ATRX and DAXX: Mechanisms and Mutations

Michael A. Dyer,¹ Zulekha A. Qadeer,^{2,3} David Valle-Garcia,² and Emily Bernstein^{2,3}

¹Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105

²Departments of Oncological Sciences and Dermatology, Icahn School of Medicine at Mount Sinai, New York, New York 10029

³Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, New York 10029

Correspondence: michael.dyer@stjude.org; emily.bernstein@mssm.edu

Recent genome sequencing efforts in a variety of cancers have revealed mutations and/or structural alterations in *ATRX* and *DAXX*, which together encode a complex that deposits histone variant H3.3 into repetitive heterochromatin. These regions include retrotransposons, pericentric heterochromatin, and telomeres, the latter of which show deregulation in *ATRX/DAXX*-mutant tumors. Interestingly, *ATRX* and *DAXX* mutations are often found in pediatric tumors, suggesting a particular developmental context in which these mutations drive disease. Here we review the functions of *ATRX* and *DAXX* in chromatin regulation as well as their potential contributions to tumorigenesis. We place emphasis on the chromatin remodeler *ATRX*, which is mutated in the developmental disorder for which it is named, α -thalassemia, mental retardation, X-linked syndrome, and at high frequency in a number of adult and pediatric tumors.

Recent whole-genome and exome sequencing efforts across a wide spectrum of cancers have unexpectedly revealed chromatin remodeling-encoding genes as being frequently altered (Jiao et al. 2011; Schwartzentruber et al. 2012; Cheung and Dyer 2013; Helming et al. 2014). The *ATRX* gene, which encodes a SWI/SNF-like chromatin remodeling protein, is frequently mutated in a variety of tumors, including adult lower-grade gliomas, pediatric glioblastoma multiforme, pediatric adrenocortical carcinoma, osteosarcoma, and neuroblastoma (Table 1). Some, but not all, of these cancers also harbor *DAXX* and/or *H3F3A* (H3.3) mutations (Schwartzentruber et al. 2012; Wu et al. 2012).

Given that *ATRX* and *DAXX* form a histone chaperone complex that deposits histone variant H3.3 into specific genomic regions, it is intriguing that mutations in the histone as well as its chaperone complex have been identified (Schwartzentruber et al. 2012). Although certain H3.3 mutations appear to be associated with altered Polycomb repressive complex 2 (PRC2) activity (e.g., H3.3K27M) (Bender et al. 2013; Chan et al. 2013; Lewis et al. 2013; Funato et al. 2014), the mechanisms by which *ATRX* and *DAXX* mutations promote oncogenesis may be distinct from those elicited by H3.3 mutation.

Given the identification of *ATRX* and *DAXX* mutations in cancer, these factors have received

Table 1. ATRX alterations identified in human cancers

Disease	Frequency (%)	Type of alteration	Age group	ALT association	Co-occurrence of DAXX/H3.3 mutations	References
Whole-genome and exome studies						
PanNets	19	Deletions, point	Adult	Yes	Yes	Heaphy et al. 2011; Jiao et al. 2011
LGG	42	Indels, point	Adult	Yes	No	Kannan et al. 2012; Johnson et al. 2014; TCGA 2015
GBM	29/16	Indels, point	Pediatric/adult	Yes	Yes/no	Liu et al. 2012b; Schwartzentruber et al. 2012
DIPG	8	Point	Pediatric	N/A	Yes/no	Khuong-Quang et al. 2012; Wu et al. 2014
NB	8/28	Indels, point, in-frame fusions	Pediatric/adolescent	Yes	No	Cheung et al. 2012; Molenaar et al. 2012; Pugh et al. 2013; Peifer et al. 2015; Valentijn et al. 2015
OS	29	Indels, point, in-frame fusions	Pediatric/adolescent	Yes	No	Chen et al. 2014
Adrenocortical tumors	32	Indels, point	Pediatric/adolescent	Yes	Yes	Assié et al. 2014; Pinto et al. 2015
mRNA/protein studies						
Melanoma	N/A	Transcriptional down-regulation, protein loss	Adult	N/A	Adult	Liau et al. 2015b
Leiomyosarcoma	33	Protein loss	Adult	Yes	Adult	Liau et al. 2015c
Angiosarcoma	18	Protein loss	Adult	Yes	Adult	Liau et al. 2015a
Liposarcoma	29	Protein loss	Adult	Yes	Adult	Lee et al. 2015
Soft tissue sarcoma	13	Protein loss	Adult	Yes	Adult	Liau et al. 2015a,b,c

PanNets, Pancreatic neuroendocrine tumors; LGG, low-grade glioma; GBM, glioblastoma multiforme; DIPG, diffuse intrinsic pontine glioma; NB, neuroblastoma; OS, osteosarcoma; ALT, alternative lengthening of telomeres; N/A, not applicable.

renewed attention, particularly in the field of pediatric oncology. To date, however, their role in promoting pathogenesis in both adult and pediatric tumors remains unclear. Here we review the functions of the chromatin remodeler ATRX and the histone chaperone DAXX in the context of cellular biology, development, and disease.

ATRX SYNDROME

ATRX was first discovered through efforts to identify the genetic lesion that contributes to the α -thalassemia, mental retardation, X-linked (ATRX) syndrome (Gibbons et al. 2003). Patients are characterized during early childhood by distinctive craniofacial features, significant developmental delays, genital anomalies and sterility, microcephaly, and severe intellectual disabilities (Gibbons et al. 2012). Coupled to these features, patients with ATRX syndrome also present with varying degrees of anemia caused by α -thalassemia, a condition caused by deficient α -globin expression (Gibbons et al. 2008). Consistent with a recessive X-linked inheritance pattern, ATRX syndrome is predominant in males. However, there are rare reports of ATRX syndrome in 46XX heterozygous females, which show an uneven pattern of X-inactivation that favors the expression of the mutant allele (Badens et al. 2006b).

Importantly, mutations in *ATRX* are the only genetic lesions that cause ATRX syndrome (Gibbons et al. 2008). This suggests that ATRX is essential for diverse developmental processes across the ectodermal (neural crest and central nervous system [CNS]), mesodermal (bone and erythroid lineage), and endodermal (genitalia) lineages. Interestingly, mutations in genes that encode proteins that interact with ATRX, such as *DAXX*, have not been identified in patients with ATRX syndrome. Moreover, ATRX patients do not have an increased incidence of cancer. Taken together, ATRX plays a unique role in development and raises questions about tissue specificity, as well as its role in promoting cancer.

Considering the monogenic origins of ATRX syndrome, it is important to identify

the type of mutations involved and their clinical severity. For example, mutations in the amino-terminal ADD (ATRX-DNMT3-DNMT3L) domain produce more severe psychomotor phenotypes than do mutations in the carboxy-terminal helicase domain (Badens et al. 2006a). Interestingly, ATRX syndrome mutations almost exclusively lie in either of these two domains (Fig. 1A). ATRX's helicase domain has DNA translocase activity via ATP-dependent hydrolysis (Xue et al. 2003; Mitson et al. 2011). In fact, inactivating mutations in this domain results in translocase defects, and one particular disease-causing mutation even uncouples ATP hydrolysis from DNA binding (Mitson et al. 2011). The ADD domain contains a GATA-like domain and a PHD (plant homeodomain) finger, which together recognize H3K9me3 when unmethylated at H3K4 (Dhayalan et al. 2011; Eustermann et al. 2011; Iwase et al. 2011). Interestingly, a nonsense mutation at residue 37 of ATRX is associated with a milder phenotype than that associated with missense mutations in the ADD and helicase domains (Guerrini et al. 2000). The nonsense mutation at residue 37 is spliced out of a subset of transcripts, which partially restores ATRX protein function. Indeed, it has been proposed that virtually all mutations in patients with ATRX syndrome represent hypomorphs caused by protein destabilizing effects of the mutations (Gibbons et al. 2008). Because mutations in numerous cancers occur outside of the ADD and helicase domains and span widely across the *ATRX* coding region (Fig. 1), they have been proposed to be loss-of-function mutations (Watson et al. 2015). Consequently, ATRX syndrome mutations may shed light on our understanding of somatic *ATRX* mutations in cancer.

MOUSE MODELS OF ATRX DEFICIENCY

Consistent with the hypothesis that ATRX is required for embryonic development, *Atrx*-null mice die at E9.5 (Garrick et al. 2006). Moreover, conditional inactivation of *Atrx* in the developing forebrain results in perinatal lethality. The frontal cortex, subiculum, and hippocampus are reduced in size, and the dentate

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Figure 1. Mutations identified in ATRX syndrome and a subset of cancers. (A) Schematic summarizing that the mutations identified in patients with ATRX syndrome are concentrated in the ADD and SNF2_N helicase domains. Mutations are represented by a black needle; larger needle head size and height indicate larger mutation frequency. (B) Needle plot depicting the distribution of mutations identified in GBM (glioblastoma multiforme), LGG (low-grade glioma), and NB (neuroblastoma) along the ATRX protein product (Gonzalez-Perez et al. 2013). The needles' head size and height indicate mutational recurrence. Note that these mutations are present throughout the gene locus and not concentrated at one particular domain. Blue bars below protein product represent in-frame fusions identified in NB (Cheung et al. 2012).

gyrus is completely absent in *Atrx^{Lox};Foxg1-Cre* mice (Bérubé et al. 2005). There is no defect in neural progenitor cell proliferation, but cells undergo apoptosis as they exit the cell cycle and initiate their differentiation programs. This effect appears to be cell autonomous. Thus, the phenotype in mice is much more severe than that in humans, because patients with ATRX mutations can survive to adulthood.

These findings in genetically engineered mouse models are consistent with the hypothesis that almost all ATRX alleles in patients with ATRX syndrome represent hypomorphs (Gibbons et al. 2008). To date, hypomorphic alleles of *Atrx* have not been modeled in mice.

There are additional cell-type-specific defects in development upon *Atrx* inactivation. For example, the amacrine and horizontal inter-

neurons are lost in *Atrx*-deficient retinæ (Medina et al. 2009). Up to 23% of patients with ATRX syndrome have visual anomalies (Medina et al. 2009), but whether these are attributable to defects in interneuron differentiation and/or survival remains unknown. The consequence of *Atrx* inactivation in skeletal muscle results in defective regenerative capacity of satellite cells (Huh et al. 2012). Skeletal muscle homeostasis requires continuous production of myoblasts from satellite cells, which then differentiate and form myotubes. Myoblasts derived from *Atrx*-deficient satellite cells are defective in cell cycle progression and show evidence of DNA damage. The proliferating myoblasts have elevated levels of both phosphorylated ATM and Chk1, which is indicative of replicative stress, and this effect is cell autonomous. In addition, the telomeres are unstable in *Atrx*-deficient myoblasts and show increased rates of merging, duplication, and bridging (Huh et al. 2012). These findings are consistent with the reported roles of ATRX in human cells (see below).

Collectively, these studies highlight the major mechanisms underlying developmental defects that result from *Atrx* inactivation during development. Specifically, they underscore the lineage-specific defects in differentiation (CNS) and in progenitor cell proliferation as a result of replicative stress-induced DNA damage (muscle). Similar defects have been reported in other cell types and tissues, such as embryonic stem cells, macrophages, Sertoli cells, and limb mesenchyme (Bagheri-Fam et al. 2011; Conte et al. 2012; Solomon et al. 2013; Clynes et al. 2014). It is possible that in lineages such as muscle wherein there is massive proliferative expansion through a stem-cell-related mechanism (i.e., satellite cells), the DNA replication phenotype is predominant. However, in the developing CNS wherein proliferation during development is primarily limited to multipotent progenitor cells, replicative stress might not be as important as lineage-specific defects during differentiation. Thus, the varied roles of *Atrx* in differentiation can be a starting point to explain the phenotypes across mesodermal, endodermal, and ectodermal lineages. Moreover, it can provide a context for understanding the role of ATRX in cancer.

DEATH DOMAIN-ASSOCIATED PROTEIN DAXX

DAXX was originally identified as a Fas death receptor binding protein that induced apoptosis via JNK pathway activation (Yang et al. 1997). Thus, it was coined the death domain-associated protein, DAXX. However, its role in apoptosis remains unclear because of conflicting reports, including the fact that the knock-out mouse resulted in extensive apoptosis and embryonic lethality, rather than the expected antiapoptotic phenotype (Michaelson et al. 1999). Moreover, DAXX was found to be primarily a nuclear protein in which it localizes to promyelocytic leukemia (PML) bodies and was suggested to promote sensitivity to the Fas receptor from its nuclear location (Torii et al. 1999; Zhong et al. 2000). DAXX was also reported to interact with a series of transcription factors (TFs) and histone deacetylase (HDAC) complexes (Hollenbach et al. 1999, 2002; Li et al. 2000). Thus, by the early 2000s, the primary role proposed for DAXX function was in regulating transcription, although the mechanism by which it did so was unclear.

Although ATRX function was studied in the context of ATRX syndrome and DAXX function in the context of apoptosis, two independent biochemical studies showed that ATRX and DAXX are in fact components of a distinct nuclear complex (Xue et al. 2003; Tang et al. 2004). It was not until the purification of histone variant chaperone complexes some years later by the groups of Hamiche and Allis that the function of the ATRX/DAXX complex would be assigned as an H3.3-specific deposition complex (see below) (Goldberg et al. 2010; Drane et al. 2010; Lewis et al. 2010). Here, we outline the molecular mechanisms of ATRX and DAXX function as a complex as well as their independent functions in chromatin.

ATRX AND DAXX REGULATE HISTONE VARIANT DEPOSITION

Biochemical investigation of H3.3 chaperone complexes identified ATRX and DAXX, and found this complex to be necessary for H3.3

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deposition at telomeres (Goldberg et al. 2010; Lewis et al. 2010) and pericentric heterochromatin (Drane et al. 2010) (Fig. 2A). Interestingly, these studies identified a second histone chaperone complex for H3.3, as the HIRA complex had been previously identified to deposit H3.3 at euchromatic regions of the genome, such as transcriptional start sites and gene bodies (Ahmad and Henikoff 2002; Tagami et al. 2004). The studies that identified the role of the ATRX/DAXX complex in H3.3 deposition not only revealed that distinct factors are responsible for H3.3 incorporation at specific genomic locations but also uncovered an unexpected role for H3.3 at heterochromatic regions of the genome.

Within the ATRX/DAXX complex, DAXX was shown as the component containing H3.3 histone chaperone activity (Drane et al. 2010; Lewis et al. 2010). For example, DAXX binds directly to H3.3 and contributes to the deposition of H3.3-H4 tetramers onto naked DNA (Drane et al. 2010; Lewis et al. 2010). Elegant structural studies resolved DAXX in complex with an H3.3/H4 dimer and showed that residue G90 (unique to H3.3 vs. canonical H3), is critical for this interaction (Elsässer et al. 2012; Liu et al. 2012a). Consistent with its role as a chromatin remodeler, ATRX binds to DAXX to incorporate H3.3 into telomeric, pericentromeric, and other repetitive DNA (Fig. 2A) (Drane et al. 2010; Goldberg et al. 2010; Wong 2010). Although the precise role of ATRX in this process remains unclear, it is well established that ATRX can recognize H3K9me3 through its ADD domain and is likely responsible for the recruitment of its binding partner DAXX to such regions.

Before studies implicating ATRX as a component of an H3.3 histone chaperone complex, its role in transcription was studied primarily at the α -globin gene cluster in erythroid cells because of the α -thalassemia phenotype observed in ATRX syndrome patients. In this particular case, ATRX promotes expression of the *HBA* genes at the α -globin locus on the subtelomere of chromosome 16 (Law et al. 2010). Our group identified a role for the transcriptionally repressive H2A variant macroH2A at the α -globin lo-

cus and telomeres (Fig. 2A). In the absence of ATRX, macroH2A is found enriched at these regions, concomitant with reduced α -globin expression—a prevalent feature of ATRX syndrome patients. Interestingly, this function for ATRX in negatively regulating macroH2A deposition at the α -globin locus is DAXX-independent (Ratnakumar et al. 2012).

ATRX BINDS TO REPETITIVE REGIONS OF THE GENOME

Initial immunofluorescence studies of ATRX found it to be localized to condensed DAPI-dense regions, suggesting that ATRX is primarily associated with heterochromatin (McDowell et al. 1999; Bérubé et al. 2000; Xue et al. 2003). More recently, ChIP-seq studies identified ATRX enrichment at telomeric, subtelomeric, and pericentric repeats, consistent with the functional studies described above (Law et al. 2010). Additional ChIP-seq analyses found that ATRX is also enriched at the silenced allele of imprinted regions, particular families of retrotransposons, a subset of G-rich intragenic regions, and the 3' exons of genes belonging to the C2H2 Zinc Finger (ZNFs) family of transcription factors (Elsässer et al. 2015; He et al. 2015; Levy et al. 2015; Sadic et al. 2015; Voon et al. 2015; Valle-Garcia et al. 2016). Some of these recent findings are outlined below.

Telomeres

The ATRX/DAXX complex is enriched at telomeric repeats where it has been shown to regulate H3.3 deposition (Goldberg et al. 2010; Law et al. 2010; Lewis et al. 2010; Wong 2010) (Fig. 2A). Although the precise function of H3.3 deposition at telomeres remains unclear, ATRX depletion leads to telomere dysfunction (Wong 2010; Watson et al. 2013; Ramamoorthy and Smith 2015; Watson et al. 2015). For example, ATRX loss leads to increased DNA damage, increased rates of telomeric end fusions, persistent telomere cohesion, and general genomic instability (Wong 2010; Watson et al. 2013, 2015; Ramamoorthy

Figure 2. ATRX has multiple functions in the chromatin landscape. (A) The SWI/SNF-like chromatin remodeler ATRX is found in various complexes throughout the chromosome to maintain distinct chromatin states. (B) Model summarizing ATRX's roles in the epigenetic landscape. (Left) ATRX has chromatin remodeling function to bind and regulate genomic regions enriched for H3K9me3 via its ADD domain and HP1 α . It also recruits EZH2 to deposit H3K27me3 to regulate transcription. (Right) ATRX functions to negatively regulate macroH2A (mH2A) deposition at the α -globin locus and telomeres and is critical in H3.3 deposition along with DAXX, particularly at repetitive regions (e.g., telomeres). (Center) ATRX also binds DNA through its SNF2_N helicase domain to resolve tandem repeats to promote transcription at the α -globin locus.

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and Smith 2015). Intriguingly, alternative lengthening of telomeres (ALT) is a frequent feature of *ATRX*-mutated cancers (see below), suggesting that *ATRX* indeed has an important role in the regulation of telomeres.

G4 Structures

ATRX syndrome patients display varying degrees of severity of α -thalassemia (Gibbons 2006). Chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq), revealed that *ATRX* directly binds to G-rich tandem repeats upstream of the α -globin genes (Fig. 2B) (Law et al. 2010). These repeats tend to form G-quadruplexes (G4) structures—secondary DNA structures that can lead to replicative stress (Huh et al. 2012, Leung et al. 2013, Clynes et al. 2014) or block transcriptional processes (Levy et al. 2015). Such structures are typically found in tandem repeats at telomeres and pericentromeric repeats, but not restricted to them (e.g., intragenic CpGs, differentially methylated regions, etc.) (Huppert and Balasubramanian 2005). Using recombinant *ATRX* protein, *in vitro* assays showed binding to preformed G4 oligonucleotides, suggesting that this property of *ATRX* is in fact direct (Law et al. 2010).

As *ATRX* binds G4 DNA, it has been suggested to resolve these structures (Law et al. 2010; Clynes and Gibbons 2013). Consistent with this, the severity of the α -thalassemia phenotype in *ATRX* syndrome patients directly correlates with VNTR (variable number tandem repeat) size at the α -globin locus (Law et al. 2010). In the absence of *ATRX*, larger VNTR regions form more G4 structures with increased impact on the transcription of the α -globin genes. This is corroborated by findings that show the phenotypes associated with *ATRX* loss are enhanced with compounds that stabilize G4 DNA (Watson et al. 2013; Clynes et al. 2014, 2015). Further, it has been suggested that one of the mechanisms by which *ATRX* suppresses recombination at telomeric repeats is by resolving G4 structures through the deposition of H3.3 (Clynes and Gibbons 2013; Clynes et al. 2015).

Transposable Elements

The *ATRX*/*DAXX* complex also mediates the deposition of H3.3 at retrotransposons, particularly those belonging to the endogenous retrovirus (ERV) family (Elsässer et al. 2015; He et al. 2015; Sadic et al. 2015). The corepressor TRIM28 (also known as KAP1) and the H3K9 histone methyltransferase SETDB1 (also known as ESET), colocalize with *ATRX* at ERVs (Fig. 2A). *ATRX*-bound retrotransposons have high levels of H3K9me3 that are dependent on the *ATRX*/*DAXX* complex. Knockdown of either component, or H3.3 itself, reduces the H3K9me3 levels at these repetitive regions and induces aberrant transcription (Elsässer et al. 2015; Sadic et al. 2015). Moreover, *ATRX*/*DAXX*-mediated silencing of ERVs is critical for preimplantation embryos during the wave of global DNA demethylation (He et al. 2015). Collectively, these studies highlight the critical role of *ATRX* in silencing repetitive elements through regulation of H3K9me3.

ZNF Genes

In line with these findings, we identified the 3' exons of ZNF genes as *ATRX* targets in human cells (Valle-Garcia et al. 2016). ZNFs are the largest family of TFs in the human genome (Nowick et al. 2011). Although these regions are not considered repetitive per se, ZNFs share high levels of DNA similarity at their 3' exons, which contain motifs encoding for their zinc finger domains. In fact, ZNF genes can encode up to 40 copies of the zinc finger motif. Unlike other *ATRX*-regulated regions (i.e., telomeric repeats, retrotransposons, etc.), ZNFs are actively transcribed and are highly enriched in both H3K36me3 and H3K9me3 at their 3' exons. Interestingly, depletion of *ATRX* decreases H3K9me3 levels at ZNF 3' exons and other regions presenting an H3K9me3/H3K36me3 atypical chromatin signature. As a consequence, cells with reduced levels of H3K9me3 show increased levels of DNA damage, suggesting that *ATRX* binds to the 3' exons of ZNFs to maintain their genomic stability through preservation of H3K9me3.

Overall, a common feature of ATRX targets is the presence of H3K9me3. The loss of H3K9me3 at repetitive regions, via ATRX loss-of-function, appears to promote genomic instability. However, it remains unclear how the loss of ATRX results in reduced H3K9me3 levels. Is ATRX required to maintain H3K9me3 through binding of its ADD domain? Does such docking of ATRX at H3K9me3-modified chromatin facilitate DAXX-mediated H3.3 deposition? What is the role of H3.3 in regulating heterochromatin dynamics? Although all evidence to date suggests that increased genomic instability is a driving feature of *ATRX*-mutated cancers, this important area of research requires further investigation.

ADDITIONAL FUNCTIONS OF ATRX

Before the discovery that ATRX cooperates with DAXX to deposit H3.3 at H3K9me3-containing repetitive regions, ATRX was reported to interact with various heterochromatin-related proteins. These protein interaction partners include HP1 α and EZH2, among others (Fig. 2) (Cardoso et al. 1998; Lechner et al. 2005). HP1 α was originally identified to interact with ATRX through a yeast two-hybrid screen (Le Douarin et al. 1996) and later shown to colocalize at pericentric heterochromatin (McDowell et al. 1999). Additional recombinant studies identified a variant PxVxL motif within ATRX that binds to the chromoshadow domain of HP1 α (Lechner et al. 2005). Curiously, this study and others were unable to copurify these two proteins from cellular extracts (Lechner et al. 2005; Rosnoblet et al. 2011). Thus, although ATRX and HP1 α co-occupy similar genomic regions and cooperate in heterochromatin regulation, their interaction may be transient, such as during the recruitment of ATRX to H3K9me3-enriched regions (Eustermann et al. 2011).

A yeast two-hybrid analysis coupled to in vitro binding assays showed that ATRX also binds to EZH2 (Cardoso et al. 1998); however, until recently, this interaction had not been characterized in cells. Sarma et al. (2014) reported that ATRX plays a role in targeting PRC2 (Polycomb repressive complex 2) in

mouse embryonic stem cells, particularly to the inactive X chromosome (Xi). In this case, the ATRX-EZH2 interaction occurs via the *Xist* RNA, which is necessary to recruit ATRX to the Xi and facilitate deposition of H3K27me3. As described above, female ATRX syndrome patients can display skewed X-chromosome inactivation (De la Fuente et al. 2011) and, therefore, H3K27me3 deposition at the Xi (and other genomic regions) may be defective in these patients.

How DAXX plays a role in the above processes remains unclear. ATRX may have particular DAXX-independent roles in the cell. As noted above, *DAXX* mutations have not been identified in patients with ATRX syndrome. Furthermore, there are certain cancers that harbor *ATRX* mutations without evidence of *DAXX* mutations (see below). We previously showed that ATRX regulates macroH2A deposition in a DAXX-independent manner as well (Ratnakumar et al. 2012). Therefore, such DAXX-independent roles of ATRX could mediate critical functions that are perturbed in *ATRX*-mutant cancers.

ATRX/DAXX MUTATIONS IN CANCER

Recent years have witnessed a myriad of whole genome/exome sequencing studies identifying mutations in *ATRX*, and to a lesser extent *DAXX*, across a spectrum of tumor types (Table 1). The first observations came from pancreatic neuroendocrine tumors (PanNETs), in which DNA sequencing studies uncovered frequent mutations in the chromatin factors MEN1, DAXX, and ATRX (Jiao et al. 2011). Notably, *DAXX* and *ATRX* mutations were found to be mutually exclusive, yet were altered in a strikingly high percentage of tumors (43%). These mutations ranged from point mutations to insertion or deletions of bases (indels) and were not localized to any particular domains of *ATRX*, suggesting loss-of-function. In accordance, both *ATRX* and *DAXX* mutations correlated with loss of protein expression by IHC analysis. Intriguingly, these mutations associated with ALT as evidenced by fluorescence in situ hybridization (Heaphy et al. 2011; Jiao et al. 2011).

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Subsequently, *ATRX* and *DAXX* mutations were identified in pediatric glioblastoma multiforme (GBM) (Fig. 1B) (Schwartzentruber et al. 2012). Remarkably, point mutations in *H3F3A* leading to critical amino acid substitutions at residues K27 (K27M) or G34 (G34R/V) of the histone tail were also identified in pediatric GBM and diffuse pontine glioma (DIPG), a rare pediatric glioma of the brainstem (Khuong-Quang et al. 2012; Wu et al. 2012). In contrast to pediatric GBM, DIPGs harbor only H3.3K27M mutations (not G34R/V) and are extremely prevalent (70%–80%) (Khuong-Quang et al. 2012; Wu et al. 2012). *ATRX* mutations are found far less frequently (~10%) in DIPG and tend to occur in older children (Khuong-Quang et al. 2012). In the context of pediatric GBM, mutations in the *ATRX*-*DAXX*-H3.3 axis occur in ~45% of patients (Schwartzentruber et al. 2012) and show a strong correlation between *ATRX* mutations and ALT (Heaphy et al. 2011; Schwartzentruber et al. 2012).

H3F3A mutations in pediatric cancers represent the first high-frequency mutations in a histone variant to be identified (Khuong-Quang et al. 2012; Schwartzentruber et al. 2012; Wu et al. 2012). Intriguingly, H3.3K27M mutations were not found to frequently co-occur with *ATRX* mutations. Several groups showed that the H3.3K27M mutation induces a dramatic global decrease in H3K27me3 levels. This may be because of sequestration and/or inhibition of the PRC2 subunit, EZH2 (Bender et al. 2013; Chan et al. 2013; Lewis et al. 2013; Funato et al. 2014). This is compelling given that *ATRX* has also been shown to interact with EZH2 (Cardoso et al. 1998; Sarma et al. 2014) and suggests that both *ATRX* and H3.3K27M mutations could be independently driving Polycomb dysfunction in cancer. In contrast, H3.3G34R/V mutations do co-occur with *ATRX* mutations, suggesting distinct functions of each in tumors harboring these mutations (Schwartzentruber et al. 2012; Wu et al. 2014). Given the young age of patients diagnosed with pediatric GBM and DIPG, *ATRX* mutations may be promoting defects in lineage differentiation in neural stem cells during brain development that culminate in aberrant cell

growth and tumor formation. This idea is corroborated by severe defects in the developing brain in the *Atrx*-deficient mouse (Medina et al. 2009) and in *ATRX* syndrome in which patients present with brain abnormalities and intellectual disabilities (Gibbons et al. 2008).

Studies in adult glioma patient cohorts showed a strong correlation of *ATRX* mutations with *IDH1* and p53 mutations in lower grade gliomas (LGGs) (astrocytomas and oligodendrogliomas) and secondary GBM (Kannan et al. 2012; Liu et al. 2012b). These mutations are mutually exclusive from (1) *FUPB1/CIC* mutations, genes that regulate cell growth and are frequently mutated in oligodendroglioma, and (2) the chromosome 1p/19q co-deletion subtype (Olar and Sulman 2015). Moreover, no *H3F3A* or *DAXX* mutations have been identified in adult GBM (Table 1). Similar to pediatric GBM, there is a strong correlation between *ATRX* mutations and ALT status and such mutations appear to be exclusive from TERT promoter mutations (Liu et al. 2012b; Ceccarelli et al. 2016). These collective findings classify *ATRX* status to a molecular subtype of adult glioma that is highly recurrent with intermediate prognosis (Olar and Sulman 2015). *IDH1* mutations promote DNA and histone hypermethylation through production of an oncometabolite 2-hydroxyglutarate (2-HG) that inhibits DNA and histone demethylases (Lu et al. 2012; Turcan et al. 2012). In adult recurrent gliomas, *ATRX* mutations are sustained in the presence of p53 loss to cooperate with mutant *IDH1* to remodel the epigenetic landscape. In contrast, *Atrx* ablation in adult mouse neurons leads to increased DNA damage and p53-mediated apoptosis (Bérubé et al. 2005), suggesting that *ATRX* loss in cancer requires additional alterations for survival.

Large-scale efforts to identify mutations in additional pediatric tumors have identified *ATRX* as frequently mutated in neuroblastoma (NB), osteosarcoma (OS), and adrenocortical tumors (Table 1) (Cheung et al. 2012; Assié et al. 2014; Chen et al. 2014; Pinto et al. 2015). Notably, no *H3F3A* and few *DAXX* mutations were detected in these patient populations. In addition to point mutations and indels, these

studies also identified large deletions near the amino-terminal region of *ATRX* leading to in-frame fusions of the gene product (Fig. 1B). Notably, such in-frame fusions remove the chromatin binding modules of *ATRX* including the ADD domain, DAXX binding domain, and the macroH2A1-, HP1 α -, and EZH2-interacting regions (Fig. 1B). *ATRX* mutations and in-frame fusions in NB associate with older age at diagnosis (28% found in adolescent patients), with poor prognosis (Table 1) (Cheung et al. 2012).

ATRX syndrome patients show craniofacial abnormalities and developmental delays (Gibbons et al. 2008), supporting the notion that *ATRX* mutations alter proper neural crest cell lineage specification during development. Thus, *ATRX* in-frame fusions and mutations in tumors in cells of this lineage may occur early in multipotent progenitors. The selective pressure to produce in-frame fusions in NB and OS would indicate a possible gain-of-function for these new *ATRX* gene products to promote a cellular growth advantage in such progenitors, although this remains to be tested.

Along with tumor sequencing efforts, several recent studies highlight the loss of *ATRX* mRNA and protein levels in multiple tumor types, including melanoma and various sarcomas (Table 1). These studies showed that as tumors become more advanced or invasive, there is a loss of *ATRX* expression by qPCR and/or IHC analysis (Qadeer et al. 2014; Liao et al. 2015a,b,c; Lee et al. 2015). Many of these studies uncovered a correlation between *ATRX* loss and ALT and proposed this co-occurrence as a prognostic factor for poor outcome (Lee et al. 2015; Liao et al. 2015a,b,c).

ATRX/DAXX LOSS AND ALT

The ALT pathway in cancer was first described in 1997 (Bryan et al. 1997). A subsequent somatic cell hybridization study showed that when cells displaying ALT were fused to normal cells, the ALT phenotype was reversed (Perrem et al. 1999). This finding suggests that ALT is caused by depletion of a factor(s) that could be rescued by normal cells. As described above,

diverse cancer types with mutations in *ATRX* and *DAXX* are associated with ALT. Approximately 80% of tumors with ALT harbor mutations in *ATRX* or *DAXX*, and conversely 70%–80% of cancers with *ATRX* mutations show ALT (Lovejoy et al. 2012).

The G-rich telomeric repeats can adopt G4 structures, and H3.3 may prevent G4 formation. Consistent with this model, *ATRX*-mediated suppression of ALT in U-2 OS cells is dependent on DAXX and H3.3 deposition at telomeres (Clynes et al. 2015). Moreover, this suppression is associated with a reduction in replication fork stalling, likely because of limited formation of G4 structures, which are a substrate for the homologous recombination (HR) at telomeres that results in ALT. An independent study suggested that ALT is dependent on sensing of replicative stress by ATR, and that the role of *ATRX* in recognizing G4 structures and preventing replicative stress is integral to the ALT phenotype in cancer cells lacking *ATRX* (O'Sullivan et al. 2014). Clynes et al. (2015) have proposed that in the absence of *ATRX*, DAXX cannot deposit H3.3 at the G-rich repeats found in telomeres, leading to formation of G4 structures, replication fork stalling, and HR of telomeres through the MRE11–RAD50–NBS1 (MRN) complex, which ultimately leads to ALT. The authors also propose that as a secondary level of regulation, *ATRX* can sequester the MRN complex to prevent it from mediating HR at telomeres (Clynes et al. 2015). In the absence of *ATRX*, the MRN complex is released to facilitate HR at telomeres. This model is supported by the findings that patients with *ATRX* syndrome have aberrant chromatin at telomeres and pericentromeric DNA (Clynes and Gibbons 2013; Clynes et al. 2013).

Ramamoorthy and colleagues have proposed an alternative mechanism to explain the contribution of *ATRX* mutations to the ALT phenotype. The telomeric poly(ADP-ribose) polymerase tankyrase 1 is required for the resolution of sister telomere cohesion during mitosis (Dynek and Smith 2004). In the absence of *ATRX*, the histone variant macroH2A1.1 negatively regulates tankyrase, thus inhibiting its ability to resolve sister telomere cohesion.

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This, in turn, allows for aberrant telomere recombination between sister chromatids (Ramamoorthy and Smith 2015).

Although evidence points toward ATRX loss of function driving tumorigenicity through aberrant DNA recombination at repetitive elements, there may be additional functional roles for ATRX mutations in cancer. This is supported by the fact that loss of ATRX alone is not sufficient to drive to ALT (Clynes et al. 2015). ATRX loss may promote cancer via transcriptional changes and/or deregulation of the DNA replication machinery, which remains to be tested.

CONCLUDING REMARKS

Given the high frequency of ATRX and DAXX mutations in cancer, these chromatin regulators likely play a key role in pathogenesis. However, it remains unclear how. Is it through promoting ALT? If loss of ATRX or DAXX alone is insufficient to promote ALT, what other key factors or mutations are required? It also remains to be determined whether histone variant deposition by the ATRX/DAXX complex is related to its tumor suppressive function. Further, we question why some pediatric cancers show mutations in ATRX, DAXX, and H3F3A (e.g., pediatric GBM), whereas others predominantly harbor mutations only in ATRX (e.g., NB and OS). Are these mutations patterns as clear-cut as reported? Or does it relate to their cell of origin? In turn, how does ATRX regulate cell fate decisions during development that go awry in cancer?

Given the diversity of mutations identified in ATRX, we also question if they are all loss-of-function. For example, in-frame deletions of ATRX, such as those found in NB and OS, may have the potential to generate new protein products devoid of critical protein interaction domains. Moreover, as complete absence of ATRX is not well tolerated in development (i.e., CNS), we question whether ATRX alterations are truly loss-of-function or hypomorphic? We also query whether ATRX controls recombination of other repetitive sequences in the genome besides telomeres (e.g., ZNFs), and whether such recombination events play a role

in tumorigenesis. Finally, can our understanding of ATRX and DAXX function be modeled in development and disease to provide clues of their roles in promoting cancer (i.e., mouse models)? To our knowledge, there are currently no in vivo models mimicking ATRX or DAXX mutations that address these important questions.

Given that ATRX has critical roles in maintenance of repetitive regions (particularly telomeres), as well as cell cycle regulation (Wong 2010; Ramamoorthy and Smith 2015; Watson et al. 2015; Huh et al. 2016), targeting these perturbed pathways could serve as therapeutic strategies for ATRX-mutant cancers. For example, Flynn et al. (2015) recently reported that ATRX null, ALT positive cells are sensitive to inhibition of the ATR kinase, a key regulator of homologous recombination. Corroborating these findings, another study showed that ATRX ablation caused increased stalled fork replication, DNA damage and subsequent hyperactivation of PARP-1 and ATM kinase. Thus, it was proposed that PARP-1 inhibitors might further exacerbate DNA damage and promote cell death in ATRX null cells (Huh et al. 2016). We posit that other strategies might include the targeting of epigenetic factors that work together with ATRX, which may also be deregulated, such as SETDB1 or EZH2. Collectively, we anticipate that addressing the important questions raised in this review will provide critical information for deciphering and treating these devastating cancers.

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