1.41	0.55	1.11
NFKB2 ENSG00000077150	0.29	-0.27	0.84
NKX2-5 ENSG00000183072	-0.41	-0.28	-0.44
NKX3-2 ENSG00000109705	0.63	-0.73	1.32
NLE1 ENSG00000073536	0.42	0.48	-0.40
NLK ENSG00000087095	0.34	0.30	-0.35
NME3 ENSG00000103024	-0.46	-0.31	-0.78
NME5 ENSG00000112981	-2.06	1.48	-6.67
NOL4 ENSG00000101746	-0.91	-1.20	0.87
NOP14 ENSG00000087269	-0.25	-0.38	-0.81
NOTCH3 ENSG00000074181	0.88	0.52	1.35
NPY1R ENSG00000164128	-2.89	0.69	0.91
NRCAM ENSG00000091129	1.02	-0.44	1.21
NRSN2 ENSG00000125841	0.31	-0.47	-0.48
NRTN ENSG00000171119	0.50	-0.82	0.69
NSFP1 ENSG00000260075	-1.27	-1.79	-1.31
NSMF ENSG00000165802	-0.41	-0.52	-0.39
NUDT16L1 ENSG00000168101	-0.57	-0.34	-0.91
NYNRIN ENSG00000205978	0.82	0.76	1.49
OSBPL1A ENSG00000141447	0.46	-0.50	0.42
OTULINL ENSG00000145569	0.59	-0.42	0.46
OXCT1 ENSG00000083720	0.89	-0.48	-0.63
OXR1 ENSG00000164830	0.53	0.62	0.69
PACSIN3 ENSG00000165912	0.61	0.48	0.67
PARM1 ENSG00000169116	-0.60	-0.99	1.42
PARP10 ENSG00000178685	1.03	1.26	0.91
PCBD1 ENSG00000166228	-0.35	-0.66	-0.79
PCDH18 ENSG00000189184	1.46	1.48	1.18
PCDHGB5 ENSG00000276547	-3.52	-5.17	-5.29
PDE1A ENSG00000115252	2.49	1.96	3.07
PDZD4 ENSG00000067840	0.56	-0.55	0.52
PEBP1 ENSG00000089220	0.25	-0.21	-0.40
PGBD5 ENSG00000177614	-0.39	-0.70	-0.55
PHF20 ENSG00000025293	0.34	0.29	0.51
PHYHIPL ENSG00000165443	-2.94	-2.18	-2.34
PIEZO2 ENSG00000154864	-2.97	-5.56	1.86
PIGG ENSG00000174227	-0.31	-0.77	-0.79
PIGS ENSG00000087111	0.39	0.31	-0.42
PIK3IP1 ENSG00000100100	1.38	1.84	1.59
PIM1 ENSG00000137193	0.51	-0.35	0.90
PLA2G12AP1 ENSG00000224680	-2.09	-2.31	-6.21
PLAC1 ENSG00000170965	1.40	0.74	0.94
PLAT ENSG00000104368	0.71	-0.59	2.16
PLEKHA4 ENSG00000105559	1.87	1.79	1.74
PLEKHA6 ENSG00000143850	1.01	1.54	1.44
PLEKHG2 ENSG00000090924	0.54	0.59	0.58
PLK4 ENSG00000142731	0.31	0.38	0.27

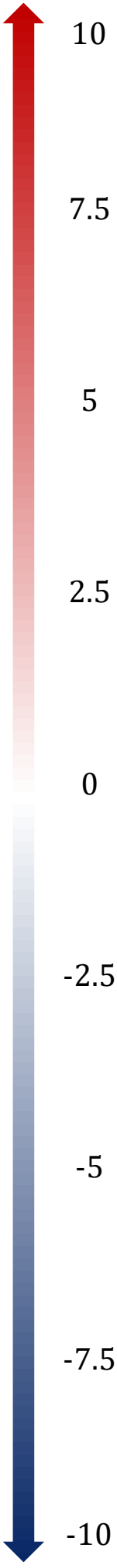


Figure S4, continued

	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK
PLOD2 ENSG00000152952	0.26	0.35	0.34
PLP2 ENSG00000102007	-1.65	-2.19	-5.08
PLS3 ENSG00000102024	0.30	0.23	0.30
PNMA2 ENSG00000240694	0.45	-0.69	0.98
PNMA8A ENSG00000182013	0.39	-0.68	0.60
PNPLA2 ENSG00000177666	-0.32	-0.24	-0.30
PNPLA4 ENSG00000006757	0.73	-0.76	-0.77
PNPO ENSG00000108439	0.30	0.42	-0.32
PPARA ENSG00000186951	0.57	0.45	0.54
PPIF ENSG00000108179	-0.45	-0.49	-0.86
PPP1R1C ENSG00000150722	2.46	2.81	3.08
PPP1R3F ENSG00000049769	-0.54	-0.75	-1.63
PRKAR1B ENSG00000188191	-0.76	-0.70	-0.90
PRR36 ENSG00000183248	-0.50	-0.93	0.60
PRRX1 ENSG00000116132	-1.87	-0.87	1.16
PRSS12 ENSG00000164099	0.45	0.60	1.55
PSMD12 ENSG00000197170	-0.29	0.29	-0.58
PTMA ENSG00000187514	-0.36	-0.28	-0.76
PTPRM ENSG00000173482	1.40	0.72	1.55
PURPL ENSG00000250337	1.64	0.46	1.50
PWWP2B ENSG00000171813	0.40	-0.49	0.69
R3HDM2 ENSG00000179912	0.46	0.46	0.58
RAB2B ENSG00000129472	0.36	0.37	0.83
RAB32 ENSG00000118508	-1.53	-1.08	-0.73
RABEP2 ENSG00000177548	-0.43	-0.38	-0.82
RARB ENSG00000077092	0.81	0.51	0.65
RBMS2 ENSG00000076067	0.51	0.67	0.81
RCC1L ENSG00000274523	-0.50	-0.54	-0.47
RCOR2 ENSG00000167771	0.98	0.83	1.96
RGS9 ENSG00000108370	0.80	0.50	1.02
RHOF ENSG00000139725	0.53	0.49	0.62
RHOT1 ENSG00000126858	0.72	0.34	0.57
RNF145 ENSG00000145860	-0.61	-0.32	-0.68
RPRM ENSG00000177519	-2.38	-1.34	1.15
RPUSD1 ENSG00000007376	-0.40	-0.67	-1.16
RRM2 ENSG00000171848	-0.24	-0.26	-0.40
RSBN1 ENSG00000081019	0.64	0.25	0.51
RUNX1T1 ENSG00000079102	1.07	-0.81	-1.11
RUNX3 ENSG00000020633	-5.20	0.51	0.87
RYR1 ENSG00000196218	4.24	3.81	4.16
S1PR5 ENSG00000180739	2.73	2.26	3.10
SAMD11 ENSG00000187634	-0.90	-0.30	-1.11
SAPCD2 ENSG00000186193	-0.38	-0.59	0.32
SASH1 ENSG00000111961	0.53	0.60	0.61
SBNO1 ENSG00000139697	0.54	0.36	0.44
SCN2B ENSG00000149575	1.61	1.76	-4.82
SCO2 ENSG00000130489	-1.69	-1.47	-1.54
SEPT3 ENSG00000100167	0.77	0.68	1.12
SERINC3 ENSG00000132824	0.29	0.32	0.25

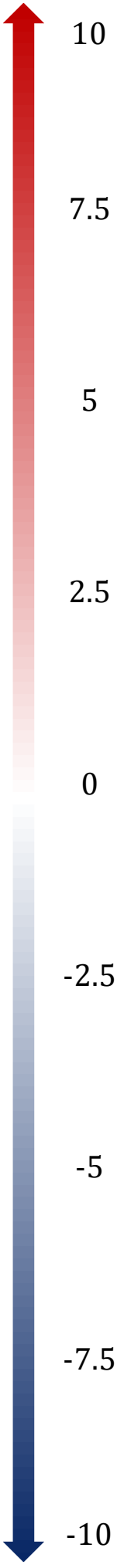


Figure S4, continued

	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK
SERPING1 ENSG00000149131	1.65	2.44	1.67
SESN2 ENSG00000130766	0.84	-0.33	0.82
SEZ6L2 ENSG00000174938	1.01	0.79	-1.33
SFRP1 ENSG00000104332	-1.78	2.69	-3.16
SKAP1 ENSG00000141293	3.90	3.93	4.60
SLC16A9 ENSG00000165449	-1.01	0.31	-0.94
SLC1A4 ENSG00000115902	0.69	-1.07	1.16
SLC25A40 ENSG00000075303	0.38	-0.53	0.94
SLC27A2 ENSG00000140284	-0.47	-0.52	0.33
SLC2A3 ENSG00000059804	2.62	1.24	1.59
SLC2A4 ENSG00000181856	1.04	1.26	1.21
SLC35F4 ENSG00000151812	-4.82	-4.32	-5.16
SLC38A3 ENSG00000188338	0.66	0.64	1.05
SLC44A2 ENSG00000129353	0.44	-0.26	0.76
SLC6A9 ENSG00000196517	1.17	0.60	1.25
SLC7A11 ENSG00000151012	1.07	1.48	1.29
SLC7A3 ENSG00000165349	1.27	-9.21	-0.98
SLC7A8 ENSG00000092068	0.53	0.72	0.98
SLC9A3-AS1 ENSG00000225138	-0.87	-0.64	-0.84
SLC9A9 ENSG00000181804	1.14	1.18	1.03
SLCO2A1 ENSG00000174640	1.95	1.89	3.11
SMPD1 ENSG00000166311	-0.51	-0.47	-0.40
SNAI2 ENSG00000019549	1.65	2.00	-1.57
SOCS3 ENSG00000184557	1.17	1.14	1.82
SOX9 ENSG00000125398	0.67	0.41	0.71
SP110 ENSG00000135899	-1.70	1.18	-3.67
SPARC ENSG00000113140	-0.98	0.63	1.17
SPINT2 ENSG00000167642	-2.51	-1.35	0.71
SPRY1 ENSG00000164056	0.40	0.45	0.83
SRD5A1 ENSG00000145545	0.47	-0.48	0.45
SRRM3 ENSG00000177679	0.63	-0.82	1.57
SSX2IP ENSG00000117155	0.55	-0.36	0.64
ST3GAL1 ENSG00000008513	0.70	0.46	1.02
STARD3 ENSG00000131748	0.35	0.30	-0.29
STUB1 ENSG00000103266	-0.36	-0.45	-1.28
SUSD4 ENSG00000143502	-0.78	-0.99	1.54
SYK ENSG00000165025	-0.93	-0.32	-0.40
SYNE1 ENSG00000131018	0.78	0.85	0.62
SYT16 ENSG00000139973	1.45	1.37	1.69
SYTL5 ENSG00000147041	1.27	0.98	0.83
TACC3 ENSG00000013810	-0.28	-0.56	-0.63
TAS2R19 ENSG00000212124	3.69	3.59	3.71
TBC1D24 ENSG00000162065	-0.48	-0.37	-0.60
TBC1D9 ENSG00000109436	0.53	0.59	0.68
TCEAL3 ENSG00000196507	-3.65	-2.31	0.88
TES ENSG00000135269	0.50	-0.46	0.95
TGM2 ENSG00000198959	1.46	1.87	1.62
THNSL1 ENSG00000185875	-0.57	-0.50	-0.50
TMEM200C ENSG00000206432	0.97	-0.81	0.74

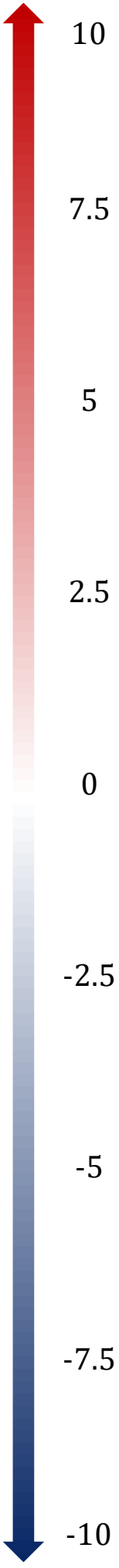


Figure S4, continued

	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK
TMEM47 ENSG00000147027	-0.37	0.32	-0.55
TMEM56 ENSG00000152078	0.43	-0.59	0.70
TMEM98 ENSG00000006042	0.44	0.49	-0.53
TNNT1 ENSG00000105048	0.74	0.64	1.57
TOP2A ENSG00000131747	0.48	0.41	0.36
TP53I3 ENSG00000115129	-0.82	1.40	-1.43
TPP1 ENSG00000166340	0.45	0.29	0.67
TPPP3 ENSG00000159713	0.76	0.83	1.05
TRAF3IP2 ENSG00000056972	0.57	0.43	0.60
TRERF1 ENSG00000124496	0.91	1.00	0.81
TRIB2 ENSG00000071575	0.46	0.67	-0.93
TRIM14 ENSG00000106785	-0.46	0.55	-0.42
TROVE2 ENSG00000116747	-0.43	0.29	0.27
TSC22D3 ENSG00000157514	0.52	-0.86	1.32
TSLP ENSG00000145777	0.81	0.70	1.39
TSPEAR-AS2 ENSG00000182912	0.90	0.51	-0.79
TTLL7 ENSG00000137941	0.58	-0.39	0.69
TYSND1 ENSG00000156521	-0.46	-0.69	-1.19
UNC13D ENSG00000092929	2.59	3.80	3.30
UNC5B ENSG00000107731	0.34	-0.32	-0.37
USH1C ENSG00000006611	1.30	1.42	1.39
USP32 ENSG00000170832	0.57	0.62	0.48
VAMP8 ENSG00000118640	-1.50	0.74	-2.93
VAV3 ENSG00000134215	1.40	0.46	1.18
WDR77 ENSG00000116455	0.22	0.35	-0.59
WNT2B ENSG00000134245	0.75	0.95	1.15
WSCD1 ENSG00000179314	-0.81	-1.70	0.56
ZFPM2-AS1 ENSG00000251003	5.06	5.30	5.55
ZIC2 ENSG00000043355	-0.76	0.46	-0.88
ZNF112 ENSG00000062370	-0.56	0.74	0.66
ZNF155 ENSG00000204920	-0.59	0.42	0.76
ZNF217 ENSG00000171940	0.53	0.45	0.92
ZNF219 ENSG00000165804	-0.53	-0.52	0.39
ZNF268 ENSG00000090612	0.33	0.38	0.67
ZNF426 ENSG00000130818	0.58	0.63	0.81
ZNF433-AS1 ENSG00000219665	-1.05	0.75	-1.89
ZNF48 ENSG00000180035	-0.29	-0.26	-0.41
ZNF492 ENSG00000229676	-1.03	1.70	1.14
ZNF503 ENSG00000165655	-0.65	0.65	-0.80
ZNF516 ENSG00000101493	0.56	0.46	0.61
ZNF703 ENSG00000183779	-0.34	0.21	0.26
ZNF772 ENSG00000197128	-0.51	0.52	1.20
ZNF783 ENSG00000204946	-0.31	-0.39	-0.39
ZNF804A ENSG00000170396	-3.00	1.34	1.21
ZNF84 ENSG00000198040	0.41	0.27	1.04
ZNF91 ENSG00000167232	-0.41	0.77	0.52
ZWINT ENSG00000122952	-0.20	-0.25	-0.84

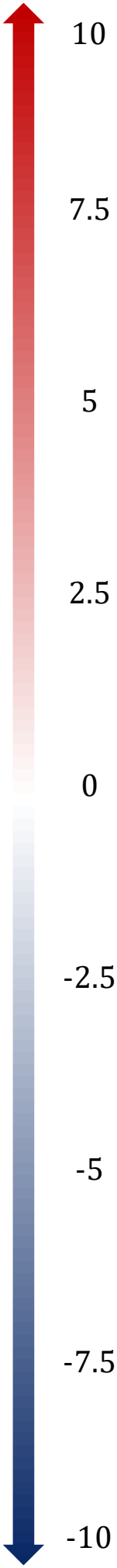
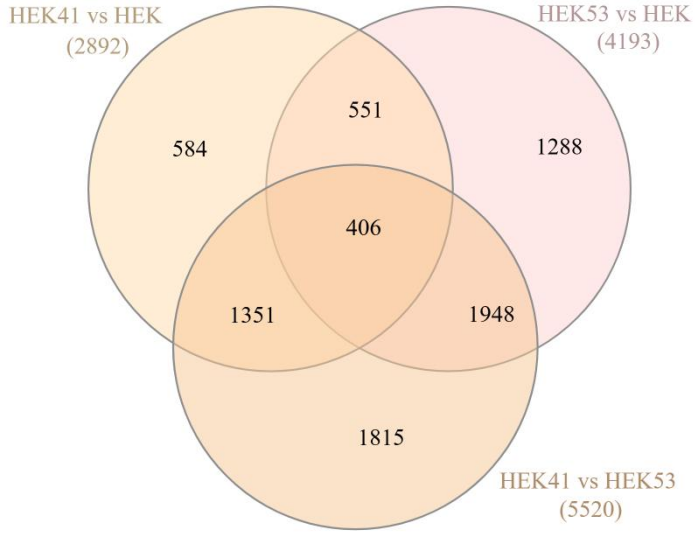
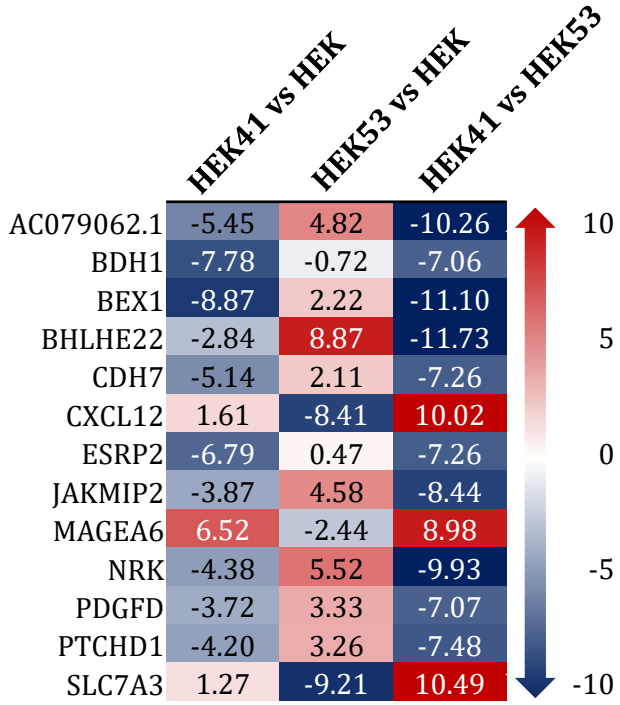
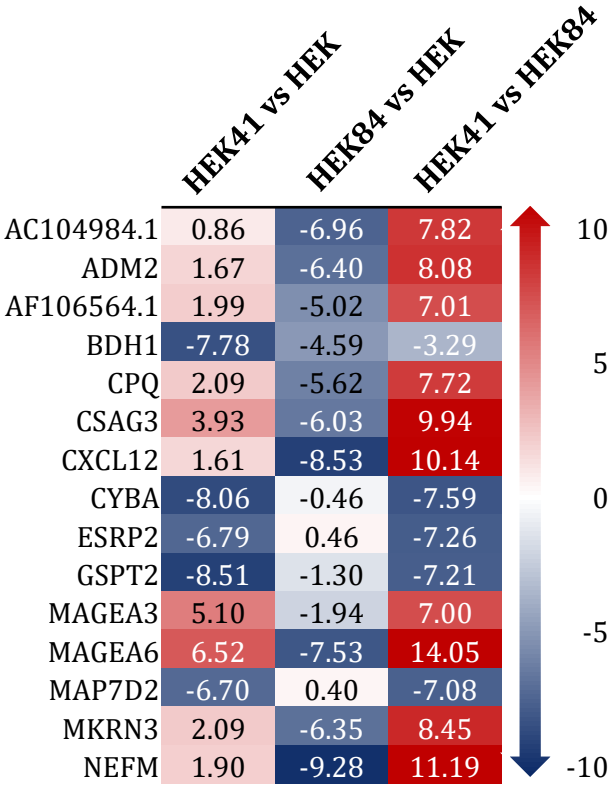
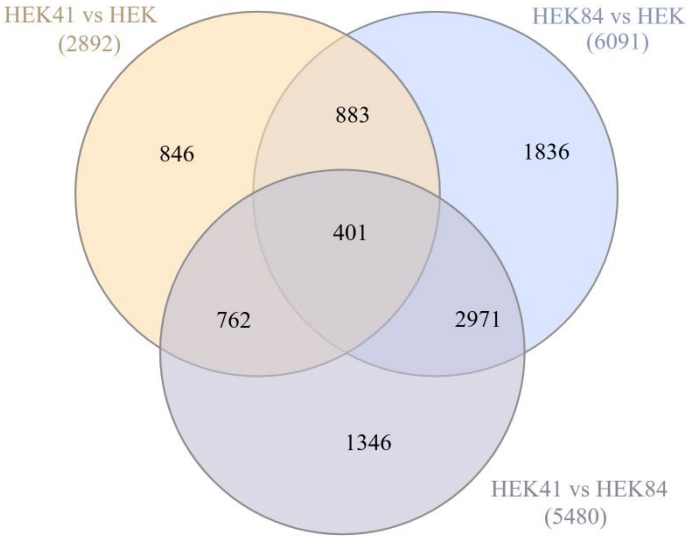


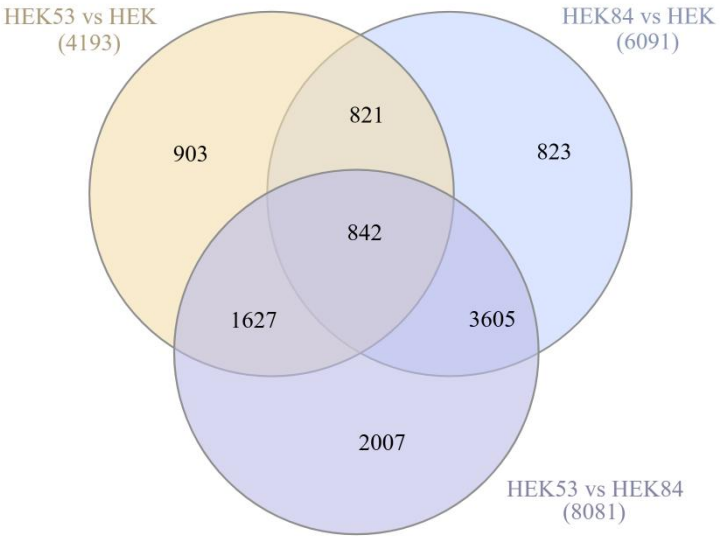
Figure S5


HEK41 vs HEK
(2892)
HEK53 vs HEK
(4193)
HEK41 vs HEK53
(5520)
584
551
1288
406
1351
1948
1815


HEK41 vs HEK
HEK53 vs HEK
HEK41 vs HEK53
AC079062.1 -5.45 4.82 -10.26
BDH1 -7.78 -0.72 -7.06
BEX1 -8.87 2.22 -11.10
BHLHE22 -2.84 8.87 -11.73
CDH7 -5.14 2.11 -7.26
CXCL12 1.61 -8.41 10.02
ESRP2 -6.79 0.47 -7.26
JAKMIP2 -3.87 4.58 -8.44
MAGEA6 6.52 -2.44 8.98
NRK -4.38 5.52 -9.93
PDGFD -3.72 3.33 -7.07
PTCHD1 -4.20 3.26 -7.48
SLC7A3 1.27 -9.21 10.49
10
5
0
-5
-10


HEK41 vs HEK
HEK84 vs HEK
HEK41 vs HEK84
AC104984.1 0.86 -6.96 7.82
ADM2 1.67 -6.40 8.08
AF106564.1 1.99 -5.02 7.01
BDH1 -7.78 -4.59 -3.29
CPQ 2.09 -5.62 7.72
CSAG3 3.93 -6.03 9.94
CXCL12 1.61 -8.53 10.14
CYBA -8.06 -0.46 -7.59
ESRP2 -6.79 0.46 -7.26
GSPT2 -8.51 -1.30 -7.21
MAGEA3 5.10 -1.94 7.00
MAGEA6 6.52 -7.53 14.05
MAP7D2 -6.70 0.40 -7.08
MKRN3 2.09 -6.35 8.45
NEFM 1.90 -9.28 11.19
10
5
0
-5
-10


HEK41 vs HEK
(2892)
HEK84 vs HEK
(6091)
HEK41 vs HEK84
(5480)
846
883
1836
401
762
2971
1346


HEK53 vs HEK
(4193)
HEK84 vs HEK
(6091)
HEK53 vs HEK84
(8081)
903
821
823
842
1627
3605
2007

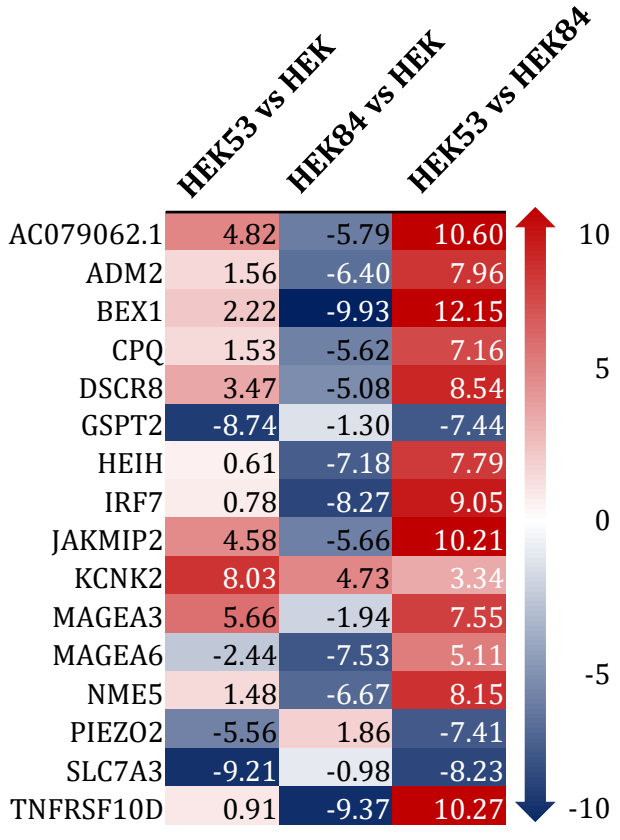

HEK53 vs HEK
HEK84 vs HEK
HEK53 vs HEK84
AC079062.1 4.82 -5.79 10.60
ADM2 1.56 -6.40 7.96
BEX1 2.22 -9.93 12.15
CPQ 1.53 -5.62 7.16
DSCR8 3.47 -5.08 8.54
GSPT2 -8.74 -1.30 -7.44
HEIH 0.61 -7.18 7.79
IRF7 0.78 -8.27 9.05
JAKMIP2 4.58 -5.66 10.21
KCNK2 8.03 4.73 3.34
MAGEA3 5.66 -1.94 7.55
MAGEA6 -2.44 -7.53 5.11
NME5 1.48 -6.67 8.15
PIEZO2 -5.56 1.86 -7.41
SLC7A3 -9.21 -0.98 -8.23
TNFRSF10D 0.91 -9.37 10.27
10
5
0
-5
-10

Figure S6

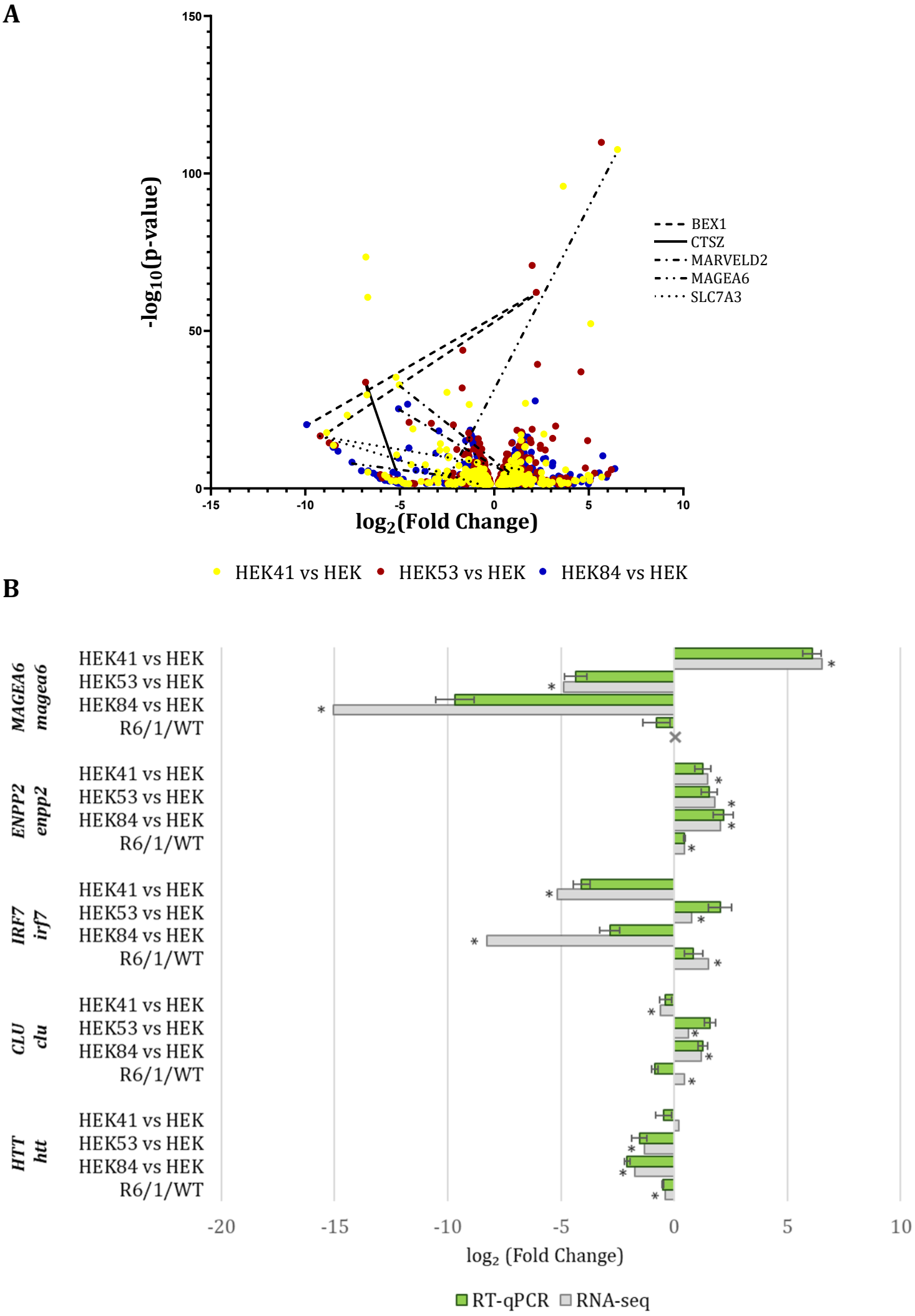
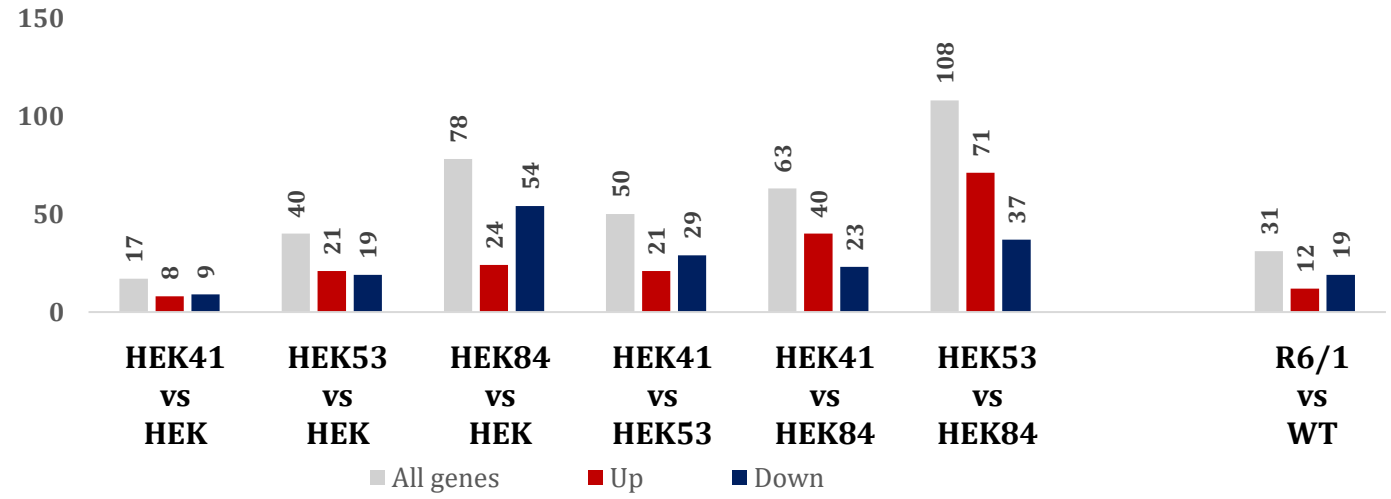

A
150
100
50
0
-log10(p-value)
-15
-10
-5
0
5
10
log2(Fold Change)
BEX1
CTSZ
MARVELD2
MAGEA6
SLC7A3
HEK41 vs HEK
HEK53 vs HEK
HEK84 vs HEK
B
MAGEA6
magea6
ENPP2
enpp2
IRF7
irf7
CLU
clu
HTT
htt
HEK41 vs HEK
HEK53 vs HEK
HEK84 vs HEK
R6/1/WT
-20
-15
-10
-5
0
5
10
log2 (Fold Change)
RT-qPCR
RNA-seq

Figure S7

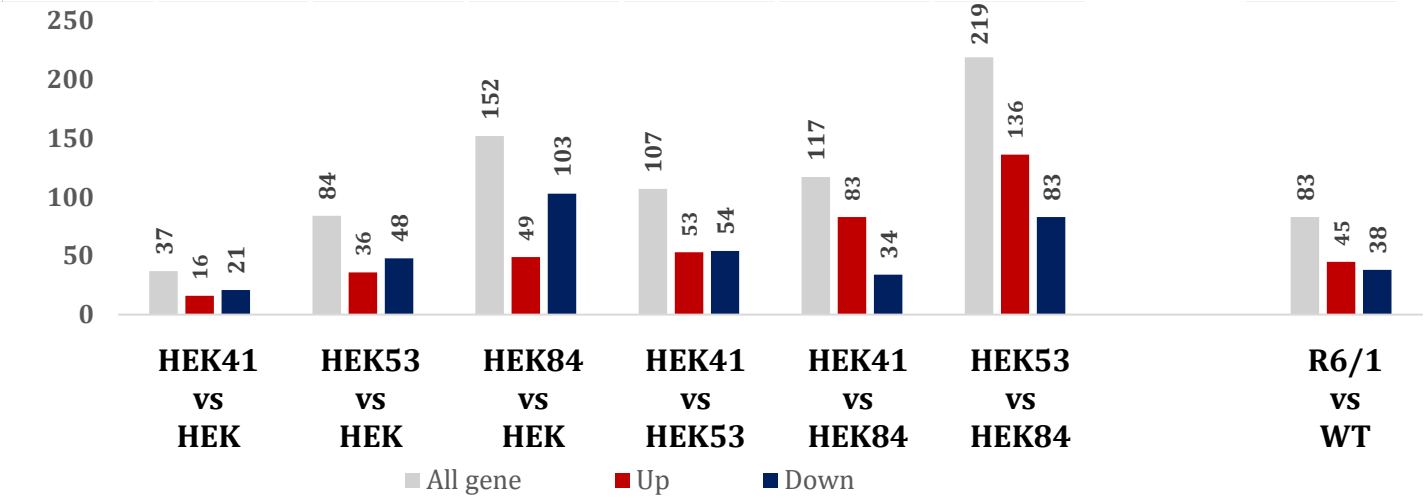
	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	17	40	78	50	63	108	31
Up	8	21	24	21	40	71	12
Down	9	19	54	29	23	37	19



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
1 - 2	1	1	3	1	3	4	0	2
0 - 1	7	20	21	20	37	67	12	1
0 - (-1)	9	18	50	29	22	35	18	-1
-1 - (-2)	0	1	4	0	1	1	1	-2
-2 - (-3)	0	0	0	0	0	1	0	-3

Figure S8

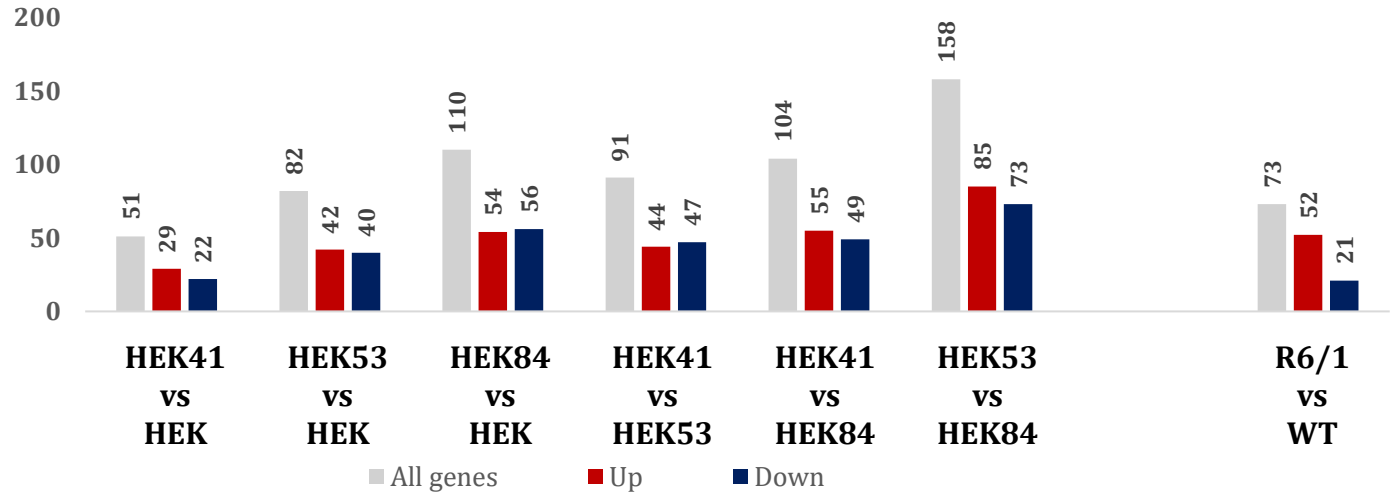
	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All gene	37	84	152	107	117	219	83
Up	16	36	49	53	83	136	45
Down	21	48	103	54	34	83	38



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
4 - 5	0	0	0	0	1	0	0	5
3 - 4	0	0	0	0	0	0	0	4
2 - 3	0	0	0	0	0	0	0	3
1 - 2	0	0	3	5	7	6	3	2
0 - 1	16	36	46	48	75	130	42	1
0 - (-1)	18	45	97	51	30	79	37	-1
-1 - (-2)	3	3	6	1	4	2	0	-2
-2 - (-3)	0	0	0	2	0	2	0	-3
-3 - (-4)	0	0	0	0	0	0	1	-4

Figure S9

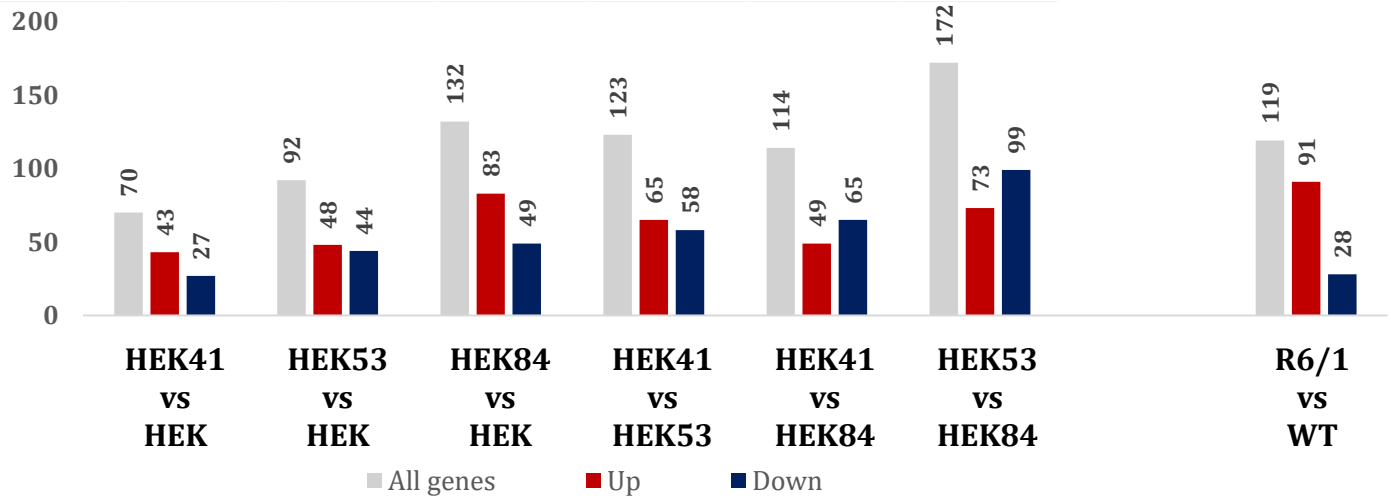
	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	51	82	110	91	104	158	73
Up	29	42	54	44	55	85	52
Down	22	40	56	47	49	73	21



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
6 - 7	0	1	0	0	0	0	0	7
5 - 6	1	0	1	0	0	0	0	6
4 - 5	0	1	1	0	0	0	0	5
3 - 4	1	0	1	2	0	1	2	4
2 - 3	0	0	1	0	0	0	0	3
1 - 2	0	2	8	4	4	7	1	2
0 - 1	27	38	42	38	51	77	49	1
0 - (-1)	20	38	50	42	40	64	21	-1
-1 - (-2)	1	1	5	3	7	6	0	-2
-2 - (-3)	1	0	1	1	2	0	0	-3
-3 - (-4)	0	1	0	1	0	2	0	-4
-4 - (-5)	0	0	0	0	0	0	0	-5
-5 - (-6)	0	0	0	0	0	1	0	-6

Figure S10

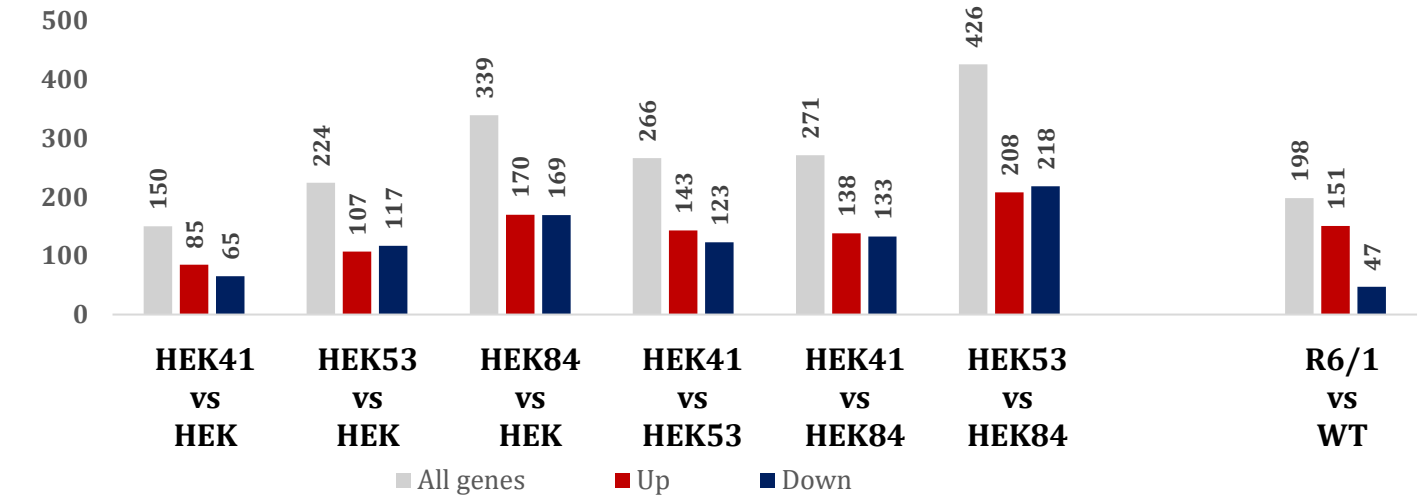
	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	70	92	132	123	114	172	119
Up	43	48	83	65	49	73	91
Down	27	44	49	58	65	99	28



Level log_2(FC)	Cellular model: HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	Animal model: R6/1 vs WT	log_2(FC)
6 - 7	0	1	0	0	0	1	0	7
5 - 6	0	2	0	0	1	2	0	6
4 - 5	1	3	3	1	2	3	2	5
3 - 4	1	2	3	0	3	2	6	4
2 - 3	2	2	6	3	0	4	5	3
1 - 2	7	5	24	7	5	7	17	2
0 - 1	32	33	47	54	38	54	61	1
0 - (-1)	15	39	36	32	33	63	26	-1
-1 - (-2)	2	2	6	13	21	20	2	-2
-2 - (-3)	5	0	1	3	4	10	0	-3
-3 - (-4)	3	1	3	4	3	3	0	-4
-4 - (-5)	1	1	1	4	1	2	0	-5
-5 - (-6)	0	1	2	1	1	0	0	-6
-6 - (-7)	0	0	0	0	1	1	0	-7
-7 - (-8)	0	0	0	0	1	0	0	-8
-8 - (-9)	1	0	0	1	0	0	0	-9

Figure S11

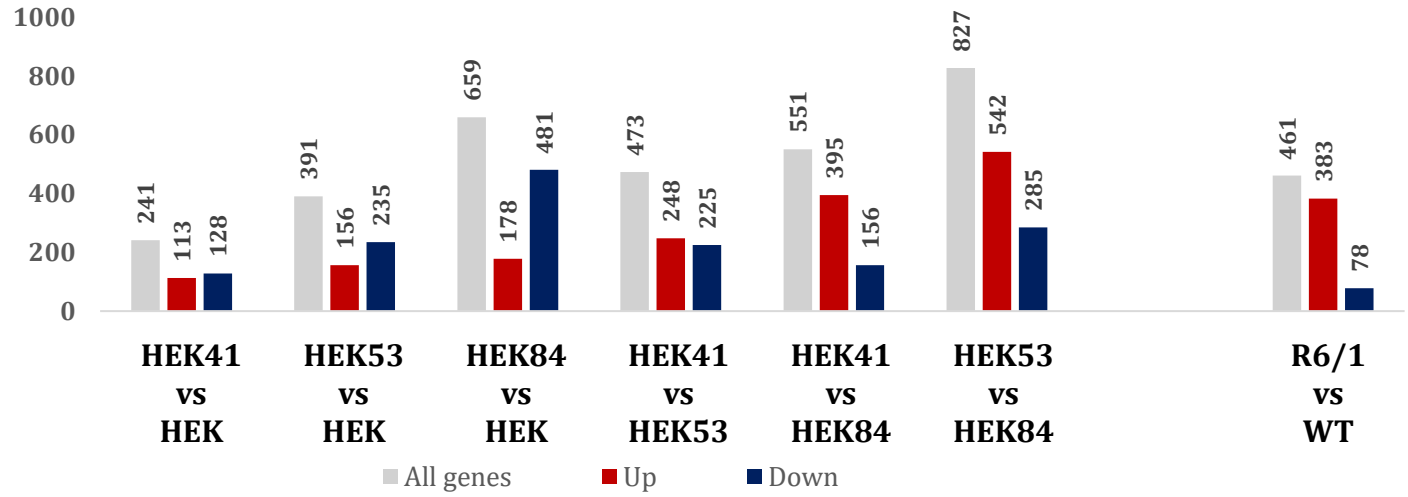
	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	150	224	339	266	271	426	198
Up	85	107	170	143	138	208	151
Down	65	117	169	123	133	218	47



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
> 10	0	0	0	0	0	1	0	> 10
9 -10	0	0	0	0	1	0	0	10
8 - 9	0	0	0	0	0	0	0	9
7 - 8	0	0	0	0	0	0	0	8
6 - 7	0	2	0	0	0	2	0	7
5 - 6	1	2	3	0	0	0	0	6
4 - 5	0	3	4	1	1	4	0	5
3 - 4	0	3	4	1	2	1	3	4
2 - 3	3	2	4	5	2	4	2	3
1 - 2	12	15	38	18	15	15	16	2
0 - 1	69	80	117	118	117	181	130	1
0 - (-1)	51	103	142	91	92	153	47	-1
-1 - (-2)	7	9	18	14	28	52	0	-2
-2 - (-3)	3	2	5	5	5	8	0	-3
-3 - (-4)	1	1	2	6	4	2	0	-4
-4 - (-5)	3	2	1	1	2	3	0	-5
-5 - (-6)	0	0	0	2	2	0	0	-6
-6 - (-7)	0	0	0	1	0	0	0	-7
-7 - (-8)	0	0	0	0	0	0	0	-8
-8 - (-9)	0	0	0	0	0	0	0	-9
-9 - (-10)	0	0	1	0	0	0	0	-10

Figure S12

	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	241	391	659	473	551	827	461
Up	113	156	178	248	395	542	383
Down	128	235	481	225	156	285	78



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
6 - 7	0	0	0	0	1	0	0	7
5 - 6	0	1	0	0	1	1	0	6
4 - 5	1	1	2	1	3	4	0	5
3 - 4	1	0	4	0	2	3	1	4
2 - 3	2	2	4	2	1	4	2	3
1 - 2	9	10	20	30	40	38	17	2
0 - 1	100	142	148	215	347	492	363	1
0 - (-1)	109	218	443	192	123	226	74	-1
-1 - (-2)	14	15	32	20	17	49	3	-2
-2 - (-3)	1	0	2	7	12	6	0	-3
-3 - (-4)	2	2	1	1	2	0	1	-4
-4 - (-5)	1	0	2	3	1	3	0	-5
-5 - (-6)	0	0	1	0	0	0	0	-6
-6 - (-7)	0	0	0	0	1	1	0	-7
-7 - (-8)	1	0	0	1	0	0	0	-8
-8 - (-9)	0	0	0	1	0	0	0	-9

Figure S13

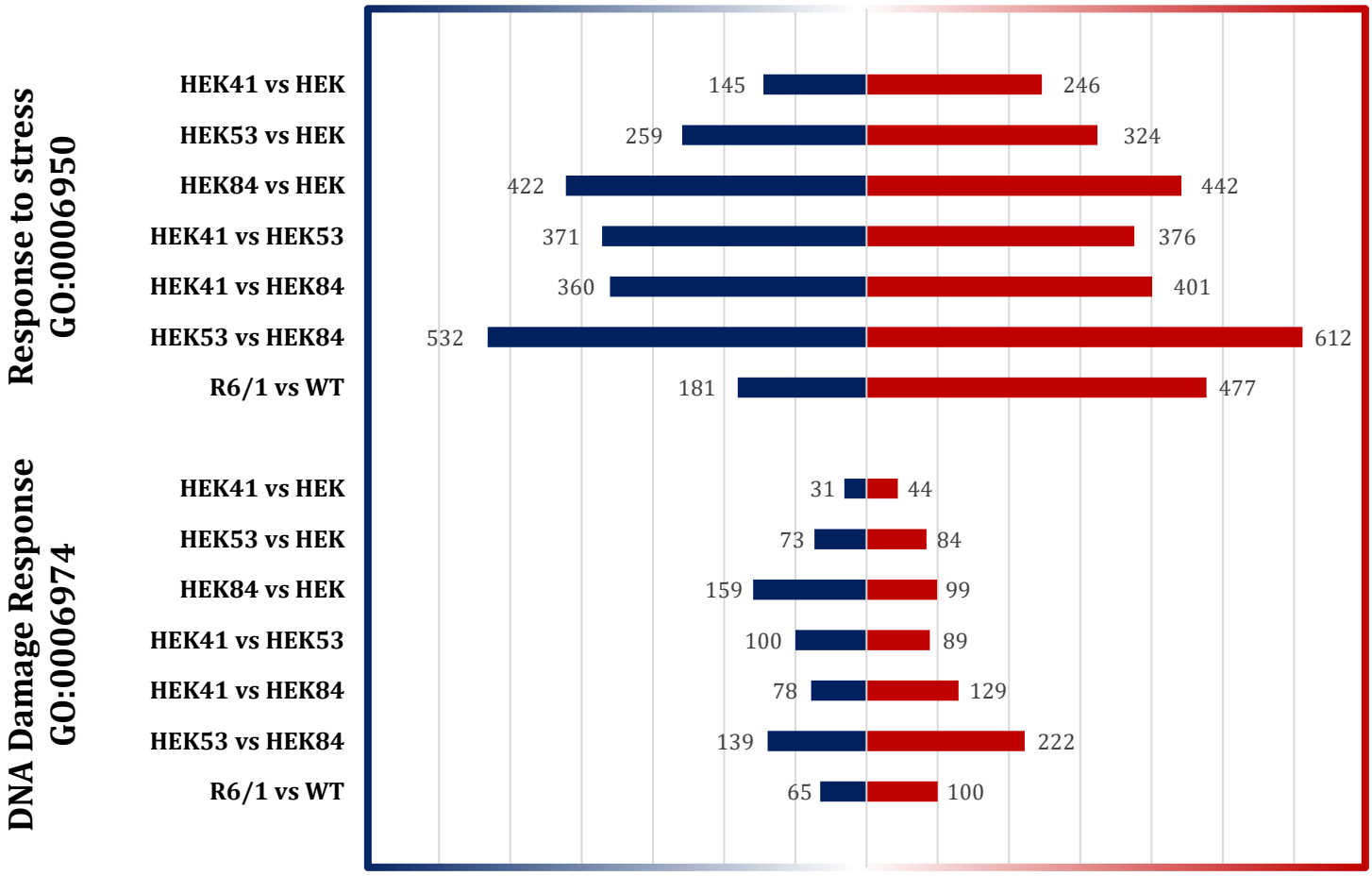
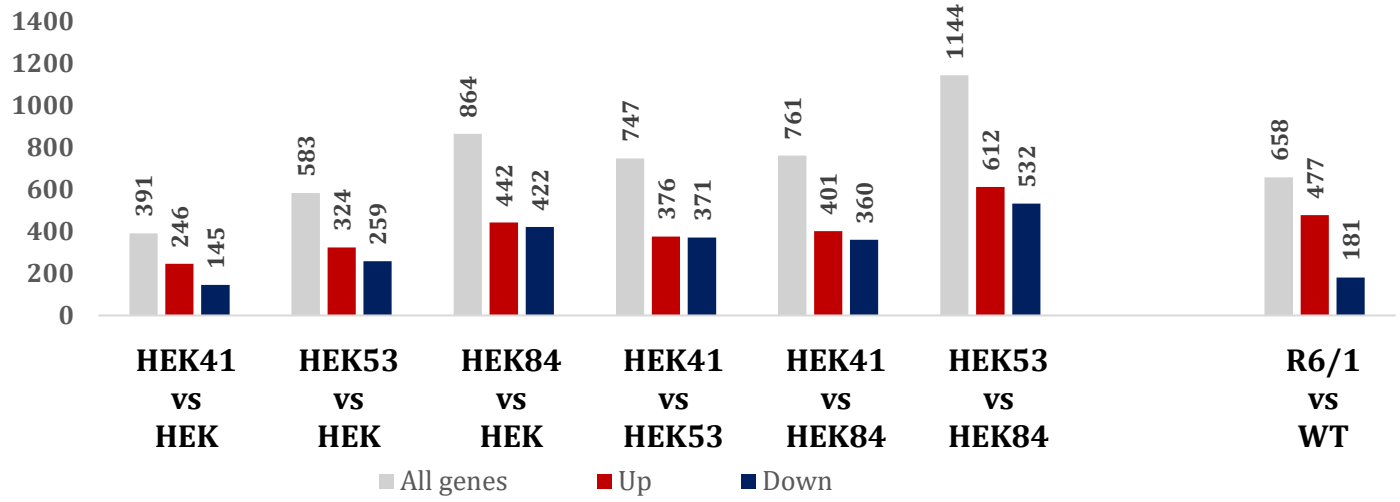

Response to stress GO:0006950
HEK41 vs HEK
HEK53 vs HEK
HEK84 vs HEK
HEK41 vs HEK53
HEK41 vs HEK84
HEK53 vs HEK84
R6/1 vs WT
145
246
259
324
422
442
371
376
360
401
532
612
181
477
DNA Damage Response GO:0006974
HEK41 vs HEK
HEK53 vs HEK
HEK84 vs HEK
HEK41 vs HEK53
HEK41 vs HEK84
HEK53 vs HEK84
R6/1 vs WT
31
44
73
84
159
99
100
89
78
129
139
222
65
100

Figure S14

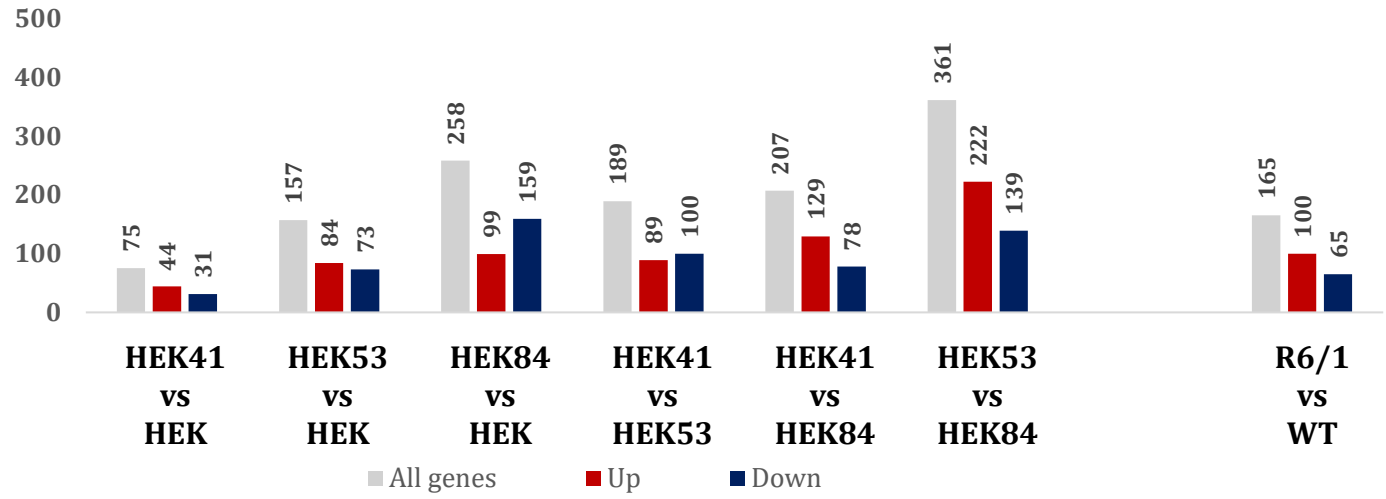
	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	391	583	864	747	761	1144	658
Up	246	324	442	376	401	612	477
Down	145	259	422	371	360	532	181



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
> 10	0	0	0	1	1	0	0	> 10
9 -10	0	0	0	0	0	1	0	10
8 - 9	0	1	0	0	0	0	0	9
7 - 8	0	0	0	0	0	0	0	8
6 - 7	0	3	0	0	0	1	1	7
5 - 6	3	3	2	0	1	5	1	6
4 - 5	3	10	12	2	5	8	2	5
3 - 4	3	7	16	2	4	5	10	4
2 - 3	4	19	18	7	1	17	10	3
1 - 2	30	38	96	53	38	51	51	2
0 - 1	203	243	298	311	351	524	402	1
0 - (-1)	107	231	372	274	244	402	173	-1
-1 - (-2)	17	18	32	45	65	87	7	-2
-2 - (-3)	12	3	7	22	29	28	0	-3
-3 - (-4)	3	2	6	16	9	9	1	-4
-4 - (-5)	4	3	1	6	5	4	0	-5
-5 - (-6)	1	1	2	3	4	1	0	-6
-6 - (-7)	0	0	0	2	3	1	0	-7
-7 - (-8)	0	0	0	2	1	0	0	-8
-8 - (-9)	1	1	2	1	0	0	0	-9

Figure S15

	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	75	157	258	189	207	361	165
Up	44	84	99	89	129	222	100
Down	31	73	159	100	78	139	65



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
9 -10	0	0	0	0	0	1	0	10
8 - 9	0	0	0	0	0	0	0	9
7 - 8	0	0	0	0	0	0	0	8
6 - 7	0	1	0	0	0	0	0	7
5 - 6	0	0	0	0	0	0	0	6
4 - 5	0	0	1	0	1	1	0	5
3 - 4	0	1	1	0	1	0	0	4
2 - 3	0	0	0	0	0	1	0	3
1 - 2	4	4	14	11	9	10	7	2
0 - 1	40	78	83	78	118	209	93	1
0 - (-1)	26	68	147	92	71	119	63	-1
-1 - (-2)	4	4	9	4	7	18	1	-2
-2 - (-3)	0	1	1	2	0	2	0	-3
-3 - (-4)	0	0	1	1	0	0	1	-4
-4 - (-5)	0	0	0	0	0	0	0	-5
-5 - (-6)	1	0	0	1	0	0	0	-6
-6 - (-7)	0	0	0	0	0	0	0	-7
-7 - (-8)	0	0	0	0	0	0	0	-8
-8 - (-9)	0	0	1	0	0	0	0	-9

Figure S16

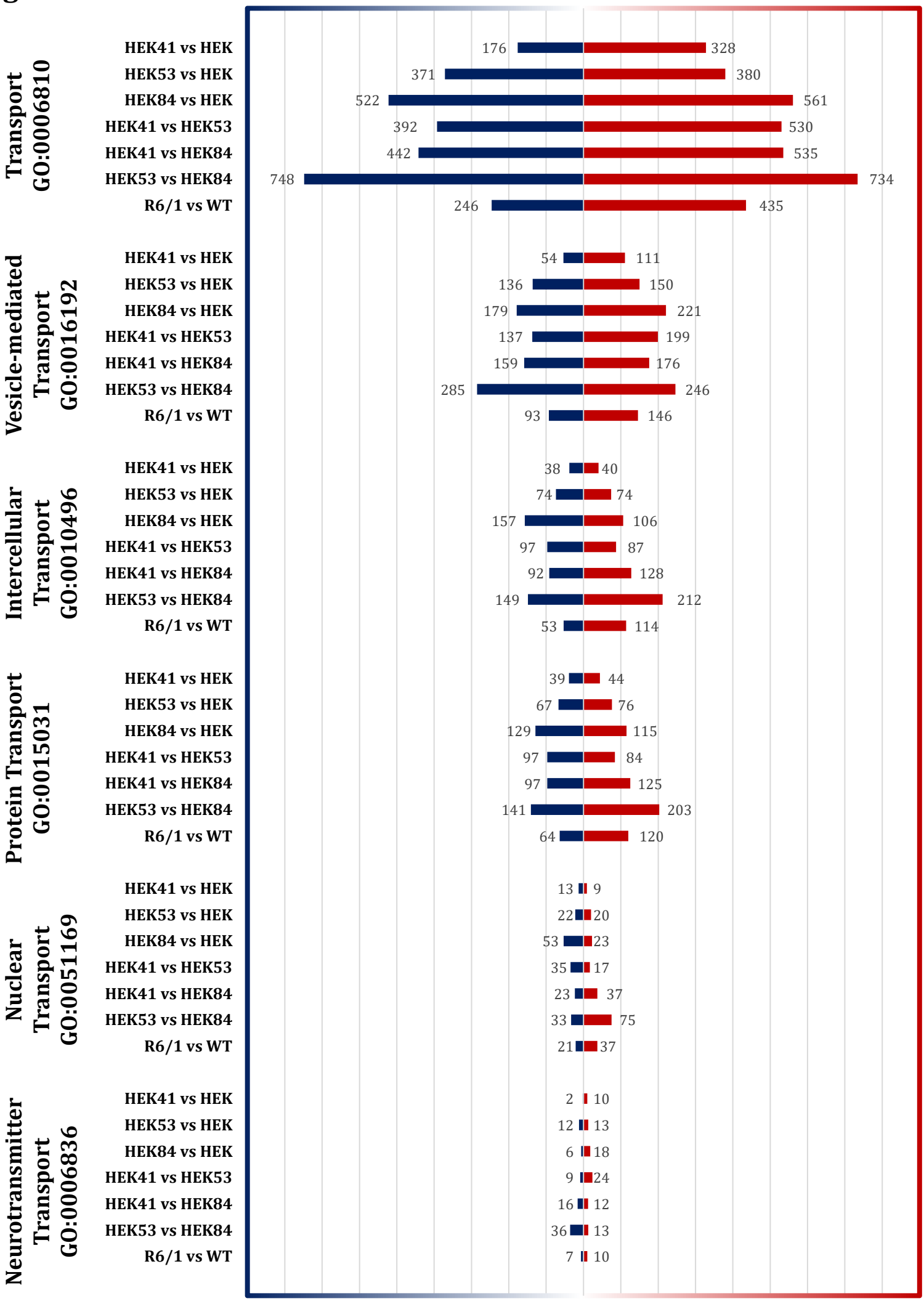
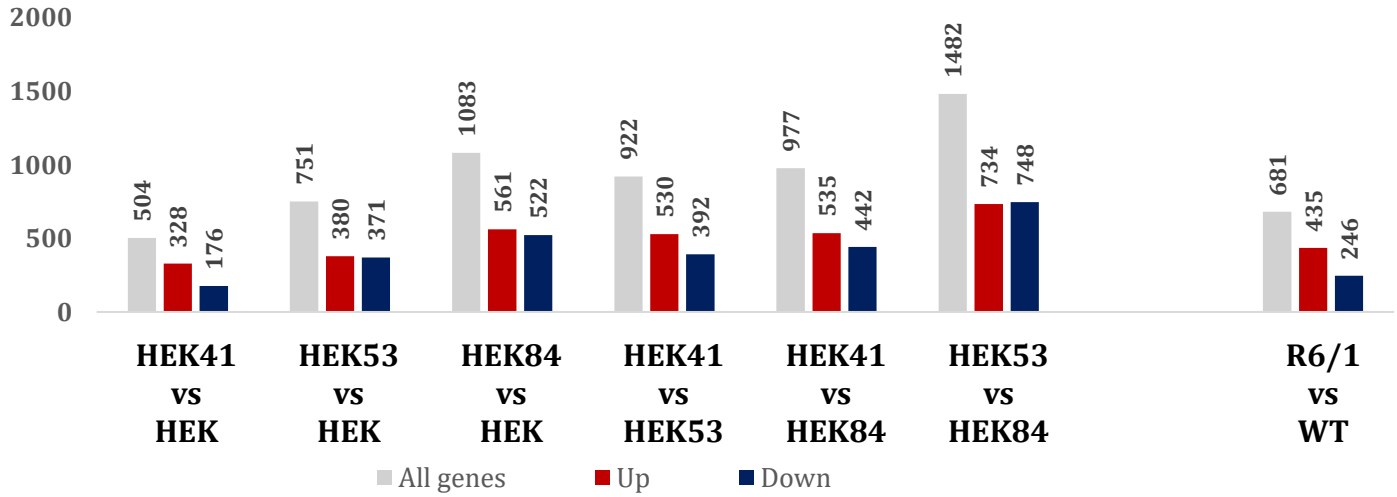

Transport GO:0006810
HEK41 vs HEK 176 328
HEK53 vs HEK 371 380
HEK84 vs HEK 522 561
HEK41 vs HEK53 392 530
HEK41 vs HEK84 442 535
HEK53 vs HEK84 748 734
R6/1 vs WT 246 435
Vesicle-mediated Transport GO:0016192
HEK41 vs HEK 54 111
HEK53 vs HEK 136 150
HEK84 vs HEK 179 221
HEK41 vs HEK53 137 199
HEK41 vs HEK84 159 176
HEK53 vs HEK84 285 246
R6/1 vs WT 93 146
Intercellular Transport GO:0010496
HEK41 vs HEK 38 40
HEK53 vs HEK 74 74
HEK84 vs HEK 157 106
HEK41 vs HEK53 97 87
HEK41 vs HEK84 92 128
HEK53 vs HEK84 149 212
R6/1 vs WT 53 114
Protein Transport GO:0015031
HEK41 vs HEK 39 44
HEK53 vs HEK 67 76
HEK84 vs HEK 129 115
HEK41 vs HEK53 97 84
HEK41 vs HEK84 97 125
HEK53 vs HEK84 141 203
R6/1 vs WT 64 120
Nuclear Transport GO:0051169
HEK41 vs HEK 13 9
HEK53 vs HEK 22 20
HEK84 vs HEK 53 23
HEK41 vs HEK53 35 17
HEK41 vs HEK84 23 37
HEK53 vs HEK84 33 75
R6/1 vs WT 21 37
Neurotransmitter Transport GO:0006836
HEK41 vs HEK 2 10
HEK53 vs HEK 12 13
HEK84 vs HEK 6 18
HEK41 vs HEK53 9 24
HEK41 vs HEK84 16 12
HEK53 vs HEK84 36 13
R6/1 vs WT 7 10

Figure S17

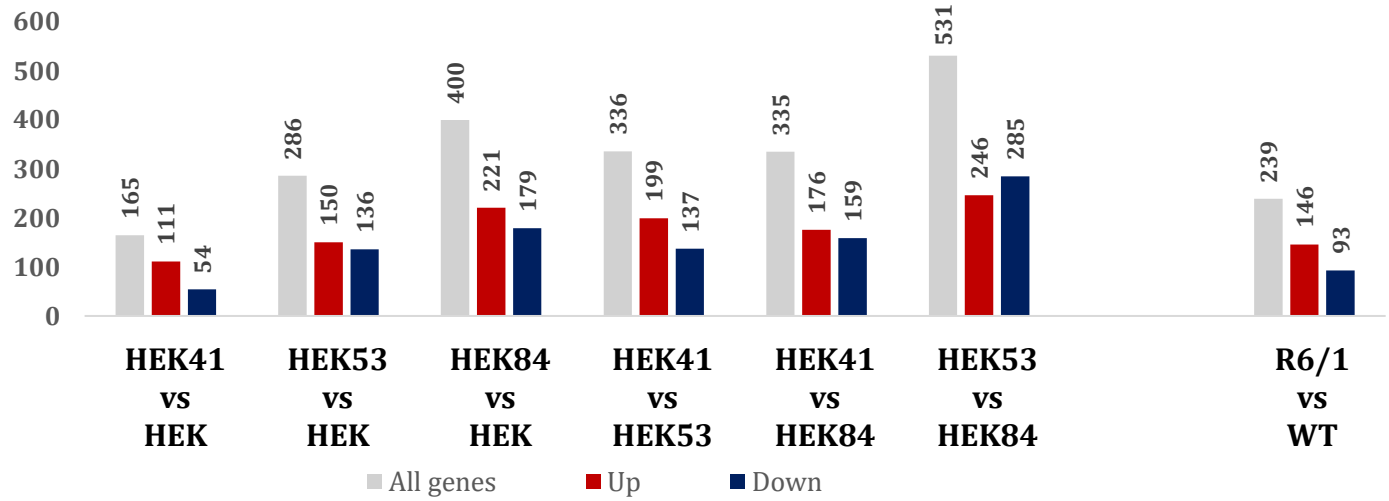
	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	504	751	1083	922	977	1482	681
Up	328	380	561	530	535	734	435
Down	176	371	522	392	442	748	246



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
> 10	0	0	0	1	1	0	0	> 10
9 -10	0	0	0	0	0	0	0	10
8 - 9	0	1	0	0	0	1	0	9
7 - 8	0	1	0	1	0	1	0	8
6 - 7	1	2	0	1	2	3	1	7
5 - 6	3	5	6	1	3	6	0	6
4 - 5	6	10	16	4	5	16	2	5
3 - 4	6	11	12	3	5	7	5	4
2 - 3	8	18	19	8	9	18	5	3
1 - 2	47	39	121	85	57	53	28	2
0 - 1	257	293	387	426	453	629	394	1
0 - (-1)	129	319	461	278	291	541	237	-1
-1 - (-2)	21	41	43	45	98	155	8	-2
-2 - (-3)	13	6	2	19	26	32	0	-3
-3 - (-4)	5	0	6	22	10	11	1	-4
-4 - (-5)	4	3	6	16	7	4	0	-5
-5 - (-6)	3	1	3	4	4	2	0	-6
-6 - (-7)	1	0	1	6	6	1	0	-7
-7 - (-8)	0	0	0	2	0	1	0	-8
-8 - (-9)	0	0	0	0	0	1	0	-9
-9 - (-10)	0	1	0	0	0	0	0	-10

Figure S18

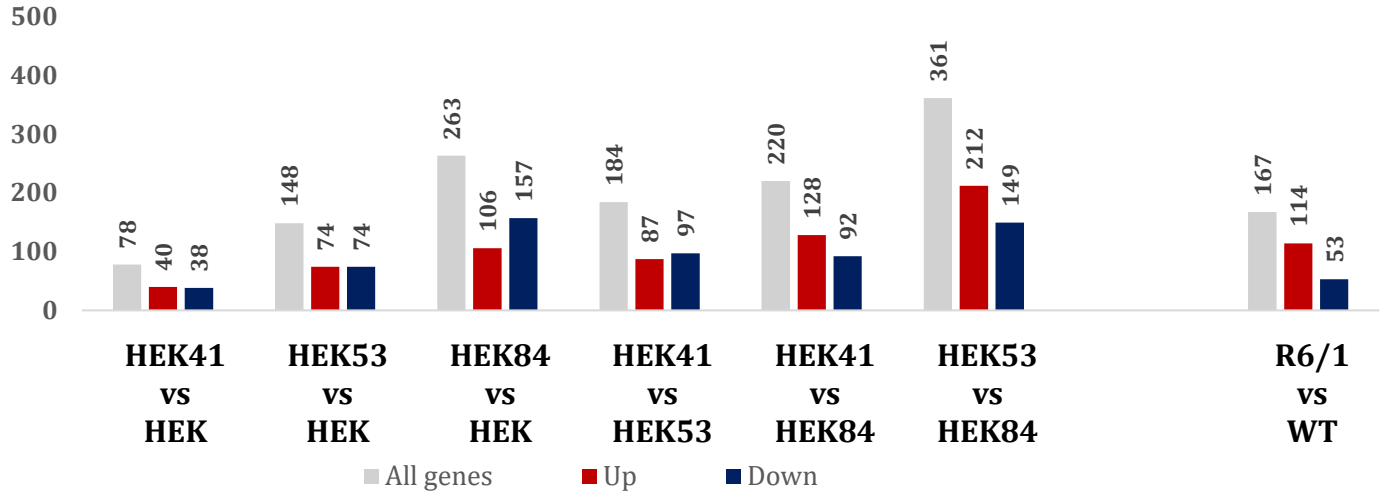
	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	165	286	400	336	335	531	239
Up	111	150	221	199	176	246	146
Down	54	136	179	137	159	285	93



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
6 - 7	0	0	0	0	0	0	1	7
5 - 6	0	1	0	1	0	1	0	6
4 - 5	0	1	3	1	1	4	0	5
3 - 4	3	3	1	0	1	2	1	4
2 - 3	2	5	7	4	1	5	4	3
1 - 2	18	9	37	30	19	11	11	2
0 - 1	88	131	173	163	154	223	129	1
0 - (-1)	40	121	158	109	117	229	89	-1
-1 - (-2)	9	13	16	11	32	47	4	-2
-2 - (-3)	3	0	1	9	6	4	0	-3
-3 - (-4)	1	0	3	5	2	3	0	-4
-4 - (-5)	1	2	1	2	1	1	0	-5
-5 - (-6)	0	0	0	1	1	1	0	-6

Figure S19

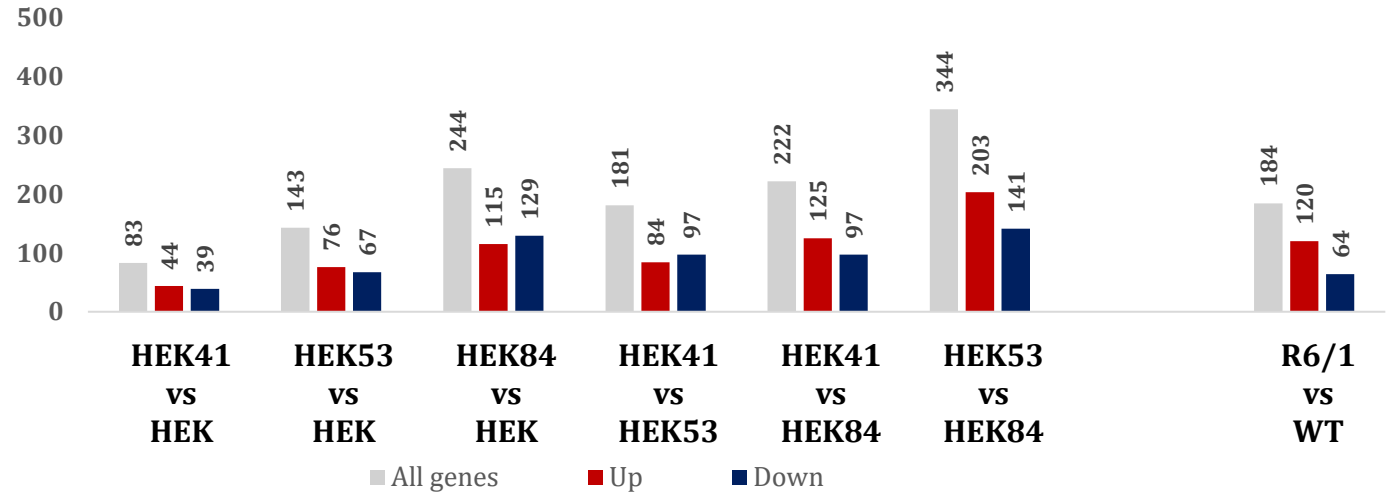
	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	78	148	263	184	220	361	167
Up	40	74	106	87	128	212	114
Down	38	74	157	97	92	149	53



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
7 - 8	0	0	0	1	0	0	0	8
6 - 7	0	0	0	0	0	0	0	7
5 - 6	1	0	1	0	2	1	0	6
4 - 5	1	2	1	0	0	1	0	5
3 - 4	0	2	2	0	1	0	0	4
2 - 3	0	0	1	0	1	1	1	3
1 - 2	2	2	10	8	5	6	4	2
0 - 1	36	68	91	78	119	203	109	1
0 - (-1)	31	68	150	85	71	124	52	-1
-1 - (-2)	4	6	5	6	15	20	1	-2
-2 - (-3)	2	0	0	3	2	2	0	-3
-3 - (-4)	1	0	1	2	3	2	0	-4
-4 - (-5)	0	0	1	1	0	0	0	-5
-5 - (-6)	0	0	0	0	0	1	0	-6
-6 - (-7)	0	0	0	0	1	0	0	-7

Figure S20

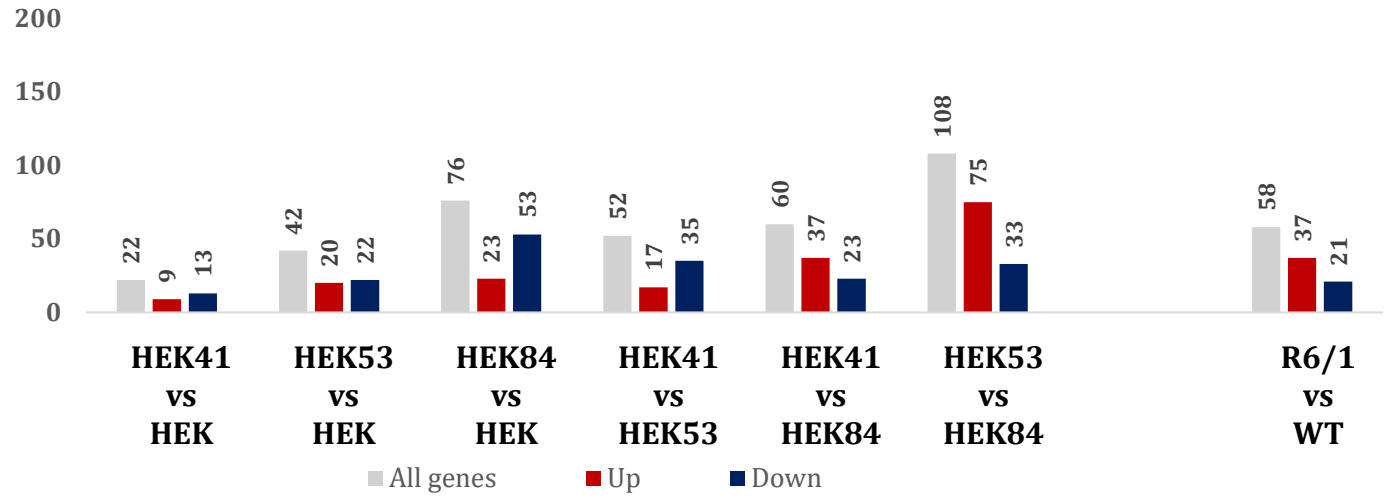
	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	83	143	244	181	222	344	184
Up	44	76	115	84	125	203	120
Down	39	67	129	97	97	141	64



Level log_2(FC)	Cellular model: HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	Animal model: R6/1 vs WT	log_2(FC)
5 - 6	0	1	1	1	0	1	0	6
4 - 5	0	1	3	0	0	2	1	5
3 - 4	1	2	4	0	0	1	0	4
2 - 3	0	1	4	0	0	1	1	3
1 - 2	7	4	17	12	9	8	6	2
0 - 1	36	67	86	71	116	190	112	1
0 - (-1)	33	59	122	83	72	111	63	-1
-1 - (-2)	4	8	7	7	15	23	1	-2
-2 - (-3)	1	0	0	1	6	5	0	-3
-3 - (-4)	1	0	0	5	2	1	0	-4
-4 - (-5)	0	0	0	1	1	1	0	-5
-5 - (-6)	0	0	0	0	0	0	0	-6
-6 - (-7)	0	0	0	0	1	0	0	-7

Figure S21

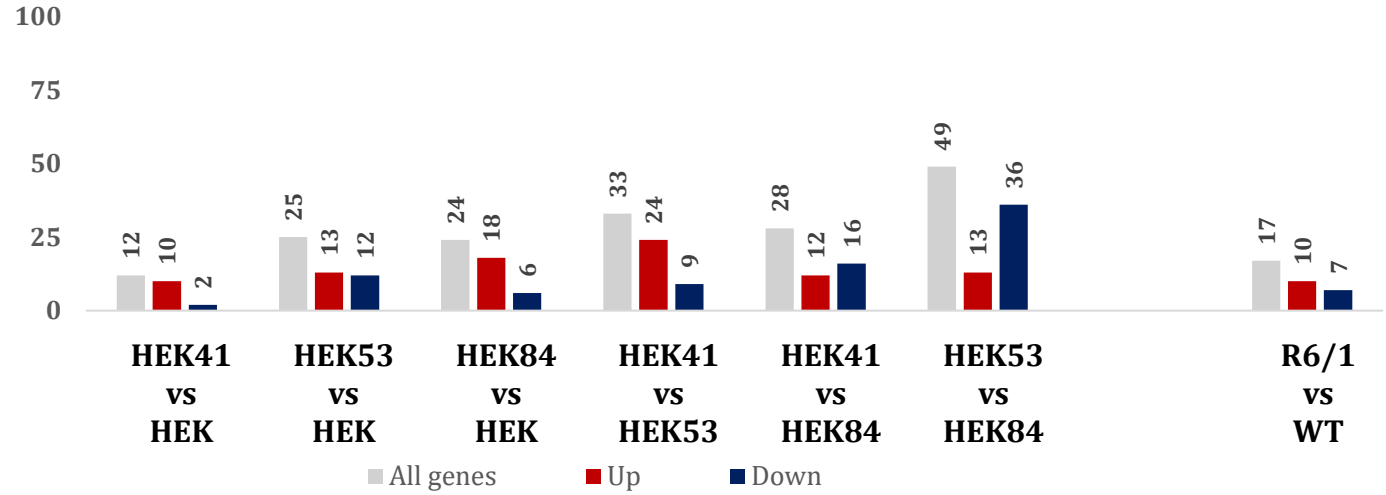
	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	22	42	76	52	60	108	58
Up	9	20	23	17	37	75	37
Down	13	22	53	35	23	33	21



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
5 - 6	0	0	1	0	0	0	0	6
4 - 5	0	0	0	0	0	0	0	5
3 - 4	0	1	1	0	0	0	0	4
2 - 3	0	0	0	0	0	0	1	3
1 - 2	1	0	2	1	1	1	2	2
0 - 1	8	19	19	16	36	74	34	1
0 - (-1)	12	21	53	33	19	27	21	-1
-1 - (-2)	1	1	0	2	3	3	0	-2
-2 - (-3)	0	0	0	0	0	1	0	-3
-3 - (-4)	0	0	0	0	0	1	0	-4
-4 - (-5)	0	0	0	0	0	0	0	-5
-5 - (-6)	0	0	0	0	0	1	0	-6
-6 - (-7)	0	0	0	0	1	0	0	-7

Figure S22

	Cellular model						Animal model
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT
All genes	12	25	24	33	28	49	17
Up	10	13	18	24	12	13	10
Down	2	12	6	9	16	36	7



Level log_2(FC)	Cellular model						Animal model	log_2(FC)
	HEK41 vs HEK	HEK53 vs HEK	HEK84 vs HEK	HEK41 vs HEK53	HEK41 vs HEK84	HEK53 vs HEK84	R6/1 vs WT	
6 - 7	0	0	0	0	0	1	0	7
5 - 6	0	0	0	0	1	0	0	6
4 - 5	0	0	0	0	0	0	0	5
3 - 4	0	2	0	0	0	0	0	4
2 - 3	0	0	1	1	0	1	0	3
1 - 2	2	1	7	3	2	1	1	2
0 - 1	8	10	10	20	9	10	9	1
0 - (-1)	1	10	5	5	9	26	7	-1
-1 - (-2)	0	2	0	3	4	8	0	-2
-2 - (-3)	1	0	0	1	2	1	0	-3
-3 - (-4)	0	0	0	0	1	1	0	-4
-4 - (-5)	0	0	0	0	0	0	0	-5
-5 - (-6)	0	0	1	0	0	0	0	-6

Oświadczenia współautorów o wkładzie w publikację

Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the HTT gene

Biochemical and Biophysical Research Communications vol. 816 (2026): 153711. doi:10.1016/j.bbrc.2026.153711

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Oświadczenie o wkładzie w publikację

Oświadczam, że mój wkład w publikację:

Szulc, A., Walter, B., Gaffke, L., Wiśniewska, K., Żabińska, M., Rintz, E., Cyske, Z., Grabski, M., Pankiv, O., Podlacha, M., Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the *HTT* gene. *Biochemical and Biophysical Research Communications, 816,* 153711. https://doi.org/10.1016/j.bbrc.2026.153711

polegał na:

- przygotowaniu i przeprowadzeniu doświadczeń w szczególności PCR, RT-qPCR, Western blot oraz pomiaru ROS
- opracowaniu i analizie całości wyników w tym analiz transkryptomicznych
- przygotowaniu figur i tabeli z wyłączeniem fig.1A, fig.1B, fig.1C oraz fig.S2A
- pozyskaniu finansowania z Programu małych grantów UG

oraz udziale w:

- sformułowaniu tematyki pracy
- przygotowaniu pierwotnej wersji manuskryptu
- redakcji i edycji pierwotnej wersji manuskryptu

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Oświadczenie o wkładzie w publikację

Oświadczam, że mój wkład w publikację:

Szulc, A., **Walter, B.**, Gaffke, L., Wiśniewska, K., Żabińska, M., Rintz, E., Cyske, Z., Grabski, M., Pankiv, O., Podlacha, M., Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the *HTT* gene. *Biochemical and Biophysical Research Communications, 816,* 153711. https://doi.org/10.1016/j.bbrc.2026.153711

polegał na:

- przeprowadzeniu modelowania strukturalnego białek
- opracowaniu i analizie wyników modelowania
- przygotowaniu figur dotyczących modelowania fig.1A, fig.1B, fig.1C oraz fig.S2A

oraz udziale w:

- przygotowaniu pierwotnej wersji manuskryptu
- redakcji i edycji pierwotnej wersji manuskryptu

Beata M. Walter



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Gdańsk 27.09.2026 r.

Oświadczenie o wkładzie w publikację

Oświadczam, że mój wkład w publikację:

Szulc, A., Walter, B., **Gaffke, L.**, Wiśniewska, K., Żabińska, M., Rintz, E., Cyske, Z., Grabski, M., Pankiv, O., Podlacha, M., Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the *HTT* gene. *Biochemical and Biophysical Research Communications, 816,* 153711. https://doi.org/10.1016/j.bbrc.2026.153711

polegał na udziale w:

- sformułowaniu tematyki pracy
- przygotowaniu i przeprowadzeniu doświadczeń w szczególności izolacji RNA
- przygotowaniu pierwotnej wersji manuskryptu
- redakcji i edycji pierwotnej wersji manuskryptu

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Gdańsk 27.09.2026 r.

Oświadczenie o wkładzie w publikację

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polegał na pomocy w:

- przygotowaniu i przeprowadzeniu doświadczeń
- redakcji i edycji pierwotnej wersji manuskryptu

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Gdańsk 27.09.2026 r.

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polegał na pomocy w:

- przygotowaniu i przeprowadzeniu doświadczeń
- redakcji i edycji pierwotnej wersji manuskryptu

Żabińska
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Gdańsk 27.09.2026 r.

Oświadczenie o wkładzie w publikację

Oświadczam, że mój wkład w publikację:

Szulc, A., Walter, B., Gaffke, L., Wiśniewska, K., Żabińska, M., **Rintz, E.**, Cyske, Z., Grabski, M., Pankiv, O., Podlacha, M., Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the *HTT* gene. *Biochemical and Biophysical Research Communications, 816,* 153711. https://doi.org/10.1016/j.bbrc.2026.153711

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- redakcji i edycji pierwotnej wersji manuskryptu

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Oświadczenie o wkładzie w publikację

Oświadczam, że mój wkład w publikację:

Szulc, A., Walter, B., Gaffke, L., Wiśniewska, K., Żabińska, M., Rintz, E., **Cyske, Z.**, Grabski, M., Pankiv, O., Podlacha, M., Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the *HTT* gene. *Biochemical and Biophysical Research Communications, 816,* 153711. https://doi.org/10.1016/j.bbrc.2026.153711

polegał na pomocy w:

- przygotowaniu i przeprowadzeniu doświadczeń
- redakcji i edycji pierwotnej wersji manuskryptu

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Zuzanna Cyske

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Gdańsk 27.09.2026 r.

Oświadczenie o wkładzie w publikację

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Szulc, A., Walter, B., Gaffke, L., Wiśniewska, K., Żabińska, M., Rintz, E., Cyske, Z., **Grabski, M.**, Pankiv, O., Podlacha, M., Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the *HTT* gene. *Biochemical and Biophysical Research Communications, 816,* 153711. https://doi.org/10.1016/j.bbrc.2026.153711

polegał na udziale w:

- przygotowaniu i zdeponowaniu wyników sekwencjonowania
- przygotowaniu pierwotnej wersji manuskryptu

Michał Grabski

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mgr inż. Oleksandr Pankiv
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Politechnika Łódzka

Łódź, 27.09.2026 r.

Oświadczenie o wkładzie w publikację

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polegał na pomocy w:

- opracowaniu i analizie wyników transkryptomicznych
- przygotowaniu pierwotnej wersji manuskryptu

Oleksandr Pankiv

Oleksandr Pankiv

mgr Aneta Szulc
Katedra Biologii Molekularnej
Wydział Biologii
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Oświadczenie o wkładzie w publikację

Oświadczam, że wkład dr hab. Magdaleny Podlachy prof. UG w publikację:

Szulc, A., Walter, B., Gaffke, L., Wiśniewska, K., Żabińska, M., Rintz, E., Cyske, Z., Grabski, M., Pankiv, O., **Podlacha, M.**, Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the *HTT* gene. *Biochemical and Biophysical Research Communications, 816,* 153711. https://doi.org/10.1016/j.bbrc.2026.153711

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Autophagy disorders and their improvement by 4′,5,7-trihydroxyisoflavone in cellular models of Huntington's disease

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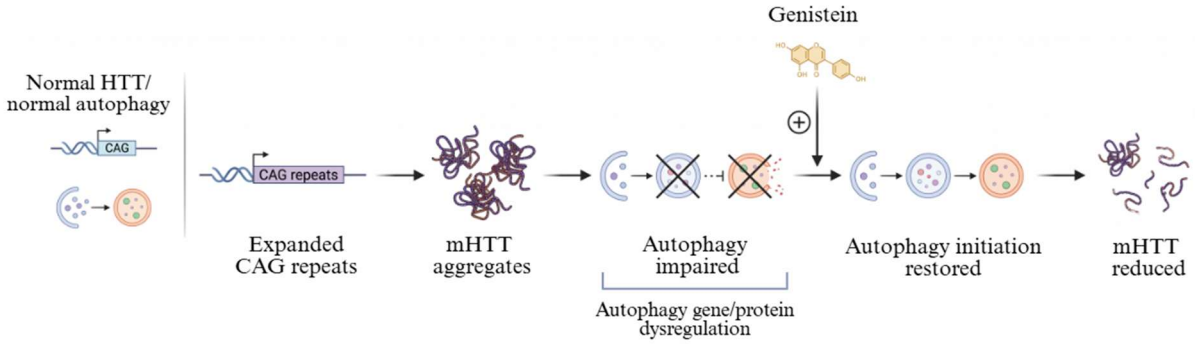
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Autophagy disorders and their improvement by 4′,5,7-trihydroxyisoflavone in cellular models of Huntington's disease



Autophagy disorders and their improvement by 4′,5,7-trihydroxyisoflavone in cellular models of Huntington's disease

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Abstract

The primary cause of Huntington's disease (HD) is the presence of a pathogenic variant of the *HTT* gene (encoding huntingtin; HTT), characterized by the extension of CAG repeats. Expression of the mutant *HTT* allele results in appearance of improper mRNA structures and production of misfolded huntingtin (mHTT) which form intracellular aggregates. However, the secondary molecular pathogenic processes, leading to cellular dysfunctions and eventually to appearance of disease symptoms in patients, are largely unknown. One of cellular processes affected in HD is autophagy, but molecular mechanisms of this disorder are poorly understood. Here, using cellular models of HD consisting of HEK293-derived lines with normal 16/17 (control) or extended (41, 53 or 84) numbers of CAG triplets, and employing transcriptomic, microscopic, and biochemical analyses, we have shown dysregulation of expression of many autophagy-related genes. More detailed analyses indicated that impaired phosphorylation of ULK1 at Ser555 and decreased levels of Atg13 might be causes of autophagy deficit in HD. The use of an autophagy stimulator, 4′,5,7-trihydroxyisoflavone (genistein), partially corrected this disorder, mostly at the stage of the initiation of this process, which correlated with normalization of expression of a battery of autophagy-related genes. In HD cells treated with 4′,5,7-trihydroxyisoflavone, levels of HTT were significantly lower or even undistinguishable from those observed in wild-type cells. In conclusion, autophagy is dysregulated in HD cells due to down-regulation of pULK1(Ser555) and Atg13, but it can be corrected by 4′,5,7-trihydroxyisoflavone at the initiation stage, resulting in reduction of mHTT abundance.

Keywords: Huntington's disease; CAG extension; cellular models; autophagy; genistein

1. Introduction

A variant of the *HTT* gene characterized by the presence of more than 36 CAG codon repeats in exon 1 is considered the cause of Huntington's disease (HD) [1]. Huntingtin (the HTT protein) is a product of the *HTT* gene, while the variant appearing as an effect of expression of the pathogenic *HTT* allele is called mHTT (for mutant HTT). Due to faulty folding (resulting from the presence of a long Gln residues' tract) mHTT forms aggregates in the cytoplasm. These aggregates, together with improper secondary structures of mutant *HTT* mRNA, which affect the splicing process due to sequestering the spliceosome components, are recognized as crucial defects triggering the cascade of cellular dysfunctions, leading to pathogenic symptoms in patients [2].

HD is a neurodegenerative disease with severe movement disorders, mental health conditions, problems with thinking and planning and shortened lifespan, despite the first symptoms appear usually in adulthood [3]. This disease is inherited in an autosomal dominant manner, as only one pathogenic variant of the *HTT* gene is sufficient to produce significant amounts of faulty folded mRNA and mHTT molecules. This makes the development of an effective therapy especially difficult, as even potential delivery of wild-type *HTT* allele is unlikely to positively influence cellular functions. Other approaches, employing either antisense RNAs or small molecules to decrease the efficiency of expression of the mutant *HTT* allele, although promising, are still of limited efficacy and/or insufficient safety which led to termination of clinical trials [4, 5]. Therefore, other therapeutic approaches appear to be also important to find effective HD treatment. However, developing any new drugs requires deep understanding of molecular mechanisms of the target disease.

Although somatic expansion of CAG repeats in the *HTT* gene was recently proposed as an important mechanism and the major cause of the late onset of HD symptoms [6, 7],

formation of intracellular aggregates, derived from mHTT, mutant mRNA of *HTT* or both, appears to be the primary pathogenic process which triggers dysregulation of expression of a battery of genes and subsequent cellular dysfunctions [8]. Therefore, elimination of such aggregates might be proposed as a potential therapeutic approach to treat HD. Apart from lowering levels of mHTT [9], stimulation of autophagy, a process of degrading partially damaged or improper molecules and cellular structures, was suggested [10]. In fact, earlier studies indicated that treatment with activators of autophagy had promising effects in cellular and animal models of HD [11]. On the other hand, strong autophagy stimulators, like rapamycin and others, despite being very useful in laboratory studies, cannot be used in clinical practice for patients with diseases requiring life-long treatment, due to adverse effects they cause when administered for a long time [10]. Therefore, a discovery that 4′,5,7-trihydroxyisoflavone (known also as genistein or 5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) can stimulate autophagy to some extent while revealing a favorable safety profile [12] and might be effective in reducing number and size of mHTT aggregate in HD cells was promising [13, 14]. Subsequent experiments indicated that this compound was effective in correcting cognitive, neuromuscular, and behavioural deficits in males [15] and females [16] of the R6/1 mouse model of HD. Furthermore, 4′,5,7-trihydroxyisoflavone was also effective in preventing HD symptoms when administered at the presymptomatic stage of the mouse development [17].

Despite demonstration of promising results of the use of 4′,5,7-trihydroxyisoflavone in animal models, the molecular mechanism of autophagy stimulation by this molecule is incompletely understood. It was reported that the process depends on activation of TFEB and FOXO3 factors [15]; however, details remain unclear. Recent study with HD models consisting of a series of human cell lines (HEK293-derivatives) bearing different numbers (16/17 in the control, and 41, 53 or 84 in HD) of CAG repeats in the exon 1 of the *HTT* gene

[18] demonstrated the dysregulations of expression of hundreds of genes in HD cells, revealing also a strong influence of the length of the repeats [19]. Importantly, among the dysregulated genes in HD cells, those coding for proteins involved in autophagy were especially well-represented (when assessing number of dysregulated genes per number of all genes related to the tested process) [19]. Therefore, the aim of this work was to assess details of autophagy impairment in HD cells and to determine specific stage(s) at which 4′,5,7-trihydroxyisoflavone improves this cellular process.

2. Materials and Methods

2.1. Cell lines and culture conditions

Cellular models of HD, based on the HEK293 line, were employed. These cell lines were constructed previously with the use of the CRISPR-Cas9 mutagenesis and consist of the control line bearing 16/17 CAG repeats in the exon 1 of the *HTT* gene (HEK), and HD lines containing 41, 53 or 84 CAG repeats (HEK 41, HEK 53 or HEK 84, respectively) [18]. DNA sequencing and PCR-based analysis were employed to confirm the presence of these genetic variants in particular cell lines, as described previously [19]. For culturing the cell lines, DMEM medium was used (Gibco™ Thermo Fisher Scientific, #11995-065). This medium was supplemented with following additives: 10% FBS (Gibco ™ Thermo Fisher Scientific, #A5256701) and 1:100 Anti-Anti (Gibco ™ Thermo Fisher Scientific, #15240062). Standard conditions were used to incubate the cultures (37°C, 95% humidity, 5% CO_2). When indicated, the cultures were treated with 50 μM genistein (4′,5,7-trihydroxyisoflavone, Pharmaceutical Research Institute, Warsaw, Poland; #446-72-0) for 24 h. In some experiments, bafilomycin A1 (Thermo Scientific Chemicals, #J61835.MCR) was added to final concentration 100 nM at the time point of 4 h before the end of cultivation.

2.2. Transcriptomic analysis

The previously obtained [19] and deposited (NCBI database; BioProject ID: PRJNA1345724) transcriptomic data from HEK, HEK 41, HEK 53 and HEK 84 cells, either untreated or treated with 50 μM genistein for 24 h, were used. The results are derived from RNA sequencing (RNA-seq) with Illumina NovaSeq X Plus platforms, where each experiment was repeated 4 times, as described earlier [19]. Following parameters and tolls were employed in the analyses: (i) mapping of raw readings with Human Ensemble Homo Sapiens GRCh38.p12 (GCA_000001405.27) reference genome; (ii); building the index of the reference genome with Hisat2 v2.0.5, and aligning to the reference genome with Hisat2 v2.0.5; (iii) predicting novel transcripts with StringTie(v1.3.3b); (iv) counting the number of reads mapped to each gene with FeatureCounts v1.5.0-p3. Differential expression analysis of two conditions/groups was conducted with DESeq2 R package (1.20.0). Benjamini and Hochberg's approach was used to adjust the resulting *p* values to control the false discovery rate (FDR). Genes were assigned as differentially expressed when $p<0.05$ (found by DESeq2). The edgeR R package (3.22.5) was used to analyze differential expression of two conditions. The threshold for significantly differential expression was fixed as corrected *p* value of 0.05 and absolute foldchange (FC) of 2. Cytoscape 3.10.4 software was employed to construct the protein interaction network, based on the STRING database with a confidence score cutoff of 0.4.

2.3. Fluorescence microscopy

Plates (each containing 6 wells) were employed to culture HEK293-derived cells. Cells (4×10^4) were passaged using coverslips and were kept overnight to allow them to

attach to the surface. The cultures were treated with either 0.1% DMSO (controls), or 50 μM genistein (dissolved in a stock solution in DMSO) for 24 h. Fixation was conducted with 2% paraformaldehyde in phosphate-buffered saline (PBS; Merck, #806552); samples were rinsed with 0.1% Triton X-100 in PBS. The blocking reaction was conducted for 1 h using 0.5% BSA (Sigma Aldrich, #A7906) and 1.5% glycine in PBS. Incubation with primary antibody (either anti-huntingtin and anti-tubulin; Supplementary Table S1), diluted 1:1000 in 0.5% BSA in PBS, was followed by 3-times rinsing with PBS and incubation with secondary antibody (Supplementary Table S1), diluted 1:500 in 0.5% BSA in PBS, for 2 h. After 5-times washing with PBS, ProLong™ Gold Antifade Mountant with the DNA dye DAPI (Invitrogen™, Thermo Scientific, #P36935) was used to mount coverslips onto microscope slides and to visualize cell nuclei. The samples were analyzed employing fluorescence microscope Leica (Germany).

2.4. Western-blotting

Cells were cultured as for fluorescence microscopy experiments, but 2 x 10^5 cells were incubated for 24 h under standard cell culture conditions until approximately 80% confluence was reached. Following trypsinization with 0.25% Trypsin-EDTA (Gibco, Thermo Scientific, #25200072.00), the cell suspension was centrifuged for 5 min at 300 × *g*, and the resulting pellet was washed twice with 1 ml of DPBS (Dulbecco's phosphate-buffered saline, Gibco, Thermo Scientific #14190144.00). Cells were lysed using a buffer consisting of 1% Triton X-100 (Sigma Aldrich, #9002-93-1), 0.5 mM EDTA, 150 mM NaCl, 50 mM Tris, pH 7.5, supplemented with protease and phosphatase inhibitors (Roche Applied Science, #05892791001 and #11873580001). Then, samples were centrifuged (15,000 x *g*, 10 min), and supernatants were used in next steps.

Most of Western-blotting experiments were performed using the capillary JESS Automated Western System (Bio-Techne/ProteinSimple, San Jose, California, USA) as described in the manufacturer's instruction. Samples for analysis were prepared according to the standard procedure for the JESS system using the 12–230 kDa EZ Standard Pack 1 (ProteinSimple, Bio-Techne # SWPS-ST01EZ-24). Primary antibodies were diluted 1:50 in Antibody Diluent 2 (ProteinSimple, Bio-Techne # 042-203). The Anti-Rabbit Detection Module (ProteinSimple, Bio-Techne, # DM-001) was used for chemiluminescence detection. Densitometric quantifications were conducted using the manufacturer's dedicated software, Compass for SW Version 6.3.

Traditional Western-blotting experiments were conducted as described previously [15]. Briefly, samples containing 80 μg of protein were mixed with 4 x SDS sample buffer and denatured at 100°C for 10 min. Separation was performed using a 15% polyacrylamide gel in the electrophoresis buffer (25 mM Tris pH 8.3, 200 mM glycine, 0.1% SDS). Transfer to a PVDF membrane was carried out using iBlot™ 3 Transfer Stacks (Invitrogen, #IB34001) in iBlot™ 3 Western Blot device. The membrane was blocked in 5% non-fat milk in TBS buffer supplemented with Tween 20 (20 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween 20). The membrane was incubated with primary antibodies overnight at 4°C or for 1 h at room temperature; in the case of peroxidase-conjugated primary antibodies against β-actin, incubation was performed solely for 1 h at room temperature. For detection, the membrane was incubated for 5 min with ECL substrate (Clarity™ Western ECL Substrate, Bio-Rad, #1705060) and subsequently visualized on a film (CL-XPosure™ Film, Thermo Scientific, #34090). Densitometric analysis was performed using an Amersham™ Typhoon™ device (Cytiva) and ImageQuant™ TL software.

Antibodies used in blotting procedures (both in JESS system and traditional blotting) are listed in Supplementary Table S2.

2.5. Statistical analysis

Experimental results are presented quantitatively as mean values ± standard deviation (SD). GraphPad Prims 11 software was employed. The Kolmogorov-Smirnov test was used to assess the normality of the distribution of variables. One-way ANOVA or two-way ANOVA and Tukey's post hoc tests were performed. Differences were assessed statistically significant when $p<0.05$.

3. Results and Discussion

3.1. Genistein (4',5,7-trihydroxyisoflavone) reduces mHTT and tubulin abundances in HEK293-derived cellular models of HD

It was demonstrated previously that treatment of either cellular (patient-derived fibroblasts or plasmid-transfected cells) or murine models of HD with genistein (4′,5,7-trihydroxyisoflavone) resulted in the stimulation of the autophagy process and reduction of levels of mHTT and numbers of aggregates [13-17]. These results raised questions about mechanisms of autophagy impairment in HD cells and about step(s) of this process that can be activated by genistein. To test if the recently constructed cellular HD models, bearing 41, 53 or 84 repeats of the CAG triplet in the *HTT* gene [18], respond to genistein in a similar way, all cell lines (HEK 41, HEK 53 and HEK 84, respectively) were treated with genistein and levels of mHTT were determined. As demonstrated in Figure 1, HD cells of all variants revealed significantly increased levels of HTT relative to control (HEK) cells. However, treatment with genistein resulted in either normalization of HTT levels or even their decreasing below the amount found in control cells (Figure 1).

Disturbed integrity of the tubulin cytoskeleton was reported previously in HD (as discussed in detail in ref. [20]). Indeed, increased levels of tubulin could be detected in HEK 41, HEK 53 and HEK 84 cells, as compared to control (HEK) cell line (Figure 1). However, in genistein-treated HD cells, levels of tubulin normalized, and were undistinguishable from those occurring in control ones (Figure 1).

These results confirmed that genistein improves cell physiology and biochemical markers in HEK 41, HEK 53 and HEK 84 models of HD. Therefore, we employed this experimental system to study autophagy and its modulation by the tested isoflavone.

3.2. Transcriptomic analysis indicates dysregulation of autophagy in HD cells and its improvement by genistein

Recent transcriptomic studies demonstrated that there are global changes in gene expression regulation in HEK 41, HEK 53 and HEK 84 models of HD [19]. When considering particular cellular processes, autophagy was among the most severely affected ones in HD, considering the percentage of the process-specific genes that are dysregulated in HEK 41, HEK 53 and HEK 84 [19]. Indeed, many autophagy-related genes were found to be up- or down-regulated in HD models relative to control cells, and the tendency was observed that the higher number of CAG repeats in the *HTT* gene, the higher number of dysregulated genes coding for proteins involved in autophagy (Figure 2). Treatment with genistein resulted in stimulation of significant number of autophagy-related genes in HD cells which exceeded that of negatively regulated genes (Figure 2). More detailed analysis showed that both stimulation and repression of expression of autophagy-related genes occurred in HD cells relative to controls, however, genes encoding negative regulators of autophagy were mostly up-regulated while genes coding for proteins required for effective autophagy were either

down- or up-regulated (Figure 3). Treatment with genistein resulted in even more pronounced changes in expression of such genes, however, number of up-regulated genes involved in positive autophagy regulation was considerably higher than that of down-regulated genes from this group (Figure 3). Similar patterns of autophagy-related genes' expression could be found when particular types of autophagy were analyzed (Supplementary Figures S1 and S2). These results led us to propose the hypothesis that in HD cells the autophagy process should be stimulated (in order to remove the accumulating mHTT), and signs of such 'attempts' to stimulation are visible as up-regulation of expression of genes of some autophagy positive regulators; however, down-regulation of genes encoding other positive autophagy regulators and up-regulation of the genes of negative regulators result in impairment of this cellular process. On the other hand, treatment with genistein results in up-regulation of a battery of genes involved in positive autophagy regulation which might overcome the above-described block and cause autophagy restoration or activation.

Such a hypothesis might be supported by another analysis, presented by comparison of especially significantly changed expression of autophagy-related genes between control and HD cells, untreated or treated with genistein, as visualized by volcano plots and heat maps (Figure 4). This kind of analysis indicated that there was a considerable number of such genes which were severely dysregulated in HD cells, but whose expression was normalized after treatment with genistein (Figure 4C). This could also be corroborated by demonstration of networks of potential interactions in particular configurations, HEK 41 vs. HEK (Supplementary Figure S3), HEK 53 vs. HEK (Supplementary Figure S4), and HEK 84 vs. HEK (Supplementary Figure S5). Comparisons of commonly dysregulated genes in the three HD models indicated that all of them reveal considerable similarity, while reflecting the tendency that higher number of CAG repeats in *HTT* causes more severe changes in expression of genes related to autophagy (Figure 5). Differences in regulations of expression

of these genes between particular HD cellular models are depicted by volcano plots (Figure 6A), heat maps (Figure 6B) and Venn diagrams (Figure 6C).

3.3. Inhibition of autophagy occurs mostly at the initiation stage in HD cells while it can be improved by genistein

Knowing that there is a dysregulation of autophagy in HD cells and its improvement by genistein, we asked at what stage(s) of this cellular process can the inhibition and the rescue of activity by genistein occur. The process of autophagy, with indicated particular stages and proteins involved in them, is depicted schematically in Figure 7. To test the specific stage(s) of autophagy which is/are affected in HD cells and restored by genistein, we investigated levels of specific proteins in HEK (control), HEK 41, HEK 53 and HEK 84 (HD models) lines, either untreated or treated with 4',5,7-triydroxyisoflavone.

When analyzing levels of proteins regulating and being involved in the initial step of autophagy (Figure 8), we found that although total level of ULK1 is changed only in HEK 41 relative to control, genistein treatment elevated the abundance of this protein in HD cells. However, not just level but phosphorylation status of ULK1 is crucial for autophagy regulation. Phosphorylation at Ser555 by AMPK results in activation of ULK1 and stimulation of autophagy [21] while phosphorylation at Ser757 by mTOR prevents AMPK-ULK1 interactions, resulting in the autophagy inhibition [22]. We found an increased level of pULK1(Ser555) in HEK 41, but significantly decreased levels of this form in HEK53 and HEK84. Levels of pULK(757) were not significantly changed in HEK41 and HEK84 while somewhat decreased in HEK53 (Figure 8). These results corroborate the proposal that initial attempts to activate autophagy, especially visible in HD cells with lower expansion of CAG repeats (in HEK 41) might be inhibited due to inefficient phosphorylation of ULK1 at Ser555

(especially when the CAG track is longer, like in HEK 53 and HEK 84). Interestingly, genistein stimulated phosphorylation of UKL1 at Ser555 in all HD cell lines, suggesting that activation of autophagy can be restored under these conditions. Although treatment with this isoflavone resulted also in increased levels of pULK1(Ser757), this potential inhibition might be compensated by higher levels of total ULK1, thus the stimulatory effects of pULK1(Ser555) could predominate. The enhancement of autophagy stimulation by genistein could also be mediated by up-regulation of Atg13 in all tested HD lines (Figure 8). This protein is a member of the autophagy initiating complex and facilitates recruitment of ULK1 to the vicinity of the membrane surface [23]. Hence, the elevation of Atg13 levels might be another step in overcoming the autophagy block, otherwise occurring in HD cells.

In this light, it is intriguing that levels of Rubicon were increased in genistein-treated cells (Figure 9). Rubicon was demonstrated to negatively regulate autophagy by interacting with the PI3K complex [24]. Thus, genistein appears to stimulate both some positive and negative autophagy regulators, while the activation of this process apparently predominates. This ostensible paradox might, nevertheless, has some benefits to cells and organisms, and could explain a lack of overstimulation of autophagy by 4',5,7-triydroxyisoflavone, reported previously [10, 12]. In fact, this was recognized as an important benefit of the potential use of this isoflavone in a clinical practice, as by gentle activating, though not overstimulating, autophagy might be administered to patients for a long time, potentially during a life-long treatment, which is crucial for therapies of neurodegenerative diseases [25]. A capability of genistein to stimulate autophagy through enhancement of ULK1 phosphorylation at Ser555 and elevation of Atg13 levels, despite up-regulation of Rubicon, can be corroborated by demonstration that proteins involved in the PI3K complex (Beclin-1, PIK3R4, PI3K Class III) were not significantly affected by genistein (Figure 9).

Contrary to proteins involved in the autophagy initiation, effects of both mutations causing HD and treatment of genistein at the stages of autophagy elongation and phagophore expansion were negligible (Figure 10). Moreover, when the cells were treated with an inhibitor of the late-stage autophagy, bafilomycin A1 [26], levels of the autophagy marker LC3-II (or the LC3-II/LC3-I ratio) were increased (due to lack of its degradation) but they were not even higher at the presence of both bafilomycin A1 and genistein; under the latter conditions they were even lower in HEK 53 and HEK 84.

In summary, the results presented in this report suggest that autophagy is disturbed in HD cells, with severity of effects roughly proportional to the length of CAG repeats in the exon 1 of *HTT*. The initiation of the autophagy process appears to be the most severely affected stage, with ULK1 phosphorylation at Ser555 and Atg13 levels being possible targets of mHTT-mediated dysregulation. Dysfunctional autophagy might then accelerate accumulation of mHTT, due to inability to remove misfolded protein molecules. If this hypothesis is true, such a process would cause a positive feedback, where misfolded mHTT down-regulates autophagy, leading to an increase in the level of mHTT, further blocking autophagic degradation, and so on. On the other hand, these deleterious effects of mHTT on autophagy could be improved by 4',5,7-trihydroxyisoflavone which stimulates autophagy. Previous works indicated that genistein can activate autophagy through stimulation of transcription factor EB (TFEB; perhaps by inhibiting the mTOR kinase) [27] or FOXO3 [15]. Here, another stimulatory pathway emerges, through elevating levels of pULK1(Ser555) and Atg13. Since ULK1 phosphorylation at Ser555 is mediated by AMPK, our discovery is in agreement with previous results, indicating that genistein may stimulate AMPK activity [28]. The novelty of our finding is that such a function of genistein was not considered previously as a mechanism for autophagy stimulation. Interestingly, up-regulation of Atg13 was reported to be capable of partially overcoming autophagy inhibition [29], the effect analogous to that

observed in this work. Intriguingly, apart from elevating pULK1(Ser555) and Atg13 levels, treatment with genistein resulted also in up-regulation of pULK1(Ser757) and Rubicon, both known as negative regulators of autophagy. However, this paradox may be only ostensible. Genistein was reported as a gentle activator of autophagy, unable to overstimulate this process, thus being recognized as a generally safe compound, contrary to other activators which may potentially cause severe adverse effects if used as therapeutics, especially for a longer time (discussed in more detail in ref. [25]). One might propose that elevated levels of pULK1(Ser757) and Rubicon in genistein-treated HD cells could prevent overstimulation of autophagy, while the genistein-mediated activation of this process (through TFEB-, FOXO3-, pULK1(Ser555)-, and Atg13-related pathways) still predominates over the inhibition, ensuring gentle but effective stimulation of autophagic degradation of deleterious particles, like mHTT. Although autophagy dysfunction in HD was reported previously [30-32], molecular mechanisms of this secondary dysfunction in this disease remained unknown. Therefore, the novelty of this work is to demonstrate the molecular targets for autophagy impairment and demonstration of newly discovered pathway of genistein-mediated stimulation of this process, with possible mechanism of preventing its overstimulation.

4. Limitations

The major limitation of this study was the use of HEK293-derived cells as the only HD models. Although these cells line have many advantages, including susceptibility to genetic modifications (which allowed to construct cell lines with different lengths of CAG repeats in the exon 1 of the *HTT* gene), easy cultivation procedures in vitro, possibility of obtaining sufficient amount of biological material for different experiments (including transcriptomic, microscopic and biochemical studies), and others, they do not represent types of cells which are crucial in pathogenesis of HD. Definitely, further studies should involve experiments with

neural cells to confirm molecular mechanisms described in this report; moreover, studies with animal models would be desirable. Second, although the models used in this work allowed to assess effects of the number of CAG repeats in the *HTT* gene, demonstrating a general rule that longer CAG tracks cause more severe dysregulations of autophagy in HD cells, the longest allele tested here contained 84 CAG triplets in the exon 1. According to recent proposals, the most severe effects of the CAG repeat extension occur when number of CAG triplets exceeds 150 [6, 7]. Therefore, studies with cellular models with longer CAG repeat regions of *HTT* could provide further important information about HD pathomechanisms. Third, the exact mechanism by which mutations in *HTT* result in lower abundance of pULK1(Ser555) and Atg13 remains to be elucidated. Finally, determining of all molecular targets of genistein, as autophagy stimulator, would be desirable in future studies.

5. Conclusions

This work demonstrates that autophagy impairment in HD occurs mostly at the stage of the initiation of this process and involves down-regulation of pULK1(Ser555) and Atg13. Genistein (4',5,7-trihydroxyisoflavone) can rescue the otherwise defective autophagy by mechanisms which include (but are not restricted to) enhancing phosphorylation of ULK1(Ser555) and elevating levels of Atg13. Overstimulation of autophagy by this isoflavone is likely prevented by simultaneous up-regulation of pULK1(Ser757) and Rubicon which are known as negative autophagy regulators.

DECLARATIONS

Authors' contributions: Conceptualization: AS, KP, LG, GW; data curation: LG; funding acquisition: GW; investigation: AS, KW, MS, MŻ, LG; methodology: AS, LG; project administration: GW; supervision: LG, GW; validation: AS, LG; visualization, AS, KW; writing – original draft: AS, LG, GW; writing – review and editing: AS, KW, MS, MŻ, KP, LG, GW.

A competing interest statement: The medical use of 5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one in the therapy of Huntington disease is a subject of the European Patent no. EP3300730.

Data availability statement: The RNA-seq raw results are deposited in the NCBI database (BioProject ID: PRJNA1345724)

Ethical approval: Not applicable

The use of generative AI statement: Generative AI or AI-assisted tools were not used to write this paper.

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FIGURE LEGENDS

Figure 1. Mutant huntingtin (HTT) and tubulin accumulate in HD cells (HEK 41, HEK 53, and HEK 84), but not in control (HEK) cells, while their abundance is normalized by 24-h treatment with 50 μM genistein (G50). Fluorescent microscopy analysis (A) is demonstrated by representative microphotographs with immuno-stained HTT and tubulin (cell nuclei are stained with DAPI), and densitometric quantification of results from 6 independent biological repeats, with at least 100 cells analysed per condition and per repetition. Quantitative results are presented as mean values ± SD. Statistically significant differences are presented as follows: *, $p<0.05$; **, $p<0.01$. Transcriptomic analyses (B) are shown as heat maps demonstrating comparison between particular cell lines in regard *HTT* and antisense-*HTT* transcripts. The scale of $\log_2$FC (fold change) is shown. Statistically significant differences between groups ($p<0.05$) are marked by asterisks (*).

Figure 2. Number of significantly dysregulated genes related to autophagy (according to Gene Ontology - QuickGO database; GO:0006914) in HD cells (HEK 41, HEK 53, and HEK 84) relative to the control line (HEK), as assessed by number of transcripts which abundance was up- or down-regulated. Treatment with 50 μM genistein for 24 h is indicated as G50.

Figure 3. Number of significantly dysregulated genes related to negative regulation of autophagy (according to Gene Ontology - QuickGO database; GO:0010507) and positive regulation of autophagy (according to Gene Ontology - QuickGO database; GO:0010508) in HD cells (HEK 41, HEK 53, and HEK 84) relative to the control line (HEK), as assessed by

number of transcripts which abundance was up- or down-regulated. Treatment with 50 μM genistein for 24 h is indicated as G50.

Figure 4. Autophagy-related genes (according to Gene Ontology - QuickGO database; GO:0006914), with especially significant changes in abundance of their transcripts in HD cells (HEK 41, HEK 53, and HEK 84) relative to the control line (HEK). The comparison is shown as volcano plots (A) or heat maps (B and C), with indication of genes whose expression in HD cells was normalized (to the level determined in control cells) after 24-h treatment with 50 μM genistein (G50) (C). Statistically significant differences between groups (p<0.05) are marked by asterisks (*).

Figure 5. Commonly dysregulated genes related to autophagy (according to Gene Ontology - QuickGO database; GO:0006914) in HD cells (HEK 41, HEK 53, and HEK 84) and control line (HEK). Treatment with 50 μM genistein for 24-h is marked as G50. The results are presented as Venn diagrams (A) and a heat map with the scale of $\log_2$FC (fold change) (B).

Figure 6. Comparison of dysregulated genes related to autophagy (according to Gene Ontology - QuickGO database; GO:0006914) between different HD cells (HEK 41, HEK 53, and HEK 84). The results are presented as volcano plots (A), heat maps with the scale of $\log_2$FC (fold change) and statistically significant differences between groups (p<0.05) marked by asterisks (B), and Venn diagram (C).

Figure 7. Schematic presentation of autophagy, with indication of the main stages of this process and crucial proteins involved in them. In this study, levels of these proteins were determined in HD cells (HEK 41, HEK 53, and HEK 84) and control line (HEK), either untreated or treated with 50 μM genistein for 24 h, as demonstrated in Figures 8, 9, and 10.

Figure 8. Levels of proteins (A) and transcripts of their genes (B) involved in the autophagy initiation in HD cells (HEK 41, HEK 53, and HEK 84) and control line (HEK), either untreated (marked as 'Untreated') or treated with 50 μM genistein for 24 h (marked as 'Genistein' or G50). Representative Western-blots and quantification of their results are shown in panel A. Quantitative results are presented as mean values ± SD. Statistically significant differences are presented as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Comparison of levels of particular transcripts is shown as a heat map with the scale of $\log_2$FC (fold change) in panel B. Statistically significant differences between groups ($p<0.05$) are marked by asterisks (*).

Figure 9. Levels of proteins (A) and transcripts of their genes (B) involved in the autophagy initiation/elongation in HD cells (HEK 41, HEK 53, and HEK 84) and control line (HEK), either untreated (marked as 'Untreated') or treated with 50 μM genistein for 24 h (marked as 'Genistein' or G50). Representative Western-blots and quantification of their results are shown in panel A. Quantitative results are presented as mean values ± SD. Statistically significant differences are presented as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Comparison of levels of particular transcripts is shown as a heat map with the scale of $\log_2$FC (fold change) in panel B. Statistically significant differences between groups ($p<0.05$) are marked by asterisks (*).

Figure 10. Levels of proteins (A) and transcripts of their genes (B) involved in the autophagy at stages of phagophore expansion and fusion with the lysosome in HD cells (HEK 41, HEK 53, and HEK 84) and control line (HEK), either untreated (marked as 'Untreated') or treated with 50 μM genistein for 24 h (marked as 'Genistein' or G50). Representative Western-blots and quantification of their results are shown in panel A. Quantitative results are presented as mean values ± SD. Statistically significant differences are presented as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Comparison of levels of particular transcripts is shown as a heat map with the scale of $\log_2$FC (fold change) in panel B. Statistically significant differences between groups ($p<0.05$) are marked by asterisks (*).

Figure 1.

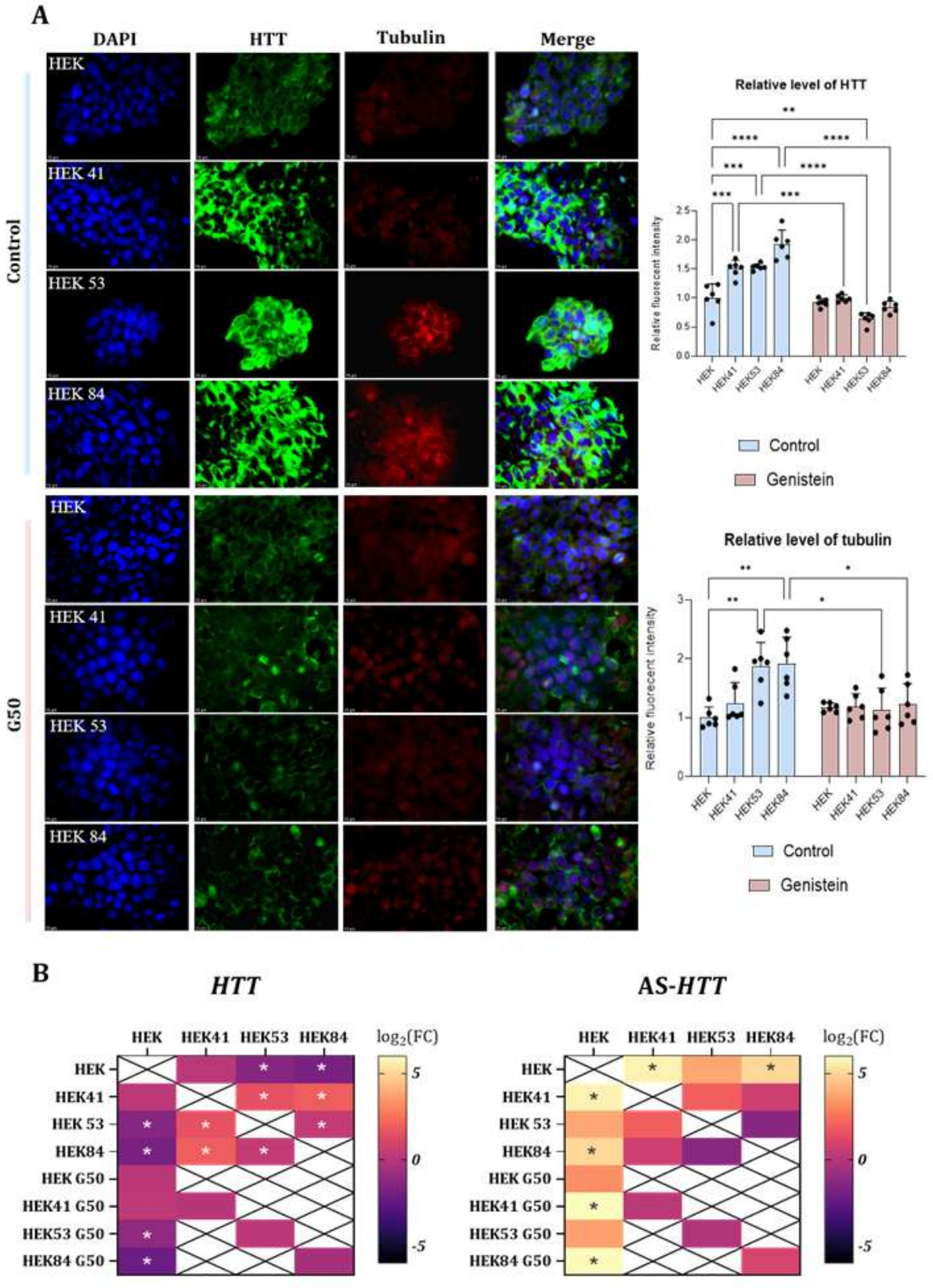

A
DAPI
HTT
Tubulin
Merge
Control
G50
HEK
HEK 41
HEK 53
HEK 84
Relative level of HTT
Relative fluorecent intensity
Control
Genistein
Relative level of tubulin
Relative fluorecent intensity
Control
Genistein
B
HTT
AS-HTT
HEK
HEK41
HEK 53
HEK84
HEK G50
HEK41 G50
HEK53 G50
HEK84 G50
log2(FC)
5
0
-5

Figure 2.

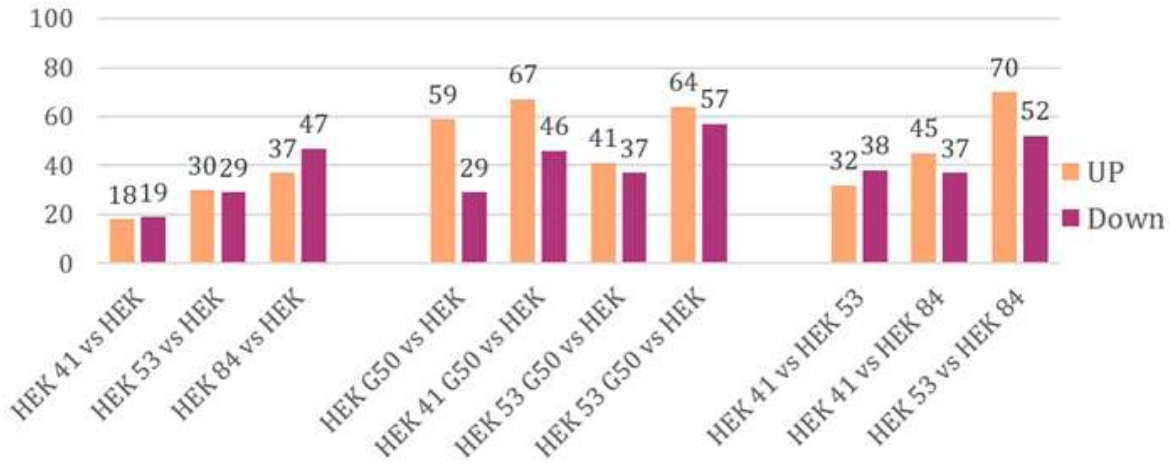


Figure 3.



Figure 4.

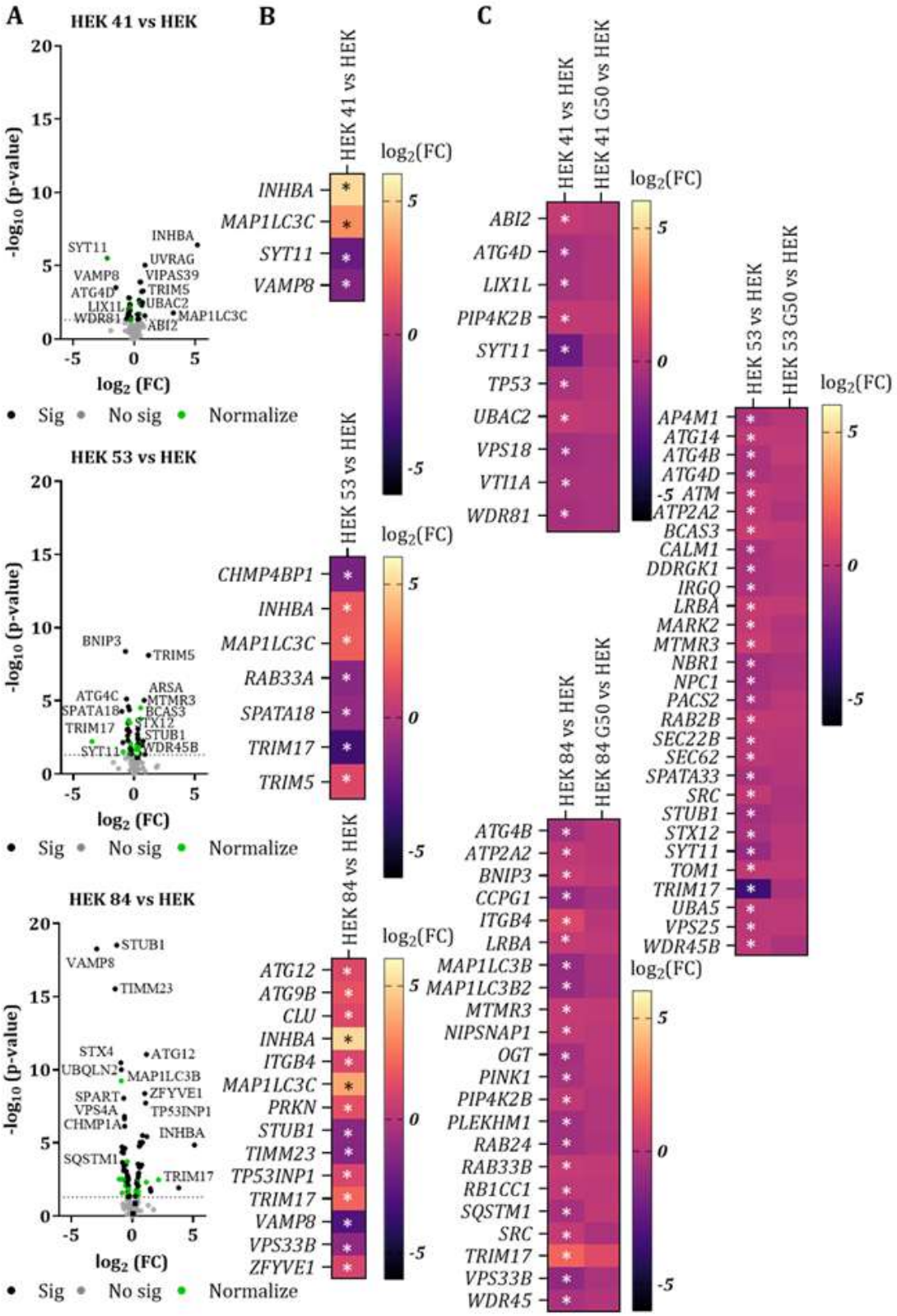

A
HEK 41 vs HEK
-log10 (p-value)
log2 (FC)
INHBA
SYT11
UVRAG
VAMP8
VIPAS39
ATG4D
TRIM5
LIX1L
UBAC2
WDR81
MAP1LC3C
ABI2
Sig
No sig
Normalize
HEK 53 vs HEK
BNIP3
TRIM5
ARSA
ATG4C
MTMR3
SPATA18
BCAS3
TRIM17
STX12
STUB1
SYT11
WDR45B
HEK 84 vs HEK
STUB1
VAMP8
TIMM23
STX4
ATG12
UBQLN2
MAP1LC3B
SPART
ZFYVE1
VPS4A
TP53INP1
CHMP1A
INHBA
SQSTM1
TRIM17
B
HEK 41 vs HEK
INHBA
MAP1LC3C
SYT11
VAMP8
log2(FC)
HEK 53 vs HEK
CHMP4BP1
INHBA
MAP1LC3C
RAB33A
SPATA18
TRIM17
TRIM5
HEK 84 vs HEK
ATG12
ATG9B
CLU
INHBA
ITGB4
MAP1LC3C
PRKN
STUB1
TIMM23
TP53INP1
TRIM17
VAMP8
VPS33B
ZFYVE1
C
HEK 41 vs HEK
HEK 41 G50 vs HEK
ABI2
ATG4D
LIX1L
PIP4K2B
SYT11
TP53
UBAC2
VPS18
VTI1A
WDR81
HEK 53 vs HEK
HEK 53 G50 vs HEK
AP4M1
ATG14
ATG4B
ATG4D
ATM
ATP2A2
BCAS3
CALM1
DDRGK1
IRGQ
LRBA
MARK2
MTMR3
NBR1
NPC1
PACS2
RAB2B
SEC22B
SEC62
SPATA33
SRC
STUB1
STX12
SYT11
TOM1
TRIM17
UBA5
VPS25
WDR45B
HEK 84 vs HEK
HEK 84 G50 vs HEK
ATG4B
ATP2A2
BNIP3
CCPG1
ITGB4
LRBA
MAP1LC3B
MAP1LC3B2
MTMR3
NIPSNAP1
OGT
PINK1
PIP4K2B
PLEKHM1
RAB24
RAB33B
RB1CC1
SQSTM1
SRC
TRIM17
VPS33B
WDR45

Figure 5.

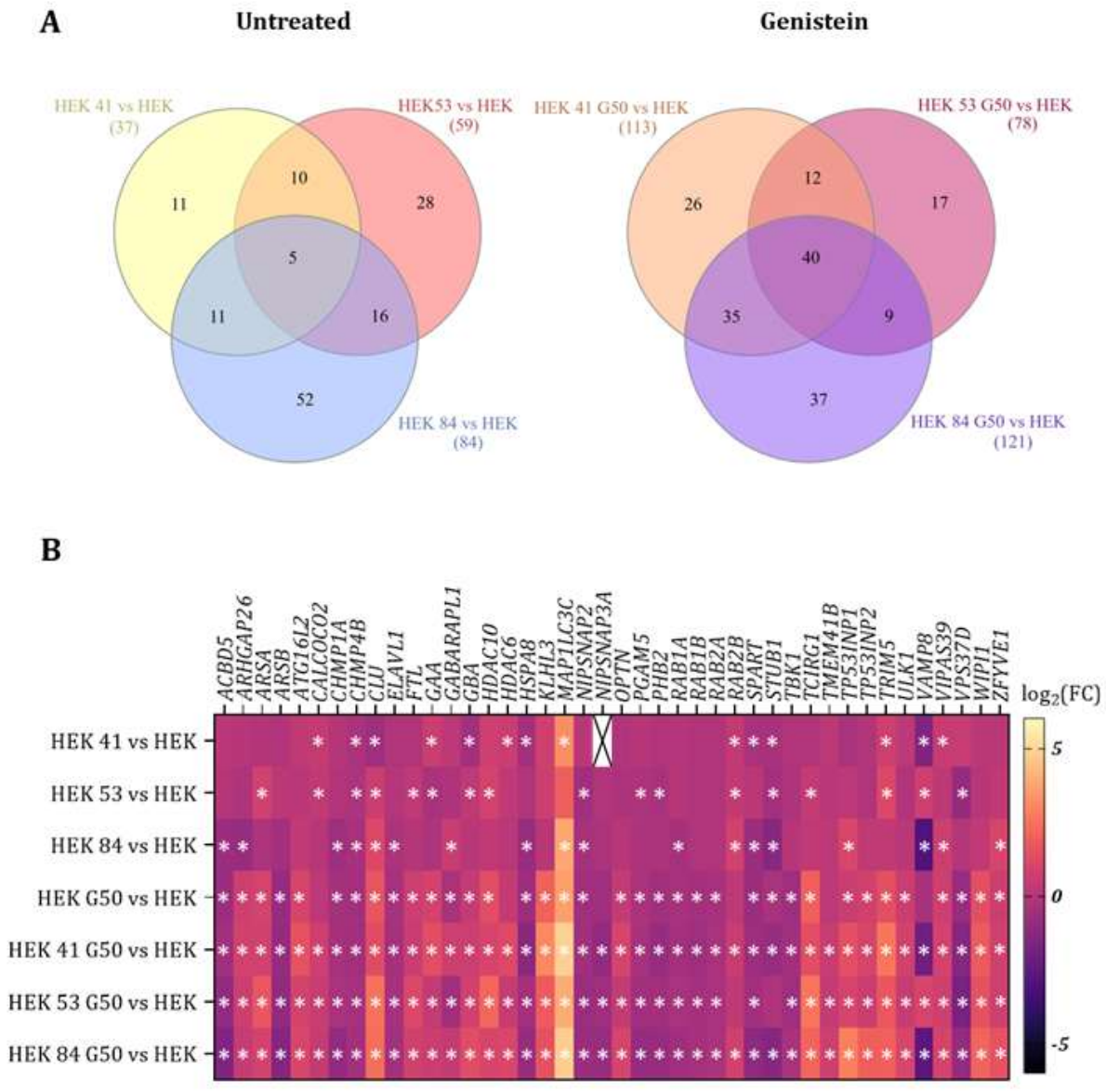

A
Untreated
Genistein
HEK 41 vs HEK
(37)
HEK53 vs HEK
(59)
HEK 84 vs HEK
(84)
11
10
28
5
11
16
52
HEK 41 G50 vs HEK
(113)
HEK 53 G50 vs HEK
(78)
HEK 84 G50 vs HEK
(121)
26
12
17
40
35
9
37
B
ACBD5
ARHGAP26
ARSA
ARSB
ATG16L2
CALCOCO2
CHMP1A
CHMP4B
CLU
ELAVL1
FTL
GAA
GABARAPL1
GBA
HDAC10
HDAC6
HSPA8
KLHL3
MAP1LC3C
NIPSNAP2
NIPSNAP3A
OPTN
PGAM5
PHB2
RAB1A
RAB1B
RAB2A
RAB2B
SPART
STUB1
TBK1
TCIRG1
TMEM41B
TP53INP1
TP53INP2
TRIM5
ULK1
VAMP8
VIPAS39
VPS37D
WIPI1
ZFYVE1
HEK 41 vs HEK
HEK 53 vs HEK
HEK 84 vs HEK
HEK G50 vs HEK
HEK 41 G50 vs HEK
HEK 53 G50 vs HEK
HEK 84 G50 vs HEK
log2(FC)
5
0
-5

Figure 6.

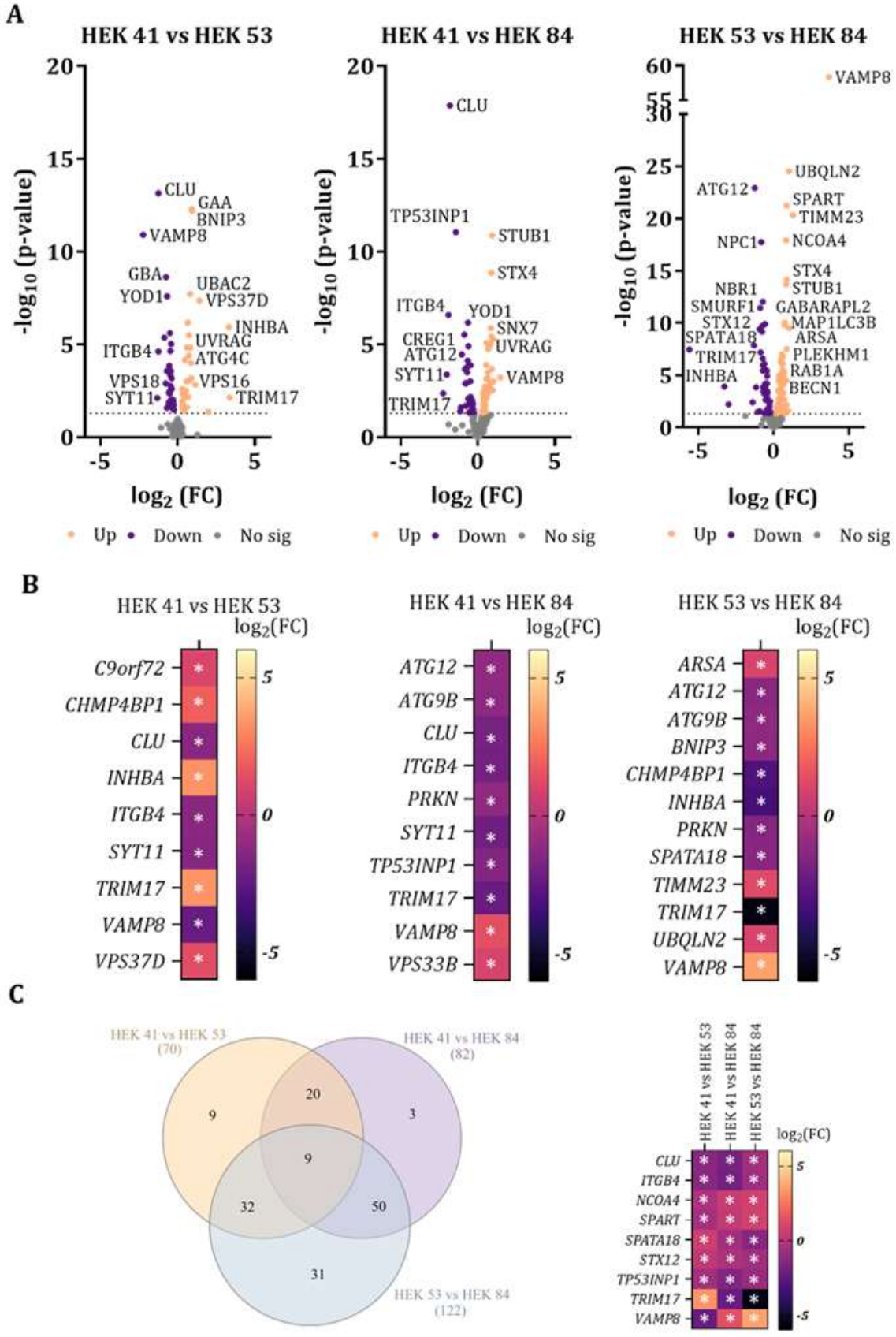

A
HEK 41 vs HEK 53
HEK 41 vs HEK 84
HEK 53 vs HEK 84
-log10 (p-value)
log2 (FC)
CLU
GAA
BNIP3
VAMP8
GBA
UBAC2
YOD1
VPS37D
INHBA
UVRAG
ITGB4
ATG4C
VPS18
VPS16
SYT11
TRIM17
TP53INP1
STUB1
STX4
ITGB4
YOD1
SNX7
CREG1
ATG12
UVRAG
SYT11
VAMP8
TRIM17
VAMP8
UBQLN2
ATG12
SPART
TIMM23
NPC1
NCOA4
STX4
NBR1
STUB1
SMURF1
GABARAPL2
STX12
MAP1LC3B
SPATA18
ARSA
TRIM17
PLEKHM1
INHBA
RAB1A
BECN1
Up
Down
No sig
B
HEK 41 vs HEK 53
HEK 41 vs HEK 84
HEK 53 vs HEK 84
log2(FC)
C9orf72
CHMP4BP1
CLU
INHBA
ITGB4
SYT11
TRIM17
VAMP8
VPS37D
ATG12
ATG9B
CLU
ITGB4
PRKN
SYT11
TP53INP1
TRIM17
VAMP8
VPS33B
ARSA
ATG12
ATG9B
BNIP3
CHMP4BP1
INHBA
PRKN
SPATA18
TIMM23
TRIM17
UBQLN2
VAMP8
5
0
-5
C
HEK 41 vs HEK 53 (70)
HEK 41 vs HEK 84 (82)
HEK 53 vs HEK 84 (122)
9
20
3
9
32
50
31
HEK 41 vs HEK 53
HEK 41 vs HEK 84
HEK 53 vs HEK 84
log2(FC)
CLU
ITGB4
NCOA4
SPART
SPATA18
STX12
TP53INP1
TRIM17
VAMP8

Figure 7.

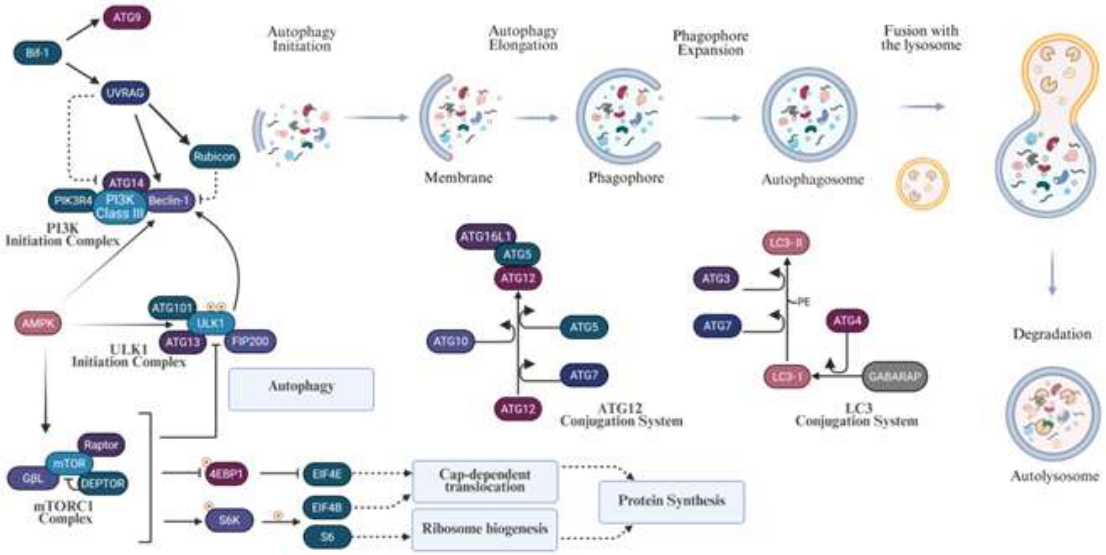

ATG9
Bif-1
UVRAG
Rubicon
ATG14
PIK3R4
PI3K Class III
Beclin-1
PI3K Initiation Complex
AMPK
ATG101
ULK1
ATG13
FIP200
ULK1 Initiation Complex
Autophagy
Raptor
mTOR
GβL
DEPTOR
mTORC1 Complex
4EBP1
S6K
EIF4E
EIF4B
S6
Cap-dependent translocation
Ribosome biogenesis
Protein Synthesis
Autophagy Initiation
Autophagy Elongation
Phagophore Expansion
Fusion with the lysosome
Membrane
Phagophore
Autophagosome
ATG16L1
ATG5
ATG12
ATG10
ATG5
ATG7
ATG12
ATG12 Conjugation System
LC3-II
ATG3
PE
ATG7
ATG4
LC3-I
GABARAP
LC3 Conjugation System
Degradation
Autolysosome

Figure 8.

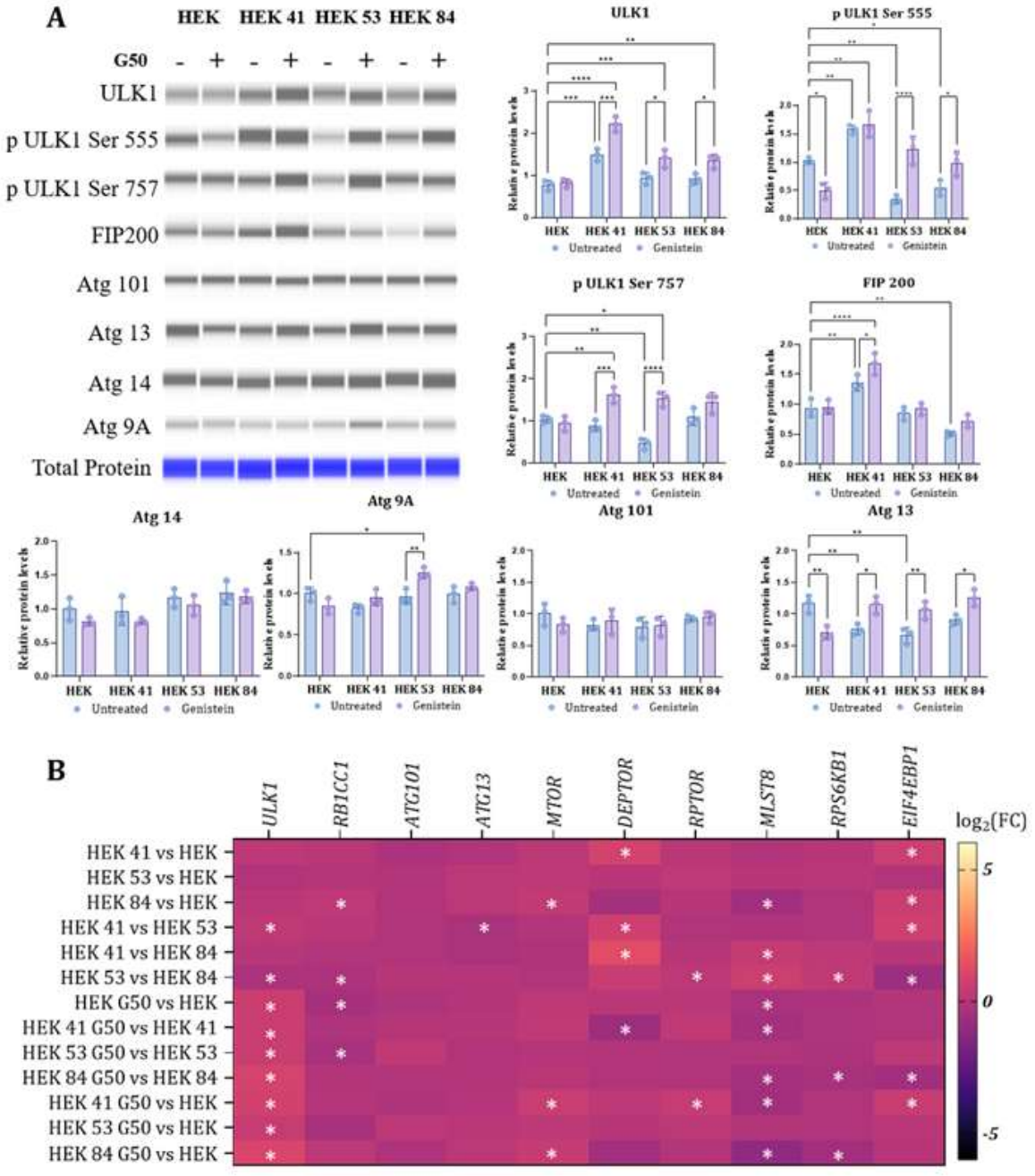

A
HEK
HEK 41
HEK 53
HEK 84
G50
ULK1
p ULK1 Ser 555
p ULK1 Ser 757
FIP200
Atg 101
Atg 13
Atg 14
Atg 9A
Total Protein
ULK1
p ULK1 Ser 555
p ULK1 Ser 757
FIP 200
Atg 14
Atg 9A
Atg 101
Atg 13
Relative protein levels
Untreated
Genistein
B
ULK1
RB1CC1
ATG101
ATG13
MTOR
DEPTOR
RPTOR
MLST8
RPS6KB1
EIF4EBP1
log2(FC)
HEK 41 vs HEK
HEK 53 vs HEK
HEK 84 vs HEK
HEK 41 vs HEK 53
HEK 41 vs HEK 84
HEK 53 vs HEK 84
HEK G50 vs HEK
HEK 41 G50 vs HEK 41
HEK 53 G50 vs HEK 53
HEK 84 G50 vs HEK 84
HEK 41 G50 vs HEK
HEK 53 G50 vs HEK
HEK 84 G50 vs HEK

Figure 9.

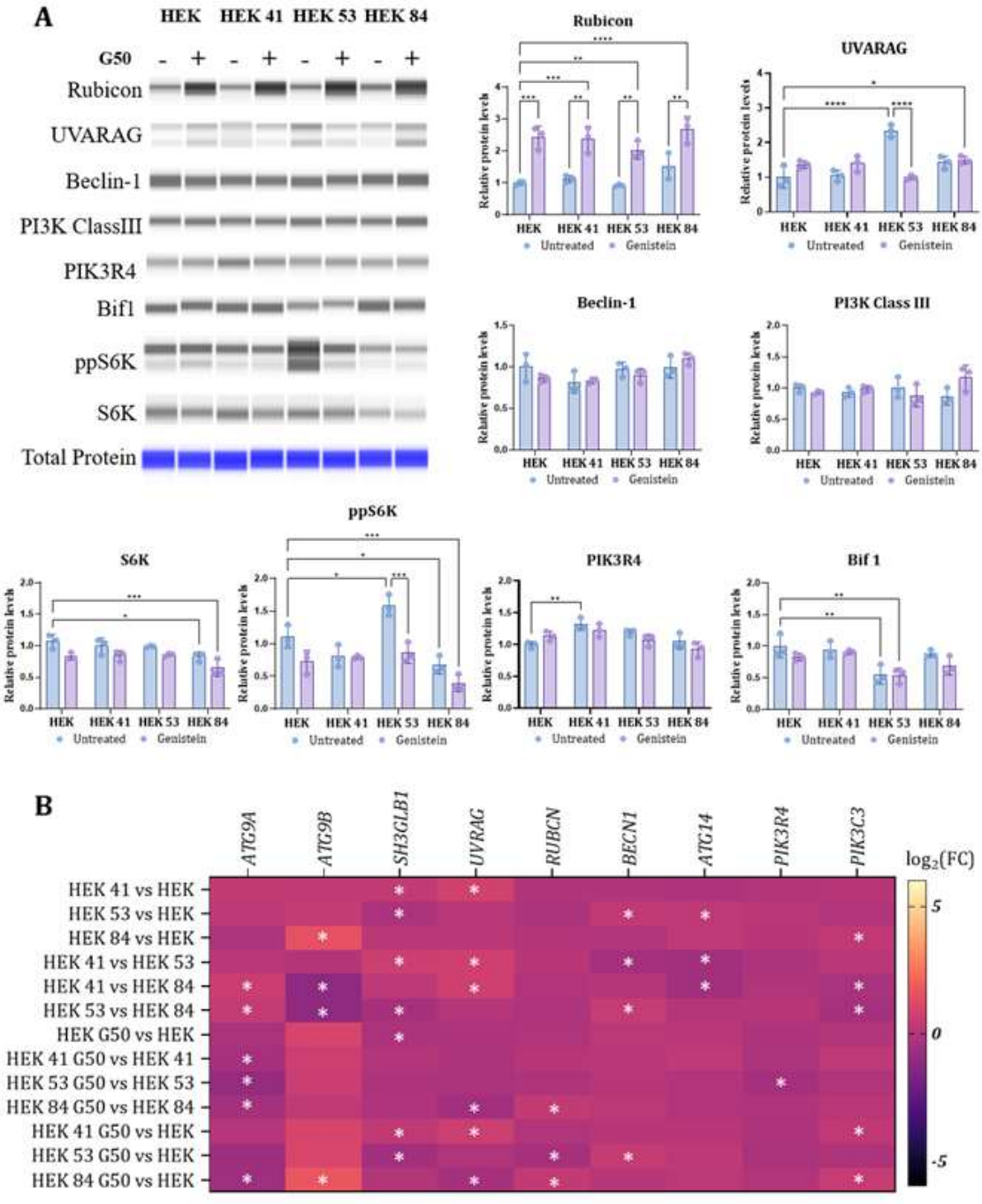
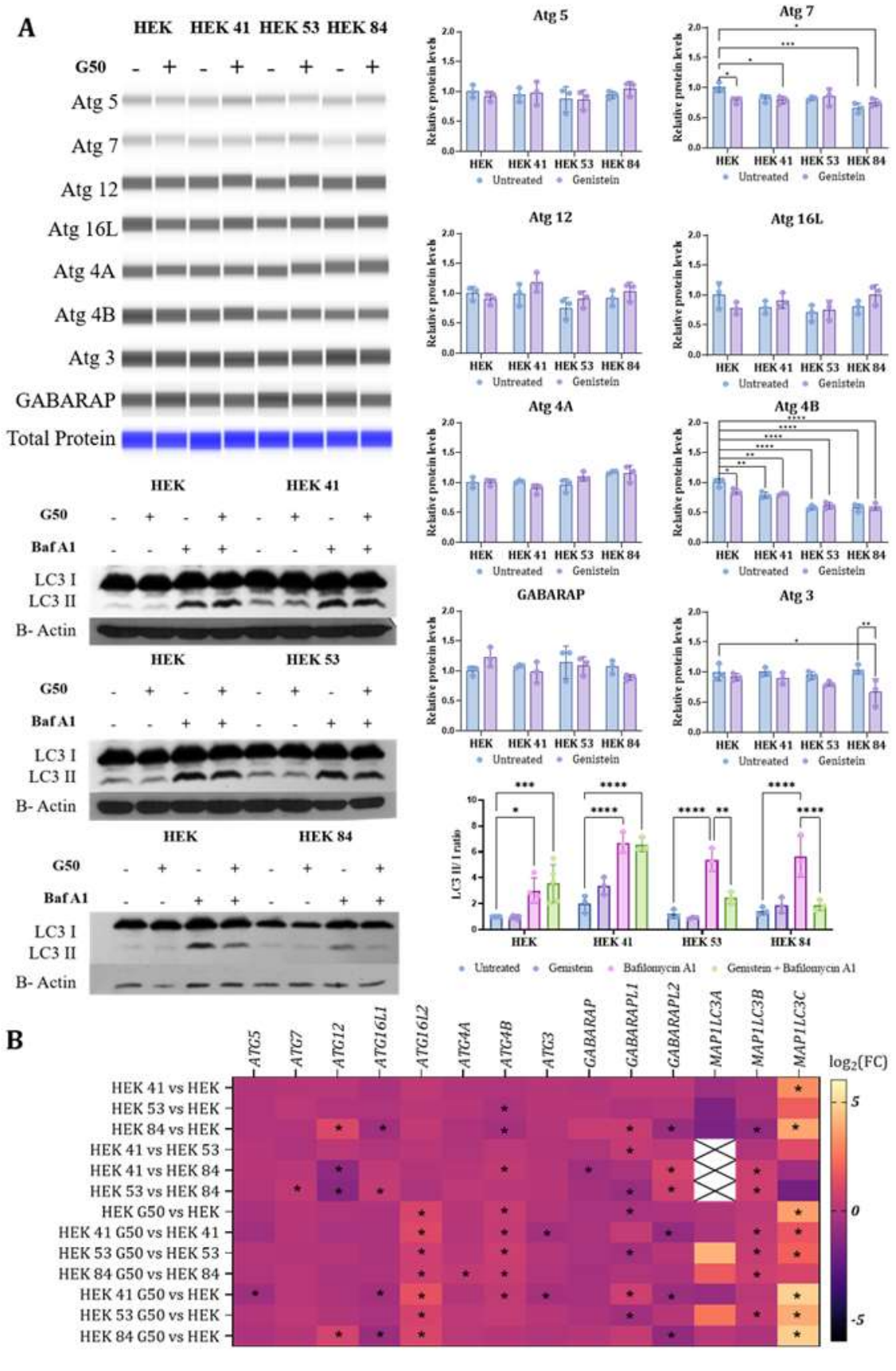

A
HEK
HEK 41
HEK 53
HEK 84
G50
Rubicon
UVARAG
Beclin-1
PI3K ClassIII
PIK3R4
Bif1
ppS6K
S6K
Total Protein
Relative protein levels
Untreated
Genistein
B
ATG9A
ATG9B
SH3GLB1
UVRAG
RUBCN
BECN1
ATG14
PIK3R4
PIK3C3
log2(FC)
HEK 41 vs HEK
HEK 53 vs HEK
HEK 84 vs HEK
HEK 41 vs HEK 53
HEK 41 vs HEK 84
HEK 53 vs HEK 84
HEK G50 vs HEK
HEK 41 G50 vs HEK 41
HEK 53 G50 vs HEK 53
HEK 84 G50 vs HEK 84
HEK 41 G50 vs HEK
HEK 53 G50 vs HEK
HEK 84 G50 vs HEK

Figure 10.


A
HEK
HEK 41
HEK 53
HEK 84
G50
Atg 5
Atg 7
Atg 12
Atg 16L
Atg 4A
Atg 4B
Atg 3
GABARAP
Total Protein
Baf A1
LC3 I
LC3 II
B- Actin
Relative protein levels
Untreated
Genistein
LC3 II/ I ratio
Bafilomycin A1
Genistein + Bafilomycin A1
B
ATG5
ATG7
ATG12
ATG16L1
ATG16L2
ATG4A
ATG4B
ATG3
GABARAP
GABARAPL1
GABARAPL2
MAP1LC3A
MAP1LC3B
MAP1LC3C
log2(FC)
HEK 41 vs HEK
HEK 53 vs HEK
HEK 84 vs HEK
HEK 41 vs HEK 53
HEK 41 vs HEK 84
HEK 53 vs HEK 84
HEK G50 vs HEK
HEK 41 G50 vs HEK 41
HEK 53 G50 vs HEK 53
HEK 84 G50 vs HEK 84
HEK 41 G50 vs HEK
HEK 53 G50 vs HEK
HEK 84 G50 vs HEK

SUPPLEMENTARY MATERIAL

Table S1. Antibody used for Immunofluorescencje

Antibody	Producer	Cat no.	Dilution
Huntingtin	abcam	ab109115	1:1000
α-Tubulin	Cell Signaling	2125S	1:1000
Anti-mouse IgG Fab2 Alexa Fluor 488	Cell Signaling	4408S	1:500
Anti-rabbit IgG Fab2 Alexa Fluor 555	Cell Signaling	4413S	1:500

Table S2. Antibody used for Wester Blot

Antibody	Producer	Cat no.	Dilution
ULK1	Cell Signaling	8054	1:50
Atg13	Cell Signaling	13273	1:50
FIP200	Cell Signaling	12436	1:50
Atg101	Cell Signaling	13492	1:50
Phospho-ULK1 Ser 757	Cell Signaling	14202	1:50
Phospho-ULK1 Ser 555	Cell Signaling	5869	1:50
Atg5	Cell Signaling	12994	1:50
Atg12	Cell Signaling	4180	1:50
Atg16L	Cell Signaling	8089	1:50
Atg7	Cell Signaling	8558	1:50
Atg4B	Cell Signaling	13507	1:50
Atg4A	Cell Signaling	7613	1:50
GABARAP	Cell Signaling	13733	1:50
Atg3	Cell Signaling	3415	1:50
PI3 Kinase Class III	Cell Signaling	4263	1:50
PIK3R4	Cell Signaling	14580	1:50
Beclin-1	Cell Signaling	3495	1:50
Atg14	Cell Signaling	5504	1:50
UVRAG	Cell Signaling	13115	1:50
Rubicon	Cell Signaling	8465	1:50
Bif-1	Cell Signaling	4467	1:50
Atg9A	Cell Signaling	13509	1:50
p70-S6K	Cell Signaling	9202S	1:50
P-p70-S6K	Cell Signaling	9234S	1:50
LC3-α/β	Santa Cruz Biotechnology	sc-39882	1:500
β-Actin–Peroxidase	Sigma Aldrich	A3854	1:25 000
Anti-mouse	Sigma Aldrich	A9044	1:4000

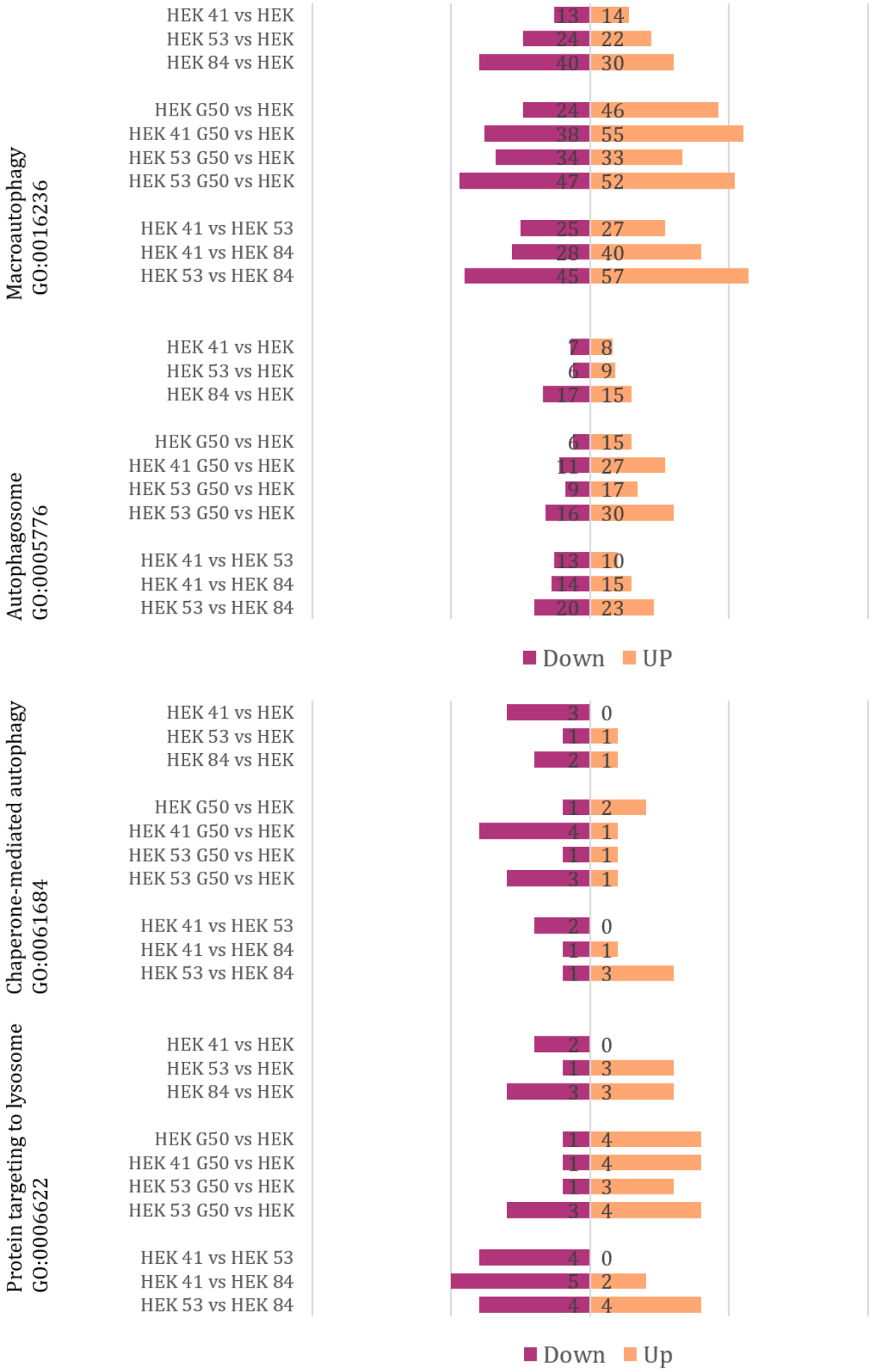


Figure S1. Number of significantly dysregulated genes related to specific types of autophagy and autophagy-related processes in HD cells (HEK 41, HEK 53, and HEK 84) relative to the control line (HEK), as assessed by number of transcripts which abundance was up- or down-regulated. Treatment with 50 mM genistein for 24 h is indicated as G50.

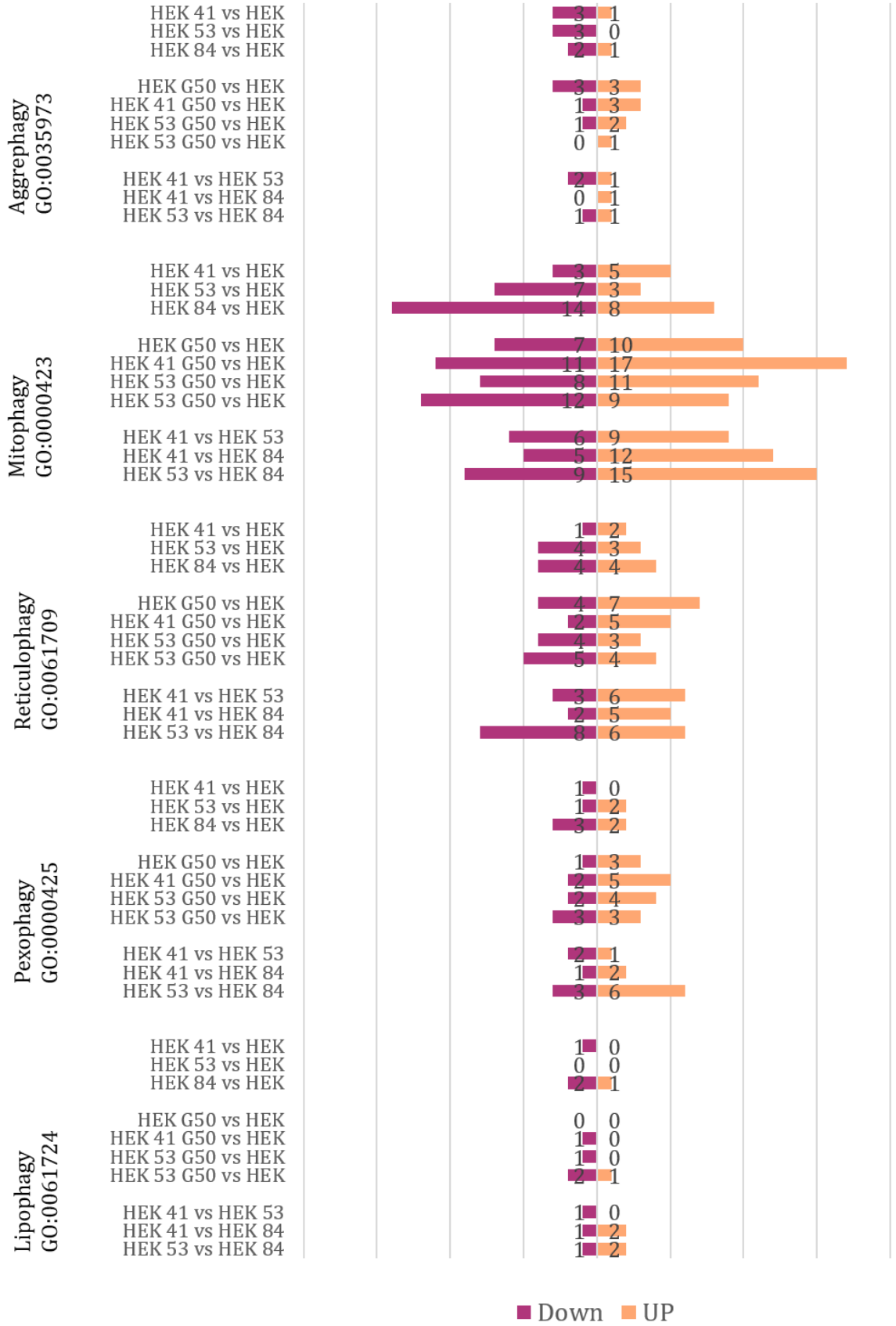


Figure S2. Number of significantly dysregulated genes related to specific types of autophagy in HD cells (HEK 41, HEK 53, and HEK 84) relative to the control line (HEK), as assessed by number of transcripts which abundance was up- or down-regulated. Treatment with 50 mM genistein for 24 h is indicated as G50.

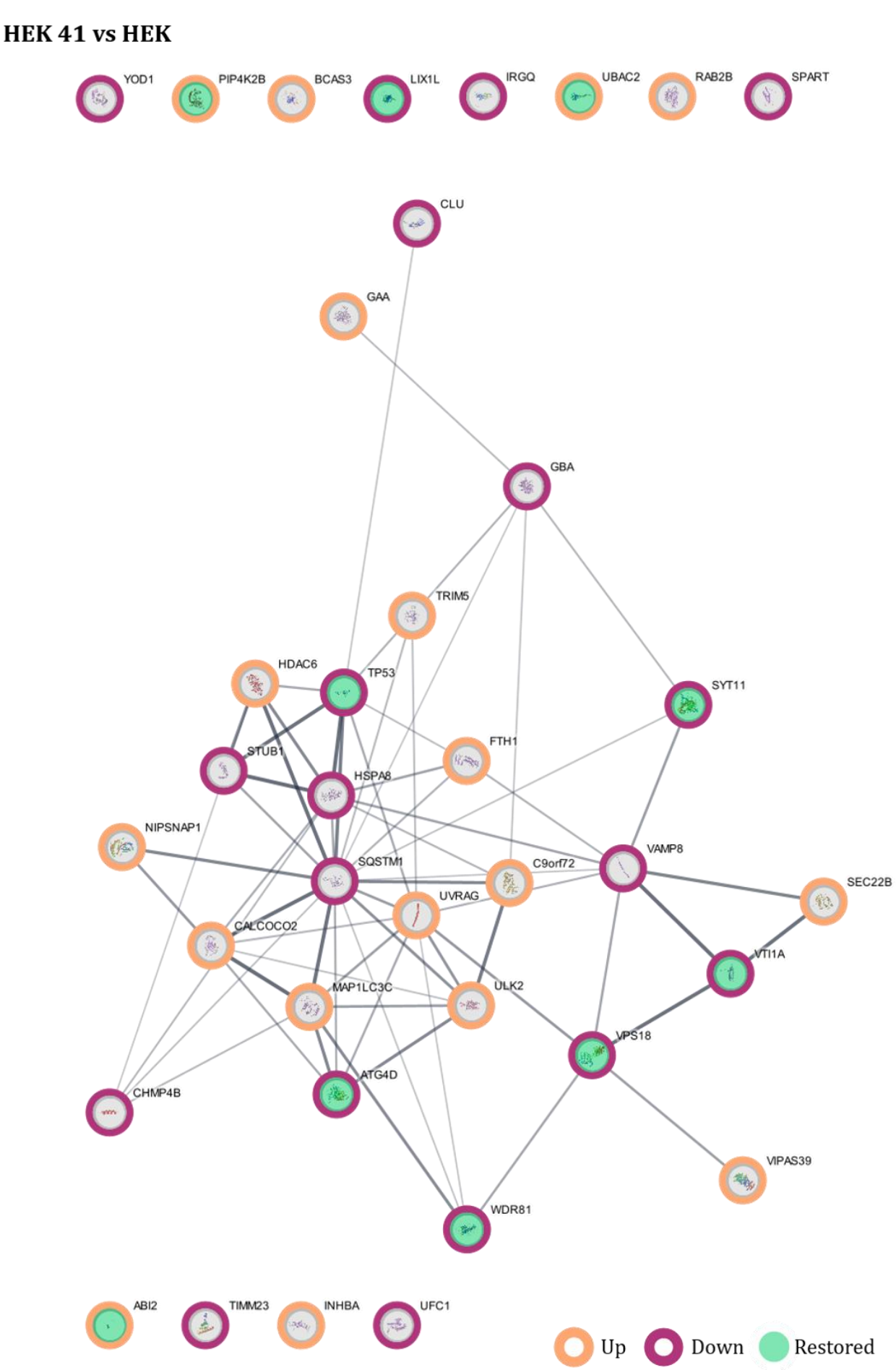


Figure S3. Interaction network between proteins involved in autophagy. Comparison between HEK 41 (HD) and HEK (control) cells. Cytoscape 3.10.4 software was used to construct the protein interaction network, based on the STRING database with a confidence score cutoff of 0.4.

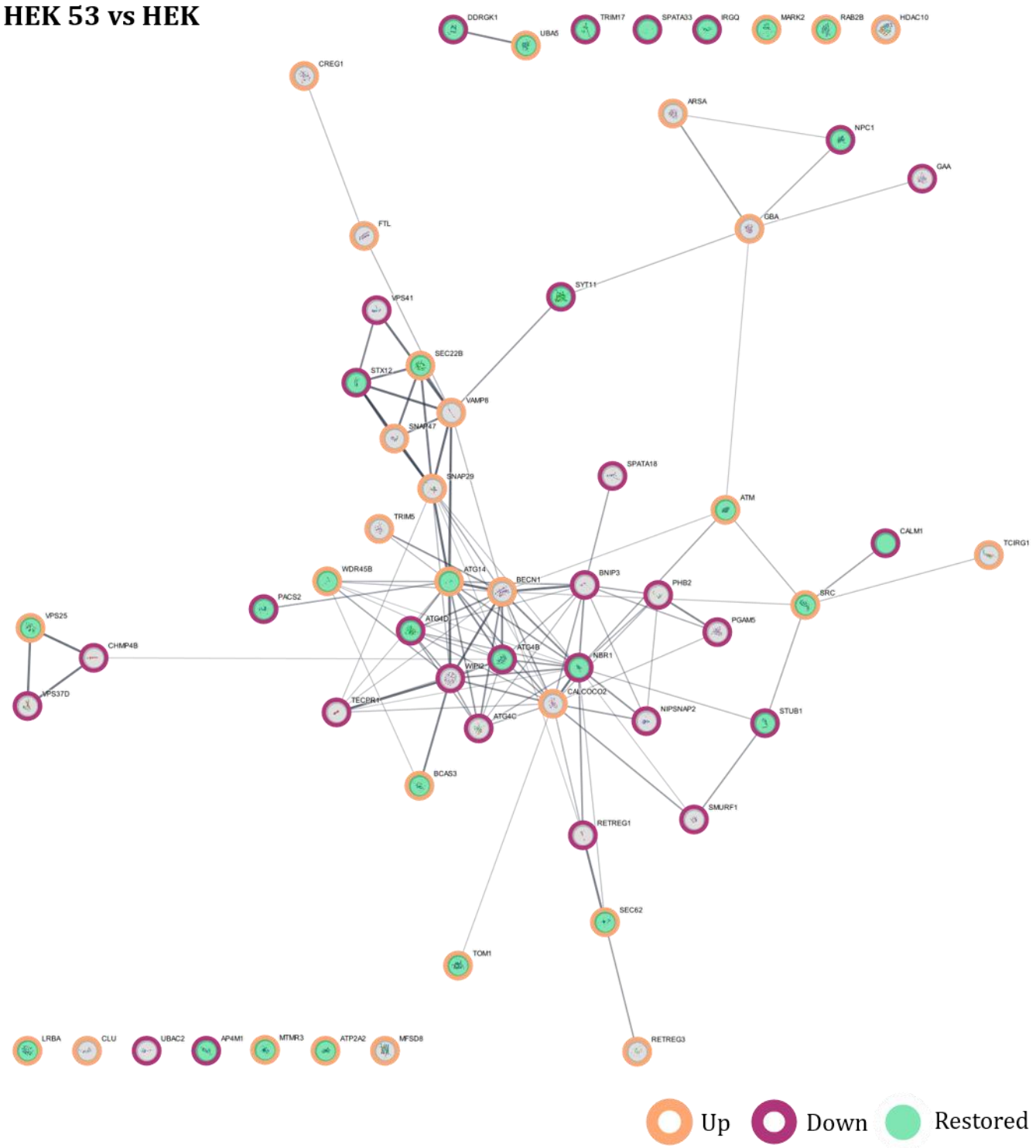


Figure S4. Interaction network between proteins involved in autophagy. Comparison between HEK 53 (HD) and HEK (control) cells. Cytoscape 3.10.4 software was used to construct the protein interaction network, based on the STRING database with a confidence score cutoff of 0.4.

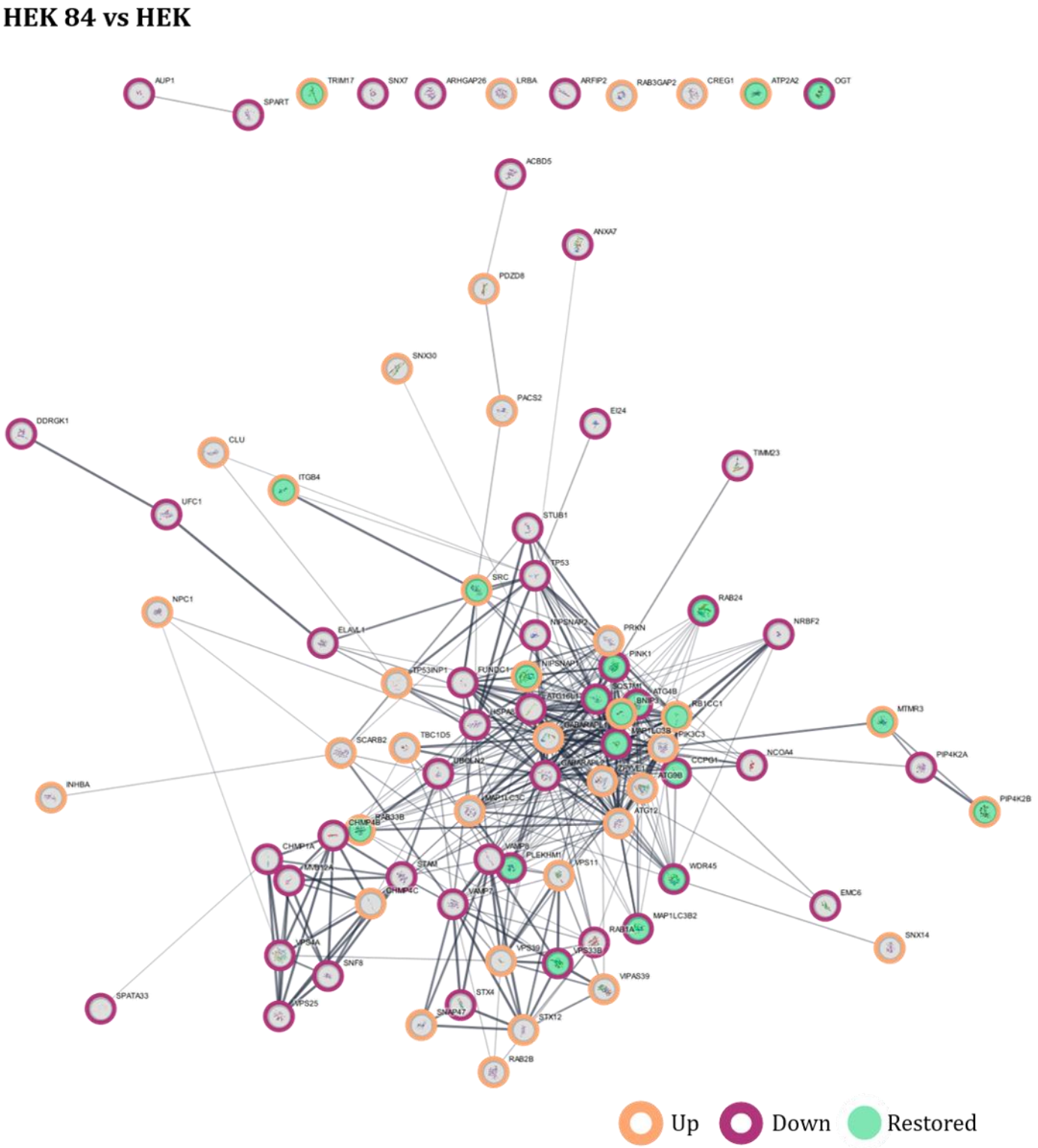


Figure S5. Interaction network between proteins involved in autophagy. Comparison between HEK 84 (HD) and HEK (control) cells. Cytoscape 3.10.4 software was used to construct the protein interaction network, based on the STRING database with a confidence score cutoff of 0.4.

Oświadczenia współautorów o wkładzie w publikację

Autophagy disorders and their improvement by 4′,5,7-trihydroxyisoflavone in cellular models of Huntington's disease

Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease

BBADIS-26-4684

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polegał na:

- przygotowaniu i przeprowadzeniu wszystkich doświadczeń
- opracowaniu i analizie wyników wszystkich doświadczeń oraz danych transkryptomicznych
- przygotowaniu figur i tabeli z wyłączeniem fig. 1A

oraz udziale w:

- sformułowaniu tematyki pracy
- przygotowaniu literatury tematu
- przygotowaniu pierwotnej wersji manuskryptu
- redakcji i edycji pierwotnej wersji manuskryptu

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- przeprowadzeniu doświadczeń
- opracowaniu wyników
- redakcji i edycji pierwotnej wersji manuskryptu

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- redakcji i edycji pierwotnej wersji manuskryptu
- redakcji i edycji pierwotnej wersji manuskryptu po recenzjach

Grzegorz Węgrzyn

prof. dr hab. Grzegorz Węgrzyn

Dorobek naukowy

Artykuły naukowe

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Doniesienia konferencyjne

1. **Szulc A.**, Walter B.M., Wiśniewska K., Żabińska M., Gaffke L., Pierzynowska K., Węgrzyn G.: Autophagy Modulation in Huntington's Disease: Insights into Pathogenesis and Therapeutic Potential, 7 Polski Kongres Genetyki, 21-14.09.2026 r. Wrocław, Polska, referat wygłoszony
2. Węgrzyn G., Podlacha M., Pierzynowska K., Gaffke L., Rintz E., Cyske Z., Wiśniewska K., **Szulc A.**, Żabińska M.: Genistein (4´,5,7-trihydroxyisoflavone) corrects behavior and improves biochemical parameters in animal models of Alzheimer's disease through autophagy stimulation, 6th Edition of Innovations and State of the Art in Alzheimer's & Dementia, 1-2.10.2025 r. Berlin, Niemcy, referat wygłoszony
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4. Pierzynowska K., Wiśniewska K., Żabińska M., Szota M., Olendzka Z., **Szulc A.**, Gaffke L., Cyske Z., Rintz E., Węgrzyn G.: The role of peroxisomes in the regulation of autophagy and ferroptosis: a novel mechanism in NBIA pathogenesis, 6th BIO Congress, 17-20.09.2025 r. Poznań, Polska, referat wygłoszony
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8. Węgrzyn G., Pierzynowska K., Podlacha M., Gaffke L., Rintz E., Cyske Z., **Szulc A.**, Wiesniewska K., Żabińska M.: Molecular mechanism and efficacy of 4',5,7-trihydroxyisoflavone in correction of symptoms of Huntington disease as revealed by studies with cellular and animal models, 49 th FEBS Congress 5-9.07.2025 r. Stanbuł, Turcja, referat wygłoszony
9. Gaffke, L., **Szulc, A.**, Cyske, Z., Podlacha, M., Pierzynowska, K., Węgrzyn, G.: Mitochondrial effects of huntingtin polyglutamine expansionina CRISPR edited HEK model, 49 th FEBS Congress 5-9.07.2025 r. Stanbuł, Turcja, poster
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13. **Szulc A.**: Zmiany w profilu ekspresji genów w komórkowym i zwierzęcym modelu choroby Huntingtona XVIII Międzynarodowa Konferencja Chorób Rzadkich "Co nowego w chorobach rzadkich", 27-29.06.2025 r., referat wygłoszony
14. **Szulc A.**, Wiśniewska K., Żabińska M., Gaffke L., Pierzynowska K., Węgrzyn G.: Initiation and elongation of the autophagy process in different types of NBIA 9th International Symposium on NBIA, 17-19.10.2024 r., Stambuł, Turcja, poster
15. **Szulc A.**, Wisniewska K., Żabińska M., Pierzynowska K., Węgrzyn G.: Changes in the Expression Profile of Genes Involved in the Autophagy Process in Huntington's Disease, VI Zjazd Polskiego Towarzystwa Biologii Medyczne 19-21.09.2024 r., Warszawa, Polska, poster
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17. Węgrzyn G., **Szulc A.**, Gaffke L., Rintz E., Cyske Z., Wiśniewska K., Żabińska M., Pierzynowska K.: Transcriptomic analysis of Huntington disease cellular models with different numbers of the CAG repeats and effects of genistein treatments, EHDN and Enroll-HD Congress, 12-14.09.2024 r., Strasbourg, Francja, referat wygłoszony
18. **Szulc A.**, Walter B.M., Glinkowska M.: Purification of bacterial proteins on the example of Pseudomonas aeruginosa DnaA replication protein, III Kongres Młodej Nauki, 6-8.07.2023r., Gdańsk, Polska, poster
19. **Szulc A.**, Walter B.M., Glinkowska M.: Rola białka GmhA w kontroli replikacji DNA w Pseudomonas aeruginosa, V Interdyscyplinarna Akademicka Konferencja Ochrony Środowiska, 15-17.09.2020 r., Gdańsk, Polska, poster
20. Walter B.M., **Szulc A.**, Glinkowska M.: The role of GmhA protein in control of DNA replication in *Pseudomonas aeruginosa,* 8th International Weigl Conference: 26-28.06.2019 r., Łódź, Polska, poster

Projekty badawcze i infrastrukturalne

“Genomika dla Polski (G4PL)” współfinansowany ze środków Europejskiego Funduszu Rozwoju Regionalnego w ramach Funduszy Europejskich dla Nowoczesnej Gospodarki
Kierownik projektu: prof. dr hab. Grzegorz Węgrzyn
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„Opracowanie systemu kompleksowej opieki nad pacjentem NBIA (neurodegeneracja z odkładaniem się żelaza w mózgu) w Polsce” finansowany w ramach programu Nauka dla Rozwoju Społeczeństwa ze środków Ministerstwa Nauki i Szkolnictwa Wyższego
Kierownik projektu: dr hab. Karolina Pierzynowska prof. UG
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Projekty finansowane przez Narodowe Centrum Nauki

OPUS 27 „Stymulacja autofagii przez genisteinę w leczeniu choroby Alzheimera: mechanizmy i efekty badanie przy użyciu genetycznych (komórkowych i zwierzęcych) modeli choroby"
Kierownik projektu: prof. dr hab. Grzegorz Węgrzyn
Sygnatura: 2024/53/B/NZ2/00225

SONATA 18 "Molekularny mechanizm zaburzeń ferroptozy w mukopolisacharydozie typu I oraz ich wpływ na przebieg choroby."
Kierownik projektu: dr hab. Karolina Pierzynowska prof. UG
Sygnatura 2022/47/D/NZ2/03095

Preludium 20 „Mechanizmy regulacji cyklu komórkowego *Pseudomonas aeruginosa* i ich rola w zdolnościach adaptacyjnych groźnej patogennej bakterii.
Kierownik projektu: **mgr Aneta Szulc**
Sygnatura: 2021/41/N/NZ2/01145

OPUS 19 “Molekularne mechanizmy stymulacji autofagii przez genisteinę oraz jej efekty komórkowe I fizjologiczne w chorobie Huntingtona”
Kierownik projektu: prof. dr hab. Grzegorz Węgrzyn
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Kierownik projektu: **mgr Aneta Szulc**
Sygnatura 1220/91/2025

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Kierownik projektu: **mgr Aneta Szulc**
Sygnatura 1220/87/2021

Projekty finansowane przez Wydział Biologii Uniwersytetu Gdańskiego

Projekty Badawcze Młodych Naukowców na Wydziale Biologii „Rola białka GmhA jako potencjalnego regulatora cyklu komórkowego *Pseudomonas aeruginosa*”
Kierownik projektu: **mgr Aneta Szulc**
Sygnatura: D001.6000.3.2021

Działalność organizacyjna

Członkostwo w Polskim Towarzystwie Biologii Medycznej
Członek Komitetu Organizacyjnego 7 Zjazdu Naukowego Polskiego Towarzystwa Biologii Medycznej (2026)
Członek Komitetu Organizacyjnego Natural Science Baltic Conference (2025)