

## Article Addendum

# Amelioration of protein misfolding disease by rapamycin

## Translation or autophagy?

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Rapamycin is an inhibitor of mTOR, a key component of the mTORC1 complex that controls the growth and survival of cells in response to growth factors, nutrients, energy balance and stresses. The downstream targets of mTORC1 include ribosome biogenesis, transcription, translation and macroautophagy. Recently it was proposed that rapamycin and its derivatives enhance the clearance (and/or reduce the accumulation) of mutant intracellular proteins causing proteinopathies such as tau,  $\alpha$ -synuclein, ataxin-3 and full-length or fragments of huntingtin containing a polyglutamine (polyQ) expansion, by upregulating macroautophagy. We tested this proposal directly using macroautophagy-deficient fibroblasts. We found that rapamycin inhibits the aggregation of a fragment of huntingtin (exon 1) containing 97 polyQs similarly in macroautophagy-proficient (Atg5<sup>+/+</sup>) and macroautophagy-deficient (Atg5<sup>-/-</sup>) cells. These data demonstrate that autophagy is not the only mechanism by which rapamycin can alleviate the accumulation of misfolded proteins. Our data suggest that rapamycin inhibits mutant huntingtin fragment accumulation due to inhibition of protein synthesis. A model illustrates how a modest reduction in polyQ synthesis can lead to a long-lasting reduction in polyQ aggregation. We propose that several mechanisms exist by which rapamycin reduces the accumulation and the potential toxicity of misfolded proteins in diseases caused by protein misfolding and aggregation.

When over 30 years ago, an antifungal metabolite of *Streptomyces hygroscopicus* from a soil sample of the Easter Island (Rapa Nui) was purified and identified as a macrocyclic lactone called rapamycin, no one could have predicted that its potent immunosuppressive and anticancerogenic actions occur through its inhibition of TOR (target of rapamycin), a kinase of the PIKK family). Heitman and colleagues<sup>1</sup>

showed that rapamycin binds the cellular co-factor FKBP12 to form a complex that binds and inhibits TOR with an impressive specificity compared to most other kinase inhibitors.<sup>2</sup> Every eukaryotic genome contains a single TOR gene (although yeast sometimes contain two).<sup>3</sup> In mammalian cells mTOR forms a specific rapamycin-sensitive mTOR complex (mTORC1) that controls many pathways, but collectively determines the mass (size) of a cell.<sup>4</sup> There is a second rapamycin-insensitive mTORC2 complex that regulates cell shape via the actin cytoskeleton.<sup>4</sup>

mTOR integrates various signals that regulate cell growth. Four major inputs have been implicated in mTOR signalling: growth factors, nutrients, energy and stress (Fig. 1). The downstream targets of the various growth-related processes of mTORC1 include ribosome biogenesis, transcription, translation and macroautophagy<sup>4</sup> (Fig. 1). Signalling pathways that regulate macroautophagy and other protein degradation systems are promising candidate mechanisms that could be targeted in order to combat neurodegenerative diseases associated with protein misfolding and aggregation. A fundamental aspect of Alzheimer's, Parkinson's, prion and polyglutamine (polyQ) diseases (proteinopathies) is the production and accumulation of extracellular and/or intracellular protein species that in their misfolded and/or aggregated state cause cellular damage. Macroautophagy has been proposed to degrade such toxic monomeric or oligomeric proteins that reside inside cells.<sup>5</sup> Given that dysfunction of protein degradation pathways may contribute to the pathology of proteinopathies, restoring these or enhancing alternative degradation pathways is a logical therapeutic strategy. Recently it was proposed that rapamycin and its derivatives upregulate macroautophagy via inhibition of mTOR (Fig. 1) and thereby enhance the clearance (and/or reduce the accumulation) of mutant intracellular proteins causing proteinopathies such as tau,  $\alpha$ -synuclein, ataxin-3 and full-length, or fragments, of huntingtin (htt).<sup>5-8</sup> Although there is evidence that this is indeed the case, these studies do not directly test whether all the effects of rapamycin occur through macroautophagic mechanisms. Another possibility is that rapamycin reduces the probability of intracellular polyQ nucleation events, and hence protein aggregation, by inhibiting protein synthesis (monomer concentration), thus enabling a reduction of aggregate load that could operate in macroautophagy-deficient circumstances. Therefore, we set out to test the hypothesis that rapamycin reduces the accumulation of aggregation-prone protein via induction of macroautophagy.

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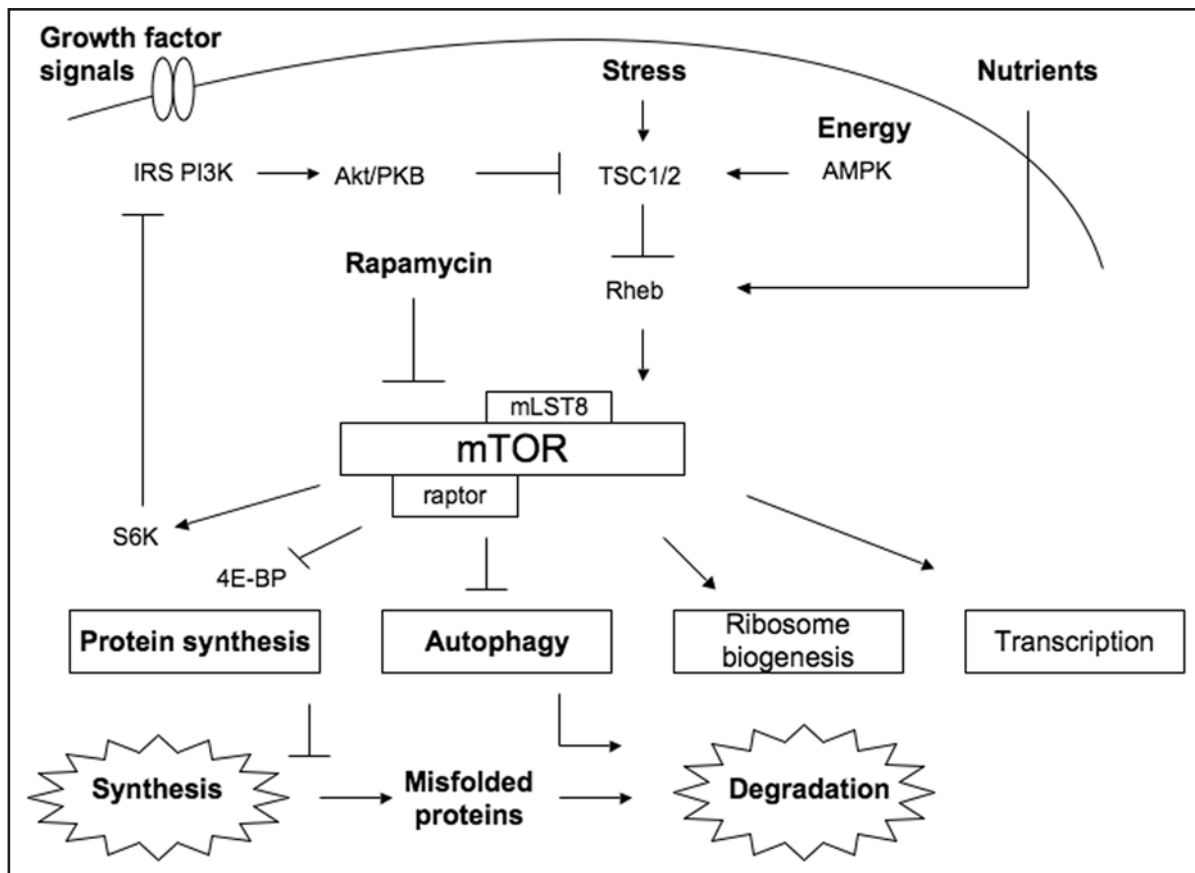


Figure 1. Upstream and downstream pathways involved in mTOR signalling in mammalian cells (adapted from ref 4). Nutrients, energy, stress and growth factors (e.g., insulin) are all converging onto mTOR via many different upstream signals. Inhibition of mTOR by rapamycin has been proposed to activate macroautophagy and hence help the turnover of misfolded, potentially toxic proteins, and thereby alleviate diseases associated with protein misfolding (see text). However, reducing protein synthesis is an alternative mechanism by which rapamycin can reduce the accumulation of polyglutamine (and possibly other) misfolded protein species. Both mechanisms could act synergistically. IRS, insulin receptor substrate; PI3K, phosphoinositide kinase; PKB, protein kinase B; TSC1/2, tuberous sclerosis complex; AMPK, AMP-activated protein kinase; Rheb, Ras homolog enriched in brain; mLST8 (mammalian)/G $\beta$ L (yeast), G protein  $\beta$ -subunit-like protein; Raptor, regulatory associated protein of mTOR; S6K, 70 kDa S6 protein kinase; 4E-BP, eIF-4E-binding protein; mTOR, mammalian target of rapamycin (protein kinase).

To address this question we studied the accumulation and clearance of polyQ control and expanded htt fragments (a peptide consisting of the first exon of htt containing 25 (control) or 97 (mutant) glutamines fused to EGFP) in macroautophagy-proficient (Atg5<sup>+/+</sup>) and deficient (Atg5<sup>-/-</sup>) mouse embryonic fibroblasts.<sup>9,10</sup> Htt fragments aggregate intracellularly in Huntington's disease animal models and in the human brain and cause cellular toxicity.<sup>11-14</sup> We quantified polyQ aggregation biochemically (by SDS-insolubility) and using cell-biological approaches (formation of inclusion bodies (IBs) also called "aggresomes") during continued synthesis of the polyQ htt fragments.<sup>15</sup> Atg5-null cells accumulate more insoluble mutant polyQ protein and form more IBs than Atg5-positive cells and this effect can be partially reversed by re-expressing Atg5 in Atg5-deficient cells.<sup>15</sup> Strikingly, we showed that inhibition of mTOR by rapamycin reduces the amount of insoluble mutant htt and the formation of htt IBs in both Atg5<sup>+/+</sup> and Atg5<sup>-/-</sup> cells by equal proportions.<sup>15</sup> Because Atg5 knockout cells are completely macroautophagy-deficient, the hypothesis that rapamycin reduces polyQ aggregation only through activation of macroautophagy is therefore not supported.

As mTOR is a 'master regulator' of the translational machinery (Fig. 2)<sup>16</sup> we tested whether rapamycin reduced polyQ aggregation

via its known inhibitory effects on protein synthesis. We found that rapamycin reduces the amount of total cellular protein (including soluble Q25 protein driven from htt-expressing control plasmids) by a modest degree (15–20%) in both Atg5<sup>+/+</sup> and Atg5<sup>-/-</sup> cells.<sup>15</sup> This degree of reduction tightly correlated with a reduction in the amount of [<sup>35</sup>S]methionine incorporation suggesting that the decrease in soluble mutant htt, and consequently the decreased accumulation of insoluble mutant htt, could be due to a reduction in protein synthesis. We then tested whether a dose of cycloheximide (CHX) that matches the decrease in protein synthesis observed by rapamycin treatment inhibited polyQ aggregation by a similar amount. CHX is a known protein synthesis inhibitor that binds to ribosomes reversibly, ensuring a response that is proportional to ribosome occupancy over extended periods of treatment. Treatment with low doses of CHX that match the decrease in protein synthesis obtained with rapamycin also mimic the reduction in SDS-insoluble mutant htt and IBs.<sup>15</sup> Therefore, under continued polyQ protein expression we propose that a rapamycin-mediated reduction in soluble protein mass decreases the probability of a polyQ nucleation event and hence the amount of aggregated polyQ load via inhibition of protein synthesis rather than by activation of macroautophagy (Figs. 1 and

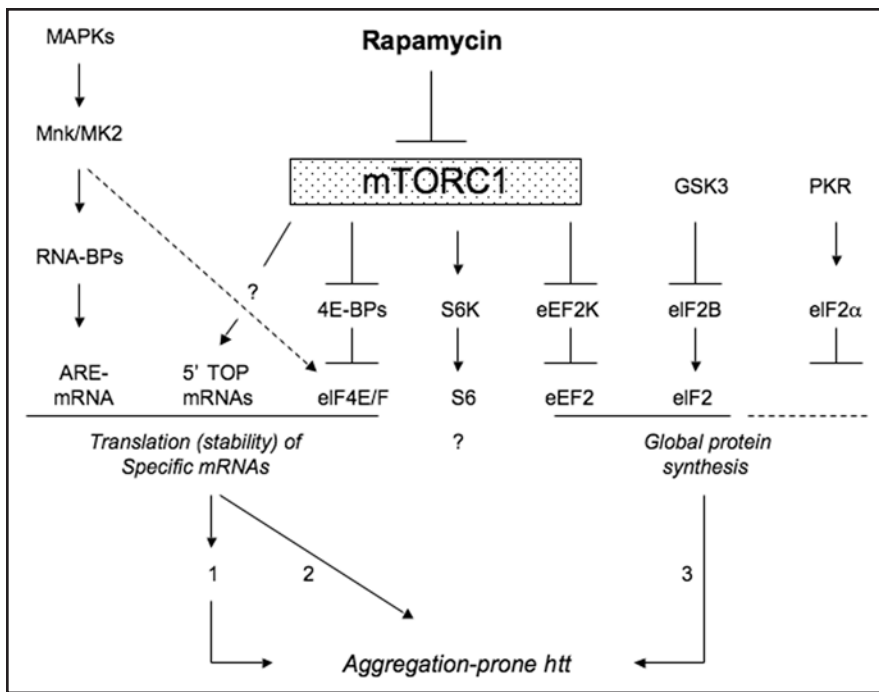


Figure 2. Rapamycin-dependent and -independent signals controlling protein synthesis (adapted from ref 10). Rapamycin can control the accumulation of misfolded, aggregation-prone proteins in various proteinopathies at the level of protein synthesis. This can be achieved by (1) inhibiting the translation of mRNAs that indirectly affect synthesis of aggregation-prone molecules such as htt (e.g., mRNAs that encode proteins which coregulate translation such as molecular chaperones) or (2) directly affect synthesis of the actual misfolding protein (e.g., htt) specifically or (3) via global effects. mTORC1, mammalian TOR complex 1; MAPK, mitogen-activated protein kinase; Mnk, MAP kinase interacting kinase; MK2, MAP kinase activated kinase; ARE, AU-rich element; 5'TOP, 5' tract of oligopyrimidine; 4E-BP, eIF4E-binding protein 1; eIF, eukaryotic initiation factor; S6K, 70 kDa S6 protein kinase; eEFL, eukaryotic elongation factor; GSK3, glycogen synthase kinase 3 beta; PKR, protein kinase RNA-activated kinase; RNA-BPs, RNA-binding proteins.

2). Figure 3 shows a model of protein aggregation that demonstrates how a small reduction in soluble polyQ molecules can lead to a significant delay in aggregation.<sup>11,17</sup>

Our study has to be followed up by exploring the precise actions of rapamycin in postmitotic neurons and animal models. The hypothesis that a beneficial effect of rapamycin is mediated by inhibition of protein synthesis can be further tested by performing mechanistic experiments where translational control is altered by either genetic knockout approaches or alteration of the various rapamycin-sensitive downstream signals that regulate translation of specific mRNAs and general protein synthesis (Fig. 2). Therefore, one question to consider is whether the reduction in polyQ aggregation by rapamycin is due to translational inhibition of a group of specific mRNAs or not. However, the situation is complex as there are mTOR-independent signalling pathways that regulate global protein synthesis (e.g., GSK3 phosphorylation of eIF2B, and PKR phosphorylation of eIF2 $\alpha$ ) or translation of specific mRNAs (e.g., ERKs and p38MAPKs Mnk-mediated signalling to eIF4E/F and ARE-mRNAs) (Fig. 2). Hence altering protein synthesis could modulate the synthesis of misfolded proteins via mTOR-dependent and -independent mechanisms. Our data does not exclude the possibility that rapamycin reduces protein aggregation via macroautophagy. Rather it opens up the opportunity to reduce aggregation via additional mechanisms. Future experiments should show the relative contribution of rapamycin in

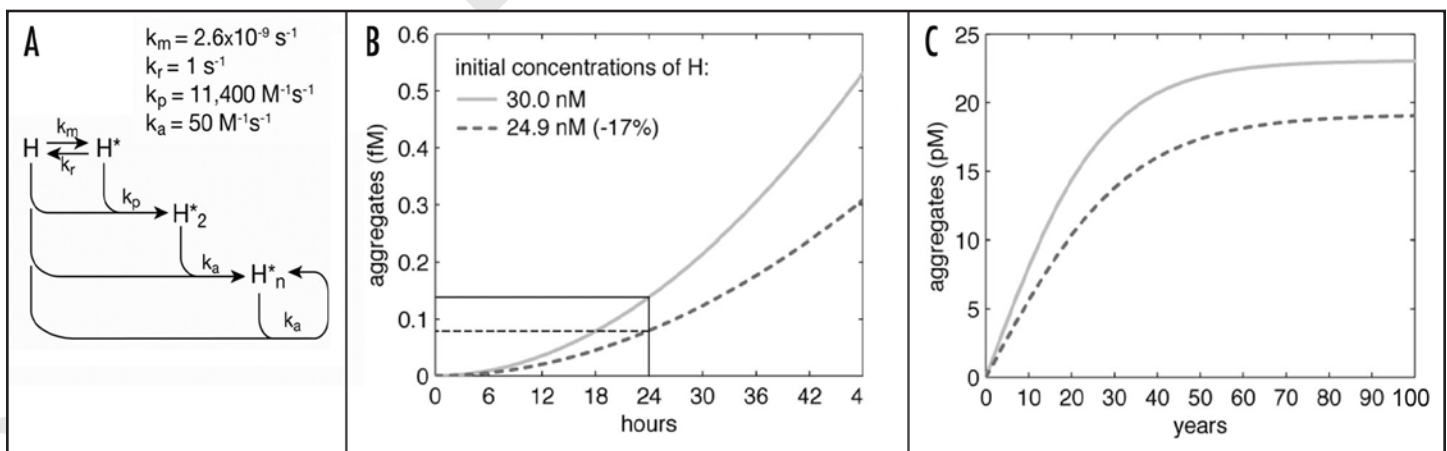


Figure 3. Model that demonstrates how, by modestly reducing protein expression, rapamycin may substantially decrease the probability of polyQ aggregation. (A) Model (extended from ref 17) supposes that monomeric Htt (H) undergoes misfolding by a reversible conformational change ( $H^*$ );  $H^*$  is then susceptible to rapid formation of a dimer with H ( $H^*2$ ), followed by stepwise aggregation ( $H^*n$ ). Rate constants were taken from ref 17 (note that the parameters for monomer stability and dimer formation in that model is based on Q47 peptides). (B) Rapamycin modestly reduces the amount of soluble protein per cell by  $\sim 17\%$  by suppressing protein synthesis. Consistent with our data, the model predicts that a 17% reduction in polyQ monomer input results in a 40% decrease in aggregate formation. (C) A modest reduction in aggregate concentration is predicted to persist throughout life (albeit one dependent on continuous exposure to rapamycin).

activating macroautophagy and/or inhibiting translation in reducing the accumulation of misfolded protein species in disease.

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