Review

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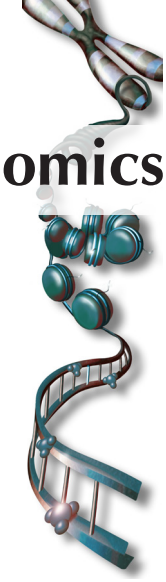
Epigenomics



Emerging roles of ATRX in cancer

ATRX was identified over 20 years ago as the gene responsible for a rare developmental disorder characterized by α-thalassemia and intellectual disability. Similarities to the sucrose nonfermentable SNF2 type chromatin remodelers initially suggested a role in transcriptional regulation. However, over the last years, our knowledge of the epigenetic activities of ATRX has expanded steadily. Recent exciting discoveries have propelled ATRX into the limelight of chromatin and telomere biology, development and cancer research. This review summarizes recent breakthroughs in understanding ATRX function in heterochromatin structure, genome stability and its frequent dysregulation in a variety of cancers.

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The ATRX protein is a chromatin remodeling factor, initially described as a putative helicase protein due to sequence homology with the DNA repair and recombination Rad54 protein [1]. The corresponding gene is comprised of 36 exons and spans approximately 300 kb of DNA sequence located on the long arm of the human X chromosome (Xq21.1) [2,3]. It is highly conserved between mouse and human (85% homology) and protein homologs have been identified in *Drosophila melanogaster* (dATRX; 66% homology) and *Caenorhabditis elegans* (XNP-1; 52% homologous) [2,4–5]. ATRX is a ubiquitously expressed nuclear protein with highest levels in fetal brain, suggesting an important role in brain development [6]. ATRX is a member of the SNF2 subgroup of the SWI/SNF protein superfamily that utilize the energy of ATP hydrolysis to remodel chromatin [2]. SWI/SNF members have been shown to modulate various cellular processes including transcription, DNA repair and mitotic recombination [2,7–8].

The ATRX polypeptide contains two highly conserved domains: a globular ATRX-DNMT3-DNMT3L (ADD) domain in the amino-terminus and a carboxy-terminal helicase-like domain [9–12] (Figure 1B). The ADD domain is cysteine-rich and contains two types of zinc finger motifs: a plant homeodomain finger flanked by a GATA-1-like C_2C_2 motif [13], which is uniquely shared between ATRX and the *de novo* DNA methyltransferases (DNMT) DNMT3A, DNMT3B and DNMT3L [10]. The helicase-like domain exhibits ATPase chromatin remodeling activity that can be stimulated by nucleosomes or DNA [14,15].

ATRX binds heterochromatin & G-quadruplex DNA

ATRX is exclusively nuclear and associates with the nuclear matrix during interphase, until it is phosphorylated and released at the onset of mitosis [16]. Early on, immunofluorescence microscopy approaches revealed that ATRX is highly enriched at DAPI-bright heterochromatin subdomains in mouse nuclei

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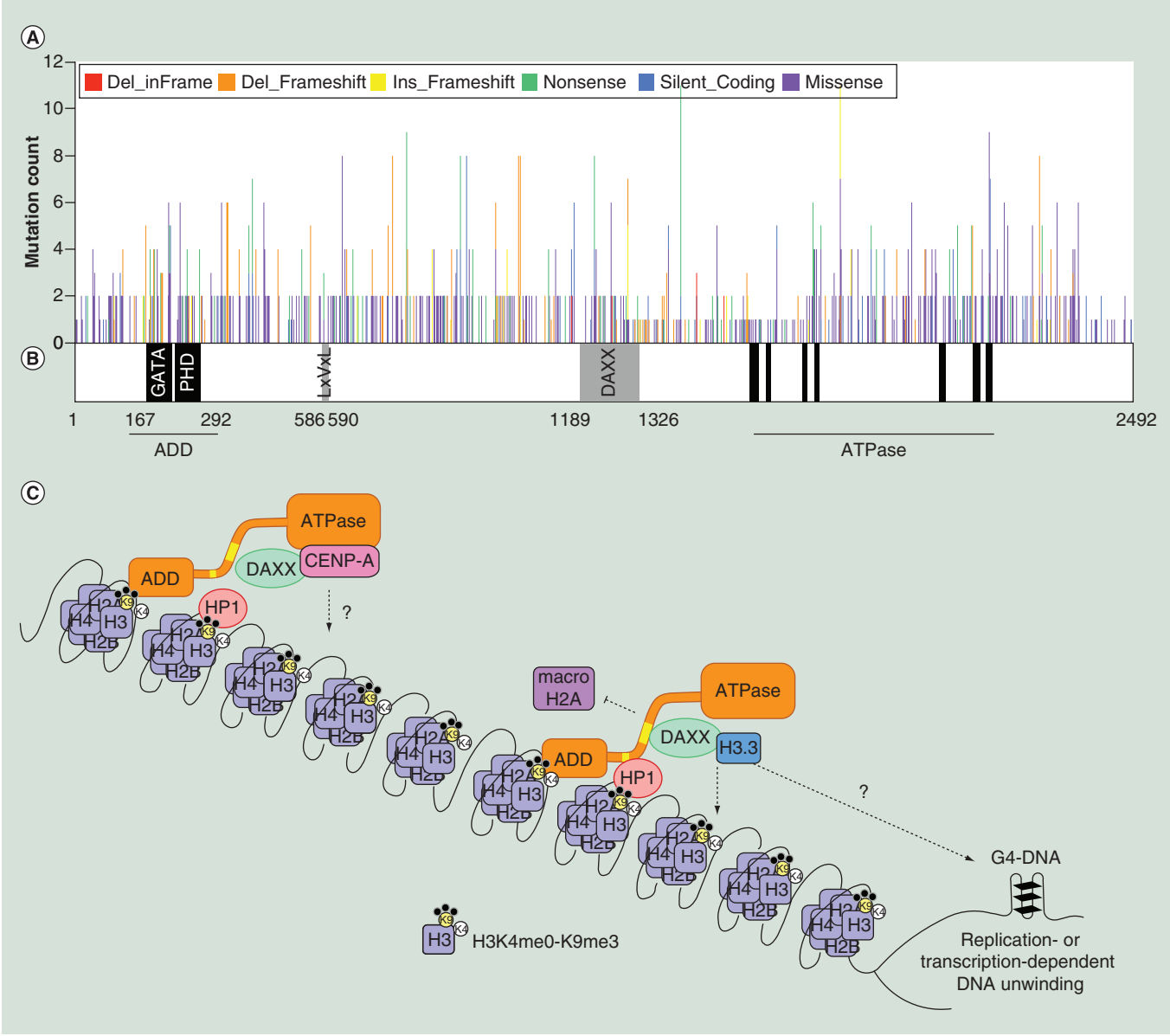

Figure 1. Mutational landscape of cancer-associated *ATRX* mutations and model depicting ATRX activities at chromatin. (A) *ATRX* mutations cataloged in the COSMIC database. Red: in frame deletion; orange: frameshift deletion; yellow: frameshift insertion; green: nonsense substitution; blue: silent coding substitution; purple: missense substitution (COSMIC: exploring the world's knowledge of somatic mutations in human cancer (Forbes *et al.* 2014). **(B)** Depiction of the *ATRX* gene. The ADD (GATA and PHD fingers) and ATPase domain are highlighted in addition to residues that mediate protein–protein interaction between ATRX/HP1 (LxVxL) and ATRX/DAXX. **(C)** ATRX can directly recognize the H3K4me0–K9me3 combinatorial modification for chromatin targeting. ATRX also interacts with the H3K9me3 reader HP1 to stabilize its association with heterochromatin, and the histone chaperone DAXX to deposit H3.3 at repetitive DNA. The deposition of H3.3 may help overcome the deleterious effects of G-quadruplex structures. Moreover, ATRX restricts macroH2A enrichment and promotes ectopic deposition of CENP-A-containing nucleosomes at noncentromeric regions in cancer cells.
ATRX:αα-thalassemia mental retardation, X-linked; DAXX: Death-associated protein 6.

throughout all stages of the cell cycle [17,18]. Since then, ATRX enrichment has been demonstrated at constitutive heterochromatic sites, such as pericentric heterochromatin, telomeres and the inactive X chromosome in female cells [19–21]. Permanent silencing of repetitive constitutive heterochromatin is linked to DNA methylation [22], as well as methylation of specific histone residues, namely histone H3 trimethylated on lysine 9 (H3K9me3) and histone H4 trimethylated on lysine 20 (H4K20me3) [23,24].

Multiple mechanisms appear to mediate ATRX targeting to heterochromatin (Figure 1C). ATRX interacts

directly with heterochromatin protein 1 α (HP1α), a chromodomain-containing reader of the H3K9me3 heterochromatin mark [16–17,25]. Depletion of ATRX has minimal effect on HP1 localization [26–28], while loss of HP1 results in abnormal targeting of ATRX to heterochromatin [29], suggesting that HP1 is involved in recruitment or stabilization of ATRX. Mutation to the LxVxL HP1 interaction motif reduces ATRX localization at heterochromatin to a lesser extent than mutations in the ADD domain [30], pointing to a stabilization role for HP1 rather than ATRX recruitment per se. ATRX itself is an efficient reader of the H3K9me3 histone mark via the ADD domain [9,20,30–33]. The ADD domain can bind both DNA and RNA, and has affinity for H3K9me3-containing nucleosomes in the absence of H3K4 methylation [9,20,30–33]. More recently, ATRX was found to be one of very few specific readers of the H3K9me3–S10ph combinatorial modification [30,34–35]. This grouping of modifications on histone H3 modulates binding of polycomb proteins during differentiation [36]. By itself, H3S10ph is induced during mitosis and under periods of high neuronal activity at the promoter of immediate early genes [37]. These findings indicate that ATRX likely targets heterochromatin primarily through the ADD domain and that binding to HP1 may reinforce or stabilize this association. The situation in neurons appears to be more complex, as the presence of the methyl CpG binding protein 2 (MeCP2) is required for ATRX to localize to DAPI-rich heterochromatic foci [18]. ATRX interacts with MeCP2 through its carboxy-terminal helicase domain and MeCP2-deficient neurons display abnormal targeting of ATRX to heterochromatin [18,38–39]. C-terminal fragments of ATRX require MeCP2 for heterochromatic localization while N-terminal fragments of the protein localize to DAPI-bright heterochromatin in an MeCP2-independent manner [18], indicating that the N-terminal ADD domain contains elements required for ATRX targeting independent from MeCP2 recruitment.

Despite the clear association of ATRX with heterochromatin, chromatin immunoprecipitation-sequencing experiments revealed that the protein also associates with numerous intergenic regions and gene bodies [40]. Many of these sites are G-rich tandem repeats with the propensity to form complex secondary structures such as hairpins/cruciforms, Z-DNA, triplexes and tetraplexes, especially when the DNA is unwound during transcription or replication [41]. Telomeric DNA is particularly prone to the formation of G-quadruplex DNA structures [42,43]. Providing the first chromatin immunoprecipitation-sequencing data for ATRX in mouse embryonic stem cells, Law *et al.* demonstrated convincingly that ATRX is enriched at predicted G-quadruplex-forming DNA sequences and that recombinant ATRX protein can interact with G-quadruplex oligonucleotides *in vitro* [40]. The link between ATRX and G-quadruplexes likely has important implications for the effects of ATRX inactivation on DNA replication and transcription. Indeed, ATRX may facilitate RNA polymerase passage through the intragenic deposition of the histone H3 replacement variant H3.3 in a subset of genes that contain predicted G-quadruplex structures [44]. Taken together, these data indicate that ATRX targeting to chromatin is likely mediated through direct interaction with G-quadruplexes, recognition of posttranslational modifications of the histone H3 tail by the ADD reader domain and stabilization of ATRX at heterochromatin through protein–protein interactions with MeCP2 and HP1 [18,30,38].

The ATRX/DAXX chromatin remodeling complex

An unbiased proteomic analysis to isolate ATRX interacting partners led to the identification of the death-associated protein 6 (DAXX) [14]. DAXX interacts with the linker region of ATRX located between the ADD and ATPase domains (Figure 1B) [15]. The ATRX/DAXX complex exhibits ATP-dependent chromatin remodeling activities such as disruption of DNA–histone interactions and translocation of histones on DNA, but does not display helicase activity [14,15]. In contrast to the HIRA complex which incorporates histone variant H3.3 at transcribed regions [45], ATRX and DAXX are responsible for replication-independent deposition of the histone variant H3.3 at telomeres, pericentric heterochromatin and ribosomal DNA repeats [46–48]. ATRX/DAXX-dependent H3.3 deposition at heterochromatic regions was surprising, as H3.3 enrichment had primarily been described at transcriptionally active chromatin [47,49–52]. The need for H3.3 incorporation at heterochromatin is still unclear, but it may contribute to the stability of repetitive chromatin structure. ATRX loss results in a failure to incorporate H3.3 and induces a DNA damage response at telomeres and pericentric heterochromatin, suggesting that H3.3 incorporation is required to maintain stability of these elements [27–28,47,53].

Transcription of repetitive elements is important for maintenance of heterochromatin structure [54,55]. Despite a failure to incorporate H3.3 at telomeres and pericentric heterochromatin in ATRX- or DAXX-depleted cells, there have been conflicting reports of transcriptional dysregulation of noncoding RNAs at these sites [44,47–48]. Telomeres express the telomere repeat-containing RNA (TERRA). A recent study demonstrated G_2/M-specific upregulation of TERRA

in HeLa cells depleted for ATRX [56], perhaps explaining the discrepancies in earlier findings of ATRX-dependent TERRA regulation [44,47]. Other repetitive DNA sequences, such as the parasitic endogenous retroviral elements, are usually silenced to prevent mutation and genomic instability. ATRX/DAXX-dependent H3.3 deposition at specific endogenous retroviral subfamilies coincides with H3K9me3-containing nucleosomes and defines a unique heterochromatin state required for silencing and repression of retrotransposition [57–59]. The emerging evidence therefore suggests that the presence of H3.3-containing nucleosomes at repetitive elements contributes to their silencing.

ATRX and DAXX also localize to promyelocytic leukemia nuclear bodies (PML-NBs), multipurpose subnuclear domains implicated in transcriptional activation, DNA replication, apoptosis and viral infection [60–62]. PML-NBs are present in most cell lines and tissue types; in a normal mammalian cell there are approximately 5–30 PML-NBs [63]. Over 100 proteins have been associated with PML-NBs, many of which show dynamic localization [64]. In contrast to heterochromatic localization, ATRX localization at PML-NBs is DAXX-dependent [61]. It is hypothesized that PML-NBs serve as platforms for heterochromatin remodeling postreplication in G_2 phase of the cell cycle, which is supported by the dynamic colocalization of ATRX/DAXX/H3.3, heterochromatin and PML-NBs during this time [61,65]. Furthermore, PML-NBs have been suggested to be sites of H3.3 storage within the nucleus and function as a gathering point for proteins involved in replication-independent H3.3 assembly [66]. In fact, PML depletion results in dissociation of PML-NBs and decreased ATRX/H3.3 at telomeres, suggesting that these subnuclear structures are important for maintaining H3.3 at telomeres [65].

ATRX inactivation causes genomic instability

Replication of heterochromatin and S phase progression are tightly linked [67], which may explain the increased DNA damage response observed in late-replicating chromatin and S phase lengthening of *Atrx*-null cells [28,53,68]. The mechanism of ATRX function in replication is not well characterized. A prevalent hypothesis is that ATRX helps to resolve or prevents the formation of G-quadruplexes to allow progression of the replication fork through regions prone to the formation of complex secondary structures, such as telomeres [69,70]. We were able to show that *Atrx*-null cells are hypersensitive to treatment with the G-quadruplex stabilizing compound telomestatin, suggesting that ATRX helps to overcome the adverse effects of these DNA structures [53]. However, resolution of G-quadruplexes by ATRX appears to be indirect since the protein performed poorly in a G-quadruplex unwinding assay [68]. Instead, ATRX may help resolve G-quadruplexes through the deposition of H3.3-containing nucleosomes. Recent evidence points to a more direct function in replicative mechanisms, as ATRX interacts with components of the MRE11-RAD50-NBS1 (MRN) complex that facilitates replication fork restart [68,71]. The MRN–ATRX interaction may occur in the context of telomeric chromatin, as the ataxia telangiectasia-mutated kinase, MRN and homologous recombination (HR) machinery are all recruited to newly replicated telomeres for reformation of the protective T-loop structure [72,73].

Replicative DNA damage can be carried over into mitosis if the damage is not severe enough to induce checkpoint activation and cell cycle arrest [74]. Ultimately, this can result in abnormal cell division, as under-replicated regions of chromosomes remain physically joined and eventually break or shatter to cause micronuclei or chromothripsis, respectively [75]. Indeed, studies have demonstrated a requirement for ATRX in both meiosis and mitosis [26,76–78]. In human HeLa cells, ATRX depletion causes chromosome congression defects and a prolonged prometaphase-to-metaphase transition that ultimately results in anaphase bridging and micronuclei formation [26,78]. In mouse oocytes, ATRX depletion disrupts alignment of chromosomes at the metaphase II spindle and causes defective chromosome cohesion, similarly resulting in micronuclei formation and centromere instability [76,77]. Therefore, the mitotic/meiotic defects seen in the absence of ATRX could be a consequence of increased stress during replication. Conversely, replicative stress and increased DNA damage can also result from abnormal cell division [79]. It remains unclear whether ATRX deficiency causes replicative stress that precedes mitotic/meiotic dysfunction or vice versa. Nevertheless, there is ample evidence to conclude that ATRX is required to maintain genomic stability.

Mouse models have been useful to investigate the effects of ATRX overexpression or inactivation in embryonic stem/neuroprogenitor cells (NPCs). Overexpression of ATRX causes neurodevelopmental and craniofacial defects, suggesting that ATRX is required in correct stoichiometric amounts for normal development [80]. Forebrain-specific deletion of the full-length *Atrx* isoform causes increased apoptosis of NPCs, resulting in decreased neonatal cortical size and hippocampal dysgenesis [81]. Generation of p53/ATRX double mutant mice effectively rescued apoptosis, implicating p53 in the apoptotic response observed in *Atrx*-null NPCs [82]. However, loss of p53 failed to completely restore neonatal forebrain size, indicating that ATRX regulates brain size through additional

mechanisms [82]. Indeed, *Atrx*-null NPCs exhibit premature differentiation, resulting in early depletion of the progenitor pool and a failure to sustain birth of late-born neurons [78]. An extended investigation of the underlying cause for p53-dependent apoptosis in *Atrx*-null NPCs revealed excessive levels of DNA damage in the embryonic brain caused by DNA replication stress [53]. Furthermore, combined inactivation of *Atrx* and *p53* led to an accumulation of cells with genomic instability *in vivo*, likely due to their inability to trigger an apoptotic response [53]. The relationship between ATRX, replication stress and p53 activation may provide a glimpse at the early events leading to tumorigenesis in individuals with acquired *ATRX* mutations in neural stem cells. Noteworthy is a recent report that H3.3 loss of function in mice causes developmental defects that resemble those observed upon loss of ATRX, such as lagging chromosomes and micronuclei, DNA damage accumulation and cell death that can be rescued by loss of p53 [83]. The striking similarities suggest that the developmental defects observed in ATRX-null cells might be explained by failure to incorporate H3.3 at heterochromatin.

ATRX misexpression & mutations are observed in various malignancies

Genetic linkage studies originally identified numerous mutations within the coding regions of *ATRX* as the cause for a rare intellectual disability syndrome (ATR-X; OMIM#301040) characterized by a wide array of developmental abnormalities, α-thalassemia and severe cognitive deficits [84]. ATR-X syndrome mutations are predominantly missense and cluster within the conserved ADD and ATPase domains of the protein, resulting in decreased amounts or function of the protein [9,85]. Patient mutations in the ATPase domain of ATRX result in abnormal targeting to PML-NBs, implicating the subnuclear structures in disease pathogenesis [86]. Additionally, patient mutations within the ADD domain result in abnormal targeting to heterochromatin [30–32]. Variability in the degree α-thalassemia presentation in ATR-X patients correlates with the length of the ψ variable nucleotide tandem repeat adjacent to the α-globin gene cluster that is predicted to form G-quadruplex structures and is enriched for ATRX binding in normal individuals [40]. The first clue that ATRX may be involved in suppressing malignancy came from the discovery that α-thalassemia myelodysplasia syndrome, somatic (OMIM#300448), a rare preleukemic condition, is caused by acquired somatic loss-of-function *ATRX* mutations [87].

Subsequently, gene expression profiling of *de novo* childhood acute myeloid leukemia patients identified reduced ATRX expression as a predictor of patient outcome [88]. Mutations in *FLT3* are a known prognosticator of poor outcome in acute myeloid leukemia patients, but further diagnostic markers were needed to distinguish between patients who were at high risk for treatment failure versus those who were not. Among those patients who harbor *FLT3* mutations, high expression of *RUNX3* and low expression of *ATRX* correlates with worse outcome and are more likely to resist treatment, whereas high *ATRX* expression correlates with a more favorable outcome [89]. Thus, this was the first study to identify *ATRX* expression levels as a predictor of cancer patient prognosis. Alterations in ATRX expression have since been described in a number of cancers: reduced expression was detected in neuroendocrine tumors [90–92], glioma [93], osteosarcoma [92,94] and melanoma [95], while overexpression of the protein was reported in colorectal cancer cell lines [96].

An inverse relationship between levels of ATRX and the histone variant macroH2A has been linked to melanoma progression, and ATRX can interact with macroH2A to negatively regulate its deposition (Figure 1C) [21,95,97]. CENP-A is another H3 variant that is canonically deposited specifically at centromeres by the Holliday junction recognition protein HJURP [98,99]. A recent report described excessive production of CENP-A in certain aggressive cancers, leading to ATRX/DAXX-dependent ectopic deposition of this variant at noncentromeric sequences (Figure 1C) [96,100]. Hence, under abnormal circumstances, the ATRX/DAXX complex can modify the histone variant composition of chromatin in a way that may promote genomic instability or gene expression changes leading to cancer.

In addition to the above scenarios, mutations in the *ATRX* gene have now been detected by exome sequencing in neuroblastoma, glioma, pancreatic neuroendocrine tumors (panNETs) and pediatric osteosarcoma [93,101–106]. The *ATRX* mutational landscape primarily includes point mutations scattered throughout the coding sequence, as well as large deletions and insertions that can result in frameshifts (Figure 1A). Collectively, this suggests that *ATRX* mutations in cancer are loss of function, however more detailed analysis of the functional consequences of *ATRX* point mutations is necessary, as those mutations may result in aberrant gain of function. Mutations in *DAXX* were also reported in these tumor subtypes but rarely co-occurred with *ATRX* mutations [101,103], confirming that they function together in the same pathway. In contrast, *ATRX* mutations commonly occurred in conjunction with mutations in the tumor suppressor *TP53* [93,103]. A model was proposed whereby epigenetic factors like ATRX may act as 'backseat drivers'

to suppress oncogenic pathways upstream of master regulators, like *TP53* [107].

PanNETs

PanNETs are the second most common pancreatic malignancy with a 10-year survival rate of only 40% [108]. Currently, surgical resection of these tumors is the front line of treatment, but many patients present with unresectable tumors or extensive metastatic disease which is not responsive to current medical therapies [108]. Whole-exome sequencing was used to identify commonly mutated genes within a subset of panNETs, and 17.6% harbored mutations in *ATRX*, 25% had alterations in *DAXX* and 3% showed *TP53* mutations [101]. They concluded that mutations in the DAXX/ATRX pathway are the second most common alteration after mutations in multiple endocrine neoplasia type 1, and *ATRX* mutations are most commonly insertion/deletions leading to frameshifts within the resulting polypeptide, presumably resulting in loss of protein function [101]. *DAXX* and *ATRX* alterations were mutually exclusive, and both correlated with an earlier age of diagnosis as well as better overall patient survival [101]. In fact, 100% of patients with metastatic disease and mutations in *DAXX/ATRX* and multiple endocrine neoplasia type 1 survived at least 10 years, versus over 60% of patients without these mutations who succumbed to their tumors within 5 years of diagnosis [101]. This indicated that *ATRX/DAXX* mutations define a molecular subgroup of panNETs and may have prognostic value. Subsequent studies of panNETs identified further alterations in ATRX and DAXX protein levels. Firstly, immunohistochemical analysis of well-differentiated neuroendocrine carcinomas, also considered panNETs, lacked ATRX nuclear staining in 36.4% of tumors and a loss of DAXX staining in 9.1% [90]. Again, loss of ATRX and DAXX immunostaining was mutually exclusive in these tumors; however, immunostaining for both ATRX and DAXX was intact in the small- and large-cell neuroendocrine carcinoma samples, which are not considered to be panNETs [90]. One conclusion is that panNETs are genetically distinct from other pancreatic neoplasms, and can be defined by *ATRX* and/or *DAXX* mutation status. More recently, analysis of a large group of panNET samples obtained from 243 patient samples across a variety of hospitals, identified loss of DAXX in 15% and a loss of ATRX in 28% of samples, with only two samples harboring a loss of both proteins [91]. This large study revealed that loss of ATRX/DAXX correlates with chromosomal instability, advanced tumor stage, development of metastases and poorer overall survival [91]. The contradictory results obtained by Marinoni *et al.* and Jiao *et al.* might be explained by the different number and composition of samples analyzed. Marinoni *et al.* analyzed more samples (243 vs 68) that were more diverse in composition (both nonmetastatic and metastatic) compared with Jiao *et al.*, where the majority of patients were metastatic. A comparison between only metastatic patients by Marinoni *et al.* in fact demonstrated a trend toward longer survival, suggesting that ATRX/DAXX loss have different roles in localized versus metastatic tumors. *ATRX/DAXX* mutations may therefore define a biologically specific subgroup of panNETs and could be useful to predict patient outcome in a stage-specific manner. Absence of ATRX or DAXX nuclear immunostaining has also been identified in lung, stomach, duodenum and rectal NETs and no samples exhibited loss of staining for both proteins [92]. Therefore, loss of ATRX and/or DAXX expression is not only relevant to pancreatic lesions, but more generally to neuroendocrine tumors across many organ systems.

Glioma spectrum tumors

Mutations in *ATRX* are also highly prominent across the glioma tumor landscape. *ATRX* mutations were initially detected in pediatric glioblastoma multiforme (GBM), where 14.3% of samples studied harbored frameshift or nonsense mutations associated with loss of nuclear ATRX expression [109]. A subsequent study surveyed four pediatric GBMs that contained mutations in the histone variant H3.3 (*H3F3A*) and identified two recurrent mutations, G34R and K27M, both located near the tail of the protein that undergoes important post-translational modifications involved with transcriptional repression or activation [103]. All four of these samples also had *ATRX* mutations, suggesting an underlying dysfunction in the ATRX/DAXX/H3.3 histone deposition pathway. Whole-exome sequencing analysis was performed in 42 additional pediatric GBMs and identified *ATRX/DAXX/H3.3* mutations in 50% of samples (15/42 *H3.3*; 14/42 *ATRX*; 2/42 *DAXX*) [103]. The majority of *ATRX* mutations were insertions/deletions leading to frameshifts, while the other mutations were either nonsense or missense and localized either within the SNF2 domain of ATRX or led to a truncation of the protein upstream of this domain (Figure 1A) [103]. Notably, mutations in *ATRX* and *H3.3* overlapped significantly with mutations in *TP53*, and eight samples had mutations in all three genes [103]. These data indicate a central role for the ATRX/DAXX/H3.3 axis in normal cellular homeostasis, and that perturbations of this axis in combination with *TP53* mutations may underlie the pathology of a subset of pediatric GBM.

Missense mutations in the gene encoding histone H3.3 (*H3F3A)* provide direct evidence that alterations

in the histone proteins themselves can promote cancer. Analysis of the H3.3K27M mutant demonstrated alterations in genome-wide H3K27me3 levels through inhibition of the Polycomb repressive complex 2 enzymatic component EZH2 [110–112]. These findings are particularly intriguing given that ATRX is responsible for H3.3 deposition at specific genomic positions, and that ATRX interacts with EZH2 [20,46–48,113]. Furthermore, the K27M mutation synergizes with p53 loss in neural progenitor cells resulting in neoplastic transformation [114]. Together, the data suggest that the ATRX-DAXX-H3.3 pathway may genetically interact with the polycomb pathway in cancer progression.

Recent studies have extended analyses of ATRX status to adult glioma samples, including WHO grade II and III astrocytomas and oligodendrogliomas, WHO grade III and IV anaplastic astrocytomas, oligoastrocytomas and WHO grade IV primary and secondary GBMs. For example, mutations in *ATRX* were identified in 93 out of 363 (26%) gliomas samples ranging across all age groups and tumor grades [102]. Specifically, *ATRX* alterations were found in 67% of grade II astrocytomas, 73% grade III astrocytomas, 57% secondary GBMs, 68% oligoastrocytomas and 20% pediatric GBMs [102]. The majority of *ATRX* mutations in pediatric GBMs were again clustered in the C-terminal SNF2 domain, while adult gliomas displayed an even distribution across the *ATRX* gene; however, both adult and pediatric gliomas showed a bias toward frameshift and nonsense mutations (Figure 1A) [102]. Another study identified *ATRX* mutations by Sanger sequencing in 47.8% of adult glioma samples distributed across age and subtype, with again the majority of mutations being insertions/deletions leading to frameshifts (Figure 1A) [93]. Immunohistochemical analysis of ATRX expression in another 96 samples demonstrated loss of nuclear ATRX staining in 32% of adult glioma samples, while ATRX staining remained intact in 34 pilocytic astrocytomas [93]. Finally, a study that examined low-grade gliomas identified *ATRX* mutations in samples ranging across grade II and III astrocytomas and oligoastrocytomas [104]. These studies established that *ATRX* mutations represent a recurring event not only in pediatric GBM, but also in adult gliomas across the tumor spectrum.

In adult gliomas, *ATRX* inactivation is often accompanied by mutations in *TP53* and *IDH1* [93,102,104], but mutually exclusive from mutations in Homolog of Drosophila capicua (CIC) as well as 1p/19q codeletion, both frequent events in oligodendroglioma [102]. CIC is a transcriptional repressor and potent regulator of cell growth downstream of mitogen-associated protein kinase signaling [115,116]. IDH is a cytoplasmic metabolic enzyme that catalyzes the reversible decarboxylation of isocitrate to α-ketoglutarate (α-KG). The majority of *IDH* mutations constitute a single amino acid substitution at R132 in the active site, resulting in decreased affinity of the enzyme for its normal isocitrate substrate and increased affinity to α-KG [117]. Mutant IDH1 transforms the latter into a so-called 'oncometabolite' referred to as 2-hydroxyglutarate (2-HG) [118]. The R132H substitution is frequent in secondary GBM tumors and in lower grade astrocytomas [117]. It is believed to be an early event in transformation, but the mechanisms involved are not completely resolved. The anomalous production of 2-HG from α-KG has three major potential outcomes that could contribute to cancer progression. First, inhibition of the Tet methylcytosine dioxygenase 2, an enzyme involved in the DNA demethylation pathway and of Jumonji-type histone demethylases leads to the hypermethylation phenotype often associated with *IDH1* mutations [119,120]. These widespread epigenetic alterations to the genome might lead to the suppression of tumor suppressor genes, thus contributing to the cancer phenotype. Second, normal production of α-KG is required for the hydroxylation of proline residues on precollagen fibrils [121]. Aberrant function of IDH1 consequently prevents collagen maturation, which could modify the extracellular matrix and blood–brain barrier, potentially modifying the migration or other properties of glioma cells. The third consequence is the ectopic activation of HIF1α signaling, although it is uncertain whether this is actually occurring in glioma cells and will require further research [122,123]. The collaborative action of *ATRX* and *IDH1* mutations is not yet understood, but might involve the transcriptional activation or suppression of cancer pathways via epigenetic alterations.

ATRX is a suppressor of ALT

Tumor samples with mutations in *ATRX/DAXX* nearly always display evidence of alternative lengthening of telomeres, or alternative lengthening of telomeres (ALT) [94,103,109,124]. Tumorigenesis, or uncontrolled cell growth, is limited by replicative senescence that occurs as a consequence of the gradual telomeric attrition that accompanies semiconservative DNA replication [125]. Most tumor cells are able to overcome this hurdle by hijacking telomerase to maintain telomere length and suppress senescence [126]. Interestingly, *TERT* promoter mutations that result in increased telomerase expression are mutually exclusive with *ATRX* mutations in glioma, suggesting that the growth advantage afforded by *TERT* mutation would be equivalent to that of *ATRX* mutation [127]. Approximately 5% of human cancers instead utilize the ALT pathway to lengthen their telomeres via HR [128]. Cells that have activated the ALT pathway exhibit numerous

characteristics that are distinct from cells that express the telomerase subunit TERT. Differences include telomere length heterogeneity [129], abundant extrachromosomal linear and circular DNA [130], high frequency of telomeric sister chromatid exchange events [131] and the presence of a specific subclass of PML bodies that contain telomeric DNA, shelterin proteins and HR factors [132]. The molecular details of the ALT pathway are uncertain, and even less is known about how the ALT pathway is initially activated in cells. Nevertheless, we do know that ALT is commonly associated with high levels of genomic instability, especially at the telomeres [94], along with remodeling of the telomere architecture [133] and is prevalent in specific cancer types with *ATRX* mutations, such as osteosarcoma and glioma [134]. The mechanism linking ATRX dysfunction to the ALT phenotype and cancer progression is presently an intense area of investigation. Although ATRX loss has been correlated with telomere dysfunction [27–28,47,53,56], exclusive loss of ATRX is not sufficient to drive ALT [56,68,94], suggesting that additional genetic or epigenetic changes are necessary. ATRX may predispose telomeric chromatin to ALT, as its loss results in persistent association of replication protein A (RPA) with telomeric ssDNA, a key intermediate of HR as well as increased TERRA levels at telomeres [56].

ALT is associated with the appearance of large PML-NBs (referred to as ALT-associated PML-NBs or APBs) that contain telomeric DNA, the DNA repair MRN complex, replication protein A (RPA) and telomere repeat-binding factor 1 and 2 (TRF1, TRF2) [132,135–137]. The appearance of APBs is a robust marker for tumors that utilize ALT, and APBs rapidly assemble upon ALT induction [138–140], suggesting that APBs may be involved in the mechanism underlying ALT. This is intriguing since ATRX/DAXX/H3.3 are PML-associated factors and mutations in this pathway drive cancers with a high frequency of ALT. It is possible that defects in the ATRX/DAXX pathway results in a loss of heterochromatic features at telomeres, such as H3.3 enrichment, leading to increased HR rates that are associated with ALT activity, but it remains unclear as to how PML-NBs are involved.

Conclusion

Accumulating evidence point to the importance of chromatin regulation in both development and cancer. ATRX is a perfect demonstration of this, as mutations of the gene can lead to syndromic ID or cancer, depending on the timing and the type of mutation. The developing embryo is unlikely to survive complete ATRX loss-of-function, but merely decreasing the amount or activity of the protein leads to severe congenital abnormalities. Conversely, de novo null mutations of the *ATRX* gene in combination with other genetic events is compatible with cancer progression in many tissues. Several lines of evidence now point to a major role for ATRX (with DAXX) in the deposition of histone H3.3 with effects on nucleosome stability, DNA replication and transcription and maintenance of telomere and heterochromatin structure and stability.

Future perspective

As *ATRX* mutations are identified in more types of cancers, research efforts have intensified to resolve the mechanisms by which ATRX regulates chromatin structure and function. Clearly, ATRX is emerging as a key suppressor of the ALT phenotype and the details of ATRX activities in HR and at the telomere will require further investigation. How replacement of nucleosomes containing the H3.3 variant at heterochromatin contributes to telomeric stability also remains unexplained. Moreover, evidence suggests that ATRX can participate in the incorporation/eviction of other histone variants, such as macroH2A and CENP-A in certain settings [21,96]. A more comprehensive screening of the effects of ATRX inactivation on histone variant incorporation across the genome at different developmental times, in various tissues and cell types and in normal versus disease states will clarify the scope of ATRX's involvement in modifying chromatin in this manner. ATRX loss is associated with altered DNA methylation at repetitive elements [141], and a recent study identified a negative correlation between *ATRX* expression and DNA methylation in astrocytic tumors [142]. Given that methylation changes have been reported in GBM and other glioma subtypes, it will be necessary to further delineate the functions of ATRX in DNA methylation dynamics [143,144].

The mechanisms leading to cancer progression in cells lacking ATRX expression are still far from resolved. Suppression of the ALT phenotype is emerging as a critical piece of the puzzle, but genomic instability caused by DNA replication stress and mis-segregation of chromosomes probably also contributes. Whereas loss of ATRX causes extensive genomic instability, it does not on its own lead to ALT or cancer. This might be because many ATRX-null cells succumb to p53-mediated apoptosis. It follows that additional mutational events that prevent DNA damage-induced cell death (i.e., *TP53* mutations) and that promote unrestricted proliferation despite extensive genetic damage are predicted to synergize with ATRX loss to promote tumorigenesis. The fact that *H3F3A/H3F3B* and *ATRX* mutations co-occur in some pediatric glioblastomas, but not in other types of cancers (i.e., PanNETs) is intriguing, and sug-

gests that the ATRX/DAXX/H3.3 histone deposition pathway functions are distinct between tissues and/or proliferative states. The cooperation between *ATRX* and *IDH1* mutations in adult gliomas is difficult to explain at the moment and will need to be investigated in the future. One of the missing pieces could involve the ability of ATRX to modulate gene expression. For example, loss of ATRX in the neonatal mouse brain resulted in altered expression of many genes, some of which have been implicated in cancer (i.e., *Igf2*) [39,44,145]. Intriguingly, ATRX protein is highly enriched at all known imprinting control regions [39,58,146] and whether the mechanism of imprinted gene regulation involves H3.3 incorporation or chromatin looping or both will need to be further explored. The generation of mouse models lacking ATRX expression in conjunction with other co-occurring mutations (i.e., *H3F3A/H3F3B, IDH1* and *TP53*) in specific cell types will provide excellent tools to decipher the molecular events that predispose *ATRX*-null cells to cancer progression and to devise targeted therapies.

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Executive summary

- α-thalassemia mental retardation, X-linked (ATRX) and death-associated protein 6 modulate heterochromatin structure via histone variant deposition.
- Absence of ATRX causes genomic instability in proliferating cells, often leading to p53-mediated apoptosis.
- ATRX loss-of-function mutations are associated with cancers that exhibit the ALT phenotype.
- ALT and cancer likely result from combined inactivation of ATRX and other mutational events that stimulate survival and proliferation.

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