Modified cell cycle status in a mouse model of altered neuronal vulnerability (slow Wallerian degeneration; Wld^s)

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Abstract

Background: Altered neuronal vulnerability underlies many diseases of the human nervous system, resulting in degeneration and loss of neurons. The neuroprotective slow Wallerian degeneration (Wld^s) mutation delays degeneration in axonal and synaptic compartments of neurons following a wide range of traumatic and disease-inducing stimuli, providing a powerful experimental tool with which to investigate modulation of neuronal vulnerability. Although the mechanisms through which Wld^s confers neuroprotection remain unclear, a diverse range of downstream modifications, incorporating several genes/pathways, have been implicated. These include the following: elevated nicotinamide adenine dinucleotide (NAD) levels associated with nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1; a part of the chimeric Wld^s gene); altered mRNA expression levels of genes such as pituitary tumor transforming gene 1 (Pttg1); changes in the location/activity of the ubiquitin-proteasome machinery via binding to valosin-containing protein (VCP/p97); and modified synaptic expression of proteins such as ubiquitin-activating enzyme E1 (Ube1).

Results: Wld^s expression in mouse cerebellum and HEK293 cells induced robust increases in a broad spectrum of cell cycle-related genes. Both NAD-dependent and Pttg1-dependent pathways were responsible for mediating different subsets of these alterations, also incorporating changes in VCP/p97 localization and Ube1 expression. Cell proliferation rates were not modified by Wld^s, suggesting that later mitotic phases of the cell cycle remained unaltered. We also demonstrate that Wld^s concurrently altered endogenous cell stress pathways.

Conclusion: We report a novel cellular phenotype in cells with altered neuronal vulnerability. We show that previous reports of diverse changes occurring downstream from Wld^s expression converge upon modifications in cell cycle status. These data suggest a strong correlation between modified cell cycle pathways and altered vulnerability of axonal and synaptic compartments in postmitotic, terminally differentiated neurons.
Background

Recent studies have highlighted the important role that vulnerability of nonsomatic neuronal compartments such as axons and synapses plays in the instigation and progression of neurodegenerative diseases, including Alzheimer’s disease, multiple sclerosis, prion disease, Huntington’s disease, and motor neuron diseases [1-4]. However, our understanding of the independent mechanisms that are required to regulate degenerative pathways in axons and synapses remains in its infancy. One powerful experimental tool that has already yielded novel insights into such pathways is the slow Wallerian degeneration (Wld\(^\alpha\)) mutation that selectively protects axons and synapses in the central and peripheral nervous systems following a wide variety of traumatic and disease-related, degeneration-inducing stimuli [5-12].

The Wld\(^\alpha\) mutation occurred spontaneously in a breeding colony of C57Bl/6 mice, resulting in a tandem triplication of an 85 kilobase region on distal chromosome 4 [13]. The Wld\(^\alpha\) gene encodes a fusion protein that comprises the full length of nicotinamide mononucleotide adenyltrasferase 1 (Nmnat1; a nicotinamide adenine dinucleotide [NAD\(^+\)] synthesizing enzyme), coupled by a unique 18-amino-acid sequence to the amino-terminal 70 amino acids of the ubiquitination enzyme ubiquitination factor E4B (Ube4b) [14]. Transgenic expression of the Wld\(^\alpha\) gene is sufficient to confer the full neuroprotective phenotype in several species, including mice, rats, and Drosophila [14-16]. Despite providing substantial protection for axons and synapses, cell bodies are not protected in Wld\(^\alpha\) mice [17-19].

The Wld\(^\alpha\)-protein product appears to be localized exclusively to neuronal nuclei, suggesting that it confers its neuroprotective effects indirectly via modification of endogenous cellular pathways [14,20-22], but there remains considerable controversy over which cellular pathways may need to be targeted to confer Wld\(^\alpha\)-mediated neuroprotection. For example, several studies have demonstrated that the NAD/Sirt1 pathway can modulate axonal degeneration as a result of increased NAD levels, driven by Nmnat1 in the chimeric Wld\(^\alpha\) gene [23-25]. However, NAD pathways alone are insufficient to confer the full neuroprotective phenotype in vivo [26,27]. Other studies have suggested that modifications of the ubiquitin-proteasome system are required for neuroprotection, in part because of the ability of Wld\(^\alpha\) to bind valosin-containing protein (VCP/p97) [28,29]. Genomic and proteomic studies have identified other downstream effects of Wld\(^\alpha\) expression in vivo and in vitro. For example, array experiments have revealed modified expression levels for a range of genes, including the robust downregulation of mRNA encoding pituitary tumor transforming gene 1 (Pttg1) [22,30]. Similarly, proteomic experiments have demonstrated modifications in the levels of mitochondrial and/or synaptic proteins such as ubiquitin-activating enzyme E1 (Ube1) [31]. However, a unified hypothesis that brings together these distinct observations is currently lacking.

We made the previously unrecognized observation that many of these downstream changes also influence cell cycle. For example, Pttg1 is an oncogene with a recently established role in regulating the G\(_1\) to S phase transition of cell cycle [32]. Similarly, Ube1 is a protein with well established roles in cell cycle [33-36], and VCP/p97 localization is intricately linked to the cell cycle, with nuclear localization only occurring during late G\(_1\) phase [37]. In addition, several studies have demonstrated that NAD-dependent pathways play important roles in regulating cell cycle [38-40]. Taken together with numerous published studies reporting that cell cycle status can play an important role in modulating neuronal vulnerability and neurodegenerative pathways [41-49], these observations suggest that cell cycle modulation may provide a unified, common pathway on which genetic and proteomic changes downstream of Wld\(^\alpha\) may act to confer neuroprotection.

Results

Increased expression of cell cycle genes and proteins in Wld\(^\alpha\)-expressing cells in vivo and in vitro

We used cell cycle pathway-specific RT2 profiler PCR arrays (see Materials and methods [below]) to quantify and compare the expression of cell cycle-related genes with high sensitivity. Initially, we used RNA extracted from the cerebellum of wild-type and Wld\(^\alpha\) mice because this tissue has proven ideal for comparative genomic experiments [22]. Wld\(^\alpha\)-cerebellar granule cells are also known to express Wld\(^\alpha\) protein at high levels and exhibit a strong neuroprotective phenotype [22]. We compared expression levels of 84 genes that regulate the cell cycle, including transitions between each of the phases, DNA replication, checkpoints, and arrest. Seventeen out of the 84 genes examined (around 20%) had expression levels increased by more than twofold in Wld\(^\alpha\) cerebellum (Figure 1 and Table 1). The array identified changes in genes associated with many different stages of the cell cycle rather than one specific stage (Table 1). Interestingly, no cell cycle related genes appeared to be suppressed greater than twofold by Wld\(^\alpha\) (Figure 1 and Table 1).
To confirm that RNA changes led to corresponding changes in protein levels, we quantified protein expression levels in the cerebellum of Wlds and wild-type mice in vivo. We chose to focus on one of the genes with a large RNA change and one with a smaller change, just above the twofold threshold, where good antibodies were available (cABL and Brca2, respectively; Table 1). The protein product for both of these genes exhibited corresponding increased expression levels, of a similar ratio to that seen for RNA (Figure 2). In addition, we examined protein levels of other known cell cycle regulators to show that the changes observed on the PCR arrays were not exclusive. Three of the four additional proteins examined (histone H2B, BRCA1, and phosphohistone H2Ax) exhibited significantly increased expression levels in Wlds cerebellum, which is in keeping with the general trend observed on the PCR arrays (Figure 2).

Next, we established that protein levels of two other cell cycle regulators, not included on the PCR array chip but previously shown to be modified in Wlds neurons, were similarly altered. Previous studies have demonstrated that protein levels of Ube1 (a protein with known cell cycle involvement [33-36]) are increased in Wld$^h$ synapses [31], and we were able to confirm this finding by showing increased total Ube1 protein levels in Wld$^h$ cerebellum (Figure 2). In addition, immunocytochemical staining for Ube1 confirmed increased nuclear expression levels in Wld$^h$-expressing neurons in vivo (Figure 3). We also found that Pttg1 protein levels (another
protein that regulates cell cycle pathways [32]) were significantly increased in Wld⁺ cerebellum (Figure 2), which is in keeping with changes in all other cell cycle regulators modified by Wld⁺. This result was surprising because although Pttg1 protein levels had not previously been examined in Wld⁺-expressing cells, several previous reports have identified reduced mRNA levels for Pttg1 [22,30].

To verify that the alterations in cell cycle gene expression were occurring as a direct result of the presence of Wld⁺, and to further confirm that RNA changes observed in Wld⁺ mouse cerebellum led to corresponding changes in protein levels, we examined the effects of Wld⁺ on cell cycle in human embryonic kidney (HEK293) cells after transfection with enhanced green fluorescent protein (eGFP)-tagged Wld⁺ constructs [22]. These experiments also provided further confirmation of cell cycle proteins. We chose to investigate the nuclear expression altered the nuclear expression (Figure 4; compare with Figures 2 and 3). Because the Wld⁺ protein is known to have a predominantly nuclear distribution [20,21], and most cell cycle proteins modulate cell cycle via interactions in the nucleus, we next tested whether Wld⁺ expression altered the nuclear expression of cell cycle proteins. We chose to investigate the nuclear distribution of phosphohistone H2A by transfection with Wld⁺-transfected HEK293 cells, which is in keeping with the general trend observed on the PCR arrays (Figure 1). These experiments also provided further confirmation that both Ube1 and Pttg1 protein levels are increased by Wld⁺ expression (Figure 4; compare with Figures 2 and 3).
both of which have previously been implicated in the cell cycle. Tritiated thymidine uptake assays were performed at 48 hours post-transfection in order to corroborate data from MTT assays generated at the same experimental time point and because this was the time point anticipated to give the maximum chance of detecting a proliferative change in these cells. These data suggest that Wld<sup>+</sup> upregulates the expression of a broad range of cell cycle regulators, pushing cells toward cell cycle re-entry, but that pathways influencing later stages of the cycle, such as mitotic cell division, remain inhibited. In order to confirm that Wld<sup>+</sup>-mediated changes in cell cycle genes/proteins were pushing terminally differentiated neurons toward cell cycle re-entry rather than inhibiting cell cycle activation, we compared the profile of Wld<sup>+</sup>-mediated protein changes with changes induced by a well known pharmacologic inhibitor of the cell cycle: the cyclin-dependent kinase inhibitor flavopiridol. Treatment of HEK293 cells with flavopiridol at an established active concentration (10 μmol/l [48]) resulted in suppression of six out of eight cell cycle proteins that were increased in Wld<sup>+</sup>-transfected HEK293 cells (Figure 7). Thus, pharmacologic inhibition of the cell cycle also induced changes in cell cycle proteins known to be altered by Wld<sup>+</sup>, but importantly these changes in expression levels occurred in the opposite direction. These data confirmed that Wld<sup>+</sup> reactivates dormant cell cycle pathways, pushing cells toward cell cycle re-entry rather than inhibiting it.

Role of Pttg1, NAD, and VCP pathways in mediating cell cycle modulation

After demonstrating that the Wld<sup>+</sup> gene robustly modifies cell cycle status in a variety of cell types in vivo and in vitro, we next investigated whether any of the previously identified downstream modifications induced by Wld<sup>+</sup> play a role in mediating cell cycle changes. First we investigated whether Pttg1 alone, as a known regulator of G<sub>1</sub> to S phase cell cycle transition [32] with increased protein levels in Wld<sup>+</sup>-expressing cells (see Figures 2 and 4), was capable of mediating Wld<sup>+</sup>-induced effects on cell cycle proteins. We compared expression levels of four previously highlighted cell cycle proteins following transfection of HEK293 cells with either a Wld<sup>+</sup> construct [22] or a Pttg1 over-expression construct [55] (Figure 8a). Three of the four proteins examined were not modified by Pttg1 expression alone (Figure 8a), suggesting that other pathways are also required to induce the full range of cell cycle related changes (see below). However, Ube1 upregulation was induced by Pttg1 over-expression to a similar extent as seen with Wld<sup>+</sup>. This suggests that elevated Ube1 protein levels previously reported in Wld<sup>+</sup> synapses [31] are occurring downstream from increases in Pttg1 protein levels.

Pttg1 is currently the only known physiological substrate for the E4 ubiquitination factor Ube4b [56], which is one of the constituent parts of the chimeric Wld<sup>+</sup> gene [13]. In order to establish whether the ability of Pttg1 to be ubiquitinated is important for the regulation of Ube1, we repeated the
experiment using an over-expression construct containing a non-ubiquitinatable form of Pttg1 [57]. The inability to be ubiquitinated completely abolished the ability of Pttg1 to increase Ube1 protein levels (Figure 8b), showing that ubiquitination of Pttg1 by Ube4b (and/or other proteins in the ubiquitin pathway) is likely to be important for Wlds-mediated cell cycle changes.

Next, we investigated whether NAD-dependent pathways play a role in mediating cell cycle changes, because several recent studies have suggested that the Nmnat1 portion of the chimeric Wld^gene plays a significant role in conferring a neuroprotective phenotype by elevating NAD levels and increasing sirtuin activity [23-25]. To examine whether NAD pathways influence cell cycle changes observed in Wld^-expressing cells, we performed cell cycle pathway specific RT² profiler PCR arrays (using human rather than mouse arrays; see Materials and methods [below]) on HEK293 cells treated with 1 mmol/l NAD applied exogenously to the culture medium. This NAD treatment has previously been shown to
confer axonal protection \textit{in vitro} \cite{23} and to mediate selected \textit{Wld}*-induced transcripational changes \cite{22}. Forty-eight out of the 84 genes examined exhibited greater than twofold changes in expression after NAD treatment. In a similar result to that obtained in the \textit{Wld}*-expressing HEK293 cell experiments, the vast majority (47 out of the 84) of modified genes had increased expression levels in the NAD treated cells (Figure 9 and Table 2). Only one cell cycle related gene appeared to be suppressed greater than twofold by NAD (Figure 9). A direct comparison of SuperArray data from \textit{Wld}*-expressing HEK293 cells compared with control cells showed similar increases in cell cycle proteins not included on the SuperArray plate, showing that increased expression of cell cycle proteins is not restricted to those included on the SuperArray. All genes were significantly increased in expression levels in \textit{Wld}*-transfected cells compared with control cells. ***\(P < 0.001\), ****\(P < 0.0001\) by unpaired \(t\)-test (two-tailed).

Figure 4
Quantitative fluorescent Western blots validate changes in cell cycle proteins in \textit{Wld}-expressing HEK293 cells \textit{in vitro}. Bar chart showing percentage change in protein expression (mean ± standard error of the mean; \(n \geq 3\) for all proteins) in \textit{Wld}-transfected HEK293 cells compared with enhanced green fluorescent protein (eGFP)-transfected control cells. As expected, \textit{Wld} protein expression was highly upregulated (left bar). The second portion of the graph shows increases in both pituitary tumor transforming gene 1 (Pttg1) and ubiquitin-activating enzyme E1 (Ube1) proteins following \textit{Wld} transfection, both of which were previously implicated in the \textit{Wld} neuroprotective phenotype \cite{22,31}. The third portion of the graph shows validation for two genes highlighted on the SuperArray analysis as being upregulated by more than twofold. The final portion of the graph shows validation for two genes highlighted on the SuperArray analysis as being upregulated by more than twofold. The final portion of the graph shows validation for two genes highlighted on the SuperArray analysis as being upregulated by more than twofold.

for 4 days confirmed that these NAD-induced changes extend beyond those included on the SuperArray, extend to the protein level, and can occur in neuronal cells (Figure 10b). These data suggest that elevated exogenous NAD levels can mimic many, but importantly not all, \textit{Wld}*-induced cell cycle changes.

Alongside identified changes in Pttg1/Ube1 expression and NAD pathways, previous studies have implicated VCP-mediated pathways (also known as p97 and CDC48) in \textit{Wld}*-mediated neuroprotection, via its interaction with the Ub48 component of the \textit{Wld} chimeric protein \cite{28}. Moreover, VCP is known to be important in early stages of cell cycle progression; VCP is normally localised in the endoplasmic reticulum during nonproliferative states (for example, terminally differentiated neurons), but relocates to the nucleus during late G1 phase in a cell cycle dependent manner \cite{37}. Thus, VCP localisation would not normally be observed in the nucleus of terminally differentiated neurons unless cell cycle had been reactivated and they are progressing toward S phase. To examine whether VCP redistribution associated with modified cell cycle status is modified by \textit{Wld}*, we examined VCP localization in the cerebellum of \textit{Wld}*- and wild-type mice. These experiments revealed an expected cytoplasmic, nonnuclear localization in wild-type neurons, but distinct, strong nuclear puncta in most cerebellar granule cells in \textit{Wld}*- mice (Figure 11). As predicted from the finding that VCP binds \textit{Wld} \cite{28}, VCP localization in the nucleus was consistently found in the same nuclear puncta as \textit{Wld} protein (Figure 11). These data provide further evidence that \textit{Wld}*-expressing cells are being pushed toward the early phases of cell cycle reentry and suggest that VCP binding may play a role in this process.

Thus, Pttg1/Ube1, NAD, and VCP pathways are all likely to be involved in mediating \textit{Wld}*-induced modifications in cell cycle status. Taken together, these findings suggest that previous observations of apparently unrelated changes in gene and protein expression/activity downstream of \textit{Wld} can in fact be unified by their ability to modify the cell cycle.

Modifications in cell stress pathways induced by \textit{Wld} in vivo and \textit{in vitro}
Changes in cell cycle status in terminally differentiated neurons are often associated with corresponding changes in cell stress pathways \cite{58-60}. To examine whether cell stress pathways were also altered in \textit{Wld}*-expressing cells, we used cell stress pathway-specific RT2 profiler PCR arrays (see Materials and methods [below]) to compare mRNA levels in the cerebellum of wild-type and \textit{Wld}*- mice (Figure 12). Fourteen out of the 84 genes contained on the array were modified greater than twofold by \textit{Wld}*, showing that a subset of cell stress pathways are also modified in \textit{Wld}*-expressing cells (Figure 12 and Table 4). In contrast to the results from cell cycle arrays, however, \textit{Wld}*- neurons revealed both increases and decreases across a range of different cell stress proteins.
Finally, in order to confirm that \textit{Wlds} altered nuclear localization, as well as expression, of cell stress proteins (as for cell cycle proteins shown in Figures 3 and 5), we investigated the expression and distribution of stress-induced phosphoprotein 1 (ST1) in \textit{Wlds}-transfected HEK293 cells (Figure 13). We chose to use ST1 as a marker of cell stress \textit{in vitro} in order to expand our coverage of cell stress modifications beyond those genes/proteins incorporated on the array chip and also
Wld\(d\) does not influence late stages of cell cycle regulating cell proliferation in NT2 cells. Bar charts showing relative proliferation rates of NT2 cells transfected with either a control vector (black bars) or a Wld\(d\) vector (white bars) at low, medium, and high concentrations. (a) Panel a shows no difference in proliferation at 48 hours after transfection using an MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) assay. (b) Panel b similarly shows no difference in proliferation at 72 hours after transfection using an MTT assay. (c) Panel c shows no difference in proliferation at 48 hours after transfection using a tritiated thymidine incorporation assay (all comparisons P > 0.05; analysis of variance with Tukey’s post hoc test).

Pharmacological inhibition of cell cycle progression (flavopiridol) versus Wld\(d\): opposing changes in cell cycle proteins. (a) Bar chart showing protein expression assayed by quantitative fluorescent western blots in HEK293 cells transfected with Wld\(d\) (black bars) or treated with exogenous flavopiridol (10 \(\mu\)mol/l; cell cycle inhibitor). Whereas Wld\(d\)-induced increases in all cell cycle proteins, flavopiridol treatment led to decreased expression of the majority of proteins examined. (b) Representative Western blots showing pituitary tumor transforming gene 1 (Pttg1) protein levels in HEK293 cells comparing control versus Wld\(d\) transfected cells (top panel) and control versus flavopiridol treated cells (bottom panel). Note how Pttg1 protein levels are increased by Wld\(d\) expression and decreased by flavopiridol treatment.
because STI1 protein levels are known to be modified in WldS synapses in vivo [31]. Anti-STI1 antibodies revealed nuclear spots of STI1 in most cells expressing WldS (Figure 13a-f). However, neighbouring cells not expressing WldS (because of less than 100% transfection efficiency) did not show any STI1 nuclear puncta. No STI1 staining was seen in control cells transfected with eGFP, indicating that stress responses were not simply occurring due to the presence of a large amount of foreign protein in the nucleus (Figure 13g-i). These findings were supported by data from quantitative Western blotting of STI1 protein levels in whole WldS cerebellum in vivo, where STI1 levels were increased by 71.6 ± 6.8% (mean ± standard error of the mean; data not shown). Interestingly, we previously showed that STI1 protein levels are decreased in synapses protected by the WldS gene in vivo [31]. The finding that nuclear STI1 immunoreactivity increases in WldS transfected HEK293 cells suggests that some stress proteins may exhibit differential compartmental expression via redistribution within WldS-expressing neurons, rather than simply having altered expression levels.

**Discussion**

Here, we show that a strong correlation exists between modified cell cycle pathways and altered vulnerability of axonal and synaptic compartments in postmitotic, terminally differentiated neurons. We have demonstrated that the neuroprotective WldS chimeric gene leads to a robust increase in expression of a broad spectrum of cell cycle-related genes in terminally differentiated neurons. These changes are indicative of an attempt to re-enter cell cycle in postmitotic neurons. Cell cycle alterations were identified in cerebellar neurons in vivo and could be replicated in HEK293 cell lines in vitro. We demonstrate that NAD, Pttg1/Ube1, and VCP pathways are all likely to be responsible for mediating distinct subsets of these downstream changes. Data from proliferation assays showing that WldS does not alter cell division or proliferation rates suggests that terminally differentiated neurons expressing WldS are pushed toward cell cycle re-entry, but do not go on to enter proliferation and growth phases. We also show that expression of the WldS gene leads to modifications in endogenous cell stress pathways that are likely to result from modifications in cell cycle status.

Contrary to previous suggestions that WldS-expressing neurons are ‘normal’, with the exception of a phenotype solely affecting axonal degeneration pathways [1], our experiments have revealed a novel cellular phenotype in WldS-expressing cells: modifications in cell cycle status. This finding brings together diverse observations from several disparate studies investigating WldS mechanisms (changes in Pttg1/Ube1, NAD, and VCP/p97 pathways), suggesting that modified cell cycle status might be a common endogenous pathway through which genomic and proteomic modifications downstream of WldS can influence neuronal vulnerability.

**Pttg1/Ube1 pathways**

Several studies have shown, using a range of experimental approaches and platforms, that WldS robustly downregulates expression of Pttg1 mRNA [22,30]. Pttg1 plays a well established role in sister chromatid separation during mitosis, but recent data have identified an important additional role as a regulator of G1 to S phase cell cycle transition [32]. In the present study we showed that Pttg1 protein levels are significantly increased in WldS-expressing cells. The most parsimonious explanation for the differences between protein and mRNA levels is that decreases in mRNA are generated by a compensatory, self-regulating feedback loop responding to elevated levels of Pttg1 protein. Because Pttg1 is the only known substrate for the Ube4b component of the WldS gene [56], it is tempting to speculate that elevated Pttg1 protein levels result from abnormal ubiquitination and targeting for degradation, caused by WldS-mediated alterations in the ubiquitin-proteasome pathway [28,29]. This finding also has implications for previous attempts to directly link Pttg1 to neuroprotection, because earlier studies examined neurodegenerative responses in Pttg1 null mice [22]. The current data suggest that repeating these experiments in Pttg1 over-
expressing mice might reveal a neuroprotective phenotype, although the undoubted contribution made by other pathways (see below) suggests that Pttg1 over-expression alone would be unlikely to confer the full levels of Wld\(^s\)-mediated neuroprotection.

We have also shown that increased Pttg1 protein levels induced by Wld\(^s\) are responsible for mediating corresponding increases in expression of another cell cycle-related protein, namely Ube1. Thus, Pttg1 is likely to be a partial mediator of other cell cycle changes induced by Wld\(^s\). We previously identified increased protein levels of Ube1 in a population of striatal synapses from Wld\(^s\) mice known to be protected from degeneration [31], suggesting that Ube1 may also play a role in directly modulating degenerative pathways in synaptic compartments of neurons. Importantly, we also found that the ability of Pttg1 to increase Ube1 protein levels was abolished if Pttg1 was expressed in a non-ubiquitinatable form. Because Pttg1 is the only known substrate for the Ube4b component of the Wld\(^s\) gene [56], these data suggest that modified ubiquitination of Pttg1 by Ube4b (either in its native form or as part of the Wld\(^s\) protein) is likely to be required to mediate downstream changes in proteins such as Ube1. However, at this stage we cannot rule out the possibility that other proteins in the ubiquitin pathway alongside Ube4b are also responsible for mediating this response.

**NAD pathways**

The most convincing evidence to date for the involvement of a single pathway in downstream mediation of the Wld\(^s\) phe-
notype has come from studies showing that the Nmnat1 portion of the chimeric Wld\(^b\) gene confers a neuroprotective phenotype via modulation of NAD levels [23-25]. Here, we have shown that experimental manipulation of NAD levels mimics many (but by no means all) of the cell cycle changes induced by Wld\(^b\). This finding raises the possibility that at least part of the NAD-mediated neuroprotective phenotype is generated by modulating cell cycle status. This suggestion is supported by several studies demonstrating that sirtuin-independent pathways (which mediate the NAD neuroprotective phenotype [23]) play important roles in regulating cell cycle [38-40]. However, as with the Pttg1 findings discussed above, it is unlikely that NAD-mediated changes alone are sufficient to induce the full range of Wld\(^b\)-mediated cell cycle changes, because NAD over-expression alone is not thought to be sufficient to confer the full neuroprotective phenotype in vivo or in vitro [26,27].

**VCP/p97 pathways**

Alongside Pttg1 and NAD, pathways mediated by VCP (also known as p97 and CDC48) have also been shown to influence cell cycle [61]. For example, although VCP is predominantly a cytoplasmic protein, it is known to enter the nucleus during late G1 phase [37]. Here, we have shown that VCP, which is currently the only known binding partner for the Ube4b portion of Wld\(^b\) [28,62], is localized to the nucleus in the majority of Wld\(^b\)-expressing neurons. This is further corroborating evidence that cell cycle has been reactivated in terminally differentiated Wld\(^b\) neurons and that they are progressing toward (or beyond) S phase. Although binding to VCP has not yet been demonstrated to be required for the Wld\(^b\) phenotype, the present study suggests that if this is an important event, VCP may be acting via regulation of the cell cycle in a similar manner to Pttg1. Cell cycle events potentially attributable to VCP pathways detected in the current experiments include changes in expression levels of BRCA proteins that are known to interact with VCP in the nucleus [63].

**Cell cycle pathways and neurodegeneration**

The hypothesis that Wld\(^b\) may be modifying neurodegenerative pathways in axons and synapses via modulation of the cell cycle is in keeping with other literature on somatic neurodegeneration, in which cell cycle is known to influence vulnerability significantly. For example, it is now known that postmitotic, terminally differentiated neurons in the adult nervous system are not 'permanently postmitotic', but rather depend upon the constant suppression of cell cycle pathways to maintain their arrested status [41]. The ability to control cell cycle pathways is therefore a critical factor in stopping neurons entering a vulnerable state, where the risk for neurodegenerative mechanisms being instigated increases significantly [41-44]. Numerous examples of cell cycle regulation gone awry, modifying neuronal vulnerability, can be found in neurodegenerative conditions such as motor neuron disease, Alzheimer's disease, and stroke [45,46]. Furthermore, pharmacologic manipulation of cell cycle progression has been used to confer somatic neuroprotection in animal models of traumatic brain injury and stroke [47,48]. The current data suggest that the influence of cell cycle status on neuronal vulnerability is likely to extend beyond neurodegenerative mechanisms resident in cell soma to incorporate independent degenerative pathways in axonal and synaptic compartments. The Wld\(^b\) gene may therefore provide an important experimental tool for future investigations into pathways through which cell cycle status modulates neuronal vulnerability.

The current data are also likely to be important for interpreting previous and future studies concerning Wld\(^b\)-mediated neuroprotection both in vivo and in vitro. Because endogenous cell cycle and cell stress pathways are robustly modified by Wld\(^b\) expression, it is difficult to imagine that Wld\(^b\)-expressing cells do not have any other covert cellular phenotypes alongside neuroprotection. These may introduce additional variables that could conceivably alter experimental outcomes (for example, comparing Nmnat over-expressing...
Table 2

Human SuperArray data showing greater than twofold cell cycle RNA expression changes in NAD-treated HEK293 cells compared with controls

| Gene name                                              | Symbol      | Acc. Number | Array cell | Fold change | SD      | Cell cycle function                                                                 |
|--------------------------------------------------------|-------------|-------------|------------|-------------|---------|--------------------------------------------------------------------------------------|
| V-abl Abelson murine leukemia viral oncogene homolog 1 | ABL1        | NM_005157   | A01        | 18.25       | 3.19    | S phase and DNA replication and regulation                                           |
| Cullin 2                                               | CUL2        | NM_003591   | D10        | 14.32       | 1.89    | G1 phase and G1/S transition and checkpoint and arrest                                |
| B-cell CLL/lymphoma 2                                  | BCL2        | NM_006333   | A09        | 12.44       | 2.12    | Regulation                                                                           |
| Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)| CDKN2B      | NM_004936   | D03        | 11.03       | 2.37    | Checkpoint and arrest and negative regulator                                         |
| Anaphase promoting complex subunit 4                  | ANAPC4      | NM_013367   | A03        | 9.17        | 0.27    | G2 phase and G2/M transition and regulation                                           |
| Cullin 3                                               | CUL3        | NM_003590   | D11        | 8.46        | 0.65    | G1 phase and G1/S transition and checkpoint and arrest                                |
| Cyclin-dependent kinase 5, regulatory subunit 1 (p35) | CDK5RI      | NM_003885   | C07        | 8.00        | 1.66    | Regulation                                                                           |
| RAD1 homolog                                           | RAD1        | NM_002853   | F09        | 7.43        | 0.48    | Checkpoint and arrest                                                                |
| SERTA domain containing 1                              | SERTAD1     | NM_013376   | G06        | 7.41        | 0.27    | G2 phase and G2/M transition                                                        |
| Ataxia telangiectasia and Rad3 related                | ATR         | NM_001184   | A06        | 6.51        | 0.36    | Checkpoint and arrest                                                               |
| Ubiquitin-activating enzyme E1                         | UBE1        | NM_003334   | G12        | 6.48        | 1.19    | S phase and DNA replication                                                        |
| Dynamin 2                                              | DNM2        | NM_004945   | E01        | 5.38        | 0.37    | G1 phase and G2/M transition                                                        |
| Cell division cycle 16 homolog                         | CDC16       | NM_003903   | C01        | 4.94        | 0.43    | M phase                                                                              |
| HUS1 checkpoint homolog                                | HUS1        | NM_004507   | E07        | 4.89        | 0.74    | Checkpoint and arrest                                                               |
| Cyclin-dependent kinase 8                              | CDK8        | NM_001260   | C11        | 4.86        | 1.25    | Regulation                                                                           |
| RAD51 homolog (RecA homolog)                           | RAD51       | NM_002875   | F11        | 4.77        | 3.09    | M phase                                                                              |
| Menage a trois homolog 1, cyclin H assembly factor     | MNAT1       | NM_002431   | F05        | 4.51        | 0.18    | G2 phase and G2/M transition                                                       |
| Tumor protein p53 (Li-Fraumeni syndrome)               | TP53        | NM_000546   | G11        | 4.40        | 1.48    | Checkpoint and arrest                                                               |
| Cyclin-dependent kinase 2                              | CDK2        | NM_001798   | C05        | 4.07        | 0.24    | Checkpoint and arrest and regulation                                                |
| Anaphase promoting complex subunit 2                  | ANAPC2      | NM_013366   | A02        | 3.76        | 0.63    | G1 phase and G1/S transition and regulation                                         |
| Growth arrest and DNA-damage-inducible, alpha          | GADD45A     | NM_001924   | E03        | 3.70        | 0.08    | Checkpoint and arrest and regulation                                                |
| Cyclin-dependent kinase inhibitor 1B (p27, Kip1)       | CDKN1B      | NM_004064   | D01        | 3.65        | 0.25    | G2 phase and G2/M transition and checkpoint and arrest                                |
| Cyclin-dependent kinase inhibitor 3                    | CDKN3       | NM_005192   | D04        | 3.36        | 0.09    | G1+G2, phase and G1/S + G2/M transition and checkpoint and arrest                     |
| CDK5 regulatory subunit associated protein 1           | CDK5R1      | NM_0016408  | C08        | 3.32        | 0.06    | G2 phase and G2/M transition                                                       |
| Cyclin F                                               | CCNF         | NM_001761   | B07        | 3.22        | 0.20    | M phase and regulation                                                              |
| Cyclin-dependent kinase 6                              | CDK6        | NM_001259   | C09        | 3.22        | 0.23    | G1 phase and G1/S transition and regulation                                         |
| DIRAS family, GTP-binding RAS-like 3                   | DIRAS3      | NM_004675   | A04        | 3.15        | 0.12    | ?                                                                                   |
| CHK1 checkpoint homolog                                | CHEK1       | NM_001274   | D05        | 2.95        | 0.15    | Checkpoint and arrest                                                               |
| Nibrin                                                 | NBN         | NM_002485   | F07        | 2.89        | 0.03    | Checkpoint and arrest                                                               |
| Retinoblastoma-like 2 (p130)                           | RBL2        | NM_005611   | G04        | 2.89        | 0.83    | Negative regulator                                                                  |
| Cell division cycle 34 homolog                         | CDC34       | NM_004359   | C04        | 2.86        | 0.14    | G1 phase and G1/S transition and checkpoint and arrest                                |
| Cyclin G2                                              | CCNG2       | NM_004354   | B09        | 2.81        | 0.18    | Checkpoint and arrest                                                               |
| BCL2-associated X protein                              | BAX         | NM_004324   | A07        | 2.76        | 0.06    | Negative regulator                                                                  |
| Proliferating cell nuclear antigen                     | PCNA        | NM_182649   | F08        | 2.76        | 0.19    | S phase and DNA replication                                                        |
| SMT3 suppressor of mitf two 3 homolog 1                | SUMO1       | NM_003352   | G08        | 2.73        | 0.10    | S phase and DNA replication                                                         |
| Cyclin-dependent kinase inhibitor 1A (p21, Cip1)       | CDKN1A      | NM_000389   | C12        | 2.67        | 0.08    | Checkpoint and arrest and regulation                                                |
Increased nuclear expression of cell cycle marker VCP corresponding with Wld<sup>e</sup> expression in mouse cerebellum. Confocal micrographs of cerebellar granule cells from (a-c) Wld<sup>e</sup> and (d-f) wild-type mice. Valosin-containing protein (VCP) is shown in green, the nuclear marker TOPRO3 is shown in blue, and Wld<sup>e</sup> protein in red (panels a and d show VCP and TOPRO3; panels b and e show Wld<sup>e</sup> and TOPRO3; and panels c and f show all three markers). Note how VCP protein can be seen in nuclear puncta with high frequency where Wld<sup>e</sup> is being expressed (arrows in panels a and c show four out of nine examples in this field of view). The majority of Wld<sup>e</sup> puncta coincided with VCP puncta. Nuclear puncta of VCP were rarely observed in wild-type cerebellar granule cells. As expected, VCP was detectable as diffuse staining in the cytoplasm of neurons in both strains of mice. Scale bar = 20 μm.
cells in vivo with cells exposed to exogenous NAD in vitro [23-27]).

Current opinion suggests that cell cycle re-entry is damaging to neurons, whereas blocking cell cycle decreases vulnerability [41-44]. The finding that the neuroprotective Wld^s gene pushes neurons toward cell cycle re-entry therefore appears at odds with this hypothesis. There are two possible explanations for this discrepancy. First, it is possible that the basic principle of re-entry is bad/suppression is good may not hold for all neurodegenerative pathways. Second, and perhaps more plausibly, it is possible that Wld^s acts to 'prime' the cell against future neurodegenerative insults by inducing early-stage cell cycle changes - and cell stress modifications - without going as far as affecting proliferation and growth stages. This potential mechanism of action is in keeping with a known role for preconditioning, sublethal 'priming' events in conferring neuroprotection by modifying endogenous stress pathways [64-67].

Cell stress pathways and Wld^s

Our finding that cell stress pathways are also modified in Wld^s mice, suggesting a possible 'primed state' of Wld^s neurons, is in keeping with the findings of other recent studies. For example, it was recently demonstrated that the NMNAT1 component of the chimeric Wld^s gene has functions alongside those involving NAD, acting as a chaperone for stress-response proteins such as heat shock protein-70 [68] (Wishart TM, Gillingwater TH, unpublished observations). In addition, we have recently shown that the mitochondrial

Figure 12

Widespread alterations in cell stress genes in uninjured/untreated Wld^s mouse cerebellum in vivo. Three-dimensional bar chart taken from SuperArray analysis software (cell stress SuperArray; see Materials and methods) showing fold difference in expression levels for 84 cell stress related genes comparing wild-type cerebellum (control sample) with Wld^s cerebellum (test sample). Individual genes with a greater than twofold expression change can be found in Table 3.
proteome is modified at a basal level in protected WldS synapses [31], suggesting intrinsic differences in the ability to respond to cell stress stimuli. This hypothesis has received experimental support from another recent study showing that NMNAT1 can protect against mitochondrial and oxidative stress [69].

Our study has identified possible individual stress-related proteins that may play an important role in the cell stress response in WldS neurons. Interestingly, several of these proteins have already been implicated in other neuroprotective situations. For example, STI1 - shown in previous work [31] and the present study to have altered levels and subcellular localization in WldS-expressing cells - appears to play an important role in neuroprotection and neuritogenesis [70] as well as in cell proliferation [71,72]. Further investigations into the in vivo role played by individual cell stress proteins modified by WldS, such as STI1, in modulating neuronal phenotypes may therefore provide important insights into mechanisms underlying axonal and synaptic vulnerability.

**Conclusion**

We have identified a strong and robust correlation between modified cell cycle pathways and altered vulnerability of axonal and synaptic compartments in terminally differentiated neurons by showing that the neuroprotective WldS gene modifies cell cycle and cell stress status in vivo and in vitro. We conclude that WldS-expressing cells have a potentially important, previously unreported cellular phenotype that is characterized by reactivation of normally suppressed cell cycle pathways in terminally differentiated neurons. We propose that multiple NAD+, Pttg1/Ubc1-, and VCP-dependent pathways are likely to be required to modulate these cell cycle changes. The data suggest that further investigations into the role of cell cycle and cell stress status induced by WldS are likely to provide insights into mechanisms that regulate axonal and synaptic degeneration in neurodegenerative disease.

**Materials and methods**

**Mouse tissue and cell lines/treatments**

Natural mutant C57Bl6/WldS (WldS) mice and C57Bl/6 (wild type) mice (all aged 6 to 8 weeks) were obtained from Harlan Olac Laboratories (Bicester, UK) and housed within the animal care facilities in Edinburgh. Mice (minimum three mice per experimental group) were killed by cervical dislocation and the cerebellum was rapidly removed.

HEK293 and NSC34 (a mouse motor neuron-like cell line [73]) cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% carbon dioxide. For transfection with eGFP-WldS [22], Pttg1 [55], and non-ubiquitinatable Pttg1 [57], 5 mg of the DNA was mixed with 10% (vol/vol) CaCl₂. An equal volume of N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid was added and the solution gently dropped onto the HEK293 cell culture [22]. For some experiments, 1 mmol/l NAD (Sigma-Aldrich, Gillingham, UK) or 10 μmol/l Flavopiridol (obtained through the National Cancer Institute, Rockville, MD, USA) was added to the medium. All cells were incubated for 4 to 5 days and were checked on a phase contrast microscope before
proceeding to either immunocytochemistry/microscopy or extraction of protein and/or RNA (at least three cultures for all transfections and treatments).

**RNA and protein extraction**

RNA was extracted from cerebella of age-matched and sex-matched mice, or HEK293 cells, in tri-reagent (Sigma) in...

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**Figure 13**
Increased nuclear expression of cell stress marker STI1 corresponding with Wld^s expression in HEK293 cells. Confocal micrographs of HEK293 cells transfected with either an (a-f) enhanced green fluorescent protein (eGFP)-Wld^s construct or an (g-i) eGFP alone control construct. Stress induced phosphoprotein 1 (STI1) is shown in red, the nuclear marker TOPRO3 is shown in blue, and Wld^s protein in green (panels a, d and g show STI1 and TOPRO3; panels b, e and h show construct and TOPRO3; and c, f and i show all three markers). Note how STI1 protein can be seen in nuclear puncta with high frequency where Wld^s is being expressed, but was never observed in non-Wld^s-expressing cells. The majority of Wld^s puncta coincided with STI1 puncta. Nuclear puncta of STI1 were never observed in eGFP transfected control cells (panels g-i). Scale bar = 10 μm.
according with the manufacturer's instructions, as previously described [22]. Protein was extracted from cerebella of age-matched and sex-matched mice, or HEK293 cells, in RIPA buffer with 10% protease inhibitor cocktail (Sigma) [31].

Super arrays
Mouse cell cycle (PAMM-020A), cell stress (PAM-003A), and human cell cycle (PAHS-020A) focused pathway arrays (Tebibio Superarrays, Peterborough, UK) in 96-well plate format, compatible with an ABI 7000 real-time PCR machine, were used to assay gene expression changes (three comparisons for each array type). Samples were added to the reaction plates and signal amplification by PCR was carried out using a Sybr-Green '1 step qRT-PCR kit' (Invitrogen). Analysis was carried out using the Analysis Suite spreadsheet provided by Tebubio Superarrays. The absence of DNA contamination and efficiency of amplification was confirmed using the analysis software provided. Gene functions listed in Tables 1 to 3 were obtained from the SuperArray product specification sheets. Raw data for all of these array experiments can be found online [74].

Quantitative Western blots
Cerebellar/cultured cell protein was separated by SDS-PAGE on 4% to 20% pre-cast NuPage 4% to 12% Bis Tris gradient gels (Invitrogen) and then transferred to PVDF membrane overnight. The membranes were then blocked using Odyssey blocking buffer (Li-COR Biosciences, Lincoln, Nebraska, USA) and incubated with primary antibodies as per manufacturers instructions (BRCA2, cAbl, CCL3 and E1 ubiquitin activating enzyme [Abcam, Cambridge, MA, USA]; anti-HDAC2 clone 3F3, anti-histone H2B and anti acetyl histone H3 [Lake Placid Biologicals, Lake Placid, NY, USA]; Pds1 Ab1 clone DCS280/Anti-Pttg1 [LabVision Corporation, Freemont, CA, USA]; and antiphosphohistone H2Axl [Upstate, Billerica, MA, USA]). Anti-Wld\* antibodies were a kind gift from Dr Michael Coleman and were used as previously described [20,21]. Odyssey secondary antibodies were added in accordance with the manufacturer's instructions (Goat anti rabbit IRDye 680 and Goat anti mouse IRDye 800). Blots were imaged using an Odyssey Infrared Imaging System (Li-COR Biosciences). The scan resolution of the instrument ranges from 21 to 339 μm, and in this study blots were imaged at 169 μm. Quantification was performed on single channels with the analysis software provided. Bands were identified according to their relative molecular weight, as detailed in the manufacturer's antibody notes. Bands were delineated using Odyssey software and the arbitrary fluorescence intensity calculated by the software. For each membrane, scans were carried out at three different intensities in order to minimize possible user error in determining correct scan intensities or over-saturation of the membrane. The average of these three separate scans (giving an n of 1 per membrane) was used for further analysis.

NT2 cell proliferation assays
MTT assays were carried out in 96-well plates. One hundred micrograms of MTT was added to 6,000 cells per well of NT2 cells transfected with control (vector only) or Wld\* constructs (200 ng [low dose], 600 ng [medium dose], and 1,200 ng [high dose]), and incubated for 3 hours. Media were then removed and formazan crystals dissolved in 50 μl dimethyl sulfoxide. The absorbance of the dimethyl sulfoxide was read at 545 nm to estimate cell number. For 3H-thymidine incorporation assays, 3 × 10^5 NT2 cells per well of a 24-well plate were transfected with control (vector only) or Wld\* constructs, as detailed above, and proliferation assayed at 48 hours. Mitogenesis was estimated from the measurement of nuclear 3H-thymidine incorporation. Cells were incubated with 0.2 Ci 3H-thymidine (specific activity 70-95 Ci/nmol; Amersham Biosciences, Little Chalfont, UK) for the last 5 hours of culture incubation. Cells were then washed twice in ice-cold phosphate-buffered saline (PBS), followed by 1 ml cold 5% trichloroacetic acid, and left on ice for 20 minutes. Then, 0.1 mol/l sodium hydroxide was added to the cells before transfer to 4 ml of scintillant. Radioactive counts were determined by scintillation counting. Experiments comprised four replicates each and were performed on at least two separate occasions.

Immunocytochemistry
Immunofluorescence staining was performed on either freshly cut (20 μm or 100 μm) cerebellar slices or HEK293 cells, fixed in 4% paraformaldehyde (Fisher Scientific, Loughborough, UK). Slices or cultured cells were incubated overnight in serum blocker consisting of 4% bovine serum albumin (Sigma) and 0.5% Triton X-100 (Sigma) in PBS. In cerebellar slices, anti-Wld\* antibodies (1:500 dilution in serum blocking solution [8]) were applied overnight and, after washing with PBS saline, a TRITC-conjugated anti-rabbit secondary antibody (DAKO, Glostrup, Denmark) was applied overnight. Primary antibodies, including Ube1 (Abcam), VCP (Abcam), Sti1 (BD Transduction Laboratories, San Jose, CA, USA), and phosphohistone H2Axl (Upstate), were also used at concentrations specified by the manufacturers. Anti-rabbit/mouse FITC-conjugated secondary antibodies (DAKO) were used on cerebellar slices and anti-rabbit/mouse TRITC-conjugated secondary antibodies (DAKO) were used on HEK293 cells. Secondary antibody only controls were also carried out and confirmed the specificity of antibodies used (data not shown). Finally, cerebellar slices and cultured cells were then washed in PBS and incubated in TOPRO 3 (Molecular Probes, Carlsbad, CA, USA) for 10 minutes before mounting in Mowiol. Staining was visualised on a laser scanning confocal microscope (BioRad Radiance 2000; BioRad, Hemel Hempsted, UK) and Z-series were merged using Lasersharp (BioRad) software.
Data analysis
All non-SuperArray data were collected in Microsoft Excel and all statistical analyses and graphs were produced using GraphPad Prism. Quantification of cytoplasmic and nuclear Ube1/TOPO3 fluorescence was undertaken on confocal micrographs captured using identical microscopic settings between images and specimens. No image manipulation was undertaken before quantification using standard fluorescence intensity tools in Image J software. Images were prepared for presentation in Adobe Photoshop.

Abbreviations
eGFP, enhanced green fluorescent protein; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; NAD, nicotinamide adenine dinucleotide; Nmnat1, nicotinamide mononucleotide adenyltransferase 1; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Pttg1, pituitary tumor transforming gene 1; ST1a, stress-induced phosphoprotein 1; Ube1, ubiquitin-activating enzyme E1; Ube4b, ubiquitination factor E4B; VCP, valosin-containing protein; Wld^s, slow Wallerian degeneration.

Authors' contributions
TMW and THG conceived, designed and coordinated the study, undertook the genomic, proteomic and immunocytochemical assays and drafted the manuscript. HNP, SRJ, and CJM carried out the cell proliferation assays. All authors read and approved the final manuscript.

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