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Mutant ATRX: uncovering a new therapeutic target for glioma

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Abstract

Introduction—ATRX is a chromatin remodeling protein whose main function is the deposition of the histone variant H3.3. ATRX mutations are widely distributed in glioma, and correlate with alternative lengthening of telomeres (ALT) development, but they also affect other cellular functions related to epigenetic regulation.

Areas covered—We discuss the main molecular characteristics of ATRX, from its various functions in normal development to the effects of its loss in ATRX syndrome patients and animal models. We focus on the salient consequences of ATRX mutations in cancer, from a clinical to a molecular point of view, focusing on both adult and pediatric glioma. Finally, we will discuss the therapeutic opportunities future research perspectives.

Expert opinion—ATRX is a major component of various essential cellular pathways, exceeding its functions as a histone chaperone (e.g., DNA replication and repair, chromatin higher-order structure regulation, gene transcriptional regulation, etc.). However, it is unclear how the loss of these functions in ATRX-null cancer cells affects cancer development and progression. We anticipate new treatments and clinical approaches will emerge for glioma and other cancer types as mechanistic and molecular studies on ATRX are only just beginning to reveal the many critical functions of this protein in cancer.

Keywords

ATRX; alpha-thalassemia X-linked mutant retardation syndrome; high grade glioma; alternative lengthening of telomeres; DAXX; DNA damage; histone chaperone; SWI/SNF2

1. Introduction

The ATRX gene was first discovered in patients with the X-linked mental retardation syndrome (ATRX syndrome) [1]. Within the past decade, its role in cancer is emerging. The

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most recent World Health Organization classification of Central Nervous System Tumors in 2016 incorporates ATRX status in its diagnostic algorithm for glioma variants [2].

The ATRX protein is part of the SWI/SNF2 (SWItch/Sucrose Non Fermentable) family of chromatin remodeling proteins, and in combination with the transcription cofactor, DAXX (death domain associated protein), maintains genomic stability through its deposition of the replication-independent histone variant H3.3 at telomeres and pericentromeric heterochromatin [3]. There is a correlation between ATRX mutations and Alternative Lengthening of Telomeres (ALT), a non-telomerase-dependent telomere lengthening mechanism [4, 5, 6, 7, 8]. ATRX loss in gliomas has been demonstrated to promote ALT. ATRX absence has also been strongly linked to DNA damage and replicative stress [9]. ATRX loss may occur by mutations, deletions, or gene fusions and correlates with other defined molecular changes such as the ALT phenotype, PDGFRA (platelet-derived growth factor receptor alpha) amplification, and TP53 (tumor protein P53) mutation [10].

While significant advances have been made in the molecular classification of brain tumors, the precise role for ATRX in gliomas remains largely unknown. There is a strong association between IDH (isocitrate dehydrogenase) mutations and ATRX mutations [11]. However, concurrence of 1p/19q co-deletion and ATRX loss is very rarely observed.

ATRX status has been shown to correlate with patient age, tumor histopathology, and prognosis. For instance, ATRX mutations confer a better progression free and overall survival in low grade glioma harboring IDH mutations without 1p/19q co-deletion [12, 13]. In genome-wide sequencing of gliomas, 30% of younger patients have ATRX mutations [14]. ATRX is mutated rarely in adult primary high grade gliomas (glioblastoma: GBMs), but it is more common in younger adult patients with lower grade gliomas [15].

With respect to glioblastoma, ATRX expression is varied. Lower ATRX expression has been observed more commonly in primary GBM and anaplastic gliomas relative to grade II gliomas, implicating its status as a marker of malignancy [9]. Current studies demonstrate important characteristics of ATRX mutations in glioma. However, most reports are correlative rather than mechanistic [12,13,16].

Recently, an ATRX-deficient genetically engineered glioma model demonstrated that loss of ATRX reduces median survival and increases genetic instability [17]. ATRX mutation was found to be associated with increased mutation rate at the single-nucleotide variant level. ATRX deficiency has been shown to impair non-homologous end joining (NHEJ), which may explain increased cellular sensitivity to DNA-damaging agents that induce double-strand DNA breaks [4]. These results are in concordance with a recent report in which authors developed an *in utero* electroporation-based method to model diffuse intrinsic pontine glioma (DIPG) with *p53* knock down alone or *p53* + *Atrx* knock downs in combination, to study the molecular mechanisms of H3.3K27M mutation on these genetic backgrounds. They found that only 29% (2/7) of the K27M-shp53 group displayed tumors at 4–6 months compared to 65% (11/17) for the K27M-shp53-shAtrx group, suggesting that *Atrx* knock down could be accelerating tumor development in this model as well [18]. Another report found that although ESCs harboring shRNAs against *p53, Atrx* and IDH

grew slower *in vitro* when compared to ESCs harboring *p53* shRNA and IDH mutation, these cells showed enhanced infiltration in an *in vivo* invasion assay, pointing to increased aggressiveness in association with ATRX knock down [19]. This mechanistic information is important for guiding novel therapeutic strategies where ATRX status or other molecular features are used to select treatment agents that are known to converge on a particular susceptibility of the tumor. Further studies are needed to fully decipher the function of ATRX in the pathogenesis of gliomas, which may inform future targeted treatment approaches.

2. Biology of ATRX

2.1 Introduction

The ATRX gene was first discovered as the genetic cause of the α-thalassemia, mental retardation, X-linked (ATRX) syndrome [1, 20]. This rare condition is characterized by typical craniofacial features, urogenital abnormalities, developmental delays, intellectual disability, hypotonia and varying grades of anemia. As an X-linked recessive genetic condition, ATRX syndrome is predominant in males, although a rare case of a heterozygous ATRX female has been reported [21].

To date, no ATRX syndrome patient with monogenic alterations in other genes has been identified. Even mutations in ATRX interacting genes were not found to cause ATRX syndrome. This indicates that ATRX plays an irreplaceable role in the central nervous system (CNS), genital, skeletal and blood cell development.

The ATRX gene spans almost 300 kbp of the × chromosome long-arm q21.1 band. The gene, containing 36 exons, encodes a 2492 amino acids protein (Figure 1) with a molecular weight of 282,586 kDa, although alternatively spliced transcript variants have been identified [22]. The protein contains an ATPase/helicase domain, belonging to the switch2/sucrose non-fermentable 2 (SWI2/SNF2) family of chromatin remodeling proteins. It also has a distinctive domain termed ADD (ATRX-DNMT3-DNMT3L, ADDATRX), which binds to histone H3 preferentially when it is lysine 9-trimethylated (H3K9me3) as opposed to lysine 4-trimethylated (H3K4me3) [23]. The ATRX ADD (ADDATRX) domain can be further divided in multiple subdomains, including a GATA-1-like domain and plant homeodomain (PHD) zinc finger [24, 25]. Also, ATRX is phosphorylated in a cell cycle-dependent manner. This cell cycle- dependent phosphorylation is concomitant with the association of ATRX with condensed chromatin in early mitosis phase [26] and its association with heterochromatin protein 1α (HPα).

A recent report found that transcription is necessary for recruitment of ATRX to telomeric repeats, and that the recruitment does not require H3K9 tri-methylation. Recruitment is based on the direct interaction between ATRX and DNA-RNA hybrid loops (R loops), an intermediate molecule of transcription. However, it remains to be addressed whether this mechanism of transcription-mediated ATRX recruitment is relevant to the interaction with other chromatin repetitive sequences [27].

Most ATRX mutations in the ATRX syndrome are located in the ADD and the ATPase/helicase domains and the mutated domain is related with the severity of the condition [28]. Interestingly, some of the mutations appearing in ATRX syndrome may be partially rescued by the synthesis of alternative splicing products that exclude the mutated exon. This has led to the hypothesis that most of the mutations in the ATRX syndrome represent hypomorphs [20] and not loss of function mutations. Attempts to target *Atrx* in mouse embryonic stem (ES) cells reveal that knock out was lethal for these cells and conditionally Atrx-null mouse embryos (Heterozygous males) do not survive more than 10 days [29]. Moreover, the conditional inactivation of *Atrx* in the developing brain results in defects in neuronal differentiation, causing a decrease in the number of cells in the neocortex and hippocampus as well as a concomitant reduction in forebrain size [30]. Comparable effects in the development of other cell lineages were also characterized, such as in muscular cells [31], where the high levels of cell proliferation highlight the effects of *Atrx*-inactivation on DNA damage and replicative stress. Similar findings are seen in macrophages [32], Sertoli [33] and limb mesenchymal cells [34]. In recent years, with the growth of available genomic and expression data in cancer, ATRX mutations have been identified in pancreatic neuroendocrine tumors (PanNETs), gliomas (Glioblastoma multiforme (GBM), diffuse pontine glioma (DIPG) and lower grade glioma (LGG)), neuroblastoma (NB), osteosarcoma and adrenocortical tumors [16, 35–39].

2.2 The ATRX/DAXX complex, a histone writer

DAXX was originally characterized as a Fas death receptor binding protein, and thus was named as death domain-associated protein (DAXX) [40]. More recently, DAXX was found to interact with ATRX as part of a histone chaperone complex [41, 42]. Within this complex, DAXX carries out the histone chaperone activity, while ATRX may play a role in recruiting the complex to certain chromatin regions through interaction with histone covalent modifications [42] (Figure 2).

More specifically, the ATRX/DAXX complex is responsible for the replication-independent deposition of H3.3 in telomeric regions [43, 44], pericentric heterochromatin and other DNA repeat regions. Although the histone variant H3.3 was initially found to be associated with transcriptionally active (open) chromatin [45], it was later discovered to be deposited into constitutive and facultative heterochromatin, revealing a connection between H3.3 and genomic stability. H3.3 histone deposition in repetitive regions may contribute to chromatin stability. ATRX loss results in a failure to deposit H3.3 in heterochromatin regions which in turn induces general genomic instability, DNA damage and telomeric end fusions.

In this context, ATRX-mutations in all cancers are associated with ALT [5, 6] (see section 4.3). DAXX mutations are commonly found in pediatric high-grade gliomas and pediatric diffuse intrinsic pontine glioma (DIPG) and are usually mutually exclusive with ATRX mutations [46]. This indicates that the oncogenic role of ATRX in these tumors may be related to its involvement in H3.3 deposition as part of the ATRX/DAXX complex. Alternatively, DAXX mutations are not commonly observed in adult low-grade gliomas, indicating that DAXX-independent mechanisms of mutant ATRX may play a role in the phenotype of these tumor cells (Figure 2).

2.3 The ATRX/DAXX complex and the promyelocytic leukemia nuclear bodies (PML-NBs)

Promyelocytic leukemia nuclear bodies (PML-NBs) are ubiquitous nuclear structures mainly composed of PML (promyelocytic leukemia protein) [47], a tumor suppressor protein. PML-NBs are found in most mammalian cell nuclei [48] and have a dynamic composition and functionality. These characteristics depend on the cell type as well as the metabolic and developmental states. PML-NBs have been implicated in diverse cellular functions, such as regulation of gene expression, tumor suppression, apoptosis, cellular senescence, genomic stability, differentiation, and immune responses. PML-NBs functions are commonly disrupted in leukemia and solid tumors. PML expression is induced in response to stress and senescence. Additionally, it is transcriptionally regulated by P53 [49]. PML is also regulated at the post-translational level, being phosphorylated in response to DNA damage to trigger translocation of PML-NBs to the nucleus or inducing apoptosis. Sumoylation of PML is also essential for the function of PML-NBs [47].

PML-NBs are attached to the nuclear matrix and are in contact with chromatin fibers. A normal chromatin structure is essential for the stability of PML-NBs. PML-NBs also associate with transcriptionally active chromatin regions [50] indicating a potential role in epigenetic regulation. Of special interest in cancer, PML-NBs associate with the *p53* locus.

PML-NBs are composed of more than a hundred proteins including ATRX, DAXX [51] and H3.3 [52]. There are two proposed mechanisms that link PML-NBs with ATRX/DAXX/H3.3 deposition. The classical mechanism suggests that PML-NBs act as reservoirs for H3.3, allowing it to interact with various chaperones [52]. A recent study [53] suggests an additional mechanism by which PML-NBs interact with defined extensive heterochromatic regions (named PML-associated domains or PADs), directing ATRX/DAXX/H3.3 deposition to these regions. Consistent with this mechanism, loss of PML results in a shift in epigenetic signatures in PADs with loss of H3K9me3 and gain of H3K27me3. Both H3K9me3 and H3K27me3 contribute to genomic stability, therefore this genetic shift is proposed to be a compensatory mechanism to preserve heterochromatin integrity when ATRX cannot be directed to PADs [53]. Interestingly, an ATRX/DAXX dependent recruitment of PRC2 (Polycomb Repressive Complex 2) might account for the H3K27 tri-methylation, which in turn may be dependent on ATRX-EZH2 binding module [54].

2.4 G-quadruplexes (G4) structures and ATRX: the case of the α-globin gene regulation

One of the features of the ATRX syndrome is deficient expression of the α-globin gene, which in turn is related to the development of alpha thalassemia-anemia [20]. The upstream region of the α-globin gene contains G-rich tandem repeats that associate to form G-quadruplexes (G4) structures. These structures can occur transiently in guanine-rich singlestranded DNA that may arise during DNA replication, transcription, and telomere elongation. In absence of ATRX, the resolution of G4 structures is hindered, and this may lead to the interruption of the aforementioned processes [55]. ATRX was found to bind to G4 structures, particularly those in the α-globin gene, a phenomenon that seems to be independent of DAXX or other proteins [56]. By binding to G4 structures, ATRX is thought to drive its resolution by means of the deposition of H3.3. ATRX was shown also to negatively regulate the deposition of macroH2A (mH2A), a member of the H2A histone

family [57] that is associated with inactivated transcription. Mutual exclusion between H3.3 and m2HA in nucleosomes has been observed, suggesting that the increased deposition of macroH2A in G4 structures in the absence of ATRX may be an indirect consequence of the lack of ATRX/DAXX-dependent H3.3 deposition. Conversely, evidence suggests that ATRX may interact directly with mH2A in a way that prevents mH2A deposition, although this hypothesis requires further studies [57].

2.5 ATRX and DNA Replication

ATRX mutation delays S-phase progression [58]. This is followed by the activation of DNA-damage responses (DDR) and mitotic failure, which in turn triggers cell death in several cell types [30–32, 58]. In this instance, ATRX is implicated in DNA replication by assisting resolution of non-B DNA structures (such as G4), thus protecting stalled replication forks from collapsing [58]. This process is also essential for the development of normal homologous recombination (HR) mechanisms in replicating cells. This is confirmed by the ATRX-dependent presence of HR proteins (such as BRCA1 and RAD51) at stalled replication forks [59]. An important component of replication instability in ARTX-null cells is the protein MRE11, a member of the Mre11-Rad50-Nbs1 (MRN) complex. The MRN complex has known functions involved with genomic stability and replication, including DNA double-strand break (DSB) repair, restart of stalled replication forks, and activation of checkpoint kinases. MRE11 may degrade DNA replication forks in ATRX knock down cells, suggesting a role for ATRX in inhibiting this reaction. This effect may be related with ATRX/DAXX-H3.3 deposition since DAXX knock down cells have the same behavior. In addition, as a consequence of fork degradation, ATRX-mutated cells activate poly (ADP-ribose) polymerase-1 *(Parp-1)*, which encodes a protein involved in DNA repair. PARP-1 protects stalled replication forks form MRE11 dependent degradation, promoting cell survival [59].

2.6 ATRX and transposable elements

Three independent studies demonstrate that ATRX-H3.3 deposition is required for the silencing of endogenous retroviral elements (ERVs), also known as long terminal repeats (LTRs) containing retrotransposons [60–62]. ChIP-seq approaches revealed that approximately one quarter of H3.3 is incorporated in condensed chromatin regions [58], which also display the repressive histone mark, H3K9me3. Approximately 59% of these heterochromatin regions are composed of interspersed repeats while ~9% are composed of intergenic regions. Since interspersed repeats are a hallmark of ERVs, it is not surprising that H3.3 was found to be enriched in some ERVs [60]. Silencing of ERVs by means of epigenetically repressive H3.3 (with H3K9me3 and H4K20me3, as well as high levels of DNA methylation) is particularly important in embryonic stem (ES) cells because these cells undergo genome-wide demethylation. Thus repressive H3.3 deposition is suggested to act as a compensatory mechanism to repress these repetitive regions [62]. Two convergent reports provide evidence of the role of the ATRX/DAXX complex in H3.3 deposition in ERVs [60, 61], one of them showing that the levels of H3K9me3 are dependent on the complex by means of the recruitment of a corepressor (Tripartite motif-containing 28 (TRIM28)) and the repressive H3K9 methyltransferase SETDB1 [61]. However, a recent report demonstrates that cells with ATRX mutations affecting the DAXX interaction domain do not show ERVs

activation, indicating that DAXX has an ATRX-independent mechanism to maintain ERVs silencing. This is supported by the identification of a DAXX complex that is ATRX-independent and includes the SETDB1 and TRIM28 heterochromatin repressor proteins. This is supported by the evidence that depletion of ATRX by shRNA or knock down in these cells does not induce ERV de-repression [63].

2.7 ATRX and the ZNF (Zinc finger domain) genes

ZNF (Zinc finger domain) C2H2-type genes are the largest family of transcription factors in humans (with more than 700 members) and one of the largest gene families in mammals [64]. Since they encode several (2 to up to 40) tandem zinc finger domain motifs, they share a high level of similarity. ZNF genes contain an atypical chromatin signature: they are enriched in H3K36me3 (mark of actively transcribed genes) and H3K9me3 (repressive mark), although they are actively transcribed. ATRX was found to be enriched at the 3' exons of ZNF genes [65]. ATRX inactivation leads to a decrease of H3K9me3 in these regions and a concomitant increase in DNA-damage [64]. Interestingly, H3K9me3 levels in ZNF genes are particularly sensitive to ATRX inactivation compared to other H3K9me3 regions, and this role of ATRX, which includes recruitment of SETDB1, TRIM28, and zinc finger protein 274 (ZNF274), appears to be DAXX-independent [65].

2.8 ATRX and the Polycomb Repressive Complex 2 (PRC2)

PRC2 is a histone methyltransferase complex involved in di and trimethylation of H3 on lysine 27 (H3K27me2/3). H3K27me3 is associated with negative regulation of gene expression. PRC2 is involved in the process of × chromosome inactivation (XCI). In this process, one of the two X-chromosomes is stochastically inactivated [66–70]. XCI is maintained by the expression of the long noncoding Xist RNA, which spreads over the inactivated chromosome in *cis*. The spread of Xist in turn recruits silencing factors that maintain the repressed state. One of the main factors is the Polycomb repressive complex 2 (PRC2), which leads to a repressed chromatin state [71, 72]. ATRX was found to be the main director for localization of the PCR2 complex to Xist occupied zones [54]. ATRX directly recognizes Xist RNA promoting PCR2 loading by interacting with the methyltransferase component protein EZH2 [54]. The role of ATRX in the PCR2 complex function at somatic chromosome level remains unknown.

2.9 Other ATRX reported interactors

ATRX also interacts with the heterochromatin protein 1 (HP1) ("Chromobox Homolog", CBX) family. This family consists of highly conserved proteins with important functions including heterochromatin maintenance, establishment of higher order chromatin structures, transcriptional regulation, and cell cycle progression. Additionally, they have been found to be deregulated in cancer. Little is understood about the role of ATRX in HP 1-protein functions.

ATRX has also been reported to interact with methyl CpG-binding protein 2 (MeCP2), one of the main epigenome readers in the cell. MeCP2 can bind CpG dinucleotides and assist in transcriptional repression by recruiting histone deacetylases and other epigenome repressor proteins [73]. This mechanism may provide a way to link DNA methylation to histone

deacetylation and may be an essential process to ensure the stabilization of silenced chromatin.

2.10 ATRX and senescence

In the last few years evidence has accumulated linking ATRX with cellular senescence. Cellular senescence refers to the irreversible arrest of cell proliferation in response to various stresses. Senescent cells secrete cytokines that can promote clearance of the tumor by the immune system; therefore, inducing senescence may be an avenue for cancer treatment [74]. Another hallmark of senescent cells is an increase in facultative heterochromatin known as senescence-associated heterochromatin foci (SAHF), a process which involves focal deposition of macro2HA and HP1 family proteins as well as H3K9 tri-methylation. ATRX is required for the development of senescence in response to cytostatic chemotherapy by playing a role in the maintenance of the SAHF. This role is dependent on the capacity of ATRX to recognize H3K9me3 and HP1 [75, 76]. ATRX was also shown to interact directly with the HRAS locus, inhibiting expression of this gene. Inhibition of the HRAS pathway is required for senescence [75]. These findings may indicate that loss of ATRX confers an advantage for developing cancer cells by means of repressing senescence pathways.

3. ATRX mutations in glioma

3.1 ATRX mutations; molecular classification of gliomas and clinical features

Historically, the classification of central nervous system (CNS) tumors has been established by resemblance of the tumor cells with their putative cells of origin. Classification was determined mainly by the morphology of the cells in histological sections combined with the identification of lineage-associated markers [77]. In addition, the proliferative capacity and biological behavior have been used to define the grade of the tumor. However, genetic and molecular characterization has proven to be an invaluable resource to understand and predict tumor phenotype and its responsiveness to treatment. Accordingly, the World Health Organization (WHO) has incorporated molecular parameters in the latest classification of CNS tumors [77].

The integrated analysis for adult diffuse gliomas in this revised classification requires the assessment of the IDH status and of the common chromosomal deletion in 1p/q19. Approximately 90% of adult classical oligodendrogliomas have IDH mutation and 1p/19q codeletion, while most adult diffuse astrocytomas are IDH-mutant but have an intact 1p/19q, allowing discriminating the two major adult groups based on their molecular features [78].

Telomere-associated mutations occurring in the telomerase reverse transcriptase (TERT) gene and in the ATRX gene are present in around 90% of IDH-mutant diffuse gliomas, and they are rarely seen together. ATRX loss-of-function mutations (detected by lack of nuclear staining by immunohistochemistry) occur in approximately 75% of astrocytomas grade II and III, and IDH-mutant secondary glioblastomas, and are exclusive with 1p/19q codeletion, so they are rarely detected in IDH-mutant oligodendrogliomas. In IDH-mutant astrocytomas, ATRX mutation does not seem to provide differential survival. On the other hand, adult WT-IDH tumors with ATRX mutations, which represent a small proportion of the total tumors of

the group (around 3%), have an increased survival compared with the ATRX-WT group [78].

Mutations in the ATRX gene in glioma were found to be distributed evenly across the gene [14, 17]. However, the mutation rate is increased twofold in the ADD domain (which binds to H3 histone) and in the C-terminal ATPase/Helicase domain (Figure 1).

3.2 ATRX mutations in pediatric high-grade glioma (HGG)

During the last 10 years great efforts have been made to unravel the hallmark genetic alterations associated with gliomagenesis [79–81]. Through the latest large-scale genomic and epigenomic studies, scientists have been able to classify the different subtypes of glioma based on their molecular features, which in combination with other patient characteristics, yielded distinct biological subgroups across all patients' ages [82, 83]. Identifying recurrent mutations and subgrouping gliomas in terms of their molecular features allow the identification of interesting targets to develop personalized therapies.

In glioma, essentially all mutations in ATRX are inactivating and can lead to loss of protein expression [84, 85]. These are rarely found in adult high grade glioma (HGG) (age ≥ 21 years) (6–7.1%) [80, 84, 85], but are frequent in grade II and III gliomas (33.2–71%) [14, 15] and secondary glioblastoma (53–57%) [14, 86] in which ATRX mutations are associated with IDH1 and TP53 mutations [15, 84, 87] (Figure 3). Mutations in IDH define a subgroup that is mostly comprised of young adults (median: 40 years, range 13–71 years) and tumors tend to arise in the cerebral hemispheres and frontal cortex [81, 82, 88]. In pediatric non-brainstem HGG (age ≤ 21 years), ATRX mutations are also common, with a frequency ranging between 14–48% [16, 85, 89, 90]. In general, ATRX mutations are found to be significantly associated with a mutation in the tail of the histone H3.3 (H3F3A gene), e.g. H3.3G34R/V [16, 89], accounting for 9–15% of pediatric HGG [16, 89, 90, 91], as well as with mutations in TP53 [16, 81, 90] (Figure 3). However, K27M mutation in the H3F3A gene, another frequent genetic alteration in the pediatric landscape (15–33%) [16, 86, 90, 91] which also usually cooccurs with TP53 mutations [16, 81], overlaps less frequently with ATRX mutations (22–60 %) [16, 89] [90]. These two types of histone mutations define two separate groups in terms of their genetic, epigenetic and clinical characteristics [83]. For instance, the H3K27M cluster consists of childhood patients (median: 10.5 years, range 5–23 years), whereas H3.3G34R/V cluster occurs more often in the adolescent population (median: 18 years, range 9–42 years) [81]. Additionally, while H3.3G34R/V mutations are predominantly restricted to tumors of the cerebral hemispheres, H3K27M mutations are usually found in midline locations, such as the thalamus (40 %), and in 78% of DIPG [90, 92].

In the pediatric-adolescent population, although mutations have also been detected throughout ATRX gene, a tendency to cluster in the helicase domain of the protein's coding region [14, 16] has been observed. In both cases, the great majority of these alterations are truncating mutations, such as frameshifts and nonsense mutations [14, 16, 17].

3.3 ATRX mutations in diffuse-pontine glioma (DIPG)

ATRX mutations are not frequently observed in diffuse intrinsic pontine glioma (DIPG), a type of infiltrating midline glioma that occurs primarily in children. There is no cure for DIPG and median survival is less than one year [93]. Through Next Generation Sequencing (NGS) technology, the histone H3K27M mutation has been identified as the most common mutation in DIPG, occurring in 78% of DIPG tumors [91]. In DIPG, the H3K27M mutation occurs more in H3F3A, the gene encoding histone variant H3.3, and less frequently in HIST1H3B/C, the genes that encode histone variant H3.1 [90] (Figure 3). ATRX mutations are present in 5–12.5% of H3.3 K27M DIPG tumors and are mainly found in older patients [90, 94, 95]. ATRX alterations are more frequently found at autopsy than at diagnosis, suggesting that the mutation may be a secondary event in these tumors [96, 97].

4. Molecular mechanisms altered in ATRX-negative glioma

4.1. ATRX as a glioma driving gene

A recent report provides insights on the role of ATRX in DIPG development [98]. In this report, the authors focus on the role of ATRX as a H3.3 depositor and study the epigenetic consequences of *Atrx* knock down. They obtained neuroephitelial progenitor cells (NPCs) from either $Tp53^{-/-}$ or $Tp53^{+I+}$ mice and knocked down *Atrx*. Profound changes in cellular morphology and gene expression were observed in $Atrx^{-/-}$ cells. They also observed a shift towards astrocytic lineage and an increase in cell migration, both characteristics of DIPG-generating cells. The changes in gene expression were associated with the role of ATRX as epigenetic regulator. ATRX was bound to gene bodies and promoters. In ATRX-null cells, H3.3 deposition at these sites was lost, causing alterations to the chromatin accessibility at these sites. These results highlight the role of ATRX as a cancer driver, encouraging the development of more accurate models to assess the role of ATRX in gliomagenesis.

4.2. ATRX and genome instability in glioma

Genomic instability is a hallmark of almost all human cancer including glioma [99, 100]. Deficiencies in mechanisms of DNA damage repair, cell cycle check point, chromosome segregation, and telomere function are sources of genomic instability. Genomic instability induces somatic mutations that contribute to malignant phenotypes in glioma [17, 101–103]. Around 90% of solid tumors show an alteration called chromosome aneuploidy, which is defined as an abnormal number of chromosomes in the cell resulting from errors of the mitotic checkpoint [104]. Glioma tumor-initiating cells (TICs) display chromosomal instability (CIN), which contributes to TIC genetic diversity [105]. However, there is a certain tolerance level for CIN beyond which the ability of TICs to form new tumors is abolished [105].

The role of ATRX in the resolution of G4 and other non-B DNA secondary structures is essential in preserving genomic stability. Consequently, the accumulation of G4 and other secondary structures in ATRX mutated cells may be a major contributor to genomic instability. ATRX appears to play a critical role in genomic stability preservation and contributes to chromosome dynamics during mitosis [58]. A study performed in HeLa cells shows that ATRX downregulation by siRNA results in abnormal chromosome congression

during mitosis [106]. RNAi knock-down of ATRX in mouse embryonic stem cells also induces a telomere-dysfunction phenotype and reduces chromobox homolog 5 (CBX5) enrichment at telomeres, suggesting that ATRX participates in telomere chromatin integrity maintenance [107]. In addition, studies in transgenic mice show that ATRX contributes to regulation of pericentric heterochromatin structure [108–110]. This is an essential mechanism that coordinates sister centromere cohesion and appropriate separation of chromatids during mitosis. The loss of this mechanism results in centromere instability and aneuploidy [104, 111].

Telomere anomalies, including ALT, also contribute to genomic instability in tumor cells [99]. ALT is found in 4% of cancers, but is particularly frequent (~ 40–60%) in subtypes of glioma with a high frequency of ATRX loss such as diffuse astrocytoma (WHO grade II), anaplastic astrocytoma (WHO II) and pediatric HGG [7]. In fact, it has been suggested that ATRX loss facilitates development of ALT [85]. The first study that associated ATRX with ALT mechanisms also identified G2/M checkpoint deficiency as a hallmark of ALT cells [6]. This work shows that ALT is associated with recurrent copy number alterations, nonreciprocal translocations, deletions, complex rearrangements, and hyper-triploid chromosome number. In addition, depletion of ATRX expression induces micronuclei formation. Therefore, ALT induced by ATRX-loss can also induce genomic instability in glioma. We recently developed a genetically engineered mouse model of ATRX-deficient glioma and demonstrated that ATRX loss induces genomic instability [17]. ATRX loss in glioma induces microsatellite instability (MSI). The microsatellites, which are repeated mononucleotides or dinucleotides sequences, are replicated in an error-prone way, resulting in increased mutation rates. This is reflected in higher levels of single nucleotides variants (SNVs), which prompt ALT [17]. Consequently, ATRX loss appears be a major event which generates CIN and MSI in tumor cells, in direct relation with ALT formation in glioma.

4.3. ATRX and DNA damage responses

DNA damage response (DDR) triggers cellular mechanisms to preserve genomic stability, including DNA damage sensing, cell cycle regulation, and DNA repair [112–114]. Anomalies observed in the karyotype and DNA sequence (such as CIN, MSI and SNVs) of ATRX-null cells indicate defects in DDR. ATRX-deficient/ALT cells have impairment of the DNA damage G1/S checkpoint [6]. This defect in checkpoint initiation is maintained after ionizing radiation, implicating deficiencies in DDR. Also, slow DSB repair kinetics after ionizing radiation (IR) is observed in ATRX-deficient/ALT cells. This is determined by the presence of tumor suppressor p53-binding protein (153BP1) foci after one day of IR. Consistent with this study, neuroprogenitor cells (NPCs) with ATRX-deficiency accumulate endogenous DNA damage, detected by an increase of activated (phosphorylated) H2AX H2AX), which binds to DNA damaged sites. The DNA damage that results from the loss of ATRX in NPCs induces ataxia-telangiectasia mutated kinase (ATM) [110]. Telomerase-fluorescence in situ hybridization (Tel-FISH) assays and γH2AX immunostaining also show that 66% of all yH2AX foci colocalize with telomeres, indicating that DNA damage in ATRX-KO cells accumulates at telomeres and telomeric DNA end fusions [110]. In addition, ATRX-deficient mouse embryonic fibroblasts are more sensitive to hydroxyurea (HU), a compound that stalls the replication fork by depleting the deoxyribonucleotide

(dNT) pool, suggesting a role of ATRX in protecting cells from replication stress [106]. ATRX was shown to be directly recruited to sites of DNA damage, and this event is necessary for efficient checkpoint activation and faithful replication restart [115]. ATRX is a binding partner of the MRN complex, also suggesting a link with DNA-repair [115]. Together, these results suggest a non-canonical function of ATRX in maintaining genomic stability.

We showed that ATRX is recruited to DNA damage sites in mouse glioma and that ATRX loss impairs non-homologous end joining (NHEJ) DNA-repair, which correlates with decreased levels of activated DNA-dependent protein kinase subunit (pDNA-PKcs) [17]. Defects in DDR can impact tumor malignancy and its response to DNA-damaging therapies. We demonstrated that ATRX-deficient mouse tumors are highly aggressive. Nevertheless, a better response to IR treatment was observed in mice harboring ATRX deficient tumors [17]. Together, these findings reveal a critical role of ATRX in DSB repair in glioma, which can be explained by its structural role and by its function as an epigenetic regulator. This highlights the importance of DDR and ATRX status in glioma when implementing an effective therapeutic approach. Depending on the subtype of glioma, ATRX deficiency can be accompanied by other genetic alterations, therefore additional research is necessary to understand the interactions between genetic lesions and their impact on DDR mechanisms and therapeutic responses.

4.4. Molecular Basis of Alternative Lengthening of Telomeres in Gliomas with loss of ATRX and DAXX

Alternative Lengthening of Telomeres (ALT) pathway occurs independently of telomerase activity to enable telomere extension. The association of ALT activation with ATRX deficiency has been observed in 14–35% of cases within a subset of high-grade pediatric gliomas [16]. In addition, 90% of *in vitro* immortalized ALT cell lines harbored loss of ATRX, suggesting inactivation of this protein is a crucial step in the ALT phenotype [6].

The normal maintenance of mammalian telomeres involves polymerization of TTAGGG repeats by telomerase reverse transcriptase. Telomere ends contain a single-stranded G-rich overhang that interacts with the nearby double-stranded region to form a lariat structure known as the telomere loop (T-loop). These G-rich telomeric repeats can form G4 structures and ATRX is thought to play an important role in recognizing them to drive DAXX-mediated H3.3 deposition. This process stabilizes the structures, preventing fork stalling and HR. In the absence of ATRX, fork-stalled structures recruit the MRN complex to telomeres, driving HR [116] (see section 2.5).

The development ALT also involves the shelterin complex, which normally represses HR at telomeres. This complex acts as a telomere cap and protects chromosome ends from the inappropriate activity of the DNA surveillance and repair pathways and also mediate the specialized mechanisms required for their replication and cohesion [5, 8]. Dysfunction in H3.3-ATRX-DAXX chromatin remodeling complex leads to reduced incorporation of H3.3 at telomeric chromatin allowing for a permissive environment for telomere-telomere recombination. The telomere sister chromatid exchange (T-SCE) model proposes that loss of ATRX suppresses the proper resolution of sister telomere cohesion that normally occurs

prior to mitosis [8]. In the absence of ATRX, the histone variant macroH2A1.1 inhibitis the ability of the telomere-associated poly-ADP ribose polymerase tankyrase to dissociate components of the shelterin complex that mediates telomere cohesion. The NuRD-ZNF827 complex anchors neighboring telomeres by partial displacement of telomeric repeat-binding factor 2 (TERF2), a component of the shelterin complex, and favors an environment for ALT through enabling the interaction of telomeres on the same or different chromosomes, the recruitment of HR proteins and deacetylating the chromatin which causes its compaction and could diminish shelterin binding to telomeres [117] (Figure 4).

Cancer cells that exploit the ALT mechanism accumulate telomere-derived homologous recombination byproducts identified as extrachromosomal telomere repeat (ECTR) DNA. ECTR in the cytosol has been shown to stimulate an innate immune stimulator of interferon genes (STING)-dependent cytosolic-DNA sensing pathway. This pathway involves a cyclic GMP-AMP synthase (cGAS) sensor and triggers an interferon expression program, including interferon beta (IF), driving inflammation *in vivo* [118]. The cGAS-STING DNA sensing pathway acts as a protective mechanism by activating anti-tumor T cell responses [119]. ATRX loss alone may promote STING activation, causing antiproliferative effects and immune response promotion *in vivo*. For this reason, ATRX-null cells develop STING inactivation to allow for survival and ALT progression after ATRX loss [119]. These findings indicate that ATRX cancer cells may be vulnerable to STING activation. This may be an important target for the development of novel immune-mediated therapeutic approaches.

5. ATRX as a therapeutic target in GBM

Genomic instability is a characteristic of almost all human cancers. Although ATRX is not commonly a driver mutation gene, it can induce genomic instability leading further genetic rearrangements and/or mutations. In this context, ATRX mutant cells have been shown to be particularly sensitive to DNA damaging agents [17] and this characteristic may be exploited clinically [17].

Over the last few years, there have been advances in the development of therapies aiming to compensate for the epigenetic dysregulation present in cancer cells [120]. Although the epigenetic landscape in ATRX mutant cells has not been elucidated and varies according with the mutational signatures of the cell, the current evidence suggests that ATRX is mainly implicated in the chromatin silencing processes. In consequence, ATRX-null cells may have a disruption in heterochromatin silencing mechanisms, with a concomitant increase of non-B structured DNA. These structures can induce genetic instability and facilitate the development of the ALT phenotype. In this sense, ATRX-null cells could be targeted by epigenetic therapies aiming to restore normal chromatin silencing levels. A series of G4 structure-interacting compounds have demonstrated a potential for cancer therapy and are currently in clinical trials [121–124]. As mentioned before, G4 structures play a key role in the genomic instability of ATRX-null cells. G4 stabilizers may block the interaction of G4 structures with recognizer proteins, possibly preventing DNA repair signaling. To our knowledge G4 destabilizing compounds have not been designed yet.

Most cancer cells acquire a telomere maintenance mechanism (TMM) to avoid shortening of the chromosome ends with each replication. As mentioned above, telomerase expression is the most common acquired mechanism, but in some cases, ALT pathways are activated to maintain telomere length via DNA repair-recombination activated processes [125]. Telomerase inhibitory therapies are being tested in clinical trials and these treatments are predicted to not work in ALT cancers [126]. Since ATRX status is strongly associated with ALT, it is particularly important to know whether the tumor is amenable for telomerase inhibitory therapies or not. Additionally, since ALT cells rely on HR to maintain the telomeres, this process is expected to be critically important for the survival of these cells. For this reason, the inhibition of different proteins involved in the HR pathway was evaluated in ALT cells. The intermediate of replication composed of single-strand DNA bound to replication protein A (RPA) triggers HR by recruiting the key regulator kinase, ATR. ATR was shown to be required for ALT, consequently rendering ALT cells selectively sensitive to the ATR inhibitors such as VE-821 [127].

As mentioned before (section 2.5), ATRX-null neuroprogenitor cells exhibit hyperactivation of poly (ADP-ribose) polymerase-1 (Parp-1) to compensate for the damage in the replication forks [59]. Therefore, ATRX-null cells may be particularly vulnerable to Parp-1 inhibition, providing a potential therapeutic approach to treat these cells. However, this approach has not been evaluated in cancer cells.

As mentioned above, a recent report shows that ATRX is required for the induction of therapy induced senescence, thus ATRX-null cells are less prone to senescence. Since senescent cells secrete cytokines which elicit a robust inflammatory response, exploring role of ATRX in senescence could provide insights in immune mediated strategies to fight cancer.

Finally, genome editing methods have been drastically improved in the last few years, and the opportunity to repair or target genes in cancer is becoming reality. For instance, ATRX susceptibilities may be exploited with gene therapy by editing genes and pathways that are specifically essential for the survival of these cells.

6. Conclusion

ATRX has a broad spectrum of functions in normal cells and its disruption has been associated with several alterations in glioma and other cancers. In the near future, we expect that the roles of ATRX in conferring advantages for cancer development and progression will continue to be investigated, providing an opportunity to unveil new therapeutic approaches based on precision medicine.

7. Expert opinion

Although the initial discovery of ATRX mutations in cancer was associated with ALT, recently uncovered functions of ATRX in different molecular processes suggest a broader and more complex role (Figure 2). Taking into account the findings over the last few years, it now seems clear that the contributions of ATRX mutations in cancer have been understated. ATRX is currently recognized not only as a heterochromatin remodeler, but also as a protein

involved in various essential mechanisms such as gene expression, replication, senescence, DNA repair, and stress responses. Additional work is needed to further dissect the role of ATRX mutations in glioma. In light of the importance of ATRX in glioma, high throughput data already available should be exploited to contrast similar samples with different ATRX mutational status. One of the main drawbacks of data interpretation is that clustering based in DNA methylation or mutations can be misinterpreted by deviations arising from localization, age, or developmental stage grouping, masking the specific gene mutations' contributions.

The interchangeability of ATRX and DAXX mutations in some gliomas subtypes (HGG) whilst not in others (such as in LGG), suggests that ATRX DAXX-dependent functions (particularly H3.3 deposition) are more relevant in pediatric CNS tumors than in adult gliomas. The co-occurrence of ATRX mutations with H3.3/H3.1 mutations and mutations in other epigenetic regulators also suggests that there may be collaborative/addictive effects among them. The study of the emergence of these mutations in the context of the tumor evolution could provide insights determining the importance of ATRX mutations in the development of cancer.

It will be essential to assess the effects of the disruption of the molecular interactions between ATRX and its binding partners. In particular, disrupted ATRX interactions with PCR-2 and HP may play a critical role on transcription and chromatin stability at a global level. Since ATRX is involved in DNA repair, it is important to determine whether ATRX mutations have an effect on increasing the mutation rate in tumor cells, which could be related with evolution of the tumor to a more malignant phenotype. However, the fact that ATRX syndrome patients do not have an increased incidence of cancer [128] indicates that ATRX may not be a conventional cancer driver gene. It is also important to determine whether ATRX mutations in cancer are loss-of-function or whether some functions are retained.

It has been shown that cells derived from an ATRX mouse glioma model are defective in NHEJ, and as a consequence, are more susceptible to DNA damaging treatments [17]. Nevertheless, the opportunities for translating these findings to the clinical arena still need to be explored.

The role of ATRX in X-chromosome inactivation reveals that ATRX interacts with the *Xist* RNA to recruit the PRC2 and inactivate one of the × chromosomes [53]. It is exciting to consider whether ATRX can interact with other non-coding RNAs to recruit chromatin-modifying enzymes to different locations in other the chromosomes, possibly regulating gene expression.

Considering that ATRX mutations are not sufficient to establish ALT, molecular studies aiming at elucidating other contributors to the mechanism and the role they play will likely provide new therapeutic targets.

An interesting area of research arises from the association of ATRX with the immunological responses. Immunotherapy is emerging as one of the more powerful resources to promote cancer rejection, and a main objective is to achieve a selective immune response against

cancer cells. This requires targeting of the vulnerabilities of these cells. The possibility of exploiting the cGAS-STING DNA sensing pathway [119] provides opportunities of eliciting an immune response against ATRX-null ALT cells. Also, as was mentioned before, ATRX mutated cells have impaired senescence activation, but at the same time they express senescence promoting features that are a consequence of their genetic instability. This implies that these cells may be particularly susceptible to the reactivation of silenced senescence. Since senescent cells secrete cytokines, this offers an opportunity to promote the clearance of the tumor by the immune system.

List of abbreviations:

153BP1	Tumor suppressor p53-binding protein
1p/19q co-deletion	Complete deletion of both the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q)
ADD	ATRX-Dnmt3-Dnmt3L (ADD) domain
ALT	Alternative Lengthening of Telomeres
ATM	Ataxia-telangiectasia mutated kinase
ATRX	Alpha Thalassemia/Mental Retardation Syndrome X-Linked protein/gene
BRCA1	Breast cancer 1
C2H2-type	Cys2His2-like type
CBX5	Chromobox homolog 5
CIN	Chromosomal instability
cGAS	GMP-AMP synthase
CNS	Central nervous system
DAXX	Death domain associated
DDR	DNA-damage responses
DIPG	Diffuse intrinsic pontine glioma
DSB	Double-strand break
ERV	endogenous retroviral element
EZH2	Enhancer of zeste homolog 2
G4	G-quadruplexes structures
GBM	glioblastoma multiforme
γH2AX	Phosphorylated H2A variant H2AX

H3.3	Replication-independent histone variant 3.3, also H3F3A
H3K4me3	H3 lysine 4-trimethylated
H3K9me3	H3 lysine 9-trimethylated
H3K27me3	H3 lysine 27-trimethylated
HGG	high grade glioma
HP1	Heterochromatin protein 1 (“Chromobox Homolog”, CBX) family
HP1α	Heterochromatin protein 1α
HR	Homologous recombination
HRAS	Harvey rat sarcoma viral oncogene homolog
HU	Hydroxyurea
IDH	Isocitrate dehydrogenase
IR	Ionizing radiation
LGG	Lower grade glioma
LTR	Long terminal repeat
mH2A	macroH2A (histone)
MeCP2	Methyl CpG-binding protein 2
MRE11	Protein member of the Mre 11-Rad50-Nbs 1 (MRN) complex
MSI	Microsatellite instability
NB	Neuroblastoma
NHEJ	Non-homologous end joining
NPC	Neuroprogenitor cell
NuRD—ZNF827	Nucleosome Remodeling Deacetylase-zinc finger protein 827
PanNETs	Pancreatic neuroendocrine tumors
Parp-1	Poly (ADP-ribose) polymerase-1
PRC2	Polycomb Repressive Complex 2
pDNA-PKcs	DNA-dependent protein kinase subunit
PDGFRA	Platelet-derived growth factor receptor alpha

PML	Promyelocytic leukemia protein
PML-NBs	Promyelocytic leukemia nuclear bodies
R loops	DNA-RNA hybrid loops
RAD51	RAD51 protein (recombinase homologous to bacterial RecA)
RPA	Replication protein A
SAHF	Senescence-associated heterochromatin foci
SETDB1	SET Domain Bifurcated 1 (histone H3-K9 methyltransferase)
shRNA	Short hairpin RNA
siRNA	Small interfering RNA (siRNA)
SNV	Single nucleotides variant
STING	Stimulator of interferon genes
SWI/SNF2	SWItch/Sucrose Non Fermentable (family of chromatin remodeling proteins)
T-SCE	Telomere sister chromatid exchange
Tel-FISH	Telomerase-fluorescence in situ hybridization
TERF2	Telomeric repeat-binding factor 2
TERT	Telomerase reverse transcriptase
TIC	Tumor-initiating cell
TMM	Telomere maintenance mechanism
TP53	Tumor protein p53
TRIM28	Tripartite motif-containing 28
WHO	World Health Organization
XCI	X chromosome inactivation
Xist	X-inactive specific transcript
ZNF	Zinc finger domain
ZNF274	Zinc finger protein 274

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*Article of special interest

**Article of outstanding interest

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Key issues

- ATRX is a chromatin remodeling protein whose main function is the deposition of the histone variant H3.3, mainly in facultative and constitutive heterochromatin.
- Mutations in ATRX were found in various cancers and the occurrence of these mutations has a close association with the development of the Alternative Lengthening of Telomeres (ALT) mechanism, a recombination-based telomere maintenance mechanism (TMM).
- ATRX mutations occur in some types of glioma, eliciting changes in the features of the cancer, from molecular to clinical perspectives. The occurrence of mutations in epigenetic regulators is frequent in glioma, indicating that epigenetic reprogramming is a critical event in glioma development.
- In addition to the association of ATRX with the ALT mechanism, ATRX has a broad spectrum of functions related with its ability to reshape the epigenetic state of the cells.
- Cancer cells harboring ATRX mutations exhibit chromatin instability and impaired DNA damage response, which make them vulnerable to DNA damaging treatments. While in animal models ATRX mutant gliomas progress faster than the ATRX wild type counterparts, they are more responsive to DNA damaging treatments. There is also a correlation between the presence of ATRX mutations and extended survival in treated patients.
- Although ATRX has been associated with a wide variety of functions, it remains to be addressed how the loss of these functions in ATRX-null cancers cells affect cancer development and progression.
- ATRX-mutated cancer cells share molecular characteristics that can be exploited to develop specific therapeutic approaches. DNA damage, chromatin instability, immune response and senescence induction emerge as the main vulnerabilities in these cells.

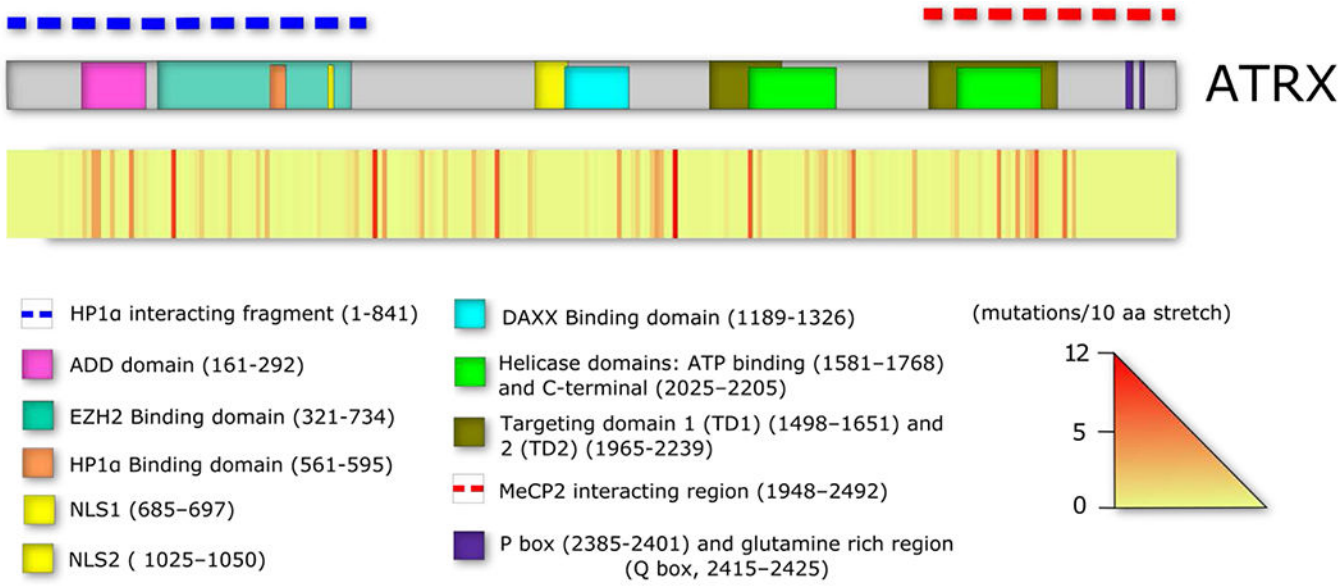

Figure 1. ATRX mutations in glioma.

Heat map of mutations found in glioma tumors using data from the Catalogue of Somatic Mutations in Cancer (COSMIC). Only mutant ATRX gliomas with DNA sequence data were included and mutation frequency per 10 amino acid stretch was analyzed. The domains of ATRX are depicted at the top of the figure, and are assigned according to previous reports [129, 130]. Mutation frequency throughout the protein is illustrated in a heat map that is aligned with the protein diagram. [**NLS1** and **NLS2**: nuclear localization signals; **TD1** and **TD2**: targeting domains to PML bodies, **P-Box**: SNF2 conserved element involved in transcriptional regulation; **Q-box**: stretch of glutamine residues which is a potential protein interaction domain.

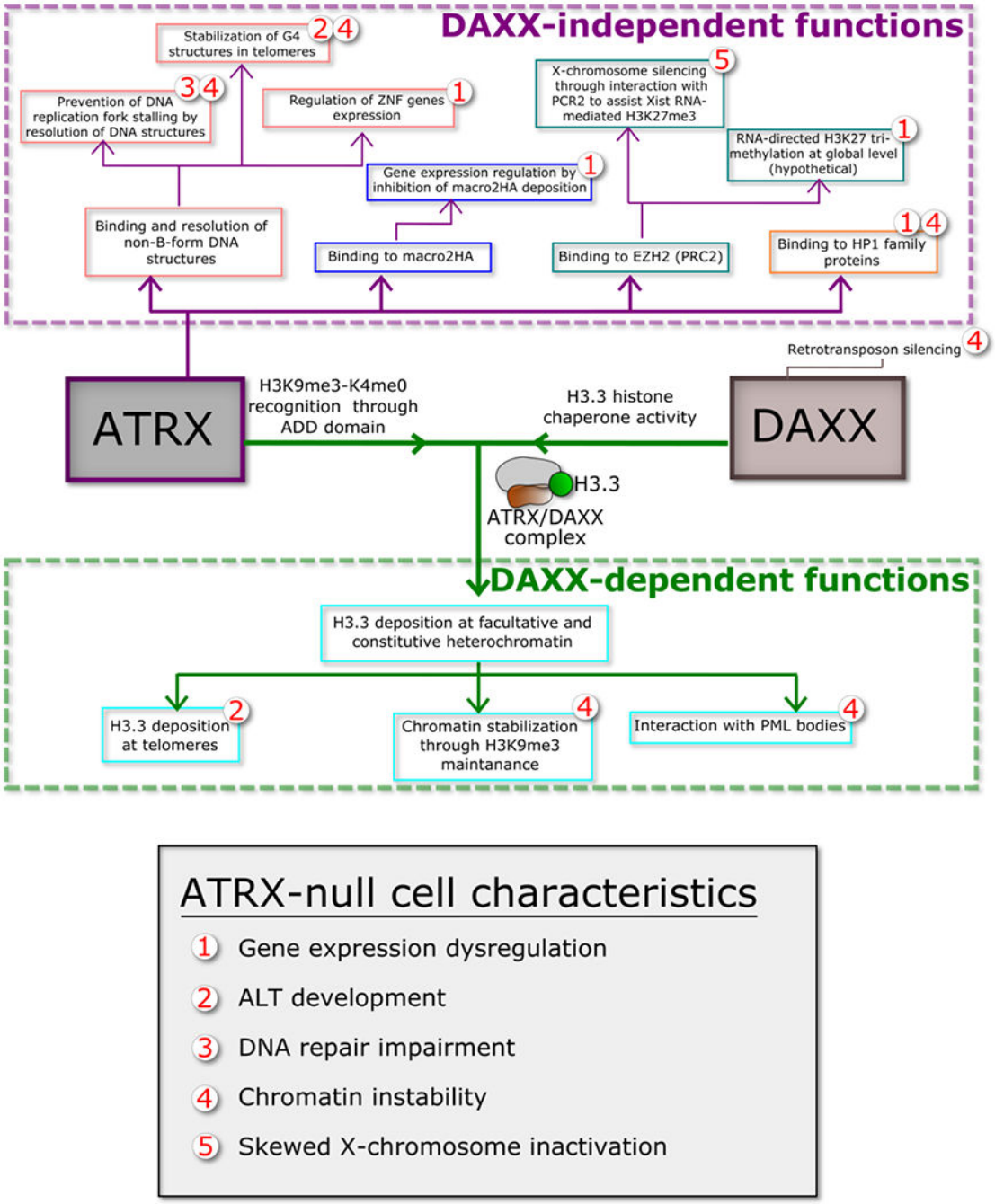

Figure 2. The various functions of ATRX

. ATRX cellular functions (shown in boxes) classified by its dependence on DAXX interaction. The disruption of each function contributes to the development of various ATRX-null characteristics seen in cancer cells. These characteristics are listed.

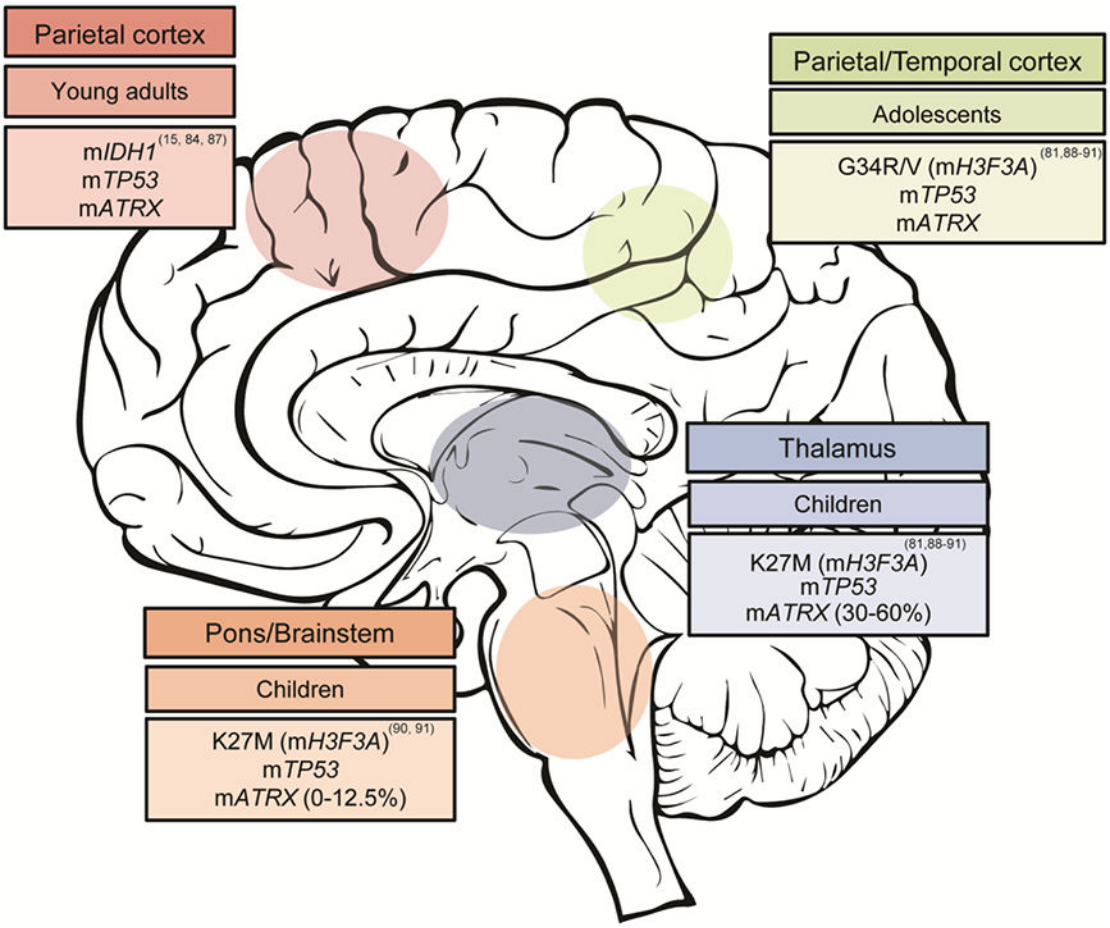

Figure 3. Neuroanatomical regions of gliomas harboring ATRX mutations.

A schematic representation of the sagittal view of a human brain highlighting the most frequent locations where gliomas with ATRX mutations are found. The most common co-occurring genetic alterations are shown in each group. H3K27M tumors are less frequently associated with ATRX mutations (frequencies of ATRX-K27M mutation co-occurrence are shown between brackets).

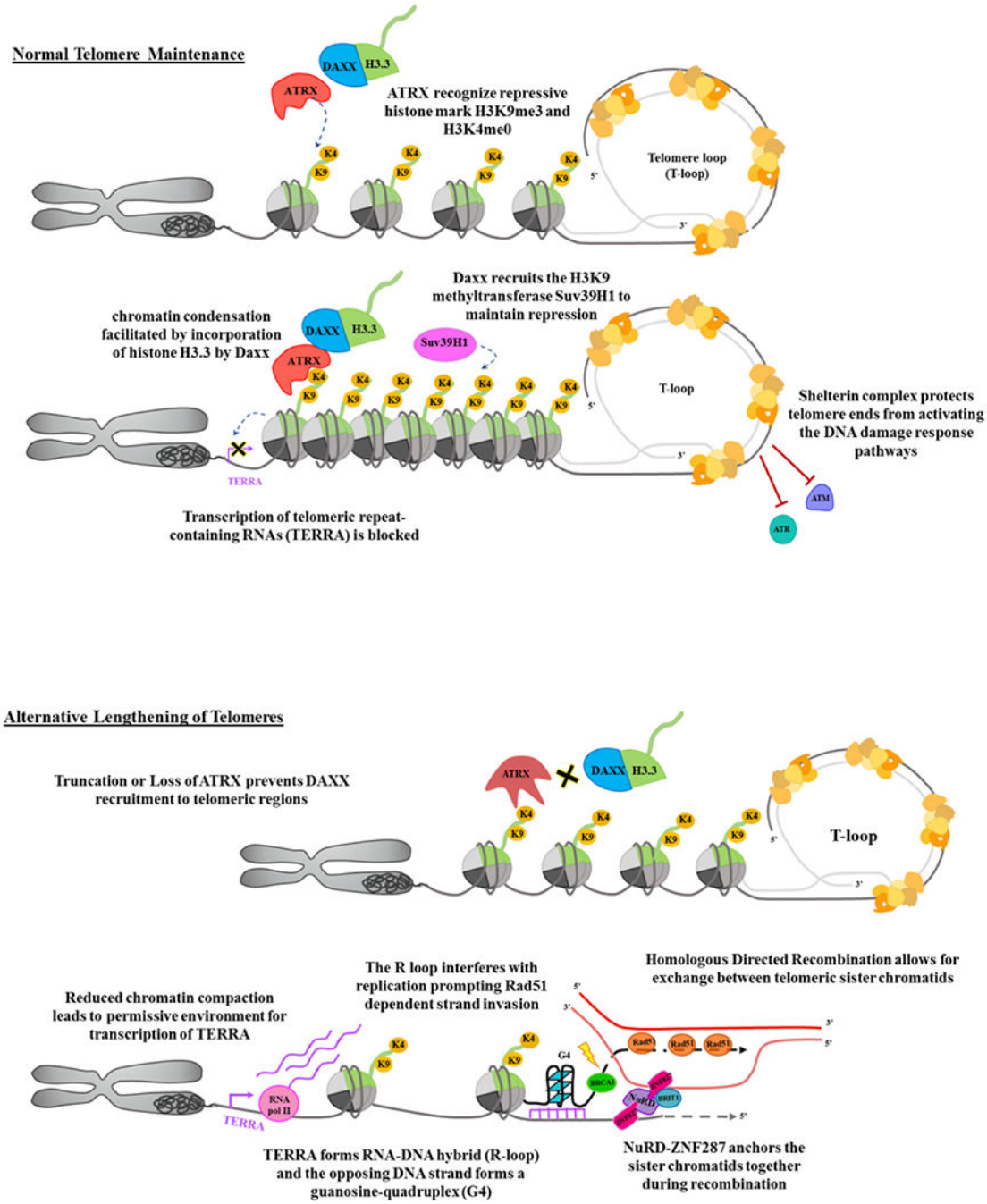

Figure 4. ATRX and ALT pathway.

A) During normal telomere maintenance, ATRX recognizes the repressive histone marks, H3K9me3 and H3K4me0, which are found near the end of telomeres. ATRX recruits DAXX, which incorporates H3.3 onto naked DNA. DAXX enlists the H3K9 methyltransferase, Suv29H1, to deposit methylation marks on the newly introduced H3.3 histones to maintain the heterochromatic state of the telomeres following mitosis. The degree of chromatin compaction prevents the expression of the long noncoding transcript of telomeric repeat-containing RNAs (TERRA). The T-loop is stabilized by the components of

the shelterin complex (TRF1, TRF2, POT1, TPP1, RAP1, and TIN2), which protects the telomere ends from activating the DNA damage response pathways. B) Alternative Lengthening of Telomeres (ALT) pathway occurs independently of telomerase activity. It relies instead upon homology directed recombination to extend telomeres. In absence of ATRX, DAXX cannot be recruited to telomeres; causing reduced chromatin compaction which allows for transcription of TERRA. TERRA interacts with telomeres generating a RNA-DNA hybrid (R-loop) structure, which restructures the complementary DNA into a G4 structure. The R-loop interferes with normal replication prompting Rad51- dependent strand invasion to the neighboring sister chromatid. The activation of HDR is stabilized by the NuRD-ZNF287 complex, which anchors the sister chromatids together throughout the exchange.