ATRX and the replication of structured DNA

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Summary

Understanding the underlying molecular basis for disease can often be a prolonged and tortuous process with many false leads and blind alleys. Relating the cause of ATR-X syndrome to the function of the protein ATRX is a case in point. In this review we attempt to bring together the diverse biological phenomena associated with ATRX dysfunction with what has recently been discovered concerning the chromatin remodeling activity of this protein. This potentially casts light on how defective DNA replication/histone replacement can impact on transcription, telomere maintenance and also possibly chromosome segregation.

Introduction

Germ line mutations in ATRX give rise to a complex human genetic disease, X-linked alpha thalassemia mental retardation (ATR-X) syndrome characterized by severe learning difficulties, a characteristic facial appearance, abnormal sexual development and a form of anemia, alpha thalassemia [1]. The latter arises because of reduced expression of alpha globin, a component of adult hemoglobin. Originally this transcriptional defect was a defining feature of the condition and the working hypothesis was of an X encoded protein facilitating the expression of a wide repertoire of genes. Subsequently, in numerous different animal models, perturbation of ATRX has been associated with a wide range of effects: altered patterns of DNA methylation (at subtelomeres, heterochromatic repeats and ribosomal DNA) [2], aberrant chromosome congression in mitosis [3] and segregation in meiosis, [4] as well as telomere dysfunction [5**]. Is there a central defect which might unify these disparate effects?
At first glance these effects at least relate to where ATRX localizes in the genome - predominantly large tandemly repeated regions such as ribosomal DNA, pericentric heterochromatin [6] and telomeres [5**, 7**]. In mouse embryonic stem (ES) cells ATRX is recruited to telomeres during S phase. If ATRX is depleted there is an increase in the presence of γH2AX, a mark of replicative stress, at these telomeric foci [5**]. This important observation strongly suggests that ATRX is playing a role in maintaining telomere integrity and given its presence during DNA synthesis it is likely that it facilitates normal telomere replication. This role for ATRX was strengthened by the findings of Huh et al., who showed that in mouse myoblasts ATRX co-localises with late replicating chromatin during S phase and when ATRX is knocked out progression through late S phase is delayed, there is a DNA damage response and telomere fragility is observed [8*].

Mammalian telomeres consist of many kilobases of the tandem repeat (TTAGGG)n. DNA replication is unidirectional commencing in the subtelomeric region and proceeding to the telomere ends [9]. Tandem repeats have the potential to fold into non B-forms of DNA (eg cruciform, triplex and hairpin secondary structures) [10]. G-rich repeats can form G quadruplex (G4) structures and there is recent evidence that these structures exist in vivo [11-13] with perhaps the best evidence at telomeres [9,14]. Many ATRX targets are predicted to form G4 [15**] and electrophoretic mobility shift assays have demonstrated that recombinant ATRX protein preferentially binds to G4 structured DNA over the unfolded G4 telomere derived sequence [15**]. G4 (and other secondary structures) are particularly likely to form if
the DNA is single stranded (ssDNA). At telomeres, the lagging strand during DNA replication and non-template strand during transcription are both G-rich and single stranded and hence offer likely substrates for G4 formation. One compelling hypothesis is that the presence of such DNA secondary structures may perturb DNA replication and that at certain tandem repeats ATRX is required to facilitate their resolution or bypass. How might this relate to the chromatin remodeling activity of ATRX?

**Chromatin remodelling activity of ATRX**

We now know that ATRX plays an important role in the incorporation of the histone variant H3.3 into telomeric, ribosomal and pericentromeric DNA and this role may extend to the smaller interstitial repeats [7,16]. ATRX works with its partner protein DAXX, the latter of which directly binds H3.3 and acts as a histone chaperone, whereas ATRX both directs where this occurs and facilitates the incorporation of H3.3 into chromatin [17]. Unlike the canonical histones, the incorporation of which is closely coupled to the progression of the replication fork, histone H3.3 is predominantly incorporated into chromatin in a replication-independent manner and appears principally to serve as a replacement variant [18,19].

One might envisage a number of ways by which ATRX helps maintain the integrity of telomere chromatin. Repetitive DNA is predicted to be an inherently unfriendly substrate for nucleosomes leading to rapid turnover [20] and a replication independent mechanism for histone replacement is therefore needed, particularly in post mitotic, terminally differentiated cells. Consistent with this, H3.3 is also
associated with actively transcribed regions where nucleosome occupancy is also known to be highly dynamic [21]. In mouse ES cells, however, H3.3 is deposited at telomeres by ATRX/DAXX predominantly during S phase, which at first sight appears paradoxical for a histone variant associated with replication independence [5**]. Is H3.3 acting as a replacement histone variant during S phase?

In human fibroblasts there is evidence for frequent stalling of replication forks, detectable by RPA34 accumulation at long tracks of ssDNA formed as a result of uncoupling of the replicative helicase and DNA polymerization [22]. The telomere specific recruitment of the low fidelity enzyme DNA polymerase β at the same time suggests that repair is associated with DNA lesion bypass. Since this repair is dissociated from the replication fork it will also be uncoupled from replication-dependent histone deposition. In order to preserve the chromatin integrity a replication independent mechanism for histone deposition is required [23]. Wong et al. [5**] showed that in mouse ES cells ATRX and H3.3 localised to telomeres at a comparable stage of S phase so it is tempting to think that ATRX/DAXX is depositing H3.3 during post-replicative repair rather than at the replication fork. This is consistent with the observations of Ray-Gallet et al. [19] who showed that although H3.3 is incorporated into chromatin during S phase the sites do not overlap with replication sites. In the absence of ATRX one might envisage that the replication stress observed could be related either to the failure to chromatinise the repaired DNA or the failure to restart a stalled fork.

Abnormal gene expression in ATR-X syndrome
How might such a problem lead to the perturbation in gene expression seen in ATR-X syndrome? Although the localization of ATRX to large structural repeats can be seen by fluorescent microscopy, chromatin immunoprecipitation of ATRX reveals the finer detail showing that ATRX also localises to GC rich sequences including CpG islands and GC-rich interstitial repeats [15**]. Looking at the well characterised end of chromosome 16 (16p13.3), wherein lies the cluster of alpha-like globin genes, it was noted that the peak of binding was at a variable number tandem repeat (VNTR), \( \psi \zeta \), within the cluster (Figure 1a). In patients with ATR-X syndrome, it was observed that the globin genes closest to the repeat were the most down regulated, and those furthest away (~10kb) least affected (Figure 1b). Furthermore, the larger the VNTR the more severe was the degree of down-regulation, even in individuals with the same ATRX mutation (Figure 1c). The same pattern was noted at another VNTR about 300kb away from the alpha cluster. The associated gene, NME4 and its near neighbor DECR2 were both affected, but NME4, which contains the VNTR, was more severely affected. Again, the degree of down-regulation was related to the size of the VNTR that lies in cis. This observation helps explain the characteristics of the genes affected in ATR-X syndrome - they happened to be located near an ATRX-binding G-rich repeat. It also explains the variable phenotypes, or at least the severity of the alpha thalassemia seen in ATR-X patients. Like the telomeres, the sequences of these repeats are able to form G4 in vitro so it is tempting to think that ATRX is acting to relieve the effects of G4 at these loci. Might the underlying problem again be with DNA replication, with the effect on local gene an associated epiphenomenon? Evidence for a link between a defect in replicating structured DNA and the perturbation of local gene expression has been demonstrated by Sarkies et
al. [23, 24*]. Using avian DT40 cells they showed that REV1, a low fidelity polymerase involved in translesional DNA synthesis, is important in replicating G4 DNA. In its absence there is an uncoupling of replication dependent histone deposition and DNA synthesis at the replication fork. As a consequence of this the pre-existing histones with their epigenetic marks were no longer efficiently recycled to the daughter strands and this leads to the loss of epigenetic memory in the region and consequently a change in local gene expression. Where previously gene expression was repressed, the genes were activated and conversely where genes were actively expressed they became silenced. Given that stalled replication forks have the propensity to collapse and generate double strand breaks, a compelling alternative explanation is that the associated silencing effects arise from epigenetic modifications originating from the site of a break. Indeed, work by Shanbhag et al., demonstrate that the induction of a double strand break can lead to the silencing of a reporter gene up to 4kb away from the break site through H2A ubiquitylation dependent pausing of RNAPII [25]. We propose that in the absence of ATRX there is a defect replicating the structured DNA associated with these G-rich tandem repeats and this may trigger fork collapse and break associated epigenetic silencing. Alternatively this may lead to the uncoupling of replication dependent histone deposition and the subsequent loss of activating histone marks at the alpha globin loci and reduced alpha globin expression causing alpha thalassaemia.

ATRX and the Alternative Lengthening of Telomere (ALT) pathway
Of considerable recent interest is the finding that ATRX mutations are often associated with a growing group of cancer types. In order to divide indefinitely, tumour cells must have a mechanism to maintain the length of their telomeres, without which they will shorten progressively with every round of DNA replication eventually leading to senescence or apoptosis [26]. In the majority of cancers, this shortening is counteracted by the expression of telomerase, a specialized reverse transcriptase which adds TTAGGG repeats; normally telomerase is only expressed in stem cells and germ cells. In 10-15% of cancers the telomeres are maintained by a telomerase independent mechanism known as the alternative lengthening of telomeres pathway (ALT) [27]. This pathway depends on homologous recombination involving either other telomeres or the extra chromosomal telomeric repeats which are a feature of ALT tumours [28]. Inactivating mutations in ATRX or DAXX have been identified in many ALT cancers and in a comprehensive study the loss of ATRX was observed in 90% of ALT cell lines [29-31*]. The recent identification of histone H3.3 mutations in addition to ATRX/DAXX mutations in paediatric glioblastomas and their correlation with ALT characteristics has reinforced this association [32] and strongly suggest that ATRX/DAXX/H3.3 normally suppresses this pathway. Depletion of ATRX appears insufficient to induce ALT suggesting that the effect is context specific [31*]. A study in pancreatic neuroendocrine tumours arising in individuals with multiple endocrine neoplasia-1 (MEN-1) syndrome showed that the loss of ATRX and/or DAXX was not apparent until the tumour size was >3cm, suggesting these are late events in tumour development [33]. It is possible that mutations in ATRX/DAXX are permissive to the continued growth of these tumours through the triggering of the ALT pathway. It is interesting to note that in one
paediatric glioblastoma three ATRX mutations were identified presumably in independent clones within the tumour [32]. How might the absence of ATRX/DAXX trigger this pathway? One possible model is that in the absence of ATRX, forks stalled by structural DNA are not efficiently processed. The unidirectional nature of replication at telomeres [9] means that stalled forks cannot be salvaged by a converging replication fork. In these circumstances fork restart is therefore solely dependent on HR, and this triggering of HR now provides a pathway for telomere maintenance and permits further tumour growth (Figure 2).

**Concluding remarks**

Defining the chromatin remodeling activity of ATRX has been a great step forward in understanding the function of this protein. The next challenge which has been outlined in this review is to explain the diverse phenomena associated with germline and acquired mutations in ATRX. Its role in the replication of telomeres has stimulated a surge of interest in its biology and it is to be expected that new and exciting leads to determining ATRX function will come thick and fast.

**Box 1. ATRX in Meiosis and mitosis**

It is tempting to speculate that, in addition to triggering replication and transcriptional silencing defects, a failure to properly chromatinise heterochromatic regions through the deposition of H3.3 could also be accountable for the chromosomal instability defects observed upon loss of ATRX function. RNAi induced knockdown of ATRX in mouse oocytes generates a range of chromosome segregation defects during the metaphase II stage of meiosis, including impaired chromosome alignment and loss
of sister chromatid cohesion [4] with similar abnormalities observed in mouse
myoblasts in which ATRX is knocked out [8*]. Consistent with this, faithful mitotic
chromosome cohesion and congression is also dependent on ATRX function [3]. Of
note studies in drosophila have shown that H3.3 is also required for faithful meiotic
chromosome segregation in male germ cells [34], lending weight to the idea that a
loss of H3.3 deposition could at least partially account for the ATRX associated
phenotypes. Indeed, ATRX has been shown to be required for the recruitment of the
H3.3 chaperone DAXX to pericentromeric heterochromatin domains in the female
germline [35], lending credence to such a model.

References and recommended reading
Papers of particular interest, published within the period of review, have been
highlighted as:
• of special interest
•• of outstanding interest

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References
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First study to demonstrate the recruitment of ATRX to telomeres during S-phase in mouse embryonic stem (ES) cells and that RNAi mediated knockdown of ATRX induces a telomere dysfunction.


Study characterising the genome wide localisation of the histone variant H3.3 in mouse ES and neuronal precursor cells. The authors demonstrate that ATRX is required for the deposition of H3.3 at telomeres.


Study characterising the role of ATRX in myoblast cells showing that ATRX colocalises to late replicating heterochromatin in S phase, with its knockout leading to an elongation in S-phase, accumulation of p53, genomic instability and telomere dysfunction.


Reports the genome wide distribution of ATRX at tandem repeat sequences (TRs) at both telomeres and euchromatin. Showed that genes associated with these TRs are often deregulated when ATRX is mutated and the change in expression is associated by the size and proximity of the associated VNTR.


17. Lewis PW, Elsaesser SJ, Noh KM, Stadler SC, Allis CD: Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-


22. Verdun RE, Karlseder J: **The DNA damage machinery and homologous recombination pathway act consecutively to protect human telomeres.** *Cell* 2006, **127**:709-720.


Showed that interruption of DNA replication at G4 sequences results in loss of the tight coupling between replication and the recycling of parental histones. This leads
to the biased incorporation of newly synthesized histones and loss of epigenetic memory.


Comprehensive characterisation of 22 ALT cell lines, of which 19 had an alteration in the expression of ATRX and/or DAXX.


33. de Wilde RF, Heaphy CM, Maitra A, Meeker AK, Edil BH, Wolfgang CL, Ellison TA, Schulick RD, Molenaar IQ, Valk GD, et al.: Loss of ATRX or DAXX expression and concomitant acquisition of the alternative lengthening of telomeres phenotype are late events in a small subset of MEN-1 syndrome pancreatic neuroendocrine tumors. Mod Pathol 2012, 25:1033-1039.


Figure legends

Figure 1. (a) Microarray analysis of ATRX ChIP DNA enrichment from human erythroblasts across the 220kb terminal region of chromosome 16p containing the α-globin genes (HBM, HBA2, HBA1, HBQ). (b) A schematic showing the relationship of the $\psi^\zeta$ VNTR (peak of ATRX binding) to the adjacent $\alpha$-globin genes. The relative
expression of the genes within the cluster are shown comparing normal controls and individuals with ATR-X syndrome. (c) ψζ VNTR length was measured in ATR-X patients with α thalassemia and the average length of the two alleles was plotted against the degree of α thalassemia as measured by % red cells showing HbH inclusions (adapted from [15]).

Figure 2. Hypothetical model of how ATRX may function to facilitate the replication of structured (G4) DNA and how in its absence the repair of double strand breaks by homologous recombination triggers the ALT pathway.
Relative gene expression

a.

b.

Increasing severity of α thalassemia

% cells with HbH inclusions
Fork arrest

- ATRX

Fork collapse
DSB
Restart by HR

ALT pathway
in malignant cells

+ ATRX

Translesional synthesis
Chromatinise DNA with H3.3
Fork restart