Regulation of protein homeostasis in acute and chronic stress

Wu, Di

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Chapter 4

FOXO1 prevents polyQ aggregation by inhibiting protein synthesis in a STAU1 and DDX18 dependent manner

Di Wu¹, Jing Yang¹, Christos Papagiannopoulos¹, Hanna M. Terpstra¹, Sybille Krauss², Harm H. Kampinga¹, Steven Bergink¹*

¹ University Medical Center Groningen, University of Groningen, Department of Biomedical Sciences of Cells and Systems, Antonius Deusinglaan 1, 9713 AV, Groningen, The Netherlands. ² German Center for Neurodegenerative Diseases (DZNE), Sigmund-Freud-Street 27, 53127 Bonn, Germany

Contribution: Di Wu contributed to Figure 1 A-C, Figure 2 I, Figure 4 C, Figure 5, Figure 6 C and Supplemental Information 2 C, D, F-H.

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Abstract:

Several proteins with an expanded CAG stretch are causal for neurodegenerative diseases such as Huntington’s disease (HD). The aberrant extended CAG repeats encode for long polyglutamine (polyQ) stretches that lead to protein aggregation. The length of the repeat is related to both the aggregation-propensity of the proteins and the onset age of the disease. Here we report that expression of the FOXO1 transcription factor results in a reduction in the aggregation of a fragment of Huntingtin containing the extended polyQ stretch. This anti-aggregation effect of FOXO1 requires the transcriptional activity of FOXO1, but surprisingly, is independent of any degradation pathway, despite that FOXO1 can induce autophagy. Finally, we identify the RNA binding proteins STAU1 and DDX18 as auxiliary factors of the suppressive effect of FOXO1. These results are in line with a model that FOXO1 facilitates DDX18/STAU1 to bind mutant Huntingtin mRNA, thereby, preventing ribosome binding and reduce the synthesis of the aggregation prone protein.

Key words: PolyQ, Aggregation, FOXO1, DDX18, STAU1
Introduction

The extension of CAG repeats in at least 9 different genes is causing neuronal dysfunction and degeneration leading to the following diseases Huntington’s disease (HD), dentatorubral pallidolysian atrophy (DRPLA), spinal and bulbar muscular atrophy (SBMA) Spino cerebellar ataxias (SCA) type 1, 2, 3, 6, 7 and 17 (1). These CAG extensions encode for glutamine (Q) and the polyQ expanded proteins have a high tendency to form amyloids or aggregates (2). Protein aggregation hallmarks many different neurodegenerative disorders (3). These aggregates reflect a loss in protein homeostasis that underlies many of these neurodegenerative diseases including the so-called polyglutaminopathies (3, 4). Protein homeostasis is maintained by the cooperative balance between the regulation of transcription, translation, folding and degradation.

A main pathway that seems to regulate protein homeostasis is the insulin/insulin like growth factor (IGF) pathway (5-7). In C. elegans, the inhibition of this (AGE-1) pathway results in both delayed protein aggregation and disease onset and in enhanced organism lifespan (8, 9). Two major transcription factors regulated by IGF/AGE-1 are HSF1/HSF-1 and FOXO/DAF-16 (10, 11).

HSF1 controls the upregulation of many (but not all) molecular chaperones that assist in protein folding and prevent unwanted protein aggregation (12). Elevated expression of HSF1 is sufficient to affect life span and reduce protein aggregation in model systems such as C. elegans (13). In line, the upregulation of the HSF1-dependent network in mammalian cells can reduce protein aggregation of mutant proteins causative for neurodegenerative diseases (14), and in fact the elevated expression of several individual heat shock proteins can do the same, although several of these are not controlled by HSF1 (15-18).

The FOXO branch of the IGF pathway also funnels into protein quality control (19, 20). In C. elegans elevated expression of DAF-16 (the C. elegans homolog of FOXO1) is sufficient to reduce protein aggregation and is associated with life span extension as well (21, 22). To do so, DAF-16 controls many downstream targets including at least one small heat shock proteins that is associated with lifespan (23).

How FOXO mediates protein homeostasis in mammals is less well understood. Mammalian FOXO includes FOXO1, FOXO3a, FOXO4 and FOXO6 (24, 25). Intriguingly, FOXO1 and FOXO3a have been shown to be able to transcriptionally upregulate the expression of several autophagy-related (Atg) genes (26). Even more so, upregulation of FOXO1 can also induce autophagy in a manner independent of its activity as transcription factor but rather via directly binding to Atg7 (27). Since boosting autophagy can help to clear protein aggregates and has been shown to reduce toxicity in several protein aggregation disease models (28, 29), we wondered if and by what mechanism, FOXO1, like Daf-16 in C. elegans, might help to reduce aggregation of mutant proteins known to be causative to neurodegenerative disease.
Here, we choose to investigate the effects of FOXO1 on the aggregation of the polyglutamine containing Huntington exon-1 fragment protein (Htt\textsuperscript{polyQ}) that is responsible for Huntington’s disease. We found that FOXO1 expression substantially decreases polyQ aggregation in a manner dependent on its transcriptional activity. However, autophagy induction by FOXO1 was found not to be essential for suppression of aggregation. Rather, we found that FOXO1 triggers a DDX18 and STAU1 dependent reduction in Htt\textsuperscript{polyQ} protein synthesis.

**Results**

**FOXO1 reduces mHtt aggregation**

To test whether FOXO1 can reduce the aggregation of amyloidogenic proteins we used a GFP tagged exon 1 fragment of the human Huntingtin protein with an extended polyQ track (GFP-mHtt\textsuperscript{Q43}). Upon expression of FLAG tagged FOXO1, GFP-mHtt\textsuperscript{Q43} protein aggregation was much reduced (Figure 1A). The suppressive effect of FOXO1 on mHtt aggregation is independent of the GFP tag as similar results were obtained using HA-mHtt\textsuperscript{Q43} (Supporting Information 1, A). Moreover, FOXO1 reduced the aggregation of a mHtt construct with significantly longer polyQ tracts, GFP-mHtt\textsuperscript{Q71} and GFP-mHtt\textsuperscript{Q119} as well, albeit to a less extent as compared to the GFP-mHtt\textsuperscript{Q43} protein (Supporting Information 1, A). We also noticed a drop in the GFP-mHtt\textsuperscript{Q43} soluble protein levels whereas it had no impact on GFP protein levels (Figure 1, B). The reduction on soluble protein levels for longer polyQ stretches (GFP-mHtt\textsuperscript{Q71} and GFP-mHtt\textsuperscript{Q119}) is less prominent (Supporting Information 1, A). Flag-FOXO1 expression also inhibited GFP-mHtt\textsuperscript{Q119} puncta formation (Figure 1, D and E). Together, these results indicate that FOXO1 can specifically reduce mHtt\textsuperscript{polyQ} aggregation. This inhibition is dependent on the polyQ length as the shorter GFP-mHtt\textsuperscript{Q43} repeat is more efficiently suppressed as the longer GFP-mHtt\textsuperscript{Q71} and GFP-mHtt\textsuperscript{Q119} (Supporting Information 1, A).

Since FOXO1 reduces mHtt aggregation, we wondered if FOXO1 can act on already formed mHtt aggregates in a so-called disaggregation reaction. To address this question we co-expressed tetracycline inducible FOXO1 with HA-mHtt\textsuperscript{Q119} or GFP-mHtt\textsuperscript{Q119} and induced FOXO1 expression 24 hours after the expression of the mHtt construct. mHtt aggregation is mostly determined by the length of the polyQ tract, the longer GFP-mHtt\textsuperscript{Q119} aggregates within 24 hours while the much shorter HA-mHtt\textsuperscript{Q43} requires more than 48 hours. FOXO1 reduced the aggregation of both HA-mHtt\textsuperscript{Q43} and GFP-mHtt\textsuperscript{Q119} (Figure 1, A, Supporting Information 1, A), when expressed simultaneously. However, expressing FOXO1 24 hours after the mHtt expression resulted in a drop in HA-mHtt\textsuperscript{Q43} aggregation while the amount of GFP-mHtt\textsuperscript{Q119} aggregation remained untouched (Figure 1, F and G). This indicates that FOXO1 reduces soluble mHtt amount but cannot remove pre-existing aggregates.
Figure 1. Foxo1 prevented mHtt aggregation by specifically reducing mHtt expression.

A-C. In HEK 293T cells, GFP-mHtt^{Q43} and GFP were co-transfected with or without FOXO1. A, Insoluble GFP-mHtt^{Q43} aggregation was detected by filter trap assay (FTA). B and C, Representative blots (lower) and quantification (upper) of soluble GFP-mHtt^{Q43} or GFP were detected by western blot. D and E, Immunofluorescence staining of HEK293T cells co-expressing GFP-mHtt^{Q119} with or without FOXO1 (D) and the quantification of GFP-mHtt^{Q119} inclusions (E). F and G, HA-mHtt^{Q43} (F) and GFP-mHtt^{Q119} (G) were co-transfected with or without FOXO1 in Flp-in™ T-Rex™ HEK293 cells. Upper, schematic representation of the experiment processing. Tetracycline was added to the culture media 24 hours after transfection to induce the expression of FOXO1 for 24h. Lower, insoluble mHtt aggregation was detected by filter trap assay (FTA). Quantification of FTA was normalized by control. Bar=100 μm. All images show typical experiments, all experiments were repeated for three times. "*" stands for P<0.05, **" stands for P<0.001, ***" stands for P<0.0001.

Transcriptional activity of FOXO1 is required to prevent mHtt aggregation

As FOXO1 is a transcription factor, we wondered if its transcriptional activity is necessary for FOXO1’s anti-aggregation function. We generated two FOXO1 truncation mutants with disabled transcriptional activity by either depleting the DNA binding domain (DBD) or depleting the transcription activity domain (TAD) (Figure 2, A). Interestingly, both FOXO1 truncation mutants were unable to significantly reduce Htt^{polyQ} expression and aggregation (Figure 2, B and C).
FOXO1 prevents polyQ aggregation by inhibiting protein synthesis

**Figure 2.** FOXO1 preventing mHtt aggregation is dependent on its transcriptional activity.

A-C. In HEK293T cells, HA-mHtt<sup>Q43</sup> (B) or GFP-mHtt<sup>Q119</sup> (C) was transfected with FOXO1 or FOXO1 truncation mutants. A, Schematic representation of the FOXO1 truncation mutants used. DBD is short for DNA binding domain, TAD is short for transcription activity domain. B and C, Soluble mHtt and insoluble mHtt aggregation were detected by western blot and filter trap assay respectively. D-F. In HEK293T cells, with or without FOXO1 overexpression, the mRNA level of proteosomal subunit (D), DNAJs (E) or sHSPs (F) was detected by qPCR. All data was normalized by GAPDH as reference and was corrected to Control. G and H, In HEK293T cells, HSPB2/HSPB4/HSPB6 was co-transfected with HA-mHtt<sup>Q43</sup>. Soluble HA-mHtt<sup>Q43</sup> was detected by western blot (G). Insoluble HA-mHtt<sup>Q43</sup> aggregation was detected by filter trap assay (FTA) (H). Quantification of FTA was normalized by control. I, In HEK293T cells, FOXO1 was transfected. The protein level of HspB2, HspB4, HspB5 and HspB6 were detected by western blot (WB). All images show typical experiments, all experiments were repeated for three times. "*" stands for P<0.05, "**" stands for P<0.001, "***" stands for P<0.0001.
Knowing that the transcriptional activity of FOXO1 is required for its ability to suppress polyQ aggregation, we wonder if FOXO1 might act indirectly by upregulating other factors that are known to suppress Htt\textsuperscript{polyQ} aggregation such as proteasome subunits and chaperones (23, 30, 31). However, the transcription of the proteasomal subunits: PSMD4, PSMB2, PSMB5, PSMB8, PSMB9 and PSMB10 are hardly increased by FOXO1 overexpression (Figure 2, D). Most chaperones and co-chaperones of the HSP70 family do not reduce Htt\textsuperscript{polyQ} aggregation, except three ubiquitously expressed co-chaperones DNAJB1, DNAJB2 and DNAJB6 and the testis specific DNAJB8. However, the three generally expressed co-chaperones were hardly or not induced by FOXO1 (Figure 2, E). Interestingly, the mRNA levels of four of the nine HSPBs (HSPB2, HSPB4, HSPB5 and HSPB6) were increased by FOXO1 (Figure 2, F). Strikingly, overexpression of three of the four HSPBs (HSPB2, HSPB4 and HSPB6) in HEK293T cells can reduce soluble HA-mHtt\textsuperscript{Q43} and insoluble HA-mHtt\textsuperscript{Q43} aggregation (Figure 2, G and H). However, we noted that the increase in protein level of these HSPBs by FOXO1 is marginal and could not be detected by western blot (Figure 2, I). Since the amount of that HSPB2, HSPB4 or HSPB6 required to suppress HA-mHtt\textsuperscript{Q43} aggregation is clearly detectable by western blot, we conclude that a role of these HSPBs in the FOXO1 mediated decrease in mHtt aggregation is unlikely.

**FOXO1 prevents mHtt aggregation independent from autophagy**

Autophagy can clear toxic protein aggregates, including polyQ amyloids (28, 29). Previously, it was shown that FOXO1 can enhance autophagy (27) raising the possibility that FOXO1 triggers the autophagy mediated clearance of polyQ aggregates. FOXO1 can indeed induce autophagy as indicated by the increase in both p62 and LC3II levels (Figure 3, A) and autophagy inhibitor cocktail (E64, pepstatin A and Bafilomycin A1) can reduce this increase (Figure 3, B). However, FOXO1 suppressed polyQ aggregation independent of autophagy as the autophagy inhibitor cocktail did not inhibit the effect of FOXO1 on HA-mHtt\textsuperscript{Q43} aggregation (Figure 3, C) nor did it reduce the drop in soluble HA-mHtt\textsuperscript{Q43} (Figure 3, C). To further confirm these results, we moved to ATG5 deficient mouse embryonic fibroblast (MEF). ATG5 is an essential factor for autophagy as it is required for LC3-PE conjugation (32), and ATG5-/− cells are deficient in autophagy (33). Autophagy was indeed impaired in the ATG5-/− cells as the lipidated LC3 form (LC3II) was completely absent in the KO cells (Figure 3, D). Similar to our results with the inhibitor, FOXO1 still reduced Htt\textsuperscript{polyQ} aggregation in both ATG5 -/- and Atg5 +/+ (wild type) cells (Figure 3, D). Moreover, FOXO1 further reduced the soluble HA-Q43 proteins levels as it did in wild type cells. Note that the overall expression of HA-mHtt\textsuperscript{Q43} was much reduced in the ATG5 -/- cells, perhaps reflecting a drop in general fitness. This drop in expression did not impact the aggregation revealing that autophagy is indeed degrading Htt\textsuperscript{polyQ} in these cells as was noted before (34, 35). To fully confirm that autophagy is absent in the ATG5-/− cells we employed the autophagy activator BAG3, a co-
chaperone that can suppress Ht\textsuperscript{polyQ} aggregation in an autophagy dependent manner (36). Whereas BAG3 reduced HA-Ht\textsuperscript{polyQ} aggregation the WT cells it did not affect the aggregation levels in the ATG5-/ cells further confirming that autophagy cannot be stimulated in the ATG5-/ cells (Figure 3, D).

**Figure 3.FOXO1 reducing mHtt aggregation is independent of autophagy.

A, In HEK293 cells, cells with or without FLAG-FOXO1 overexpressing were harvested 1 or 2 days post-transfected. Autophagy was detected by LC3 I/LC3 II and p62. B and C, Cells were co-transfected with HA-mHtt\textsuperscript{Q43} with or without FLAG-FOXO1. Autophagy inhibitors were added 18 h after transfection. Protein expression and autophagy indicators were tested by western blot (B). Soluble HA-mHtt\textsuperscript{Q43} and insoluble HA-mHtt\textsuperscript{Q43} aggregation were tested by filter trap assay (FTA) (C). D, In MEF Atg5 (+/+) and Atg5 (-/-) cells, HA-mHtt\textsuperscript{Q43} was co-transfected with or without FOXO1/MYC-BAG3. Soluble and insoluble HA-mHtt\textsuperscript{Q43} were detected by western blot. All images show typical experiments, all experiments were repeated for three times. "*" stands for P<0.05, "***" stands for P<0.001, "****" stands for P<0.0001.

**FOXO1 does not accelerate the degradation of mHtt**

Given the notion that FOXO1 prevents mHtt aggregation in an autophagy independent manner, we asked whether FOXO1 acts through other degradation pathways such as the UPS (ubiquitin proteosomal system). Besides stimulating autophagy, it has been reported that FOXO1 can also stimulate degradation through the activation of the UPS (37). However, two independent proteasome inhibitors, Bortezomib
and MG132 were incapable of reducing either the FOXO1 mediated drop in soluble HA-mHttQ43 nor on the drop in aggregation (Figure 4, A and B). Ubiquitylated proteins in cell extracts accumulated after treatment with both inhibitors indicating that the UPS was successfully inhibited (Figure 4, B).

Since the UPS and autophagy are the most prominent degradation pathways in the cell but seem to be indispensable for reducing both the soluble and insoluble HA-mHttQ43 levels mediated by FOXO1, we wonder if other degradation pathways are activated by FOXO1. However, neither the rate of degradation of GFP-mHttQ74 nor GFP-mHttQ119 was changed by FOXO1 (Supporting Information 2, A and B) after treatment with the translational inhibitor cycloheximide (CHX). CHX blocks translation in general, potentially masking subtle effects on HA-mHttQ43 degradation by FOXO1. To further test a hypothetical role of FOXO1 on GFP-HttQ43 degradation, we turned to a 35S pulse chase experiment (Figure 4, C). Similar as what we noted in the experiments using CHX, Htt polyQ constructs are stable proteins that are slowly degraded and FOXO1 has no impact on the rate of degradation (Figure 4, C and Supporting Information 2, C). In summary, FOXO1 reduces both the soluble and insoluble mHtt protein levels without of any measurable effect on protein degradation pathways.

**Figure 4, FOXO1 didn’t change the degradation ratio of mHtt.**

A and B. In HEK293T cells, HA-mHttQ43 was transfected with or without FOXO1, followed by proteosome inhibitor treatments for 6 hours. HA-mHttQ43 aggregation was detected by FTA (A). Soluble HA-mHttQ43 and ubiquitylated protein were detected by WB (B). C. HEK293T cells were co-transfected with GFP-mHttQ43 with or without FOXO1, and then pulse labelled with 35S for 40 min. The decay of the radioactive signal during chasing was monitored. Representative blots were presented on the left. Autoradiography (AR) indicated the 35S signal for each time point. Data were quantified and normalized to the time point 0.5 h (right). All images show typical experiments, all experiments were repeated for three times. \* stands for P<0.05, \*** stands for P<0.001, \**** stands for P<0.0001.
FOXO1 decreases the synthesis ratio of mHtt independently from Ltn1

To exploit an alternative explanation for the drop in polyQ expression levels we reasoned that FOXO1 acts on protein synthesis rates and turned to pulses of S\textsuperscript{35} labelled methionine in Htt\textsuperscript{polyQ} expressing cells with or without elevated FOXO1. Interestingly, the amount of radioactive labelled GFP-mHtt\textsuperscript{Q43} accumulated much slower in cells expressing FOXO1 as compared to control cells (Figure 5, A and Supporting Information 2, D and E). This effect was specific as the accumulation of S\textsuperscript{35} labelled GFP was unchanged (Supporting Information 2, E).

The drop in labelled Htt\textsuperscript{polyQ} can either be explained by accelerated degradation or by a reduction in synthesis. Although we already showed that the degradation rate of full length Htt\textsuperscript{polyQ} was untouched by FOXO1 (Figure 4, C), we wanted to explore whether or not nascent polyQ chains may be (preferentially) degraded in response to the FOXO1 induced pathway. Ribosomal quality control s (RQC) depends on the E3 ligase LTN1 that sits at the ribosome and ubiquitylates unwanted nascent polypeptides (38, 39). However, inhibiting LTN1 did not suppress the anti-aggregation function of FOXO1 (Figure 5, B and Supporting Information 2, F). We therefore conclude that FOXO1 specifically regulates Htt\textsuperscript{polyQ} synthesis in a LTN1 independent manner.

**Figure 5, FOXO1 reduced the synthesis ratio of mHtt without directly changing mHtt transcription.**

A. In HEK293T cells, GFP-mHtt\textsuperscript{Q43} was co transfected with or without FOXO1, and then pulse labelled with \textsuperscript{35}S for indicated time points. Autoradiography (AR) indicated the \textsuperscript{35}S signal for each time point (left). Data were quantified and normalized to time point 0 h (right). B. GFP-mHtt\textsuperscript{Q74} was co transfected with or without FOXO1, after 24 h, Ltn1 siRNA was transfected. GFP-mHtt\textsuperscript{Q74} aggregation was detected by filter trap assay (FTA), representative blot was presented on the lower. Quantification of FTA was normalized by control (upper). All images show typical experiments, all experiments were repeated for three times. "*" stands for P<0.05. "**" stands for P<0.001.

FOXO1 mediated suppression of polyQ aggregation depends on STAUI and DDX18

Extension of CAG in the alleles of patients who suffer from polyglutaminopathies not only leads to toxic proteins with too long polyQ tracks, but also results in an altered mRNA\textsuperscript{polyQ} structure. The extension of CAG in the mRNA severely impacts the 3D folding of the mRNA forming double hairpin (40). This altered structure results in differential binding of RNA-binding proteins (41, 42). To test whether FOXO1 altered the composition of proteins that can bind to Htt\textsuperscript{polyQ}-mRNA, we performed pull downs with in vitro transcribed biotinylated Htt\textsuperscript{Q47}-mRNA as bait in cells with or without FOXO1 expression (Figure 6, A). Bioinformatical analysis of the identified hits revealed that mRNA processing and translation initiation factors as GO terms were reduced in cells expressing FOXO1 while mRNA
binding was enriched (Figure 6, ). In total, we identified 23 proteins (Table S1) that were significantly enriched in Htt\textsuperscript{Q47} binding in cells that express FOXO1 (n=3). Six of these proteins, STAU1, IGF2BP3, FUS, DDX18, DDX41 and TAF15, were predicted to bind to RNA (DAVID 6.8 database). Knock down using CRISPRi technology (43) of either STAU1 or DDX18 significantly reduced the FOXO1-mediated inhibition of GFP-mHtt\textsuperscript{Q74} aggregation (Figure 6, C and Supporting Information 2, H for knock down efficiency). Under these experimental conditions knock down of IGF2BP3, FUS and TAF15 did not affect the FOXO1-dependent reduction in GFP-Htt\textsuperscript{Q74} aggregation (Figure 6, C and Supporting Information 2, H).

Discussion

In this study, we found that FOXO1, a transcription factor downstream of the insulin/IGF1 signalling pathway, specifically reduces mHtt aggregation. Surprisingly, the anti-Htt\textsuperscript{polyQ} aggregation effect of FOXO1 was found to be independent of either autophagy or UPS. CHX treatment, turning off all protein synthesis, and \textsuperscript{35}S pulse chase experiments show that no potential protein degradation pathway is involved in the action of FOXO1 on Htt\textsuperscript{polyQ}, but uncovered that reducing Htt\textsuperscript{polyQ} synthesis rate is how FOXO1 specifically reduce proteotoxic Htt\textsuperscript{polyQ} aggregation. Reducing the expression of STAU1 and DDX18, two RNA binding proteins, reversed the FOXO1’s anti-aggregation function.
The translational rate of mRNA is monitored and is determined by many factors including the occupancy of ribosomes on the mRNA. Consequently the limited ribosome binding positions can be occupied or competed by RNA-binding proteins and therefore these may repress translation (44, 45). Our hypothesis is that FOXO1 facilitates the binding of either STAU1 or DDX18 to Htt\textsuperscript{polyQ}-mRNA thereby inhibiting polysome binding and resulting in a reduced protein synthesis.

DDX18 (DEAD box-18) is a RNA binding protein, or a potential RNA chaperone (46). Similar to the folding of poly-peptides, RNA also folds. But unlike polypeptide folding that is dominated by multiple forces and factors, RNA folding is mainly dictated by the nucleotide acid composition which make RNA folding vulnerable to mis-folding or dropping in kinetic traps (47). However, more and more evidence support a model of RNA folding chaperones (47). RNA chaperones are RNA binding proteins that can specifically bind to mRNA and destabilize the RNA helices structure for refolding (47). The DEAD box domain containing RNA binding protein family is regarded as a family of such RNA chaperones with helicase activity. By hydrolysing ATP, DEAD box proteins can disrupt a misfolded RNA structure in order to facilitate a correct refolding (46). Although several pieces of the puzzle are missing, recent studies indicate DEAD box proteins are involved in RNA splicing, ribosome biogenesis RNA export, translation and so on (48). DDX18 has been found to participate in the regulation of tumour death and cell proliferation (49), but more information about this DEAD box family member is still remain uncovered.

STAU1 (Staufen 1) is known as a double-strand DNA (dsRNA) binding protein containing multiple dsRNA binding domains. By binding to RNA, STAU1 contributes to RNA transport, localization and mRNA metabolism (50, 51). For example, binding of STAU1 to the 3’-UTR of ADP ribosylation factor (ARF) mRNA directs it to the mRNA decay pathway (52). In another translationally repressed mRNA model, STAU1 can up-regulate the translation under a situation of binding to the 5’ end of the mRNA (53). Interestingly, it was also reported that STAU1 was recruited to mutant ATXN2 aggregates which is the disease protein of Spinocerebellar ataxia type 2 (SCA2) and related to SCA2 transcription regulation (54).

For FOXO1-induced translational inhibition of Htt\textsuperscript{polyQ}, DDX18 and STAU1 are required, whether they are each sufficient for the FOXO1 mediated effects is yet unclear. It is very well possible that FOXO1 activates a repertoire of factors that together are needed to culminate in the specific translational inhibition reported here. This is currently being explored. However, although the detailed mechanisms are still not fully uncovered, our findings revealed that activation of the FOXO1 related pathway may be of potential value for HD therapy.
Material and methods

Cell cultural

HEK293T cell (ATCC), Flp-In™ T-Rex™ HEK293 cell line (Invitrogen) and Mouse embryonic fibroblast (MEF, ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) with 10% fetal calf serum (FCS, Greiner Bio-one, Longwood, FL, USA) in a 37 °C incubator with 5% CO2. MEF cells or siRNA were transfected or transfected by using Lipofectamine (Invitrogen) according to the manufacturer’s instruction. Other cell lines were transfected using polyethylenimine (PEI) (Invitrogen) following the manufacturer’s protocol.

Chemicals and reagents

Bortezomib, MG132, E64, Pepstatin A, Bafilomycin A1 and Cycloheximide were purchased from Sigma. siRNA against Ltn1 was bought from Ambion.

DNA constructs

HA-mHtt51 encoding a HA-tagged Huntingtin exon 1 fragment with 43 CAG repeats (55), GFP-mHtt019 (31), GFP-mHtt019 (31), MYC-BAG3 (56), PCDNA3-FLAG-FOXO1 (27), PCDNA3-FLAG-FOXO3a (27), GADD34 (292-590) (34), Rab9AC (consistently active) (57), Rab9DN (Dominant negative) (57), Ub-R-GFP and ODC-GFP (58) were described previously.

The V5-FOXO1 fragment was amplified from PCDNA3-FLAG-FOXO1 construct with BamH1 and XhoI restriction sites on 5’ and 3’ terminal respectively (The following primers were used: F-GATCTTGGATCCATGGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGCGCC AGGGCCCTCAGGGTTGGAGATC, R-GATTACCCTCGAGTCAGCCTGACACCCAGCTATGTCGTG, and the reaction had to contain 5% DMSO.) Then, the fragment was inserted into FRT/TO vector. The sequence was checked by Starseq (Germany).

Immunoblot

For Western Blot, cells were lysed in FTA sample buffer (10mM Tris-HCl, 150mMNaCl, 2% SDS, pH=8.0) and were boiled with equal amount of 2× Laemmli buffer. Samples were then loaded and run on a 12% SDS page gel and transferred (Bio-Rad). After transfer, membranes were blocked and incubated with primary and secondary antibodies followed by detection using the ChemiDoc Touch Imaging system (Bio-Rad).

Filter trap assays (FTA) were performed as reported before (59). In short, 3.5×10^3 HEK293 cells were seeded on 6 well plates. Cells in each well were lysed in 200 μl FTA sample buffer (10 mM Tris-
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Cl, pH 8.0, 150 mM NaCl, 2% SDS). 100 μg, 20 μg and 4 μg samples were diluted in FTA sample buffer supplied with 50 mM dithiothreitol (DTT). Boiled samples were then applied onto a pre-washed (by FTA wash buffer, 10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% SDS) 0.2 μM cellulose acetate filter (GE Water and Process Technologies, Trevose, PA, USA) with 2 Whatman papers (Bio-Rad, Hercules, CA, USA) in a Bio-Dot microfiltration apparatus (Bio-Rad). Gentle suction was applied to filter the samples followed by three times washing using FTA wash buffer. After blocking the membrane for 1 h in 5% milk, incubated with primary (overnight) and secondary antibodies (1 h), membrane was exposed using ChemiDoc Touch Imaging system (Bio-Rad).

Primary and secondary antibodies used: mouse anti-v5 primary antibody (invitrogen) at a 1:1000 dilution; anti-GFP primary antibody (invitrogen) at1:5000 dilution; anti-HA primary antibody (abcam) at 1:1000 dilution; anti-FLAG (sigma) at a 1:2000 dilution; anti-GAPDH primary antibody (Fitzgerald) at 1:10000 dilution; anti-α-tubulin primary antibody (abcam) at 1:1000 dilution; anti-p62 primary antibody (invitrogen) at a 1:2000 dilution; anti-LC3(CST) at a 1:2000 dilution; anti-HSPB2 primary antibody (abcam) at 1:1000 dilution; anti-HSPB4 primary antibody (StressMarq) at 1:1000 dilution; anti-HSPB5 primary antibody (StressMarq) at 1:1000 dilution; anti-HSPB6 primary antibody (abcam) at 1:1000 dilution; anti-polyglutamine primary antibody (MAB1574, Sigma) at 1:1000 and sheep anti-mouse secondary antibody (GE Healthcare life science) at 1:5000 dilution.

**Immunofluorescence**

48 hours after transfection, cells on coverslips were fixed in 2% formaldehyde for 15 minutes, washed 2 times with PBS (pH=7.5) and permeabilized with PBS-TritonX100 (0.1%). Then cells were washed with PBS and with PBS plus (PBS with 0.5% BSA, 0.15% glycine), followed by primary antibody incubation overnight at 4℃. Then cells on coverslips were washed with PBS plus for 4 times and were incubated with secondary antibody. After washing with PBS plus and PBS DNA was stained with DAPI (Thermo Fisher Scientific) for 5 minutes followed by washing with PBS. Cover slips were mounted in vectashield (Agar Scientific). Mouse anti-v5 primary antibody (invitrogen) at a 1:10000 dilution, Alexa 594 anti-mouse (Invitrogen) secondary antibody was diluted as 1:1500. Images were taken by a Leica sp8 confocal microscope and edited by Fiji.

**RT-qPCR**

Total RNA was isolated by RNA isolation kit (Stratagene) followed by cDNA generating using Moloney murine leukemia virus reverse transcriptase (Invitrogen). RT-qPCR was performed using SYBR green and iQ5 (Bio-Rad).

Primers for qPCR:
GAPDH, F-TGCACCACCAACTGCTTAGC, R- GGCGATGGGACTGTGGTCAAGATTGAGAGGAG;  
HSPB1, F- ACGGTCAAGACCAAGGATGG, R- AGCGTGATATTATCCGCGTGA;  
HSPB2, F- ACCGCCGATGCAATTTGTATGAG;  
HSPB3, F- ATAGAGATTCCAGTGCTAC, R- CAGGCAGTGCAATATTAAAGCATGA;  
HSPB4, F- ACCGGCAAGTTCTGTCAT, R- CTGGTTGTCTTTCCGCTGAG;  
HSPB5, F- AGGTGGTTGGGAGATGTGATTGA, R- GAGATGAAGTAATGGTGAGAGGT;  
HSPB6, F- TGCTAGACGTGAAGCACTTCT, R- ACCACCTTGACAGCAATTTCC;  
HSPB7, F- AAGGCCCTGAGCATGTTTTCC, R- ACTTGTGAGCGAAGGTGTTCA;  
HSPB8, F- CTCCCTGCCACTCTCAGCAG, R- GGCGATGGGACTGTGGTCAAGATTGAGAGGAG;  
HSPB9, F- ACCATGCCAGACGTTCT, R- CATGCCGTAATGACCTTTTC;  
HSPB10, F- ATGGCTGCACTGAGTTGTCTC, R- GCACCGTGTGCTAAATTCGT;  
HSPB11, F- CTCTGAAGGGTCCGAAGTGAT, R- ATTCCTGTGGTGGTCCAAAAC;  
PSMB2, F- TGTCAGAGACGCTTGACATC, R- CCTCTCAACACACAGGAGTAAT;  
PSMB5, F- AGGAACGCTCCTCCTGTAACAG, R- AGGCGCTCCTTATCCACAG;  
PSMD4, F- GCCTATCTCTGCCTACA, R- CGGCCATTCGAGCCAGAT;  
PSMB9, F- GGTTCATGATTCCCGAGTGCT, R- CAGCCAAACAAATGGAGGTT;  
PSMB10, F- TCCTTGAGAATCAGCTTC, R- ATCCTTTGCTTCCCTAAGAAC;  
PSMB11, F- GCAGGCTGTACTATCTGCGAA, R- AGAGCCGAGTCCATTTCGCTAT;  
DNAJA1, F- ACTGGAGCCAGCGATATTAT, R- CTTCCTTCTGCGAGTAAAACACA;  
DNAJB1, F- AAGGCATGGACATTGATGCC, R- GGCGAAAACAAATGGAGGTT;  
DNAJB2, F- CAGTGCCACCCAGACAAACAAC, R- CGGCCATTCGAGCCAGAT;  
DNAJB6, F- AAGTGCTGTCCGAGTCAAGA, R- GGTACGGAATGTGAAGGCAA;  
HTT exon1: F- CCGCTCAGGTTCTGCTTTC, R- AGGACTTGGGAGGACTCGAG;  
DDX18, F- TGAATGTGCTGCTGGGTTCC;  
DDX41, F- CAGGAGGAACGGAAGGACCAGCAGG;  
FUS, F- GTGGTGAACCTGGAGTGGCTG;  
IGF2BP3, F- AGCTTCTCAGGTTGCTGGA, R- GCCCTGAACCTGAGGCTCTGG;  
STAU1, F- TCTCGGATGCAGTCCACACT, R- GTGTGTGCAGCAGCTTGTTG;  
TAF15, F- GATCGCGCAAGGCGACATC, R- CAAGAGAAGGCACCAGAGGAA.

**CHX pulse/chase**

HEK293T cells were transfected with indicated plasmids. 12 hours post-transfection, 50 μg/ml cycloheximide was added into cultural medium and was incubated for indicated time before harvesting sample.
35S pulse/chase

35S pulse experiment

HEK293T cells were transfected with GFP-mHtt\(^{Q43}\) with or without Foxo1. Cells were labelled with methionine \(^{35}\)S and cysteine \(^{35}\)S for different time. GFP-mHtt\(^{Q43}\) in cell lysis was pulled down by GFP-trap. Autograph intensity reflects the amount of GFP-mHtt\(^{Q43}\) containing \(^{35}\)S in pull down or whole cell extraction. Total amount of GFP-mHtt\(^{Q43}\) were measured by Western Blot using anti-GFP antibody. \(\alpha\)-tubulin was used as loading control.

35S chase experiment

HEK293T cells were transfected with GFP-mHtt\(^{Q43}\), with or without FOXO1. Cells were labelled with methionine\(^{35}\)S and cysteine\(^{35}\)S for 1.5h. After washing, cells were incubated in normal cultural media for different time. GFP-mHtt\(^{Q43}\) in cell lysis was pulled down by GFP-trap. Autograph intensity reflects the amount of GFP-mHtt\(^{Q43}\) containing \(^{35}\)S in pull down or whole cell extraction. Western Blot using anti-GFP antibody measured total amount of GFP-mHtt\(^{Q43}\). \(\alpha\)-tubulin was used as loading control. For quantitative analysis, autograph intensity were normalized by loading control or total pulled down GFP. Then, all the later time points were normalized against 0h.

RNA pull down-MS

In HEK293T cells, mHtt\(^{Q47}\) was transfected with or without FOXO1. Cells were lysed 48 hours post-transfection. Biotinylated mHtt\(^{Q47}\) was generated by \textit{in vitro} transcription and was then incubated with cell lysis. RNA binding proteins were co-precipitated with biotinylated mHtt\(^{Q47}\) mRNA by streptavidin, followed by mass spectrum.

Bioinformatics

GO-term enrichment analysis was performed using DAVID 6.8. Fold enrichment and p-value were corrected by Benjamini methods.

CRISPi

CRISPi was following the protocol from Matthew H Larson, 2013 (43). Briefly, dCas9-KRAB (#71237) and sgRNA (#44248) were obtained form Addgene. dCas9-KRAB was cloned into mammalian expression vector pcDNA 3.1. sgRNA was designed by CRISPR-ERA v1.2 (http://crispr-era.stanford.edu/) following the standard protocol. sgRNAs sequences are listed below:

- STAU1: GGC\text{CGCTGGGCGC}\text{GCTCTCCTCT}
- IGF2BP3: G\text{GAAGACTGGGATGCGTT}
- FUS: G\text{CGTCGGTACTCAGCGGTGTT}

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DDX18: GCCTATTCTAGGCACCTGTT
DDX41: GACTGGCGTGTGGCTTTGCAGC
TAF15: GCTTTTCGTATTCGTTCT

Statistical analysis

Results were statistically compared using the Student’s $t$ test was performed for unpaired or paired groups. A $p$ value of $< 0.05$ was considered significant (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). All experiments were repeated for at least three times.
References


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Table S1. Mass spectrum detected protein that pulled down with mHttQ47 mRNA when Foxo1 is overexpressed.

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FOXO1 prevents polyQ aggregation by inhibiting protein synthesis

### Table S2. Mass spectrum detected protein that pulled down with mHttQ47 mRNA without Foxo1 overexpression.

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Supporting Information 1.

A. In HEK293T cells, different mHtt(314) were co-transfected with or without FOXO1. Insoluble mHtt(314) aggregation was detected by filter trap assay (FTA) (upper). Soluble mHtt(314) was detected by western blot (WB) (lower). B and C, Refers to Figure 1, F and G, presenting expression level of different proteins was detected by Western blot (WB). All images show typical experiments, all experiments were repeated for three times. *** stands for P<0.05, **** stands for P<0.001.
Supporting Information 2.

In HEK293T cells, A and B, GFP-mHttQ119 (A) or GFP-mHttQ43 (B) was co-transfected with or without FOXO1 for 12 h, followed by cycloheximide (CHX) chase experiment for indicated time. Representative bolts of soluble mHtt were detected by western blot (lower), quantification is on the top. C and D, Refer to Figure 4C (C) and Figure 5A (D). Cells were co-transfected with indicated mHtt polyQ with or without Foxo1. Cells were labelled with 35S for indicated time (D). Then, the decay of the labelled protein while chasing with unlabelled precursor (C). Autoradiography (AR) represents 35S signal of total extracts. All quantification in C were normalized to time point 0 h, all quantification in D were normalized to 0 h. E, Refers to Figure 5A, 35S pulse experiment was used to measure the synthesis rate of GFP, HA-mHttQ43 and v5-mHttQ71. Autoradiography (AR) represents 35S signal of precipitated polyQ in each time. F and G, Refers to Figure 5B (F) and Figure 6C, presenting expression levels of indicated proteins. H, Refers to Figure 6C, knock down efficiency of CRISPRi targeting on indicated protein was checked by RT-qPCR on transcriptional level. All data were normalized by Control --. "**" stands for P<0.001.