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Parkin reverses TDP-43-induced cell death and failure of amino acid homeostasis

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Abstract

The E3 ubiquitin ligase Parkin plays a central role in the pathogenesis of many neurodegenerative diseases. Parkin promotes specific ubiquitination and affects the localization of TDP-43, which controls the translation of thousands of mRNAs. Here we tested the effects of lentiviral Parkin and TDP-43 expression on amino acid metabolism in the rat motor cortex using high frequency ¹³C NMR spectroscopy. TDP-43 expression increased glutamate levels, decreased the levels of other amino acids, including glutamine, aspartate, leucine and isoleucine, and impaired mitochondrial TCA cycle. TDP-43 induced lactate accumulation and altered the balance between excitatory (glutamate) and inhibitory (GABA) neurotransmitters. Parkin restored amino acid levels, neurotransmitter balance and TCA cycle metabolism, rescuing neurons from TDP-43-induced apoptotic death. Furthermore, TDP-43 expression led to an increase in 4E-BP levels, perhaps altering translational control and deregulating amino acid synthesis; while Parkin reversed the effects of TDP-43 on the 4E-BP signaling pathway. Taken together, these data suggest that Parkin may affect TDP-43 localization and mitigate its effects on 4E-BP signaling and loss of amino acid homeostasis.

Keywords

Parkin; TDP-43; amino acids; ¹³C NMR; ALS

Introduction

Parkin is an E3 ubiquitin ligase involved in protein degradation, while *Park2* mutations result in loss of Parkin function, leading to autosomal recessive early onset Parkinson disease (Cookson & Bandmann 2010, Kitada *et al.* 1998, Lucking *et al.* 2000, Shimura *et al.* 2000). Transactivation response (TAR) DNA-binding protein 43 (TDP-43) is a 414 amino acid protein with DNA/RNA binding protein properties (Ou *et al.* 1995). TDP-43 binds to *Park2* mRNA and regulates its expression (Polymenidou *et al.* 2011). TDP-43 increases

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Parkin expression (Hebron et al. 2012, Polymenidou et al. 2011, Lagier-Tourenne et al. 2012), while TDP-43 depletion down-regulates Parkin mRNA in human stem-cell derived from motor neurons bearing TDP-43 aggregates (Lagier-Tourenne et al. 2012). In healthy neurons, TDP-43 is predominantly nuclear, but in neurodegeneration, including Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD-TDP, FTD hereafter), TDP-43 is translocated to the cytosol where it is ubiquitinated and/or phosphorylated and cleaved into smaller fragments (Hasegawa *et al.* 2008, Mackenzie *et al.* 2007, Neumann *et al.* 2007a, Neumann *et al.* 2007b, Neumann *et al.* 2006, Zhang *et al.* 2009, Yoshiyama *et al.* 2007, Leigh *et al.* 1991, Arai *et al.* 2006, Mackenzie *et al.* 2010). Lentiviral TDP-43 increases nuclear and cytosolic TDP-43 protein levels, while Parkin co-expression mediates TDP-43 ubiquitination, leading to translocation of nuclear TDP-43 to the cytosol (Hebron et al. 2012). These findings suggest that Parkin may mediate TDP-43 localization and modulate its role in many cellular processes.

Alterations of amino acid metabolism are recognized in neurodegenerative diseases (Greenamyre *et al.* 1985, Ellison *et al.* 1986, Young & Penney 1984, Plaitakis & Carosco 1987, Perry *et al.* 1987). Glutamate accumulation in cortical neurons increases nuclear TDP-43, which returns to its basal level following glutamate-induced injury (Zheng et al. 2012), and induction of glutamate excitotoxicity does not lead to cytosolic TDP-43 inclusions in cultured organotypic slices (Leggett et al. 2012). However, elevation of TDP-43 in the mouse forebrain reduces the level of glutamate dehydrogenase and γ -amino butyric acid (GABA) (Tsai et al. 2010), suggesting that perturbation of amino acid and neurotransmitter metabolism results from alterations of TDP-43 localization. We previously showed that up-regulation (Herman et al. 2011) or lentiviral expression of human wild type TDP-43 (Herman et al. 2012) can lead to pathological changes, including cleavage, aggregation and phosphorylation. Parkin affects TDP-43 localization (Hebron et al. 2012) and reverses amyloid-induced changes in mitochondrial tricarboxylic acid (TCA) cycle (Khandelwal *et al.* 2011, Algarzae *et al.* 2012). To determine the role of TDP-43 in amino acid metabolism and Parkin effects, we used lentiviral gene delivery that allows examination of the early effects of TDP-43 expression on brain metabolism *in vivo*. We examined the role of Parkin on TDP-43-mediated changes on amino acid and neurotransmitter metabolism using high frequency ^1H -decoupled ^{13}C nuclear magnetic resonance (NMR) spectroscopy.

Materials & Methods

Stereotaxic injection

Stereotaxic surgery was performed to inject the lentiviral constructs encoding either LacZ, Parkin and/or TDP-43 into the primary motor cortex of two-month-old male Sprague-Dawley rats weighing between 170-220g as previously described (Burns et al. 2009). Viral stocks were injected through a microsyringe pump controller (Micro4) using total pump (World Precision Instruments, Inc.) delivery of 6 μl at a rate of 0.2 $\mu\text{l}/\text{min}$. The needle (26 gauge) was slightly withdrawn every 2 min to avoid mechanical damage and remained in place at the injection site for an additional minute before slow removal over a period of 2 min. Animals were injected into left side of the motor cortex with 2×10^9 m.o.i (Multiplicity of infection (m.o.i) is the ratio of the number of lentiviral particles to the approximate number of target cells that can be potentially infected with at least one viral particle) Lv-LacZ and into the right side with 1×10^9 m.o.i Lv-Parkin+ 1×10^9 m.o.i Lv-LacZ; or 1×10^9 m.o.i Lv-TDP-43+ 1×10^9 m.o.i Lv-LacZ; or 1×10^9 m.o.i Lv-Parkin+ 1×10^9 m.o.i Lv-TDP-43. All animals were sacrificed two weeks post-injection and the left cortex was compared to the right cortex. A total of 8 animals for each treatment were used for Western blot (WB) and caspase-3 activity, 8 animals of each treatment (32 animals) for IHC, and 5 animals for NMR spectroscopy. This manuscript uses the same models that that we

previously published (Hebron et al. 2012) and the same lentiviral titer was used and the experiments were all conducted at the same time. Wild type C57BL/6 and homozygous Parkin^{-/-} mice generated on C57BL/6 background (Goldberg *et al.* 2003) were used for WB. Hemizygous *TDP-43* transgenic mice harboring human TDP-43 under the control of mouse Thy1 promoter and generated on C57BL/6 background (Wils *et al.* 2010) were bred according to Jackson's laboratories' protocol and used for WB. All studies were approved and conducted according to Georgetown University Animal Care and Use Committee (GUACAC).

WB analysis

The motor cortex was dissected out and homogenized in 1×STEN buffer then centrifuged at 5.000g and the supernatant was collected. Total TDP-43 was probed either with human-specific anti-TDP-43 (1:1000) mouse monoclonal (2E2-D3) antibody generated against N-terminal 261 amino acids of the full-length protein (Abnova) or (1:1000) rabbit polyclonal (ALS10) antibody (ProteinTech, catalog no. 10782-2-AP). Rabbit polyclonal anti-Parkin (PRK8) antibody (Millipore) was used (1:1000) for WB. Rabbit polyclonal antibodies for total 4E-BP1 (1:1000), Thr 37/46 phosphorylated 4E-BP1 (1:500), Ser 209 phosphorylated eIF-4E (1:500) and Thr 389 phospho-p70S^{6K} (1:500) were used (Translational control sampler kit, Cell Signaling Technology). Rabbit polyclonal antibodies for total mTOR (1:1000) and Ser 2448 phosphorylated mTOR (1:1000) were used (Translational control sampler kit, Cell Signaling Technology). A rabbit polyclonal V5 (1:1000) epitope (Invitrogen) and rabbit polyclonal anti-actin (Thermo Scientific) were used (1:1000). Rabbit polyclonal anti-excitatory amino acid transporter (EAAT)-1 (1:1000) antibody (Cell Signaling Technology), and rabbit polyclonal anti-EAAT2 (1:1000) antibody (Cell Signaling Technology), and rabbit polyclonal anti-EAAT3 (1:1000) antibody (Cell signaling Technology) were used. WB were quantified by densitometry using Quantity One 4.6.3 software (Bio Rad). Densitometry was obtained as arbitrary numbers measuring band intensity. Data were analyzed as mean±SEM, using ANOVA with Neumann Keuls multiple comparison between treatments.

Histology was performed on 20µm-thick brain sections to assess neural disintegrative degeneration in animal models using the FD NeuroSilver™ staining kit II (FD NeuroTechnologies, Inc., Baltimore, MD), which provides high contrast and rapid silver staining for the microscopic detection of neuronal and fiber degeneration *in vivo*. Total cell counts in cortical subfields were obtained by a blinded investigator using unbiased stereology analysis (Stereologer, Stereology Resource Center, Chester, MD). A minimum of six 20-µm sections were analyzed from 400 µm in each direction from the injection site in the ipsilateral area and an equivalent size area within the same region in the contralateral area. The multilevel sampling design in the Stereologer software, based on the optical fractionator sampling method, was used to estimate positive cell numbers detected by Nissl and Silver (Khandelwal et al. 2011).

Caspase-3 fluorometric activity assay

To measure caspase-3 activity in the animal models, we used EnzChekw caspase-3 assay kit #1 (Invitrogen) on cortical extracts and Z-DEVD-AMC substrate and the absorbance was read according to the manufacturer's protocol.

High frequency ¹³C/¹H Nuclear Magnetic Resonance (NMR) Spectroscopy

Animals were fasted overnight with free access to tap water and were intra-peritoneally (I.P) injected with [1-¹³C] glucose solution (0.5 mol/L) over 10s (0.3 ml/25–30 g body weight; 200 mg/kg). 45 min later, animals were sacrificed by cervical dislocation and hemispheres were isolated and immediately homogenized in 6% ice-cold perchloric acid (PCA), 50 mM

NaH₂PO₄. After homogenization, the PCA-de-proteinized supernatant were separated by centrifugation at 14000×g for 30 min, frozen on dry ice and lyophilized overnight. Extracts were then re-suspended in 0.65 mL D₂O containing 2 mM sodium [¹³C]formate as internal intensity and chemical shift reference (δ 171.8). Metabolite pool size was identified on ¹H {¹³C-decoupled} NMR spectra. Peak areas were adjusted for nuclear Overhauser effect, saturation and natural abundance effects and quantified by reference to [¹³C] formate. Metabolite pool sizes were determined by integration of resonances in 400 MHz {¹³C-decoupled} ¹H spectra using N-acetylaspartate as internal intensity reference. Incorporation of ¹³C into isotopomers was measured in reference to [¹³C]formate. ¹³C[¹H-decoupled] spectra typically 30000-33000 transients, pulse width is 4 seconds, 83,300 data points were acquired at 9.7 Tesla Varian Spectrometer with dual ¹³C/¹H probe. {¹³C-decoupled}-¹H spectra were acquired with 3000 scans, pulse angle 45°, relaxation delay 1s, line broadening 0.5 Hz, acquired data points 13.132 and transformation size 32K at room temperature. Spectra were integrated and quantified using MestReNova (Master Lab Research).

Statistical analysis

Data were analyzed using GraphPad PRISM software (San Diego, CA) and statistical analysis was performed using ANOVA, Neumann Keuls, $p < 0.05$ or as indicated, n is indicated in the text, and graphs were plotted mean±SEM.

Results

Parkin reverses TDP-43-induced cell death in rat motor cortex

We previously showed that injection of a lentiviral vector driving wild type TDP-43 expression in the rat motor cortex (Herman et al. 2012) leads to protein cleavage, aggregation and phosphorylation 2 weeks post-injection (Herman et al. 2012). We also demonstrated that TDP-43 is expressed in neurons as well as microglia and Parkin translocates TDP-43 from the nucleus to the cytosol (Hebron et al. 2012). These studies were extended to analyze amino acid metabolism via ¹³C NMR. Lentiviral injection significantly increased (44%, $n=5$, $P < 0.05$) TDP-43 levels in the rat motor cortex (Fig. 1B) compared to LacZ (Fig. 1A) and Parkin (Fig. 1C). However, Parkin co-expression led to TDP-43 localization within the cytoplasm in DAPI (4',6-Diamidino-2-Phenylindole, Dilactate)-stained cells (Fig. 1C), as we previously showed in these animal models (Hebron et al. 2012). Counterstaining TDP-43-stained cortical sections with 3,3'-Diaminobenzidine (DAB) showed TDP-43 expression throughout the motor cortex in TDP-43 (Fig. 1F&G) compared to LacZ (Fig. 1E) and Parkin (Fig. 1H). To determine TDP-43 effects on cell survival, Nissl-stained cells were quantified by stereological counting. TDP-43 significantly reduced (31%, $n=8$, $p < 0.03$) the number of Nissl-stained cells in the rat cortex (Fig. 1J&V) compared to LacZ (Fig. 1I&V) and Parkin (Fig. 1L&V); while Parkin co-injection (Fig. 1K&V) reversed Nissl-stained cell loss compared to TDP-43 alone. Co-staining showed endogenous TDP-43 and Parkin expression in LacZ cortex (Fig. 1M), but co-expression of Parkin with TDP-43 (Fig. 1O) resulted in less TDP-43 in DAPI-stained nuclei and more co-localization with cytosolic Parkin compared to TDP-43 (Fig. 1N) or Parkin alone (Fig. 1P, $n=8$). Neurodegenerative death was also demonstrated via silver staining, which showed significantly increased staining (56%, $n=8$, $p < 0.02$) in TDP-43 brains (Fig. 1R&V) compared to LacZ (Fig. 1Q&V) and Parkin (Fig. 1T&V). High magnification (Fig. 1Q) shows silver staining within cell bodies and axonal processes, suggesting that TDP-43 induces neuronal and axonal degeneration. Parkin reduced the number of silver-stained cells back to LacZ level (Fig. 1S&V). Caspase-3 activity was also measured via ELISA and showed that TDP-43 significantly increases caspase-3 levels (Fig. 1U, 94%, $n=8$, $p < 0.001$) compared to LacZ and Parkin; but Parkin co-expression prevents the increase in caspase-3 (Fig. 1V). Taken together, these data indicate that TDP-43 induces cell death in the rat

cortex and Parkin reverses TDP-43 effects. To verify equal gene expression via lentiviral delivery, WB blot analysis of lentiviral V5 epitope showed equal amount of V5 expression (Fig. 1W, 3rd blot) relative to actin in all injected animals. However, TDP-43 levels were significantly increased (44%, n=8, p<0.02) relative to actin (Fig. 1W, 2nd blot) in TDP-43 compared to LacZ or Parkin alone, while Parkin levels were increased (31%, n=8, p<0.04) relative to actin (Fig. 1W) compared to LacZ.

Parkin restores TDP-43-induced alteration of amino acid and mitochondrial TCA cycle metabolism

High frequency $^{13}\text{C}/^1\text{H}$ NMR spectroscopy was performed in cortical extracts to compare the effects of Parkin on TDP-43 in the right hemisphere (ipsilateral) compared to LacZ or Parkin in left hemisphere (contralateral) 2 weeks post-injection. ^1H -decoupled/ ^{13}C MRS show that TDP-43 significantly increased (50%, n=5, p<0.0001) the concentration of ^{13}C label in glutamate (Glu) C4 compared to LacZ and Parkin (Fig. 2A). Parkin co-expression with TDP-43 reversed ^{13}C concentration in Glu C4 back to LacZ level (Fig. 2A). TDP-43 decreased ^{13}C in glutamine (Gln) C4 (34%, n=5, p<0.002) compared to LacZ and Parkin; while Parkin co-expression with TDP-43 restored ^{13}C back to LacZ level (Fig. 2A). ^{13}C -decoupled ^1H MRS showed that TDP-43 induced a significant increase in total glutamate (21%, p<0.05, n=5) and a decrease in glutamine (28%, p<0.05, n=5) compared to LacZ and Parkin, which reversed glutamate and glutamine levels back to control (Fig. 2B). Aspartate levels were also measured because it is an excitatory amino acid that can be transported by EAATs similar to glutamate. TDP-43 significantly decreased ^{13}C concentration in aspartate (Asp) C2 and C3 (47% and 42% respectively, n=5, p<0.002) compared to LacZ and Parkin (Fig. 2C), but this effect was restored by Parkin (Fig. 2C). TDP-43 significantly decreased (21%, p<0.05, n=5) aspartate pool size compared to LacZ and Parkin, which reversed aspartate level back to control (Fig. 2D). Measurement of TCA cycle metabolism showed that TDP-43 decreased ^{13}C concentration in succinate (Succ) C2/C3 and citrate (Cit) C3 (53% and 44%, respectively, n=5, p<0.002) compared to LacZ and Parkin (Fig. 2E) and Parkin co-expression reversed ^{13}C concentration in Succ C2/C3 and Cit C2 back to LacZ level (Fig. 2E). The decrease of ^{13}C flux into TCA cycle intermediates succinate and citrate was echoed by a decrease in metabolite concentration (62% and 51%, respectively, p<0.0001, n=5), while Parkin reversed mitochondrial TCA metabolism back to control (Fig. 2F). Because leucine and isoleucine are branched-chain amino acids that feed into the TCA cycle (Sears *et al.* 2009), their metabolism was examined to determine whether they are affected by TCA cycle perturbation. TDP-43 significantly decreased (21% and 18%, respectively, n=5, p<0.05) ^{13}C concentration in leucine (Leu) C4 and isoleucine (Isoleu) C4 compared to LacZ and Parkin (Fig. 2G). Parkin co-expression with TDP-43 reversed ^{13}C concentration in Leu C4 and Isoleu C4 back to LacZ level (Fig. 2G). TDP-43 also decreased (32% and 22%, respectively, p<0.05, n=5) the pool sizes of leucine and isoleucine compared to LacZ and Parkin, which returned leucine and isoleucine levels back to control (Fig. 2H).

Parkin abrogates TDP-43-induced oxidative stress and loss of GABA neurotransmitter

Lactate metabolism was measured to determine whether TDP-43 alteration of mitochondrial TCA cycle induces oxidative stress and cytosolic lactate accumulation. TDP-43 induced a significant increase (29%, n=5, p<0.04) in ^{13}C concentration in lactate (Lac) C3 compared to LacZ and Parkin (Fig. 3A). However, Parkin expression with TDP-43 reversed alteration of ^{13}C levels in Lac C3, consistent with Parkin effects on TCA cycle intermediates. Additionally, TDP-43 significantly increased lactate level (38%, n=5, p<0.001) and Parkin restored lactate back to LacZ and Parkin levels (Fig. 3B). Taken together, these data indicate oxidative stress and lactate accumulation as a result of TDP-43-induced mitochondrial TCA cycle changes. GABA levels were also measured to determine whether the changes in excitatory glutamate affected the inhibitory neurotransmitter GABA, which is in part

derived from glutamate. TDP-43 significantly decreased ^{13}C concentration in GABA C2 (Fig. 3C, 50%, $n=5$, $p<0.0001$) compared to LacZ and Parkin, but Parkin restored the decrease of ^{13}C in GABA C2. TDP-43 also decreased the total level of GABA (62%, $n=5$, $p<0.0001$) compared to LacZ (Fig. 3D). These data suggest that TDP-43 expression alters neurotransmitter levels and disturbs the balance between inhibitory and excitatory neurotransmitters. However, Parkin reverses GABA level when co-expressed with TDP-43, but GABA remains significantly less than LacZ level (21%, $p<0.004$).

To ascertain that TDP-43 did not change glutamate and aspartate metabolism via alterations of EAAT levels, which transport glutamate as well as aspartate, WB was performed and showed no difference in the protein levels of EAAT1, 2 and 3 relative to actin (Fig. 3E), indicating that the changes in glutamate and aspartate were not due to altered levels of transporters.

TDP-43 activates 4E-BP, independent of mTOR, signaling to affect amino acid levels

We examined the effects of TDP-43 expression on markers of translational control, including the mammalian target of Rapamycin (mTOR) pathway that can affect amino acid metabolism. Inhibition of mTOR inhibits the translation factor eIF-4E that is modulated by eIF-4E-binding proteins (4E-BPs) phosphorylation and cap-dependent translation (Gingras *et al.* 2001, Grolleau *et al.* 2002). Kinase signaling via mTOR affects mRNA translation through phosphorylation of downstream kinases such as 4E-BPs and p70S^{6k} (Gingras *et al.* 2001, Grolleau *et al.* 2002, Shima *et al.* 1998, Mamane *et al.* 2004). No differences were observed in total mTOR (Fig. 4A, 1st blot) or phosphorylation at Ser 2448 (Fig. 4A, 2nd blot) between TDP-43, Parkin±TDP-43 and LacZ. The phosphorylation of p70S^{6k} at Thr389 (3rd blot) was also unaffected between different treatments, suggesting lack of mTOR activation. However, a significant increase in total levels (55%, $n=8$, $p<0.001$) and Thr 37/46 phosphorylated (42%, $p<0.01$) 4E-BP1 were observed (Fig. 4B&C), indicating increased kinase activity in TDP-43 brains compared to LacZ. A significant decrease (51%, $n=8$, $p<0.001$) in eIF-4E phosphorylation at Ser 209 was observed downstream of 4E-BP1 (Fig. 4B&C), suggesting that TDP-43 increases total 4E-BP1 and may modulate translational control, independent of mTOR activity. Parkin co-expression with TDP-43 significantly reversed total (27%, $n=8$, $p<0.04$) and phosphorylated (39%, $p<0.01$) 4E-BP1 (Fig. 4D&E), while significantly increasing (19%, $n=8$, $p<0.05$) eIF-4E phosphorylation (Fig. 4D&E), suggesting that Parkin expression modulates TDP-43 effects on 4E-BP signaling.

These results were verified in 4-5 months old male control C57BL/6, homozygous Parkin^{-/-} (Goldberg *et al.* 2003) and hemizygous *TDP-43* transgenic mice harboring human TDP-43 (Wils *et al.* 2010) all generated on C57BL/6 background. Significantly higher TDP-43 levels (190%, $n=3$, $p<0.0001$) were observed in TDP-43 transgenic mice relative to actin compared to control (Fig. 4F&G). However, Parkin^{-/-} mice (1st blot) had significantly increased levels of TDP-43 relative to actin (85%, $n=3$, $p<0.002$) compared to control (Fig. 4F&G). A significant increase in the levels of total (165%, $n=3$, $p<0.002$) and phosphorylated (91%, $P<0.01$) 4E-BP1 (Fig. 4F&G) were observed in TDP-43 transgenic mice, indicating increased kinase activity. Similarly, a significant increase in the levels of both total (70%, $n=3$, $p<0.02$) and phosphorylated (43%, $p<0.05$) 4E-BP1 (Fig. 4F&G) were observed in Parkin^{-/-} mice. Furthermore, a significant decrease (71%, $p<0.001$, $n=3$) in eIF-4E phosphorylation (Fig. 4F&G) was observed in TDP-43 mice. Phosphorylated eIF-4E was reduced (19%) in Parkin^{-/-} mice. Taken together, these results suggest that Parkin attenuates the effects of TDP-43 on perturbations of translational control.

Discussion

These studies reveal the early effects of TDP-43 on amino acid metabolism. TDP-43 increased glutamate levels and decreased the levels of other amino acids, including its precursor glutamine and the excitatory amino acid aspartate. The increase in glutamate level was associated with apoptotic cell death and increased oxidative stress, including decreased mitochondrial TCA cycle metabolism and cytosolic lactate accumulation. The level of cytotoxic glutamate remained elevated after TDP-43 over-expression despite the decrease in TCA metabolism and glutamine level, suggesting lack of glutamate re-cycling via EAATs. The current data suggest that the effects of TDP-43 on brain metabolism, may be due to neuronal and/or astrocytic changes as lentiviral TDP-43 is expressed in both cell types (Herman et al. 2012). We previously demonstrated that alterations of glutamate transport significantly change ^{13}C flux through the TCA cycle and affect glutamate/glutamine cycling rates in guinea pig brain slices (Moussa *et al.* 2007, Moussa Cel *et al.* 2002). Principal component analysis of ^{13}C -labeled metabolome suggested that selective inhibitions of individual EAATs lead to characteristic changes in brain metabolism. This observation indicates that each EAAT may have a very distinctive role in brain function and small perturbations in its expression and/or location could have far reaching - possibly adverse - consequences (Moussa et al. 2007, Rae *et al.* 2006, Rae *et al.* 2005).

Parkin reversed TDP-43 effects on TCA cycle and amino acid metabolism, perhaps due to Parkin role in nuclear TDP-43 translocation (Hebron et al. 2012). Nuclear TDP-43 regulates thousands of gene expression and is implicated in many steps of RNA expression and transport (Polymenidou *et al.* 2011, Tollervey *et al.* 2011), so its nuclear translocation may affect control of amino acid synthesis. TDP-43 binds to *Park2* mRNA and regulates its expression (Polymenidou et al. 2011), and we demonstrated that lentiviral TDP-43 over-expression increases *Park2* mRNA and protein levels *in vitro* and *in vivo* (Hebron et al. 2012). Inversely, TDP-43 depletion down-regulates *Park2* mRNA in human stem-cell derived motor neurons in sporadic ALS (Lagier-Tourenne et al. 2012). Parkin stability is also altered in TDP-43 mice harboring the A315T mutation (Hebron et al. 2012). In post-mortem brains of human ALS and FTD, Parkin level is altered in motor neurons containing TDP-43 aggregates (Lagier-Tourenne et al. 2012). TDP-43 modulation of Parkin levels (Shimura et al. 2000) and detection of ubiquitin-positive TDP-43 inclusions in neurodegeneration (Hasegawa et al. 2008, Mackenzie et al. 2007, Neumann et al. 2007a, Neumann et al. 2007b, Neumann et al. 2006, Zhang et al. 2009, Yoshiyama et al. 2007), suggest that Parkin may mediate TDP-43 localization via ubiquitination. We showed that co-expression of lentiviral TDP-43 and Parkin promotes K-48 and K-63-linked ubiquitin to TDP-43, leading to translocation of nuclear TDP-43 (Hebron et al. 2012). Together these data suggest that Parkin mediates TDP-43 localization via ubiquitination.

In healthy neurons, TDP-43 is predominantly nuclear, but in neurodegeneration, including ALS and FTD, TDP-43 is translocated to the cytosol where it is ubiquitinated and/or phosphorylated and cleaved (Hasegawa et al. 2008, Mackenzie et al. 2007, Neumann et al. 2007a, Neumann et al. 2007b, Neumann et al. 2006, Zhang et al. 2009, Yoshiyama et al. 2007, Leigh et al. 1991, Arai et al. 2006, Mackenzie et al. 2010). It is still unknown whether nuclear or cytosolic (or both) TDP-43 is the culprit in ALS/FTD pathology. Patients with sporadic ALS, as well as familial ALS due to TDP-43 mutations, show abnormal accumulations of TDP-43 in the cytoplasm of spinal cord motor neurons (Neumann et al. 2006, Mackenzie et al. 2007). TDP-43 also accumulates in ubiquitin-positive cytoplasmic or nuclear inclusions in FTD and ALS (Kwiatkowski *et al.* 2009, Vance *et al.* 2009). Therefore, TDP-43 localization may be essential for its biological function. The sheer length of motor neurons in the descending spinal motor tracts, which degenerate in ALS (Dickson *et al.* 2007, Geser *et al.* 2008, McCluskey *et al.* 2009, Mitchell & Borasio 2007), may make

these neurons more vulnerable to alterations in TDP-43 localization compared to other neurons bearing TDP-43 aggregates. Many RNA binding proteins exhibit dual functions, including nuclear, such as mRNA splicing, and cytoplasmic, such as mRNA transport and silencing (Sau *et al.* 2011). Deregulation of axodendritic transport is associated with gene mutations causing motor neuron disorders (Sau *et al.* 2011). Thus, TDP-43 transport from the soma to distant synapses may be required for generation of synaptic proteins. Relevant to the current studies, TDP-43 binds to mRNA of several synaptic proteins and may regulate the level of vesicular glutamate transporters (vGluT), glutamate receptors, and synaptotagmin, which controls synaptic vesicle dynamics (Polymenidou *et al.* 2011). Thus, future studies should focus on Parkin-mediated TDP-43 localization to the synapse and maintenance of glutamate metabolism in motor versus non-motor neurons. Recent findings suggest that glutamate increases nuclear TDP-43 (Zheng *et al.* 2012), while glutamate excitotoxicity does not lead to cytosolic TDP-43 inclusions (Leggett *et al.* 2012). Defects in glutamate transmission are widely implicated in neurodegeneration (Rahn *et al.* 2012). The level of some plasma membrane glutamate transporters is altered in ALS, perhaps leading to excitotoxicity and motor neuron degeneration in human ALS patients (Rothstein 1995, Wilson & Shaw 2007, Quinlan 2011). Taken together, these findings and our data suggest that TDP-43 translocation may depend on glutamate levels, which could exacerbate TDP-43 pathology. Additionally, Parkin role in reversing mitochondrial TCA cycle metabolism may also be due to its effects on mitochondrial dynamics (Ziviani *et al.* 2010, Poole *et al.* 2010, Deng *et al.* 2008, Tanaka 2010), which promotes mitochondrial fragmentation during mitophagy (Tanaka *et al.* 2010), thus leading to replenishment of functional mitochondria and increased TCA cycle metabolism.

Parkin plays an important role in synaptic transmission and Parkin deficient animals display defects in synaptic function. Parkin over-expression reverses overgrown neuromuscular synapses and reduces evoked and miniature excitatory junction potentials in motor neurons axons of Parkin mutant larvae (Vincent *et al.* 2012). Parkin regulates the function and stability of glutamatergic synapses, and Parkin knockdown induces glutamatergic synapse proliferation, leading to excitotoxicity (Helton *et al.* 2008). Proteomic analysis of Parkin^{-/-} brain lysates show alterations in proteins that are essential for synaptic function (Periquet *et al.* 2005). Synaptotagmin XI is a member of the synaptotagmin family that is well characterized for vesicle formation and docking (Schiavo *et al.* 1997, Fukuda *et al.* 2000). Parkin regulates the function of synaptotagmin that controls storage, docking and release of vesicular glutamate (Huynh *et al.* 2003). Synaptic stimulation triggers local protein synthesis at the synapse (Jiang & Schuman 2002); while inhibition of protein synthesis disrupts synaptic plasticity, including long-term facilitation, long-term potentiation (LTP), and long-term depression (LTD) (Kang & Schuman 1996, Martin *et al.* 1997, Huber *et al.* 2000). Therefore, local translational control impacts at the level of individual synapses. TDP-43 availability at the synapse may be important to maintain homeostatic level of synaptic proteins. TDP-43 binds to the mRNA of a large number of proteins that are integral to synaptic function and formation (Polymenidou *et al.* 2011), including synaptotagmin, glutamate transporters, glutamate receptors and astrocytic glutamate transporters (Polymenidou *et al.* 2011). One form of LTP, long-lasting late-phase LTP (L-LTP), requires both gene transcription and RNA translation (Klann & Dever 2004). For example, hippocampal LTD mediated by metabotropic glutamate receptors requires rapid translation of preexisting mRNA (Huber *et al.* 2000). Thus, Parkin may affect TDP-43 localization and increase its bio-availability at synapses to maintain synaptic glutamate metabolism.

The effects of TDP-43 expression on the balance between excitatory and inhibitory neurotransmitters are novel. The decrease in GABA was concurrent with increased glutamate, which is one precursor of GABA synthesis via glutamate dehydrogenase activity. These results are consistent with prior studies indicating that TDP-43 expression reduces the

level of glutamate dehydrogenase and GABA levels in the mouse brain (Tsai et al. 2010). Additionally, GABA may be derived from succinate, further suggesting that TDP-43 expression targets neurotransmitter synthesis. The simultaneous change in glutamate and GABA are indicative that TDP-43 expression affects neurotransmitter balance, resulting in increased excitatory and decreased inhibitory mechanisms. The change in neurotransmitter levels may affect brain regions that are associated with specific neurodegenerative changes, leading to excito-toxic damage and modulation of neurotransmitter systems in a disease-dependent manner. Furthermore, these studies tested the early effects of TDP-43 expression, independent of the developmental effects of the transgene in animal models expressing TDP-43 or its mutations, therefore other changes may be associated with neurotransmitter synthesis. TDP-43 may affect translational control via regulation of splicing and transcription of many mRNAs. The effects of TDP-43 expression on 4E-BP activity may affect translational control and amino acid metabolism (Sears et al. 2009), including leucine and isoleucine, which affect the mTOR pathway (Herningtyas *et al.* 2008, Matsumura *et al.* 2005). In the current studies, expression of TDP-43 did not change mTOR activity or P70S^{6k} phosphorylation 2 weeks post-injection. TDP-43 up-regulated the level of total 4E-BP and increased its phosphorylation, leading to decreased phosphorylation of eIF4E, suggesting that TDP-43 affects translation initiation downstream of mTOR. TDP-43 may stimulate the phosphorylation of 4E-BP1 to decrease interaction between eIF4E and 4E-BP, and consequently activate cap-dependent translation (Gingras et al. 2001, Grolleau et al. 2002). Interestingly, TDP-43 over-expression increased glutamate levels, but decreased all other amino acids, including aspartate, leucine, isoleucine and glutamine, suggesting a role for translational control in TDP-43 induced alterations of amino acid and neurotransmitter levels.

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References

- Algarzae N, Hebron M, Miessau M, Moussa CE. Parkin prevents cortical atrophy and Abeta-induced alterations of brain metabolism: (1)(3)C NMR and magnetic resonance imaging studies in AD models. *Neuroscience*. 2012; 225:22–34. [PubMed: 22960314]
- Arai T, Hasegawa M, Akiyama H, et al. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun*. 2006; 351:602–611. [PubMed: 17084815]
- Burns MP, Zhang L, Rebeck GW, Querfurth HW, Moussa CE. Parkin promotes intracellular Abeta1-42 clearance. *Hum Mol Genet*. 2009; 18:3206–3216. [PubMed: 19483198]
- Cookson MR, Bandmann O. Parkinson's disease: insights from pathways. *Hum Mol Genet*. 2010; 19:R21–27. [PubMed: 20421364]
- Deng H, Dodson MW, Huang H, Guo M. The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in *Drosophila*. *Proc Natl Acad Sci U S A*. 2008; 105:14503–14508. [PubMed: 18799731]
- Dickson DW, Josephs KA, Amador-Ortiz C. TDP-43 in differential diagnosis of motor neuron disorders. *Acta Neuropathol*. 2007; 114:71–79. [PubMed: 17569066]
- Ellison DW, Beal MF, Mazurek MF, Bird ED, Martin JB. A postmortem study of amino acid neurotransmitters in Alzheimer's disease. *Ann Neurol*. 1986; 20:616–621. [PubMed: 2878639]
- Fukuda M, Moreira JE, Liu V, Sugimori M, Mikoshiba K, Llinas RR. Role of the conserved WHXL motif in the C terminus of synaptotagmin in synaptic vesicle docking. *Proc Natl Acad Sci U S A*. 2000; 97:14715–14719. [PubMed: 11114192]

- Geser F, Brandmeir NJ, Kwong LK, Martinez-Lage M, Elman L, McCluskey L, Xie SX, Lee VM, Trojanowski JQ. Evidence of multisystem disorder in whole-brain map of pathological TDP-43 in amyotrophic lateral sclerosis. *Arch Neurol*. 2008; 65:636–641. [PubMed: 18474740]
- Gingras AC, Raught B, Gygi SP, et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev*. 2001; 15:2852–2864. [PubMed: 11691836]
- Goldberg MS, Fleming SM, Palacino JJ, et al. Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. *J Biol Chem*. 2003; 278:43628–43635. [PubMed: 12930822]
- Greenamyre JT, Penney JB, Young AB, D'Amato CJ, Hicks SP, Shoulson I. Alterations in L-glutamate binding in Alzheimer's and Huntington's diseases. *Science*. 1985; 227:1496–1499. [PubMed: 2858129]
- Grolleau A, Bowman J, Pradet-Balade B, Puravs E, Hanash S, Garcia-Sanz JA, Beretta L. Global and specific translational control by rapamycin in T cells uncovered by microarrays and proteomics. *J Biol Chem*. 2002; 277:22175–22184. [PubMed: 11943782]
- Hasegawa M, Arai T, Nonaka T, et al. Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Ann Neurol*. 2008; 64:60–70. [PubMed: 18546284]
- Hebron ML, Lonskaya I, Sharpe K, Weerasinghe PP, Algarzae NK, Shekanyan AR, Moussa CE. Parkin ubiquitinates Tar-DNA binding protein-43 (TDP-43) and promotes its cytosolic accumulation via interaction with histone deacetylase 6 (HDAC6). *J Biol Chem*. 2012
- Helton TD, Otsuka T, Lee MC, Mu Y, Ehlers MD. Pruning and loss of excitatory synapses by the parkin ubiquitin ligase. *Proc Natl Acad Sci U S A*. 2008; 105:19492–19497. [PubMed: 19033459]
- Herman AM, Khandelwal PJ, Rebeck GW, Moussa CE. Wild type TDP-43 induces neuroinflammation and alters APP metabolism in lentiviral gene transfer models. *Exp Neurol*. 2012
- Herman AM, Khandelwal PJ, Stanczyk BB, Rebeck GW, Moussa CE. beta-amyloid triggers ALS-associated TDP-43 pathology in AD models. *Brain Res*. 2011; 1386:191–199. [PubMed: 21376022]
- Herningtyas EH, Okimura Y, Handyaningsih AE, Yamamoto D, Maki T, Iida K, Takahashi Y, Kaji H, Chihara K. Branched-chain amino acids and arginine suppress MaFbx/atrogen-1 mRNA expression via mTOR pathway in C2C12 cell line. *Biochim Biophys Acta*. 2008; 1780:1115–1120. [PubMed: 18616983]
- Huber KM, Kayser MS, Bear MF. Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. *Science*. 2000; 288:1254–1257. [PubMed: 10818003]
- Huynh DP, Scoles DR, Nguyen D, Pulst SM. The autosomal recessive juvenile Parkinson disease gene product, parkin, interacts with and ubiquitinates synaptotagmin XI. *Hum Mol Genet*. 2003; 12:2587–2597. [PubMed: 12925569]
- Jiang C, Schuman EM. Regulation and function of local protein synthesis in neuronal dendrites. *Trends Biochem Sci*. 2002; 27:506–513. [PubMed: 12368086]
- Kang H, Schuman EM. A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. *Science*. 1996; 273:1402–1406. [PubMed: 8703078]
- Khandelwal PJ, Herman AM, Hoe HS, Rebeck GW, Moussa CE. Parkin mediates beclin-dependent autophagic clearance of defective mitochondria and ubiquitinated Abeta in AD models. *Hum Mol Genet*. 2011; 20:2091–2102. [PubMed: 21378096]
- Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*. 1998; 392:605–608. [PubMed: 9560156]
- Klann E, Dever TE. Biochemical mechanisms for translational regulation in synaptic plasticity. *Nat Rev Neurosci*. 2004; 5:931–942. [PubMed: 15550948]
- Kwiatkowski TJ Jr, Bosco DA, Leclerc AL, et al. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science*. 2009; 323:1205–1208. [PubMed: 19251627]
- Lagier-Tourenne C, Polymenidou M, Hutt KR, et al. Divergent roles of ALS-linked proteins FUS/TLS and TDP-43 intersect in processing long pre-mRNAs. *Nat Neurosci*. 2012
- Leggett C, McGehee DS, Mastrianni J, Yang W, Bai T, Brorson JR. Tunicamycin produces TDP-43 cytoplasmic inclusions in cultured brain organotypic slices. *J Neurol Sci*. 2012; 317:66–73. [PubMed: 22459357]

- Leigh PN, Whitwell H, Garofalo O, Buller J, Swash M, Martin JE, Gallo JM, Weller RO, Anderton BH. Ubiquitin-immunoreactive intraneuronal inclusions in amyotrophic lateral sclerosis. Morphology, distribution, and specificity. *Brain*. 1991; 114(Pt 2):775–788. [PubMed: 1646064]
- Lucking CB, Durr A, Bonifati V, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene. *N Engl J Med*. 2000; 342:1560–1567. [PubMed: 10824074]
- Mackenzie IR, Bigio EH, Ince PG, et al. Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. *Ann Neurol*. 2007; 61:427–434. [PubMed: 17469116]
- Mackenzie IR, Rademakers R, Neumann M. TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia. *Lancet Neurol*. 2010; 9:995–1007. [PubMed: 20864052]
- Mamane Y, Petroulakis E, Rong L, Yoshida K, Ler LW, Sonenberg N. eIF4E--from translation to transformation. *Oncogene*. 2004; 23:3172–3179. [PubMed: 15094766]
- Martin KC, Casadio A, Zhu H, Yaping E, Rose JC, Chen M, Bailey CH, Kandel ER. Synapse-specific, long-term facilitation of aplysia sensory to motor synapses: a function for local protein synthesis in memory storage. *Cell*. 1997; 91:927–938. [PubMed: 9428516]
- Matsumura T, Morinaga Y, Fujitani S, Takehana K, Nishitani S, Sonaka I. Oral administration of branched-chain amino acids activates the mTOR signal in cirrhotic rat liver. *Hepatol Res*. 2005; 33:27–32. [PubMed: 16169275]
- McCluskey LF, Elman LB, Martinez-Lage M, Van Deerlin V, Yuan W, Clay D, Siderowf A, Trojanowski JQ. Amyotrophic lateral sclerosis-plus syndrome with TAR DNA-binding protein-43 pathology. *Arch Neurol*. 2009; 66:121–124. [PubMed: 19139310]
- Mitchell JD, Borasio GD. Amyotrophic lateral sclerosis. *Lancet*. 2007; 369:2031–2041. [PubMed: 17574095]
- Moussa CE, Rae C, Bubb WA, Griffin JL, Deters NA, Balcar VJ. Inhibitors of glutamate transport modulate distinct patterns in brain metabolism. *J Neurosci Res*. 2007; 85:342–350. [PubMed: 17086545]
- Moussa Cel H, Mitrovic AD, Vandenberg RJ, Provis T, Rae C, Bubb WA, Balcar VJ. Effects of L-glutamate transport inhibition by a conformationally restricted glutamate analogue (2S,1'S,2'R)-2-(carboxycyclopropyl)glycine (L-CCG III) on metabolism in brain tissue in vitro analysed by NMR spectroscopy. *Neurochem Res*. 2002; 27:27–35. [PubMed: 11926273]
- Neumann M, Kwong LK, Sampathu DM, Trojanowski JQ, Lee VM. TDP-43 proteinopathy in frontotemporal lobar degeneration and amyotrophic lateral sclerosis: protein misfolding diseases without amyloidosis. *Arch Neurol*. 2007a; 64:1388–1394. [PubMed: 17923623]
- Neumann M, Mackenzie IR, Cairns NJ, et al. TDP-43 in the ubiquitin pathology of frontotemporal dementia with VCP gene mutations. *J Neuropathol Exp Neurol*. 2007b; 66:152–157. [PubMed: 17279000]
- Neumann M, Sampathu DM, Kwong LK, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science*. 2006; 314:130–133. [PubMed: 17023659]
- Ou SH, Wu F, Harrich D, Garcia-Martinez LF, Gaynor RB. Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs. *J Virol*. 1995; 69:3584–3596. [PubMed: 7745706]
- Periquet M, Corti O, Jacquier S, Brice A. Proteomic analysis of parkin knockout mice: alterations in energy metabolism, protein handling and synaptic function. *J Neurochem*. 2005; 95:1259–1276. [PubMed: 16150055]
- Perry TL, Hansen S, Jones K. Brain glutamate deficiency in amyotrophic lateral sclerosis. *Neurology*. 1987; 37:1845–1848. [PubMed: 2891083]
- Plaitakis A, Caroscio JT. Abnormal glutamate metabolism in amyotrophic lateral sclerosis. *Ann Neurol*. 1987; 22:575–579. [PubMed: 2892463]
- Polymenidou M, Lagier-Tourenne C, Hutt KR, et al. Long pre-mRNA depletion and RNA missplicing contribute to neuronal vulnerability from loss of TDP-43. *Nat Neurosci*. 2011; 14:459–468. [PubMed: 21358643]
- Poole AC, Thomas RE, Yu S, Vincow ES, Pallanck L. The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. *PLoS One*. 2010; 5:e10054. [PubMed: 20383334]

- Quinlan KA. Links between electrophysiological and molecular pathology of amyotrophic lateral sclerosis. *Integr Comp Biol.* 2011; 51:913–925. [PubMed: 21989221]
- Rae C, Moussa Cel H, Griffin JL, Bubb WA, Wallis T, Balcar VJ. Group I and II metabotropic glutamate receptors alter brain cortical metabolic and glutamate/glutamine cycle activity: a ¹³C NMR spectroscopy and metabolomic study. *J Neurochem.* 2005; 92:405–416. [PubMed: 15663488]
- Rae C, Moussa Cel H, Griffin JL, Parekh SB, Bubb WA, Hunt NH, Balcar VJ. A metabolomic approach to ionotropic glutamate receptor subtype function: a nuclear magnetic resonance in vitro investigation. *J Cereb Blood Flow Metab.* 2006; 26:1005–1017. [PubMed: 16395280]
- Rahn KA, Slusher BS, Kaplin AI. Glutamate in CNS neurodegeneration and cognition and its regulation by GCPII inhibition. *Curr Med Chem.* 2012; 19:1335–1345. [PubMed: 22304712]
- Rothstein JD. Excitotoxicity and neurodegeneration in amyotrophic lateral sclerosis. *Clin Neurosci.* 1995; 3:348–359. [PubMed: 9021256]
- Sau D, Rusmini P, Crippa V, Onesto E, Bolzoni E, Ratti A, Poletti A. Dysregulation of axonal transport and motoneuron diseases. *Biol Cell.* 2011; 103:87–107. [PubMed: 21250942]
- Schiavo G, Stenbeck G, Rothman JE, Sollner TH. Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. *Proc Natl Acad Sci U S A.* 1997; 94:997–1001. [PubMed: 9023371]
- Sears DD, Hsiao G, Hsiao A, Yu JG, Courtney CH, Ofrecio JM, Chapman J, Subramaniam S. Mechanisms of human insulin resistance and thiazolidinedione-mediated insulin sensitization. *Proc Natl Acad Sci U S A.* 2009; 106:18745–18750. [PubMed: 19841271]
- Shima H, Pende M, Chen Y, Fumagalli S, Thomas G, Kozma SC. Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. *EMBO J.* 1998; 17:6649–6659. [PubMed: 9822608]
- Shimura H, Hattori N, Kubo S, et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat Genet.* 2000; 25:302–305. [PubMed: 10888878]
- Tanaka A. Parkin-mediated selective mitochondrial autophagy, mitophagy: Parkin purges damaged organelles from the vital mitochondrial network. *FEBS Lett.* 2010; 584:1386–1392. [PubMed: 20188730]
- Tanaka A, Cleland MM, Xu S, Narendra DP, Suen DF, Karbowski M, Youle RJ. Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *J Cell Biol.* 2010; 191:1367–1380. [PubMed: 21173115]
- Tollervey JR, Curk T, Rogelj B, et al. Characterizing the RNA targets and position-dependent splicing regulation by TDP-43. *Nat Neurosci.* 2011; 14:452–458. [PubMed: 21358640]
- Tsai KJ, Yang CH, Fang YH, et al. Elevated expression of TDP-43 in the forebrain of mice is sufficient to cause neurological and pathological phenotypes mimicking FTL-D. *J Exp Med.* 2010; 207:1661–1673. [PubMed: 20660618]
- Vance C, Rogelj B, Hortobagyi T, et al. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science.* 2009; 323:1208–1211. [PubMed: 19251628]
- Vincent A, Briggs L, Chatwin GF, et al. parkin-induced defects in neurophysiology and locomotion are generated by metabolic dysfunction and not oxidative stress. *Hum Mol Genet.* 2012; 21:1760–1769. [PubMed: 22215442]
- Wils H, Kleinberger G, Janssens J, et al. TDP-43 transgenic mice develop spastic paralysis and neuronal inclusions characteristic of ALS and frontotemporal lobar degeneration. *Proc Natl Acad Sci U S A.* 2010; 107:3858–3863. [PubMed: 20133711]
- Wilson JM, Shaw CA. Late appearance of glutamate transporter defects in a murine model of ALS-parkinsonism dementia complex. *Neurochem Int.* 2007; 50:1067–1077. [PubMed: 17095122]
- Yoshiyama Y, Higuchi M, Zhang B, et al. Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. *Neuron.* 2007; 53:337–351. [PubMed: 17270732]
- Young AB, Penney JB. Neurochemical anatomy of movement disorders. *Neurol Clin.* 1984; 2:417–433. [PubMed: 6152481]
- Zhang YJ, Xu YF, Cook C, et al. Aberrant cleavage of TDP-43 enhances aggregation and cellular toxicity. *Proc Natl Acad Sci U S A.* 2009; 106:7607–7612. [PubMed: 19383787]

- Zheng M, Liao M, Cui T, Tian H, Fan DS, Wan Q. Regulation of nuclear TDP-43 by NR2A-containing NMDA receptors and PTEN. *J Cell Sci.* 2012; 125:1556–1567. [PubMed: 22526419]
- Ziviani E, Tao RN, Whitworth AJ. Drosophila parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. *Proc Natl Acad Sci U S A.* 2010; 107:5018–5023. [PubMed: 20194754]

Figure 1. Parkin reverses TDP-43-induced apoptotic cell death in rat motor cortex

TDP-43 and DAPI staining of 20 μ m thick brain sections injected with lentiviral vector driving expression of A) LacZ, B) TDP-43, C) TDP-43 and Parkin and D) Parkin. Low magnification shows TDP-43 expression in DAB counterstained 20 μ m thick brain sections injected with lentiviral vector driving expression of E) LacZ, F) TDP-43, G) TDP-43 and Parkin, and H) Parkin. Nissl staining of 20 μ m thick brain sections injected with lentiviral vector driving expression of I) LacZ, J) TDP-43, K) TDP-43 and Parkin, and L) Parkin alone. TDP-43 and Parkin co-staining of 20 μ m thick brain sections injected with lentiviral vector driving expression of M) LacZ, N) TDP-43, insert shows silver staining in cell body and axonal processes, O) TDP-43 and Parkin and P) Parkin. Silver staining of 20 μ m thick brain sections injected with lentiviral vector driving expression of Q) LacZ R) TDP-43 S) Parkin and TDP-43 and T) Parkin alone injected brains. Histograms represent U) caspase-3 activity and V) stereological counting of Nissl and silver-positive cells in lentiviral LacZ, TDP-43, Parkin+TDP-43. WB analysis on 4-12% SDS NuPAGE gel shows W) TDP-43 levels relative to actin (1st blot) and Parkin levels relative to actin (2nd blot). V5 epitope level showing equal amount of V5 expression (3rd blot) relative to actin in LacZ, TDP-43 and Parkin. Asterisk is significantly different to LacZ, mean $\pm$ SEM, ANOVA, Neumann Keuls, p as indicated, n=8.

Figure 2. High frequency ^{13}C NMR spectroscopy reveals alteration in the levels of amino acid and TCA cycle intermediates
 Histograms represent **A)** ^{13}C concentration derived from glucose in Glu C4 and **B)** pool size of total glutamate and glutamine in rat hemispheres injected with lentiviral vector driving the expression of LacZ, TDP-43, Parkin or Parkin+TDP-43. **C)** ^{13}C concentration in Asp C2 and C3 and **D)** pool size of total of aspartate rat hemispheres injected with vector driving the expression of LacZ, TDP-43, Parkin or Parkin+TDP-43. **E)** ^{13}C concentration in TCA cycle intermediates Succ C2/C3 and Cit C2 and **F)** pool size of total succinate and citrate in rat hemispheres injected with lentiviral vector driving the expression of LacZ, TDP-43, Parkin or Parkin+TDP-43. **G)** ^{13}C concentration in Leu C4 and Isoleu C4 and **H)** pool size of total leucine and isoleucine in rat hemispheres expressing LacZ, TDP-43, Parkin or Parkin+TDP-43. Asterisk indicates significantly different, mean $\pm$ SEM, ANOVA, Neumann Keuls, n=5. p as indicated.

Figure 3. Parkin reserves lactate accumulation, loss of GABA levels and restores EAATs function

Histograms represent **A)** ^{13}C concentration in Lac C3 and **B)** pool size of total lactate in rat hemispheres injected with lentiviral vector driving the expression of LacZ, TDP-43, Parkin or Parkin+TDP-43. **C)** ^{13}C concentration in GABA C2 and **D)** pool size of GABA in rat hemispheres injected with lentiviral vector driving the expression of LacZ, TDP-43, Parkin or Parkin+TDP-43. * indicates significantly different, mean $\pm$ SEM, ANOVA, Neumann Keuls, p as indicated, n=5. **E)** WB analysis on 4-12% SDS NuPAGE gel shows EAAT1 (1st blot) and EAAT2 (2nd blot) and EAAT3 (3rd blot) relative to actin in LacZ, TDP-43 and Parkin. * indicates significantly different, mean $\pm$ SEM, ANOVA, Neumann Keuls, p as indicated, n=6.

Figure 4. TDP-43 activates 4E-BP signaling

WB of cortical brain lysates in gene transfer animals on 10% SDS NuPAGE gel showing **A**) total mTOR levels (1st blot) phosphorylated mTOR at Ser 2448 (2nd blot) and phosphorylated p70S^{6k} levels at Thr 389 (3rd blot) relative to actin (4th blot). **B**) WB of cortical brain lysates on 4-12% SDS NuPAGE gel showing total 4E-BP1 levels (1st blot) phosphorylated 4E-BP1 at Thr 37/46 (2nd blot) and phosphorylated eIF-4E at Ser 209 (3rd blot) relative to actin (4th blot). **C**) Histograms represent densitometry, WB on brain lysates on 4-12% SDS NuPAGE gel showing **D**) total 4E-BP1 levels (1st blot) phosphorylated 4E-BP1 at Thr 37/46 (2nd blot) and phosphorylated eIF-4E at Ser 209 (3rd blot) relative to actin (4th blot). **E**) Histograms represent densitometry. **F**) WB on brain lysates from TDP-43 transgenic and Parkin^{-/-} mice showing total 4E-BP1 levels (1st blot) phosphorylated 4E-BP1 at Thr 37/46 (2nd blot) and phosphorylated eIF-4E at Ser 209 (3rd blot) relative to actin (4th blot). **G**) Histograms represent densitometry of Western blots, * indicates significantly different, mean±SEM, ANOVA, Neumann Keuls, p and n as indicated.