Compromised genomic integrity impedes muscle growth after Atrx inactivation

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ATR-X syndrome is a severe intellectual disability disorder caused by mutations in the ATRX gene. Many ancillary clinical features are attributed to CNS deficiencies, yet most patients have muscle hypotonia, delayed ambulation, or kyphosis, pointing to an underlying skeletal muscle defect. Here, we identified a cell-intrinsic requirement for Atrx in postnatal muscle growth and regeneration in mice. Mice with skeletal muscle-specific Atrx conditional knockout (Atrx cKO) were viable, but by 3 weeks of age presented hallmarks of underdeveloped musculature, including kyphosis, 20% reduction in body mass, and 34% reduction in muscle fiber caliber. Atrx cKO mice also demonstrated a marked regeneration deficit that was not due to fewer resident satellite cells or their inability to terminally differentiate. However, activation of Atrx-null satellite cells from isolated muscle fibers resulted in a 9-fold reduction in myoblast expansion, caused by delayed progression through mid to late S phase. While in S phase, Atrx colocalized specifically to late-replicating chromatin, and its loss resulted in rampant signs of genomic instability. These observations support a model in which Atrx maintains chromatin integrity during the rapid developmental growth of a tissue.

Introduction

Males inheriting germline mutations in the ATRX gene develop α-thalassemia mental retardation X-linked (ATR-X) syndrome (1). ATR-X is a human developmental disorder characterized by severe intellectual disabilities, α-thalassemia, urogenital dysfunction, skeletal abnormalities, and neonatal hypotonia. This characteristic collection of symptoms in patients suggests a critical role for ATRX in these tissues. Similarly, in mice, the survival of neurons in the CNS and the development of reproductive tissue also requires Atrx (2–5).

The ATRX gene encodes a 280-kDa chromatin remodeling protein with an N-terminal ATRX-DNMT3-DNMT3L (ADD) domain that forms a histone binding pocket and a C-terminal SNF2 ATPase domain (6–9). Like most SNF2 chromatin remodelers, ATRX is part of a larger complex that includes the death domain–associated protein (Daxx) (10, 11). Chromatin remodeling complexes in general use the energy derived from ATP hydrolysis to reorganize nucleosome position, promote disassembly/incorporation of nucleosomes during DNA replication, and actively facilitate histone variant exchange (12, 13). Histone variants are incorporated into nucleosomes throughout the cell cycle, unlike the replication-dependent canonical histones. Structural incorporation of histone variants often accompanies a functional change in chromatin. For example, deposition of histone variant macroH2A is concomitant with facultative silencing of the female X chromosome (14). In contrast, histone variant H3.3 is highly enriched at transcribed genes and in the constitutive heterochromatin found at pericentromeres and telomeres (15–19). Atrx-Daxx complexes are required for the deposition of histone variant H3.3 at pericentromeres and telomeres, but, strangely, not in transcribed genes (17). Furthermore, Atrx ChIP sequencing experiments by Law et al. demonstrated an affinity for G-rich and simple tandem repeats (TRs) found in telomeres, α-globin locus, and throughout the genome (20). Genome-wide occupancy at TRs by Atrx suggests a global role in regulating chromatin structure and genome integrity. Intriguingly, somatic mutations in Atrx have been found in acquired α-thalassemia myelodysplastic syndrome (ATMDS) and, more recently, in pancreatic neuroendocrine tumors (PanNETs), where 61% of PanNETs examined exhibited abnormal telomeres reminiscent of tumors that activate the alternative lengthening of telomeres (ALT) pathway (21–24).

Here, we explored the role of Atrx in skeletal muscle development, as neonatal hypotonia is diagnosed in 85% of all ATR-X syndrome patients (25). Due to their reduced muscle function, patients do not walk until later in childhood, while some remain incapable for a lifetime. We showed a primary defect in muscle growth and regeneration caused by an accumulation of genomic damage in Atrx-deficient satellite cells. Despite normal resting numbers, Atrx-knockout satellite cells were incapable of sustained expansion in ex vivo muscle fiber cultures. 2-parameter flow cytometry BrdU pulse-chase analysis of Atrx-knockout satellite cell–derived myoblasts revealed a delay in the progression through mid to late S phase, implicating a replication-dependent chromatin defect. Consequently, we observed fragmented and aberrant nuclei, elevated levels of γ-H2AX foci, telomeric fragility, and activation of the p53–ataxia-telangiectasia mutated (p53-ATM) DNA damage response (DDR). Taken together, these observations indicate that Atrx is required for replication-dependent chromatin maintenance in rapidly proliferating myoblasts.

Results

Atrx conditional knockout mice have delayed muscle growth and severe deficits in muscle regeneration. Skeletal muscle hypotonia, delayed ambulation, and kyphosis are commonly associated with ATR-X syndrome patients (1). Therefore, we postulated that Atrx func-
The progeny generated from this cross resulted in no significant deviation from the expected Mendelian ratios for all the predicted genotypes (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI63765DS1). Since Atrx cKO mice were viable, it appeared that embryonic and fetal myogenesis was generally unaffected in our model.

Nonetheless, Atrx cKO mice were consistently smaller in size than their Atrx<sup>+/y</sup> littermate controls from birth (Figure 1B). Analysis at 3 weeks of age demonstrated a 20% reduction in body mass, which correlated with an overall reduction in limb musculature and exaggerated curvature of the spine (Figure 1, B and C). These observations indicate that Atrx is important for the growth and maturation of skeletal muscle in young mice. Interestingly, by adulthood, differences in body mass became insignificant between Atrx cKO mice and Atrx<sup>+/y</sup> littermate controls (Figure 1C).

Postnatal growth delay may indicate that satellite cell number or function is compromised. Satellite cells are also responsible for the robust regenerative capacity of adult skeletal muscle. We therefore assessed the recovery of adult Atrx cKO muscle from an injury. Acute injury was induced in the tibialis anterior (TA) muscle by cardiotoxin (CTX) injection in Atrx cKO and Atrx<sup>+/y</sup> littermate controls. Recovery from CTX injury was clearly abnormal in Atrx cKO mice. Atrx cKO TA muscles displayed an abundance of poorly formed microfibers and open interstitial cavities in the area of regeneration 2 weeks after CTX injury, in contrast to the fully regenerated fibers from Atrx<sup>+/y</sup> littermates (Figure 1, E and F). A significantly lower proportion of muscle fibers contained centrally located nuclei 2 weeks after CTX injection (Figure 1D), indicative of a reduction of regenerating muscle fibers. Furthermore, fiber caliber measurements before and after CTX demonstrated an inability of Atrx cKO mice to efficiently increase both the overall proportion (Atrx cKO, 9% increase; Atrx<sup>+/y</sup>, 22% increase) and size (Atrx cKO, 25% increase; Atrx<sup>+/y</sup>, 83% increase) of muscle fibers with cross-sectional area greater than 0.1 mm<sup>2</sup> (Table 1). The presence of highly fibrotic regeneration was also strikingly evident by Masson’s Trichrome staining in Atrx cKO, but not Atrx<sup>+/y</sup>, TA muscles (Figure 1, E and F). The early postnatal growth delay and poor muscle regeneration after CTX injury point to possible defects in satellite cell number or function in Atrx cKO mice.

Figure 1
Atrx inactivation in skeletal muscle leads to delay in muscle growth and severe regeneration deficit after CTX-induced acute injury. (A) Confocal double-immunofluorescent micrograph of Atrx and Pax7 from a freshly isolated EDL myofiber. (B) 3-week-old Atrx cKO and Atrx<sup>+/y</sup> control (WT) mice. (C) Body mass comparison between Atrx<sup>+/y</sup> and Atrx cKO mice at 3 weeks and 8 weeks (Adult) of age. Values represent mean ± SEM (n = 9). *P < 0.05, t test. (D) Quantification of regenerating muscle fibers as assessed by the proportion of nuclei located centrally in muscle fibers 2 weeks after CTX injection. **P < 0.01, t test. (E and F) Masson’s Trichrome staining of the TA muscle 2 weeks after CTX injection from Atrx<sup>+/y</sup> (E) and Atrx cKO (F) mice. Poor muscle regeneration in Atrx cKO mice is shown by the excessive amounts of fibrotic tissue (blue collagen staining) in the damaged region (F, inset). Original magnification, ×630 (A); ×100 (E and F). Insets in E and F are enlarged ×3.
Atrx cKO muscle has normal numbers of satellite cells, but compromised growth properties. Satellite cells represent the primary source of muscle progenitors for postnatal muscle growth and regeneration (28). We therefore set out to determine the presence and functionality of the satellite cells in our Atrx cKO mouse model. Myf5-Cre-driven targeted excision was assessed in a Cre-activated ROSA-YFP reporter mouse (Myf5-Cre:ROSA-YFP). Staining of freshly isolated muscle fibers from Myf5-Cre:ROSA-YFP mice revealed coexpression of Atrx in YFP+ satellite cells (Supplemental Figure 1A).

Atrx immunostaining on freshly isolated Atrx cKO fibers verified complete Cre excision in the skeletal muscle lineage (Figure 2A). Despite the absence of Atrx, the percentage of Pax7+ satellite cells was not significantly different in bulk hind limb muscle digests from 2-week-old Atrx cKO mice and Atrxf/y littermate controls (Supplemental Figure 1B). Furthermore, direct counts of Pax7+ nuclei from freshly isolated extensor digitorum longus (EDL) muscle fibers confirmed the presence of normal numbers of satellite cells in both Atrx cKO and Atrxf/y animals (Figure 2B).

Our satellite cell counts were consistent with the numbers reported in other studies, which range between 8 and 10 satellite cells per EDL fiber (29, 30).

Since the absolute numbers of resident satellite cells did not appear to vary significantly in Atrx cKO mice, we proceeded to characterize the growth properties of activated satellite cells from live single myofiber cultures. Myofibers were maintained in a non-adherent floating culture system supplemented with BrdU. Satellite cell activation and subsequent myoblast division were marked by BrdU incorporation. Each individual fiber represented a single unit giving rise to an isolated population of daughter myoblasts. After 48 hours in culture, Atrx cKO fibers generated a modest number of BrdU+ myoblasts, albeit significantly lower than that of Atrxf/y controls.

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<th>Fiber caliber measurements of uninjured and CTX-injured TA muscle in adult Atrx cKO mice and Atrxf/y littermate controls</th>
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<td>Large (&gt;0.1 mm²)</td>
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<td>Area (mm²) Frequency</td>
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<td>Atrx cKO CTX</td>
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<td>0.142 ± 0.03 30%</td>
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Area values are mean ± SEM.

**Table 1**

Atrxf/y, 88 fibers (n = 4); Atrx cKO, 66 fibers (n = 3). **P < 0.01, t test. (C) Myoblast counts from single muscle fibers after 6 days in culture. Single EDL muscle fibers from Atrxf/y and Atrx cKO mice were plated onto a Matrigel substrate and fixed after 6 days in culture. Desmin+ myoblasts were enumerated for each fiber (inset). Atrxf/y, 58 fibers (n = 2), Atrx cKO, 64 fibers (n = 4). Box plots represent quartile distribution of Desmin+ myoblasts per fiber. Original magnification, ×400 (A); ×200 (C).
trols (Figure 2B). Dramatic defects in myoblast expansion became evident when Atrx cKO fibers were left in culture for longer periods. For these experiments, myofibers were adhered to a Matrigel substrate and maintained in culture for 6 days. The muscle-specific intermediate filament protein Desmin was used to mark myoblasts for enumeration. Indeed, Atrx deletion was only evident in Desmin+ myoblasts, whereas Desmin– fibroblastic cells all expressed Atrx (Supplemental Figure 1C). Quantitative assessment of Desmin+ myoblasts confirmed a 9-fold reduction in numbers generated by Atrx cKO fibers (Figure 2C). On average, the Atrx−/− controls generated 1,700 myoblasts per fiber, compared with 190 from Atrx cKO mice. We therefore concluded that inefficiency of myoblast expansion is the primary cause of the reduction in postnatal limb musculature and the severe regeneration deficit in Atrx cKO mice.

De novo deletion of Atrx does not affect terminal differentiation of myoblasts. The defective growth properties of Atrx cKO myoblasts precluded our attempts to establish primary cultures for further characterization. We therefore used an alternate methodology to

Figure 3
De novo deletion of Atrx reduces the expansion rate of myoblasts, but does not affect their capacity to terminally differentiate. (A) Immunostaining for Atrx in uninfected, Atrx+/Y:Ad-LacZ, and Atrx+/Y:Ad-Cre primary myoblasts. (B) RT-PCR analysis of Atrx exon 18 excision after Ad-Cre infection. RNA was harvested from myoblasts in growth conditions (GM) or after 1 day in differentiation media (D1). Ribosomal gene L32 was used as a loading control transcript. (C) Immunoblot analysis of Atrx protein levels after Ad-Cre infection. (D) Cumulative population doubling growth curve of uninfected (Ctrl), Atrx+/Y:Ad-LacZ, and Atrx+/Y:Ad-Cre myoblasts. Equal numbers of cells were initially seeded for each time point. Cumulative population doublings (CPDs) were obtained from total viable cell numbers at each time point. Error bars represent ± SEM (n = 4). (E and F) Differentiation time course of Atrx+/Y:Ad-LacZ (E) and Atrx+/Y:Ad-Cre (F) myoblasts. Myoblasts were induced to differentiate in low-mitogen differentiation media (DM) and subsequently fixed after 24 and 72 hours. Differentiation capacity was assessed by immunostaining for the early differentiation marker myogenin and the terminal differentiation marker MHC. Original magnification, ×200 (A); ×400 (E and F).
isolate Atrx-knockout primary myoblasts and study their functional properties. Primary myoblast cultures were established from Atrx<sup>−/−</sup> animals and subsequently infected with Cre recombinase-expressing adenovirus (Ad-Cre). The resulting myoblasts (referred to herein as Atrx<sup>−/−</sup>:Ad-Cre) exhibited near-complete elimination of Atrx, as assessed by immunofluorescent staining, RT-PCR, and Western blot analysis (Figure 3, A–C).

A slower rate of expansion was clearly evident after infection in Atrx<sup>−/−</sup>:Ad-Cre myoblasts compared with Ad-LacZ–infected (Atrx<sup>−/−</sup>:Ad-LacZ) myoblasts and uninfected controls (Figure 3D). Indeed, these results remained consistent with what was observed in the Atrx cKO fiber culture experiments. As such, we used these cells to assess whether loss of Atrx function has an inhibitive effect on myogenic differentiation. Upon withdrawal from high-mitogen growth conditions, Atrx<sup>−/−</sup>:Ad-Cre myoblasts were capable of fusing to form multinucleated syncytial myotubes. Furthermore, Atrx<sup>−/−</sup>:Ad-Cre myoblasts were fully capable of inducing the differentiation program, as indicated by the expression of both the early (myogenin) and terminal (myosin heavy chain [MHC]) markers of differentiation (Figure 3, E and F).

Atrx depletion delays S phase and induces accumulation of p53 protein. While previous work has highlighted a growth deficit in Atrx-null ES cells and siRNA knockdown cell culture models, there remains some confusion regarding the underlying mechanism. Proposed mechanisms include enhanced apoptosis, chromatic cohesion and congression defects during mitosis, and defective loading of histone H3.3 during transcription or replication (3, 17, 19, 20, 31). To begin to assess the mechanism underlying the poor proliferative capacity of Atrx cKO myoblasts, we first asked whether loss of Atrx function could deregulate the expression of cell cycle genes in our myoblast cultures. 3 biologically independent Atrx<sup>−/−</sup> myoblast lines were infected with control Ad-LacZ or Ad-Cre virus and subsequently harvested for RNA 48 hours after infection. Quantitative real-time RT-PCR (QPCR) expression analysis detected no deregulation of cell cycle genes such as Ccnd1, Ccne1, Ccna2, Ccnb1, Cdk2, Cdk4, p107, p130, E2f4, and E2f2 at the RNA level. However, we observed 1.3-fold upregulation of the cyclin-dependent kinase (Cdk) inhibitor Cdkn2a, while E2f1, E2f3, and Ccnb were downregulated 1.3-fold, and Ccna1 and Ccnb2 were downregulated 1.5- and 1.3-fold, respectively (Supplemental Figure 2A). Interestingly, protein immunoblot analysis detected changes not seen in our RNA analysis for the cell cycle regulators p107, cyclin E, cyclin A, and the Cdk inhibitor p27 (Supplemental Figure 2B). In addition to aberrant cell cycle gene regulation, we consistently observed accumulation of p53 protein in Atrx<sup>−/−</sup>:Ad-Cre myoblasts (Figure 4A), which suggests that one or more cell cycle checkpoints may be activated in the absence of Atrx.
Atrxf/y

point, we treated exclude the S phase population, delineating a BrdU– G1 and G2/M 2-parameter flow cytometric analysis. The BrdU pulse served to der was in G2/M (Figure 4B). Orderly passage of the control cells The Journal of Clinical Investigation

Atrxf/y

functions.

p53 accumulation would be associated with colchicine treatment, this was a surprising result, as we anticipated that loss of Atrx triggers the p53 checkpoint during the S phase of (Supplemental Figure 3). Similarly, the passage of the G2/M populations failed to detect any effect of Atrx loss on cell cycle dis- tribution, consistent with work in -null ES cells (27). To deter- mine whether Atrx loss results in a more subtle defect in cell cycle progression, a BrdU pulse-chase protocol was used (32). Briefly, Atrx f/y:Ad-LacZ and Atrx f/y:Ad-Cre myoblasts were pulsed with BrdU for 1 hour and subsequently collected at 4-hour intervals for 2-parameter flow cytometric analysis. The BrdU pulse served to exclude the S phase population, delineating a BrdU– G1 and G2/M population at the 0-hour time point (immediately following the 1-hour BrdU pulse). Cell cycle progression from 0 to 24 hours was assessed in the BrdU– population as they transited from G1 through S and into G2/M. Approximately 85% of the BrdU– cells were in the G1 phase of the cell cycle at the 0-hour time point, while the remain- der was in G2/M (Figure 4B). Orderly passage of the control cells from G1 and G2/M through the cell cycle occurred during the chase period, while a small but statistically significant delay in the pas- sage of Atrx f/y:Ad-Cre myoblasts from G1 through S and G2/M into the subsequent G1 phase of the cell cycle was observed (Figure 4B). These results indicated that the absence of Atrx delayed the com- pletion of S phase and consequently produced a transient disequi- librium in the cell cycle profile at the 16- and 20-hour time points (Supplemental Figure 3). Similarly, the passage of the G2/M popu- lation of BrdU Atrx f/y:Ad-Cre myoblasts was delayed compared with Atrx f/y:Ad-LacZ controls (Figure 4B). In light of these findings, we examined Atrx localization with respect to sites of DNA replica- tion during S phase. Active sites of DNA replication were marked with a brief 20-minute BrdU pulse in asynchronously proliferating myoblasts. Immunolocalization revealed a strikingly similar pat- tern between late-replicating chromatin domains and Atrx (Figure 4C, red box). These lines of evidence support a putative function for Atrx in the processing of late-replicating chromatin.

Loss of Atrx results in genomic instability and aberrant mitotic events. Replication errors frequently lead to chromosomal abnormalities that affect native chromatin structure and associated functions. Atrx f/y:Ad-Cre myoblasts presented an abnormal cyto- logic phenotype characterized by an increase in irregular nuclear morphology, poorly formed mitotic spindles, binucleation of cells, and supernumerary (1-2) γ-tubulin–staining microtubule organizing centers (Figure 5A). Quantification of the proportion of myo- blasts with aberrant nuclear morphology (micronuclei, binuclei, or multinuclei) revealed a significantly higher percentage in Atrx f/y:Ad-Cre than in Atrx f/y:Ad-LacZ myoblasts (Figure 5A). Furthermore, visualization of the mitotic spindle revealed substantial contrast other studies (5, 31). Our results prompted us to specifically examine cell cycle kinetics in Atrxf/y:Ad-Cre myoblasts by flow cytometry. A static 1-parameter analysis of asynchronous populations failed to detect any effect of Atrx loss on cell cycle dis- truction, consistent with work in Atrx-null ES cells (27). To deter- mine whether Atrx loss results in a more subtle defect in cell cycle progression, a BrdU pulse-chase protocol was used (32). Briefly, Atrxf/y:Ad-LacZ and Atrxf/y:Ad-Cre myoblasts were pulsed with BrdU for 1 hour and subsequently collected at 4-hour intervals for 2-parameter flow cytometric analysis. The BrdU pulse served to exclude the S phase population, delineating a BrdU– G1 and G2/M population at the 0-hour time point (immediately following the 1-hour BrdU pulse). Cell cycle progression from 0 to 24 hours was assessed in the BrdU– population as they transited from G1 through S and into G2/M. Approximately 85% of the BrdU– cells were in the G1 phase of the cell cycle at the 0-hour time point, while the remain- der was in G2/M (Figure 4B). Orderly passage of the control cells from G1 and G2/M through the cell cycle occurred during the chase period, while a small but statistically significant delay in the pas- sage of Atrxf/y:Ad-Cre myoblasts from G1 through S and G2/M into the subsequent G1 phase of the cell cycle was observed (Figure 4B). These results indicated that the absence of Atrx delayed the com- pletion of S phase and consequently produced a transient disequi- librium in the cell cycle profile at the 16- and 20-hour time points (Supplemental Figure 3). Similarly, the passage of the G2/M popu- lation of BrdU Atrxf/y:Ad-Cre myoblasts was delayed compared with Atrxf/y:Ad-LacZ controls (Figure 4B). In light of these findings, we examined Atrx localization with respect to sites of DNA replica- tion during S phase. Active sites of DNA replication were marked with a brief 20-minute BrdU pulse in asynchronously proliferating myoblasts. Immunolocalization revealed a strikingly similar pat- tern between late-replicating chromatin domains and Atrx (Figure 4C, red box). These lines of evidence support a putative function for Atrx in the processing of late-replicating chromatin.

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between control $\text{Atrx}^{+/y}$:Ad-LacZ and $\text{Atrx}^{+/y}$:Ad-Cre myoblasts. In mitotic $\text{Atrx}^{+/y}$:Ad-LacZ myoblasts, $\alpha$-tubulin staining of the spindle fibers revealed a well-formed and organized spindle apparatus (Figure 5B). However, despite possessing a bipolar organizing center, $\alpha$-tubulin staining of the spindle fibers was weaker and more disorganized in mitotic $\text{Atrx}^{+/y}$:Ad-Cre myoblasts. The defect in mitotic spindle formation was also associated with a scattered alignment of metaphase chromosomes (Figure 5B). Interestingly, visualization of phospho–histone H3–stained metaphase chromosomes revealed what appeared to be fragmented microchromosomes (Figure 5B), indicative of genomic instability. The presence of genomic instability prompted us to examine the potential activation of DDR pathways by $\gamma$-H2AX staining (Figure 6A). The percentage of cells with $\gamma$-H2AX foci were significantly increased in $\text{Atrx}^{+/y}$:Ad-Cre versus $\text{Atrx}^{+/y}$:Ad-LacZ myoblasts (Figure 6B).

$\text{Atrx}$ is highly enriched in PML bodies and pericentromeric heterochromatin (33). Moreover, recent studies have revealed an association between histone variant H3.3 and $\text{Atrx}$ on telomeres in mouse ES cells (17, 19). Dysfunctional centromeres and telomeres often lead to a multitude of cellular responses, such as chromosomal instability, activation of cell cycle checkpoints, and hyperactivated DDR (34–36). Inability to reestablish function of these crucial structures can result in mitotic catastrophe and eventually cell death. We therefore examined the telomeres and centromeres of $\text{Atrx}^{+/y}$:Ad-Cre myoblasts for evidence of dysfunction. Visualization of the telomeres using FISH revealed an increased frequency of aberrant telomeric signals in $\text{Atrx}^{+/y}$:Ad-LacZ and $\text{Atrx}^{+/y}$:Ad-Cre myoblasts. Values represent percent total ± 95% confidence interval. $\text{Atrx}^{+/y}$:Ad-LacZ, 661 chromosomes ($n = 3$); $\text{Atrx}^{+/y}$:Ad-Cre, 1,096 chromosomes ($n = 3$). Original magnification, $\times630$ (A); $\times1,000$ (C and D).
was increased approximately 2-fold in progression and maintenance of genomic stability. Consistent proteins, such as Brca1 and Rad51, are essential for proper S phase homologous recombination (HR) repair pathway. HR repair protein was activated and the percent total of aberrant nuclei diminished to very low levels by 72 hours. Under high-mitogen growth conditions. Infected myoblasts were left untreated or were irradiated (10 J/m² UV pulse), and Atrx depletion activates the p53-ATM DDR pathway and concomitant reduction in Rad51. Activation of ATM and p53 is concomitant with reduction of Rad51 in Atrx-depleted myoblasts. Properly functioning DDRs are critical for maintaining genomic stability during the cell cycle. ATM protein kinases, along with ATM- and Rad3-related (ATR) protein kinases, are activated in response to replication stress caused by collapsed or stalled DNA replication forks (37). We therefore assessed ATM/ATR signaling in Atrx-depleted myoblasts to determine the presence of excessive replicative stress.

Western blot analyses of Atrx<sup>f/y</sup>:Ad-Cre myoblasts revealed an average 3-fold increase in phospho-ATM<sup>Ser1981</sup> and a 1.5-fold increase of phospho-Chk1<sup>Ser345</sup> an ATR kinase substrate (Figure 7A and Supplemental Table 2). Activation of ATM simultaneously targets downstream effectors such as p53, while mobilizing the homologous recombination (HR) repair pathway. HR repair proteins, such as Brc1 and Rad51, are essential for proper S phase progression and maintenance of genomic stability. Consistent with our earlier observation, p53 protein was increased approximately 2-fold in Atrx<sup>f/y</sup>:Ad-Cre myoblasts (Figure 7A and Supplemental Table 2). Interestingly, levels of the HR repair protein Rad51 were reduced by 2-fold in Atrx<sup>f/y</sup>:Ad-Cre compared with Atrx<sup>f/y</sup>:Ad-LacZ myoblasts (Figure 7B and Supplemental Table 2). We further examined the capacity of proliferating myoblasts to recover from acute exposure to UV-induced DNA damage in the absence of Atrx. Atrx<sup>f/y</sup>:Ad-Cre and Atrx<sup>f/y</sup>:Ad-LacZ myoblasts were pulsed with 10 J/m² UV and harvested 24, 48, and 72 hours later. Under high-mitogen growth conditions, Atrx<sup>f/y</sup>:Ad-Cre myoblasts maintained elevated levels of both p53 and the DNA damage marker γ-H2AX 72 hours after UV irradiation (Figure 7C). In contrast, Atrx<sup>f/y</sup>:Ad-LacZ controls showed recovery from UV irradiation by 48 hours with p53, and γ-H2AX levels diminished to very low levels by 72 hours. Finally, we further validated the intrinsic requirement for Atrx in rapidly proliferating myoblasts by adenoviral reintroduction of ATRX (Ad-ATRX) into Atrx<sup>f/y</sup>:Ad-Cre myoblasts (Supplemental Figure 5). After Ad-ATRX infection, we observed reduced levels of activated Chk1 compared with Atrx<sup<f/y</sup>:Ad-Cre myoblasts, although p53 levels remained elevated and the percent total of aberrant nuclei was not significantly reduced (Supplemental Figure 5, A and B).

Table 2

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<th>Diffuse</th>
<th>Bridge</th>
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Values denote number of chromosomes per classification (n = 3 experiments per group).

( Supplemental Figure 4). Taken together, these data demonstrate that proliferating Atrx<sup>f/y</sup>:Ad-Cre myoblasts accumulate DNA damage, and thus suggest that Atrx is involved in the processing or maintenance of late-replicating heterochromatin.

Atrxf/y infection, we observed reduced levels of ATRX knockdown in human primary myoblasts results in growth deficits and p53 accumulation. Due to a high degree of homology between mouse and human ATRX (6), one would expect satellite cell expansion deficiencies to be similar in the absence of functional ATRX in humans. In order to validate this assumption, we transfected human primary myoblasts with either nonspecific control siRNA (siScrambled) or ATRX-specific siRNA (siATRX) (Figure 8, A and B). Western blot analysis demonstrated an increase in p53 protein levels in siATRX-knockdown myoblasts compared with siScrambled controls (Figure 8B). Moreover, loss of ATRX in human primary myoblasts significantly reduced proliferation, as determined by a 36% reduction in WST-1 cleavage by mitochondrial dehydrogenases (Figure 8C). We also examined a panel of 3 ATR-X patient-derived lymphoblast lines harboring different mutations in ATRX (Figure 8D). Line 1 contained a 2-kb deletion spanning the int34/ex35 junction, resulting in a short C-terminal truncation; line 2 contained a Gly249Cys substitution in the ADD domain; and line 3 carried a 100-kb deletion spanning exons 2–7, producing an internally deleted protein lacking a functional ADD domain that was detectable by a C-terminal specific Atrx antibody (M.S. Huh, unpublished observations). Immunoblot detection using a monoclonal antibody directed toward an N-terminal epitope of ATRX detected signals in both UV-treated and untreated controls as well as patient lines 1 and 2 (Figure 8D).

Importantly, we observed in

Figure 7

Atrx depletion activates the p53-ATM DDR pathway and concomitant reduction in Rad51. (A and B) Western blot analysis of Atrx<sup>f/y</sup>:Ad-LacZ (−) and Atrx<sup>f/y</sup>:Ad-Cre (+) myoblasts exposed to the replication inhibitor hydroxyurea. (A) Immunoblots were probed with antibodies specific for Atrx, phospho-ATM<sup>Ser1981</sup>, Chk1, phospho-Chk1<sup>Ser345</sup>, p53, and γ-H2AX; β-actin was used as loading control. (B) Immunoblots were probed with antibodies specific for Atrx, PAR, and Rad51; β-actin was used as loading control. (C) Western blot analysis of UV-induced DDR in Atrx<sup>f/y</sup>:Ad-LacZ and Atrx<sup>f/y</sup>:Ad-Cre myoblasts under high-mitogen growth conditions. Infected myoblasts were left untreated or were irradiated (10 J/m² UV pulse), and protein was harvested at 24, 48, and 72 hours. Immunoblots were probed with antibodies specific for p53 and γ-H2AX; β-actin was used as loading control.
Discussion

Animal model of Atrx deficiency in skeletal muscle. ATR-X syndrome is a severe intellectual disability disorder with a wide clinical spectrum. While this implies a complex developmental role, it has generally been presumed that many of the clinical features arise indirectly from CNS deficiencies. In this study we identified, for the first time to our knowledge, a cell-intrinsic requirement for Atrx in the hyperexpansion of satellite cells, thus placing an extensive demand on the remaining satellite cells for regeneration (29, 49). Our analysis of CTX-injected Atrx cKO muscle demonstrated a profound deficit in regeneration, as revealed by poorly formed residual microfibers at the site of injury in addition to large vacuolar spaces and a high degree of fibrosis. The fatty/fibrotic phenotype is most likely a direct result of a combination of poor satellite cell expansion and increased compensation by the resident fibro/adipogenic progenitors that expand and differentiate to fill the void (50). Our findings further demonstrated the requirement for Atrx in the hyperexpansion of myogenic progenitor cells.

Delayed S phase progression activates the DDR and leads to genomic instability. It has been previously documented that Atrx-null cells have growth deficiencies, yet the precise mechanism underlying this deficit remained elusive. In particular, a competitive coculture assay between WT and Atrx-null ES cells demonstrated a precipitous disappearance of the Atrx-null population after 4 passages, subsequently, the developmental growth of embryonic and later fetal muscle tissue is dependent on a different population of progenitor cells that are Pax3+Pax7+ (44, 45). Moreover, Relaix et al. demonstrated that 93% of Pax3+Pax7+ muscle progenitors were Myf5+, undergoing massive proliferation prior to MRF expression and differentiation (44). Consequently, the developmental inactivation of Atrx in our Atrx cKO model occurred after the rapid expansion of Pax3+Pax7+ progenitors, resulting in viable offspring.

In the first 3 weeks of life, muscle mass accumulates rapidly through the accretion of myonuclei from activated muscle satellite cells in the mouse (46, 47). Despite their small size, Atrx cKO mice had normal numbers of resident satellite cells that were capable of activating and entering a proliferative phase. However, fewer myoblasts were evident within 48 hours, and profound deficiencies in myoblast expansion were realized after culturing fibers for 6 days. It was dramatically evident from the final numbers that Atrx-null myoblasts were incapable of sustaining a rapid growth rate. Consequently, under natural physiological growth demands, muscle mass accumulation was delayed in Atrx cKO mice. Beyond 3 weeks to full adulthood, addition of myonuclei ceases, and muscle mass increase is achieved through hypertrophic growth of the muscle fiber (47). It was during this period that we observed the gradual compensation of body mass in Atrx cKO mice compared with their Atrx+ littermates.

Activation and expansion of satellite cell–derived myoblasts is also fundamental for proper muscle regeneration after exercise or injury (48). CTX ablation of muscle is an extreme experimental paradigm that destroys differentiated muscle fibers and 90% of satellite cells, thus placing an extensive demand on the remaining satellite cells for regeneration (29, 49). Our analysis of CTX-injected Atrx cKO muscle demonstrated a profound deficit in regeneration, as revealed by poorly formed residual microfibers at the site of injury in addition to large vacuolar spaces and a high degree of fibrosis. The fatty/fibrotic phenotype is most likely a direct result of a combination of poor satellite cell expansion and increased compensation by the resident fibro/adipogenic progenitors that expand and differentiate to fill the void (50). Our findings further demonstrated the requirement for Atrx in the hyperexpansion of myogenic progenitor cells.

Collectively, these data support an ATR-X disease model whereby rapid myoblast proliferation results in the accumulation of DNA damage during replication, which in turn activates the p53-ATR DDR pathway and increases the frequency of genomic instability.

Figure 8

ATRX knockdown in human primary myoblasts leads to p53 induction and reduced proliferative capacity. (A) Double-immunofluorescent staining for skeletal muscle–specific intermediate filament proteins Desmin and ATRX 72 hours after transfection with siATRX. (B) Western blot analysis of human primary myoblasts transfected with siScrambled and siATRX. Immunoblots were probed with antibodies specific for ATRX and p53; β-actin was used as loading control. (C) WST-1 proliferation assay. Values represent mean ± SEM (N = 4). **P < 0.01, t test. (D) Western blot analysis of immortalized ATRX mutant patient lymphoblast lines (numbered 1–3). UV, 10 J/m2 UV irradiation of WT lymphoblasts; NT, untreated WT lymphoblasts. Immunoblots were probed with antibodies specific for ATRX, p53, phosphorylated p53Ser37, and γ-H2AX; β-actin was used as loading control. Lanes were run on the same gel but were noncontiguous (white lines). Original magnification, ×200 (A).
disrupts telomeric replication, leading to telomeric dysfunction and genomic instability (62). Interestingly, Atrx has been shown to have an affinity for G4 structures in vitro (20). Thus, one could envision Atrx acting to alleviate these G4 structures in an ATPase-dependent manner during telomere replication. However, further studies are required to determine whether there is a higher prevalence of G4 structures in the absence of Atrx.

The ATPase domain of Atrx is most similar to that of the HR protein Rad54, which suggests that Atrx may be involved in resolving stalled replication forks (6). The higher frequency of replication stalling in heterochromatin necessitates the recruitment of homology-directed DNA repair proteins (Rad51, Brc, Rad52, and Rad54) in order to resume DNA replication in an error-free manner (63). In this regard, Atrx and Rad54 are both capable of remodeling mononucleosomes as well as exhibiting a translocase activity (10, 11). Despite these similarities, it is unknown whether Atrx can stabilize Rad51 onto presynaptic filaments or resolve DNA intermediate structures ( Holliday junctions or stalled replication forks) by driving branch migration, as has been demonstrated for Rad54 (64–67). Nonetheless, our results are congruent with a model proposed by Karlseder and colleagues that demonstrates the recruitment of the MRN complex, ATM kinase, and HR machinery to the newly replicated telomere for the reformation of the protective t-loop (68, 69). Coincidently, our results showed that loss of Atrx caused a delay in mid to late S phase that was concurrent with ATM activation and Rad51 reduction. As such, Atrx may provide a Rad54-like activity in promoting the stability of Rad51 on telomeric overhangs, thus facilitating the formation of the protective t-loop.

We demonstrated here that Atrx deficiency in the mouse model was applicable in a clinical context. Much like in mouse myoblasts, loss of ATRX in human myoblasts caused proliferative deficiency and p53 accumulation. Furthermore, we have shown that lymphoblasts from ATR-X patients induced the ATM/ATR DDR. Patient mutations are far less severe than the Atrx knockout model, which validates our animal model. In addition, these lines of evidence support a model in which intrinsic deficiencies in human satellite cells lead to muscle weakness, giving rise to hypotonia, kyphosis, and ambulation difficulties, which are all common features of ATR-X patients.

Our studies have defined the physiological and cellular consequences of Atrx dysfunction in skeletal muscle. More importantly, we highlighted the importance of Atrx in maintaining genomic stability and telomeric integrity in rapidly proliferating myoblasts. These findings support a general function for Atrx in rapidly proliferating progenitor cells.

**Methods**

**Mice and CTX injury protocols.** Myf5-Cre mice were obtained from P. Soria-no (Mount Sinai School of Medicine, New York, New York, USA; ref. 26) and maintained on a 129/Sv background. Atrx exon 18 floxed mice (3) were maintained on a C57BL/6 background. See Results for the breeding strategy. CTX regeneration experiments were performed as previously described (70). TA muscles of adult mice were injected with 25 μl of 10 μM CTX (Latoxan). Muscles were harvested 1 and 2 weeks after injection. H&E and Masson’s Trichrome histology were performed at the University of Ottawa pathology laboratory.

**Primary myoblast and single fiber isolation protocols.** Primary myoblasts were isolated from hind limbs of 4-week-old Atrx floxed male mice as previously published (39). Primary myoblasts were maintained in Hams F-10 (Wisent) growth media (GM) supplemented with 2.5 ng/ml human recombinant bFGF (Invitrogen), 20% FBS (PAA), and 2% penicillin/streptomycin (Invitrogen).
Ad-LacZ and Ad-Cre were used at an MOI of 10 on Atrx<sup>−/−</sup> primary myoblasts, as described previously (71), and Ad-ATRX was used at an MOI of 1.

Live myofibers were dissociated and cultured from the EDL muscle (72). Floating muscle fiber cultures were maintained in 60-mm bacterial plates (Fisher) coated with horse serum (Invitrogen) in DMEM (Invitrogen) supplemented with 10% horse serum, 0.5% chick embryo extract (catalog no. CE650T; Accurate Chemical and Scientific Corp.), and 10 μM BrdU (Sigma-Aldrich). For adherent fiber cultures, individual fibers were seeded in 8-well chamber slides (catalog no. 177445; Lab-Tek) coated with Matrigel (catalog no. 40234; Becton Dickinson) and maintained in DMEM supplemented with 10% horse serum and 0.5% chick embryo extract.

**Immunostaining and Western blots.** Primary myoblasts were trypsinized and resuspended in GM. Myoblasts were cytopsin (Cytospin4; Thermo Shandon) onto slides and fixed in 2% PFA or ice-cold methanol/acetic acid at a 1:1 ratio. For immunostaining, myoblasts were permeabilized with 0.1% Triton-X in PBS and incubated in blocking solution (20% horse serum, 0.1% fetal bovine serum, and 0.03% sodium azide in PBS) for 2 hours at room temperature. Primary antibodies were diluted in PBS and incubated overnight at 4°C. Myoblasts were stained with antibodies to Pax7 (1:3 dilution; Developmental Studies Hybridoma Bank [DSHB]), Atrx (1:500 dilution, clone D33; Dako), myogenin (1:10 dilution, F5D; DSHB), desmin (1:500 dilution, clone D3; Dako), myogenin (1:10 dilution, F5D; DSHB), α-tubulin (1:0000 dilution, clone DM1A; Sigma-Aldrich), γ-tubulin (1:500 dilution, ab11317; Abcam), phospho-H2AX (1:250 dilution, ab2056; Cell Signaling), and CENPA (1:500 dilution, ab1587; Cell Signaling). Images were taken using either an Axio (1:10,000 dilution, AC-15; Sigma-Aldrich), phospho-ATM Ser1981 (10H11. Chk1Ser345 (2341; Cell Signaling), E12; Millipore), Chk1 (1:1,000 dilution, 2G1D5; Cell Signaling), phospho-Chk1 (1:500; 2341; Cell Signaling), γ-H2AX (1:1,000 dilution, 20E3, Cell Signaling), Rad51 (1:1,000 dilution, R-1582; Sigma-Aldrich), PAR (1:2,000 dilution, 4335-MC-100; Trevigen), MHC (1:50 dilution, MF20; DSHB), cyclin E (1:3,000 dilution, 06459; Upstate), p107 (1:18) (1:2,000 dilution; Santa Cruz), cyclin A (1:19) (1:2,000 dilution; Santa Cruz), and Ki67 (1:4,000 dilution, BD610241; BD Biosciences). Western blots were quantified by densitometry analysis using Image J software. See complete undetected blots in the supplemental material.

**BrdU pulse-chase protocol.** Primary myoblasts were infected with Ad-LacZ or Ad-Cre and the following day seeded at 4 x 10<sup>5</sup> cells per 10-cm plate. Seed ed myoblasts were maintained in GM for an additional 48 hours and then pulsed with 10 μM BrdU for 1 hour. Myoblasts were trypsinized and washed 3 times with PBS, and fresh GM was reapplied. Myoblasts were trypsinized and fixed on ice-cold 70% ethanol for 4-hour intervals up to 24 hours. Fixed pulse-chased samples were processed for flow cytometry analysis as described previously (32). BrdU antibody (1:100 dilution, clone B44; BD Biosciences) and Alexa Fluor 488 secondary antibody (1:500 dilution; Invitrogen) were used. Fluorescence-activated flow cytometry was performed on a Beckman Coulter FACSc Station, and cell cycle data were analyzed by ModFit software (Verity Software House).

**siRNA knockdown.** Human primary myoblasts were seeded into 60-mm tissue culture plates in myoblast GM. On the day of transfection, myoblasts were transfected in serum-free OptiMEM media (Invitrogen) with nontargeting siScrambled (siGENOME Non-Targeting siRNA Pool #2; Dharmacon) or siATRX (siGENOME SMARTpool, Human ATRX; Dharmacon) at a concentration of 100 nM with INTERFERin siRNA transfection reagent (Polyplus-transfection) according to the manufacturer’s instructions.

**Metaphase chromosome telomeric FISH.** 48 hours after infection, primary myoblasts were treated with 10 μg/ml colchicine (Sigma-Aldrich) for 5 hours. Myoblasts were harvested and processed for telomeric PNA FISH (BioSynthesis) according to a previously published protocol (73).

**SYBR QPCR analysis.** RNA was harvested 48 hours after infection (Qiagen), and 1 μg RNA was reverse transcribed (SuperScript III; Invitrogen). cDNA was used for expression analysis of cell cycle genes by qPCR SuperArray (PAMM-020, SBiosciences). QPCR was performed at a Stratagene Mx3000P system.

**WST-1 proliferation assay.** Mouse or human myoblasts were seeded into a 96-well flat-bottomed cell culture plate (Costar) at 2 x 10<sup>4</sup> cells/well. Cells were maintained in GM for 4 days (mouse) and 5 days (human), at which time WST-1 assay (ab65473; Abcam) was performed according to the manufacturer’s instructions.

**Statistics.** Statistical analyses were performed using Microsoft Excel. 2-tailed t test and 95% confidence intervals were used to determine significance of differences. A P value less than 0.05 was considered significant.

**Study approval.** All mice used in our studies were housed and cared for according to Canadian Council on Animal Care (CCAC) guidelines and University of Ottawa Animal Care Committee protocols. Human lymphoblastoid cell lines were obtained after receipt of written informed consent from participants and guardians as outlined in the approved Ottawa Hospital Research Ethics Board Protocol no. 199577-01H. The authors thank Vanessa Seale for technical assistance with CTX muscle injections; Jeff Hamill for technical assistance with the flow cytometry; and M.A.M. Todd, F.J. Dilworth, and L. Megeny for comments on the manuscript. This work was supported by operating grants to D.J. Picketts from Canadian Institutes of Health Research and Association Francaise Contre les Myopathies.

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