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ATM and the epigenetics of the neuronal genome

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Abstract

Ataxia-telangiectasia (A-T) is a neurodegenerative syndrome caused by the mutation of the ATM gene. The ATM protein is a PI3kinase family member best known for its role in the DNA damage response. While repair of DNA damage is a critical function that every CNS neuron must perform, a growing body of evidence indicates that the full range of ATM functions includes some that are unrelated to DNA damage yet are essential to neuronal survival and normal function. For example, ATM participates in the regulation of synaptic vesicle trafficking and is essential for the maintenance of normal LTP. In addition ATM helps to ensure the cytoplasmic localization of HDAC4 and thus maintains the histone 'code' of the neuronal genome by suppressing genome-wide histone deacetylation, which alters the message and protein levels of many genes that are important for neuronal survival and function. The growing list of ATM functions that go beyond its role in the DNA damage response offers a new perspective on why individuals with A-T express such a wide range of neurological symptoms, and suggests that not all A-T symptoms need to be understood in the context of the DNA repair process.

Keywords

Histone deacetylase; cell cycle; neurodegeneration; synaptic vesicle; LTP

Genomic integrity in the neuron

The neurons of the CNS are extreme examples of a problem faced by all highly differentiated cells. Their morphological and functional specialization is so extensive that they have become completely incapable of cell division. The advantage for the cell, and indeed the organism, is that this complex shape renders the neuron capable of highly sophisticated feats of information processing, storage and transfer that are mostly likely unavailable to a morphologically less complex cell such as a skin fibroblast. This advantage comes with a price, however. Skin cells can easily heal a wound by entering a cell division process and increasing their numbers until the breach is healed. Perhaps because of their complex shape, however, neurons are not capable of this seemingly simple act of self-preservation. And the wounds that cannot be healed result from all manner of injury, including DNA damage. Because they cannot replace themselves, neurons of the CNS cannot rely on cell division either to replace a 'wounded' neighbor or to enhance DNA

repair through the use of homologous recombination (a mechanism heavily reliant on DNA replication to work properly). They must guard their genome carefully and, in the case of human beings, they must do so for 8 or more decades.

Paradoxically, while their extreme polarization and differentiation prevent neurons from successfully engaging in a complete cell division process, we have come to learn that virtually all neurons are capable of starting the process. Indeed, the neurons of the vertebrate CNS survive only by suppressing an intrinsic urge to reenter a cell cycle. Those neurons that fail in this suppression begin a process that leads not to mitosis but to death. The strongest evidence in favor of this cell cycle/cell death hypothesis comes from the developing nervous system, and includes the following observations.

1. During normal target-related neuronal death, the target-deprived neurons die after entering a cell cycle. They express cell cycle proteins, synthesize new DNA and die within hours (1).
2. If CNS neurons are genetically incapable of arresting the cell cycle, death occurs in association with DNA replication as they enter their normal maturation process. This has been shown for mutations in the tumor suppressor, retinoblastoma (RB) (2–4), various DNA damage response enzymes (5–9) and in cyclin dependent kinase 5 (Cdk5) knockouts (10). As with target deprivation, death-by-cycle is rapid, occurring within hours.
3. If developing CNS neurons are forced to enter a cell cycle by expression of an oncogene, they will pass into S-phase and synthesize DNA, but rather than divide they will die (11–14).
4. In vitro, withdrawal of trophic support or exposure to certain stressors (e.g., oxidative stress, excitotoxic injury or DNA damaging agents), leads neurons to enter a cell cycle and die (15–23).
5. The cycling cells are the dying cells, as illustrated by the finding that cells can be double-labeled with cell cycle and cell death markers (10, 20).
6. Critically, blocking the cell cycle, either pharmacologically or genetically, prevents the cell death (21–23)

In the aggregate, these data have led to the hypothesis that initiation of a cell cycle in a post-mitotic neuron will cause its death. The corollary of this hypothesis is that blocking the cell cycle will block cell death. There is additional supportive evidence for this cell cycle/cell death relationship in adult neurons, and the volume of this evidence is quite impressive. In a variety of human neurodegenerative conditions –Alzheimer's disease (AD), Parkinson's disease (PD) (24–26), amyotrophic lateral sclerosis (ALS) (27–29), stroke (30, 31), as well as in genomic instability conditions such as ataxia-telangiectasia (A-T) (32, 33) – neurons in populations at high risk for degeneration show clear evidence of having re-initiated a cell cycle process. Indeed the cell cycle/cell death concept was first proposed to explain the unexpected presence of mitotic forms of tau protein in the neurons of patients who had died with AD (34, 35). Since then, a number of laboratories have reported the re-expression of various cell cycle proteins in neurons from patients with Alzheimer's. The proteins include cyclins (36–40), Cdk kinases (34, 41, 42), PCNA (36, 38), Ki67 (38, 43) and Cdk inhibitors (42, 44–46). Unscheduled neuronal cell cycle activity is found at all stages of AD (40, 47). This protein expression is part of a true cell cycle process that proceeds through most of S-phase as DNA replication has been documented by cytogenetic techniques (25, 39, 47–49). Perhaps most compelling of all is the evidence from a cross-sectional analysis of 42 human subjects that suggests in AD it is the cycling neurons that die (47). Cell cycle regulation in the post-mitotic neuron is thus a matter of considerable significance for a healthy neuron.

In the adult, however, the mechanisms that link the cell cycle to the cell death process are more complicated than in the embryo. Neurons in the adult brain can begin a cell cycle, synthesizing the needed cell cycle proteins and replicating their DNA. But in adult neurons the process of cell cycle progression is uncoupled from cell death. The neurons do not die, but neither can they complete the cell cycle (no evidence of M-phase has ever been reported). Further, the neurons of the adult mouse or human CNS can exist in this abnormal 'hyperploid' state for months if not years (50, 51). The evidence for this is found in the counts of cycle-positive neurons in the AD brain. By either immunocytochemistry for cell cycle proteins or FISH for DNA replication, 5–10% of the neurons in at-risk neuronal populations are 'cycling'. But since a typical apoptotic process takes only ~12 hours to complete, if the cycling cells were truly apoptotic, half of the population should be dead in a week and 95% should be dead in less than a month. As an average course of AD is 10 years from first symptoms to death, this is clearly not the case. Thus death by cell cycle in adult neurons must be a very slow process. This protracted time period is unexpected, but the AD mouse models indicate that 6–12 months might be an underestimate of the length of the process (32, 51, 52).

Neuronal death by cycle in ataxia-telangiectasia

Alerted to the paradoxical association of unscheduled cell cycle initiation as a cause of neurodegeneration, it seems nearly self-evident that the loss of neurons in A-T should be associated with cell cycle processes in the neuronal populations at risk. This is because the gene that is mutated in A-T (ATM) encodes a large member of the PI3K kinase family that is activated by DNA damage. Once activated, ATM regulates both the process of DNA double strand break repair itself and the cell cycle through the activation of various cell cycle check-point proteins. In the absence of ATM, cells are tumor prone as they are more likely to proceed through cell division even if their DNA is damaged. Similarly, it turns out that the neurons that are at risk for death in the brains of individuals with A-T are highly likely to ectopically express cell cycle proteins. These cells include cerebellar Purkinje and granule cells as well the occasional striatal neuron. Cells in areas that are unaffected by the disease such as the temporal cortex show no such ectopic expression. Underscoring the centrality of the cell cycle events to the process of cell death, and their relationship to the human disease, mice in which the endogenous *Atm* gene has been mutated show a similar distribution of ectopic cell cycle events. Purkinje cell cycling has now been verified in mice carrying either one of two different engineered *Atm* alleles (32, 33, 53). The neuronal cell cycle phenotype even appears in ATM-deficient fruit flies (54). The concordance of the cell cycle phenotype in fly, mouse and man is especially noteworthy since for reasons we do not understand none of the mouse alleles suffers from the extensive cerebellar cell loss that is observed in humans. Other phenotypes such as sterility and immune system defects are reproduced far more faithfully. The cell cycling reassures us that despite its incomplete nature in the mouse, the cell death process has begun at the correct time and in the correct cells (32).

From nucleus to cytoplasm: additional roles for ATM protein in neurons

The observations linking cell cycle checkpoint problems brings the phenotype of neurodegeneration in closer register with that observed in other tissues. DNA damage is a known cause of neuronal cell cycle activity (55–57) and since cell cycle initiation in a neuron initiates a process of cell death, a pathway to the neurological symptoms seems clearer for these observations. The situation is far more complex, however. The loss of ATM protein does indeed impair a neuron's ability to hold its cell cycle in check, but it also impacts other functions beyond DNA damage.

These functions have been less well studied than others, in part because they are related to a growing list of ATM cytoplasmic functions. ATM has long been described associated with vesicles (58, 59) and vesicle proteins (60, 61). Our lab has pursued this linkage further (33) and shown that ATM, as well as its sister kinase ATR, are important for the proper functioning of neuronal synaptic vesicles. For example *Atm*$^{-/-}$ neurons show a significantly slowed rate of spontaneous vesicular release as measured by the loss of the fluorescent FM4-64 dye. The mechanism behind this phenotype is intriguing. ATM phosphorylates synapsin-I on S656, and when it does so it enables synapsin to participate in a complex with ATR and its own downstream kinase target, VAMP2 (synaptobrevin). The functional importance of these physical interactions can be appreciated by the observation that when a non-phosphorylatable VAMP2 (T35A) mutant is transfected into neurons it acts as a dominant-negative and decreases the rate of spontaneous vesicle release to a rate indistinguishable from *Atm*$^{-/-}$ neurons (33). The finding was made on neurons dissociated from cerebral cortex, not from cerebellum. This suggests that the consequences of ATM deficiency for the nervous system extend more widely than may be generally assumed. This latter suggestion is supported further by our findings from field recordings from the hippocampus.

Electrophysiologists often use a phenomenon known as long term potentiation (LTP) as a cellular assay of memory. The best studied preparation in which such studies are conducted is the hippocampal projection known as the Schaeffer collateral system that connects the pyramidal neurons of area CA3 with those in area CA1. Widely spaced test pulses are given to fibers from area CA3 to determine the baseline responsiveness of the CA1 cells. Then, an experimental burst stimulation is given (typically twelve bursts of four pulses each). Following the sequence, the responsiveness of the CA1 cells to a test pulse increases by 2–3 fold; and while this initial potentiation decays, there is a period lasting several hours where the ‘memory’ of the burst continues to potentiate the response of the CA1 neurons: long term potentiation. In *Atm*$^{-/-}$ mouse hippocampus, although the early events of LTP can be induced, the plateau phase that normally lasts for hours is virtually wiped out (33). This suggests that the vesicular problems identified in the dye-release experiments are part of a larger phenotype that includes systems-level problems in the properties of the neuronal networks in many brain regions. Indeed it has recently been reported that when *Atm*$^{-/-}$ neurons interconnect in vitro, they demonstrate reduced synchronization persistence compared to wild type after DNA damage (62). It is also worth noting, in keeping with its expanding list of functions in the nervous system, that ATM is now believed to play a key role in mitochondrial homeostasis (63). Since the energy demands of neurons are quite high, it is highly likely that this additional deficit increases the physiological malfunctions of the mutant neurons. In the aggregate, therefore, the problems associated with the loss of the synapsin-I/VAMP2/ATM/ATR complex are significant; they fit easily into a larger picture of multiple ATM-induced neuronal defects and thus are likely to have effects that are felt throughout the CNS. This furthers the view that the impact of ATM deficiency is felt well beyond its primary effects in the cerebellar cortex. This expanded view does not demand that we the role of ATM as a DNA damage response protein is of lesser importance in the CNS. Rather, the LTP work should be seen in the context of the observation that activation of glutamate receptors in neurons can significantly enhance the DNA damage response (64).

The vesicular studies involving F4-64 dye release were performed in cultured neurons from the neocortex. The LTP experiments revealed deficits in hippocampal circuits. Thus, even though the most obvious neurodegeneration occurs in the cerebellum, the problems brought on by ATM deficiency may possibly have consequences that are experienced by every neuron. Indeed the structurally obvious and massive cerebellar degeneration found in A-T may have distracted us from degenerative changes in other brain regions. Many of the neurological signs of A-T do not fit with a ‘classic’ cerebellar syndrome, and increasingly

the notion that the IQ of A-T children is normal has been questioned (65–67). Thus it seems that in keeping with the diverse roles of ATM in multiple biological processes, the neurological deficits in A-T can be found in several different domains.

From cytoplasm back to the nucleus: the epigenetics of ATM

The neurophysiological processes in which ATM is involved are broad-based. They are not obviously related to the functions of ATM as a DNA damage response protein. This reminds us that of the full 370 kD protein, we remain in the dark about the function of most of its regions. The PI3K-like kinase domain is located near the C-terminus, yet the primary amino acid sequence contains few hints as to what the remaining 80–90% of protein is for. The wide range of targets identified by Matsuoka et al. (68) and the un-obvious direct relationship of these targets to DNA repair suggests that there are many ATM targets whose cellular functions remain to be determined. Therefore, one possible way in which to view the large diversity of targets is to imagine the large potential of ATM to serve as an adaptor protein – a linker that brings its kinase activity in close proximity to a designated target based protein-protein interactions. These adaptor functions are most likely not mediated through traditional WD40 or SH2 type structures; there are none apparent in the ATM sequence. This requires us to assume a more specialized set of interactions with these targets that are unique to ATM. It is from this perspective that recent work from our laboratory on the involvement of ATM in the establishment of the histone acetylation ‘code’ is most easily viewed.

There are three main classes of histone deacetylase (HDAC) proteins. The most typical are those of the Class I group (HDAC1, 2, 3, and 8) contains structural homologues of the yeast enzyme encoded by the *Rpd3* gene. These are predominantly nuclear proteins and group members such as HDAC1 and HDAC2 have clear roles in the epigenetic regulation of histone chemistry and genome structure. The most unexpected class of HDACs contains the sirtuins. By structure and known enzyme activity, these cytoplasmic deacetylases are involved in the regulation of the metabolic pathways, due in part to their NADH-sensitivity. They were identified on genetic grounds as part of the constellation of genes involved in regulating the aging process, but subsequent work demonstrated that they were true HDACs. Between these two groups lies the Class II histone deacetylases (HDAC4, 5, 6, 7, 9 and 10). Members of this group are homologues of the yeast *Hda1* gene and are further divided into two sub-groups based on their structural properties.

In general, Class II HDACs display cell type-restricted patterns of expression and appear to have a role in skeleton formation and muscle differentiation (69–71). HDAC4 a member of the Class IIa family of HDACs and is highly abundant in the brain. It is a predominantly cytoplasmic protein in neurons (69, 72, 73). Its deficiency is marked in part by an early postnatal atrophy of the cerebellum with surviving Purkinje cells that are notably reduced in dendritic complexity (74). HDAC4 is normally phosphorylated by calcium/calmodulin-dependent kinases (CaMK) enabling its binding to the 14-3-3 family of protein chaperones (72, 75–78). Binding to 14-3-3 results in the retention of HDAC4 in the cytoplasm. Dephosphorylation of HDAC4 is accomplished by PP2A. This leads to its dissociation from 14-3-3 and its migration to the nucleus where it leads to the de-repression of specific target genes. HDAC4, like other class IIa HDACs, associates with MEF2A (myocyte enhancer factor 2A) or CREB repressing its transcriptional activity (79–81).

The mechanism by which the ATM kinase exerts an influence on this system is indirect. HDAC4 itself is not an ATM target; neither are the kinases that phosphorylate it (CaMKII and CaMKIV). Rather, ATM phosphorylates the HDAC4 phosphatase, PP2A. The specific target is the A-subunit, which is regulatory rather than catalytic in function. When PP2A-A

is phosphorylated on its ATM site, it is no longer able to physically associate with HDAC4 (in co-immunoprecipitation experiments) and therefore cannot dephosphorylate it. This allows HDAC4 to remain phosphorylated and bound to 14-3-3 in the cytoplasm. In ATM deficiency, however, PP2A-A is unphosphorylated and hence free to associate with HDAC4. This leads to HDAC4 dephosphorylation, failure to bind to 14-3-3 and nuclear accumulation.

It is in this ectopic nuclear location that HDAC4 does significant damage to neuronal cells. HDAC4 is particularly abundant in Purkinje cells where it is normally found almost exclusively in the cytoplasm. In the absence of ATM, however, HDAC4 levels rise and, as predicted, the bulk of this increase occurs in the nucleus. Nuclear HDAC4 is associated with a reduction in the levels of MEF2A and CREB signaling and with a large, nearly genome wide, de-acetylation of histone proteins H3 and H4. ChIP-sequencing analysis showed that the altered acetylated histone 'marks' changed in very specific ways in the mutant in a manner that was consistent with the observed changes in mRNA and protein levels. In the aggregate the effects of ATM deficiency on histone acetylation suggested that these epigenetic alterations are partially responsible for the altered phenotype of the mutant neurons. De-acetylation is observed in regions of more condensed chromatin, which is in turn associated with a reduced rate of transcriptional activity. Consistent with this relationship, in the region of genes whose function is important for neuronal function or survival, the mutant chromatin tends to show a decreased number of histone acetylation 'marks'.

These changes are more that simple correlations; they have true functional significance. The most extensive evidence of this comes from a variety of manipulations performed on cultures of dissociated CNS neurons. When HDAC4 with an exogenous nuclear localization signal is transfected into wild type neurons, the enhanced levels of nuclear HDAC4 drives neuronal cell cycling and caspase-3 cleavage. By contrast, when RNAi is use to reduce HDAC4 levels, neuronal cell cycle re-entry of cultured $Atm^{-/-}$ neurons decreases and survival improves. A similar result is found with pharmacological agents such as Trichostatin A (TSA) that block HDAC activity. The role of PP2A can also be demonstrated in culture. The model predicts that in $Atm^{-/-}$ neurons, the initiating problem is a de-repression of the activity of the PP2A phosphatase. This prediction is borne out by the finding that pharmacological inhibition of PP2A activity with endothal is effective in blocking HDAC4 nuclear accumulation, neuronal cell cycle activity and subsequent degenerative changes. Similarly, transfection of an ATM-non-phosphorylatable PP2A-A mutant acts as a dominant negative and reverses the cell cycle and degenerative phenotype of $Atm^{-/-}$ neurons.

These changes are significant at the systems level as well. When $Atm^{-/-}$ mice are fed TSA for 10 days, the number of cell cycle-positive Purkinje cells decreases, as do the levels of activated caspase-3. If behavioral tests are run before sacrifice, the treatment with TSA significantly improves the behavioral performance of $Atm^{-/-}$ mice on two measures of motor activity – the rota-rod and activity in an open field paradigm.

ATM epigenetics and the DNA damage response

The story of ATM and its impact on the state of histone acetylation tend to focus attention on various actions of ATM that are not directly related to the DNA damage response. Yet the consequences of HDAC4 nuclear translocation, like those of ATM itself, are multifaceted. And when the correct question is posed, an interrelationship among the various HDAC4 functions is revealed. When treated with even low levels of etoposide to induce DNA double strand breaks, $Atm^{-/-}$ neurons are hypersensitive compared to wild type

(measured by the activation of caspase-3). This poor DNA damage response is not a function of ectopic HDAC4 activity since transfecting the mutant cells with shRNA against HDAC4 before etoposide treatment does not improve the response. The wild type controls, however, reveal a more nuanced picture. At the low levels of etoposide that induce degenerative changes in $Atm^{-/-}$ neurons, wild type neurons show virtually no increase in caspase-3 activation. Nonetheless, if HDAC4 shRNA is transfected into the wild type neurons the degenerative changes are dramatic. The suggestion is that part of the repair process that ensues in normal neurons after DNA damage requires HDAC4, presumably in the cytoplasm, to block a program of neurodegeneration that might otherwise be unleashed.

Summary

The actions of ATM in the nervous system reveal a complex story that is closely related to genomic integrity and the DNA damage response, yet goes well beyond it. There is little question that DNA damage is an existential problem for a non-mitotic CNS neuron. It is thus critical for a nerve cell to maintain a functional DNA damage response network, including adequate levels of functional ATM protein. Yet evidence is increasing that the function of ATM in neurons includes activities that are unrelated to its role in the DNA damage repair pathway. These functions include cytoplasmic activities related to vesicle trafficking, in particular synaptic vesicles. The importance of functional ATM in these situations can be seen in the poor vesicle release property of $Atm^{-/-}$ neurons in culture and in the failure of LTP in $Atm^{-/-}$ hippocampal slices. Still further evidence of the cytoplasmic function of ATM can be found in the requirement of cytoplasmic HDAC4 for the full restorative effect of DNA double strand break repair after etoposide treatment.

On the one hand, this large array of seemingly disparate functions is disconcerting. It is much more gratifying to view a single protein such as ATM as having a single targeted role in the normal physiology of a cell. If DNA repair, mitochondrial dynamics and synaptic vesicle release are all ATM functions, then we are left without a simple way of conceptualizing the gene, its protein and the human disease that results from their deficiency. It is as if ATM were more a Swiss Army knife – with different blades for different situations – than a simple paring knife with but a single use. On reflection, however, the large size of the protein and its complex transcriptional regulation, including the splicing of 64 exons, is almost easier to reconcile with the view of ATM as a multifaceted protein. Given that so many regions of this 370 kD protein have no obvious domain structure or known function in relationship to the DNA damage response, the Swiss Army knife perspective may in the end prove to be the more reasonable view of ATM action.

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Highlights

- The role of ATM in the nervous system is reviewed
- ATM has functions in the neurons beyond the DNA damage response
- ATM has functions in the neuronal cytoplasm required for neuronal health and survival
- ATM alters the epigenetic code by maintaining histone deacetylase 4 in the cytoplasm