Compromised genomic integrity impedes muscle growth after Atrx inactivation

Michael S. Huh,1 Tina Price O’Dea,1 Dahmane Ouazia,1 Bruce C. McKay,2,3 Gianni Parise,4 Robin J. Parks,1,3 Michael A. Rudnicki,1,3 and David J. Picketts1,3

1Regenerative Medicine Program and 2Cancer Therapeutics Program, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada. 3Department of Medicine, University of Ottawa, Ottawa, Ontario, Canada. 4Department of Kinesiology and Department of Medical Physics and Applied Radiation Sciences, McMaster University, Hamilton, Ontario, Canada.

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 hypertonia, 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) mice were viable, but by 3 weeks of age presented hallmarks of under-developed musclecuture, 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 muscle.

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–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 nucleosomes 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 on 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-

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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 (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 Atrxd/2 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 Atrxd/2 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).

Atrxd/2 mice have normal numbers of satellite cells, yet is unable to efficiently expand the myoblast population when activated in culture. (A) Double-immunofluorescent micrograph of Atrx and Pax7 from freshly isolated Atrxd/2 and Atrxd/2 EDL myofibers. Pax7+ satellite cells were still present in Atrx cKO myofibers (yellow arrowheads). (B) Myoblast proliferation index of activated satellite cells from single muscle fiber cultures in suspension. Single muscle fibers isolated from Atrxd/2 and Atrxd/2 EDL muscles were immediately fixed (0 hours) or were grown in BrdU-supplemented GM, then harvested and fixed after 6 and 48 hours in culture. Immunostaining for Pax7 (0 hours) and BrdU (6 and 48 hours) was performed, and positive nuclei were enumerated. Values represent mean ± SEM. Atrxd/2, 89 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 Atrxd/2 and Atrxd/2 cKO mice were plated onto a Matrigel substrate and fixed after 6 days in culture. Desmin+ myoblasts were enumerated for each fiber (inset). Atrxd/2, 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).

Table 1

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Area values are mean ± SEM.
controls (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 Atrxy 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

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**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, Atrxy:Ad-LacZ, and Atrxy: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), Atrxy:Ad-LacZ, and Atrxy: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 Atrxy:Ad-LacZ (E) and Atrxy: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 Atrxf/y animals and subsequently infected with Cre recombinase-expressing adenovirus (Ad-Cre). The resulting myoblasts (referred to herein as Atrxf/y: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 Atrxf/y:Ad-Cre myoblasts compared with Ad-LacZ–infected (Atrxf/y: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, Atrxf/y:Ad-Cre myoblasts were capable of fusing to form multinucleated syncytial myotubes. Furthermore, Atrxf/y: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, chromatid 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 f/y 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, 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 Atrxf/y:Ad-Cre myoblasts (Figure 4A), which suggests that one or more cell cycle checkpoints may be activated in the absence of Atrx.

Figure 4
Atrx depletion delays S phase and induces accumulation of p53 protein. (A) Immunoblot analysis of p53 protein levels after infection with Ad-LacZ (+) or Ad-Cre (+). Infected cells were untreated (NT) or treated with hydroxyurea (HU) or colchicine (Col) for the indicated times, and then protein samples were harvested. β-Actin was used as loading control. (B) Flow cytometry cell cycle progression analysis of BrdU–population from Atrxf/y:Ad-LacZ and Atrxf/y:Ad-Cre myoblasts. Graphs represent percent BrdU–cells in the G1, S, and G2/M gates at the indicated time points after the moment of BrdU removal (0 hour). Data are mean ± SEM (n = 4). *P < 0.05, t test. (C) S phase–specific double immunostaining for Atrx and BrdU. Atrxf/y myoblasts were pulsed for 20 minutes with BrdU and immediately fixed. Atrx colocalized to late replicating regions of DNA (red box), but did not colocalize with early replicating DNA (blue box). Original magnification, ×630 (C). Boxed regions are enlarged ×9 at right.
To test whether loss of Atrx elicits a stage-specific cell cycle checkpoint, we treated Atrx\(^{+/−}\):Ad-Cre myoblasts with the DNA replication inhibitor hydroxyurea or the mitotic inhibitor colchicine. Interestingly, p53 accumulation was observed with hydroxyurea treatment, but not in the presence of colchicine (Figure 4A), which suggests that loss of Atrx triggers the p53 checkpoint during the S phase of the cell cycle. This was a surprising result, as we anticipated that p53 accumulation would be associated with colchicine treatment, as suggested by other studies (5, 31). Our results prompted us to specifically examine cell cycle kinetics in Atrx\(^{+/−}\):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 distribution, consistent with work in Atrx-null ES cells (27). To determine whether Atrx loss results in a more subtle defect in cell cycle progression, a BrdU pulse-chase protocol was used (32). Briefly, Atrx\(^{+/−}\):Ad-LacZ and Atrx\(^{+/−}\):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 remainder was in G2/M (Figure 4B). Orderly passage of the control cells from G1 and G2/M through the chase period, while a small but statistically significant delay in the passage of Atrx\(^{+/−}\):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 completion of S phase and consequently produced a transient disequilibrium in the cell cycle profile at the 16- and 20-hour time points (Supplemental Figure 3). Similarly, the passage of the G2/M population of BrdU– Atrx\(^{+/−}\):Ad-Cre myoblasts was delayed compared with Atrx\(^{+/−}\):Ad-LacZ controls (Figure 4B). In light of these findings, we examined Atrx localization with respect to sites of DNA replication during S phase. Active sites of DNA replication were marked with a brief 20-minute BrdU pulse in asynchronously proliferating myoblasts. Immunofluorescence revealed a strikingly similar pattern 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\(^{+/−}\):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 (≥2) γ-tubulin–staining microtubule organizing centers (Figure 5A). Quantification of the proportion of myoblasts with aberrant nuclear morphology (micronuclei, binuclei, or multinuclei) revealed a significantly higher percentage in Atrx\(^{+/−}\):Ad-Cre than in Atrx\(^{+/−}\):Ad-LacZ myoblasts (Figure 5A). Furthermore, visualization of the mitotic spindle revealed substantial contrast...
between control $Atrx^{+/+}$:Ad-LacZ and $Atrx^{+/+}$:Ad-Cre myoblasts. In mitotic $Atrx^{+/+}$: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 $Atrx^{+/+}$: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 $Atrx^{+/+}$:Ad-Cre versus $Atrx^{+/+}$:Ad-LacZ myoblasts (Figure 6B).

$Atrx$ is highly enriched in PML bodies and pericentromeric heterochromatin (33). Moreover, recent studies have revealed an association between histone variant H3.3 and $Atrx$ 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 $Atrx^{+/+}$:Ad-Cre myoblasts for evidence of dysfunction. Visualization of the telomeres using FISH revealed an increased frequency of aberrant telomeric signals in $Atrx^{+/+}$:Ad-LacZ and $Atrx^{+/+}$:Ad-Cre myoblasts. Arrowheads denote chromosomes with aberrant telomeric signals. Represented images of normal (top left), deleted (top middle), merged (top right), bridged (bottom left), duplicated (bottom middle), and diffused (bottom right) telomeric signals. Percent fragile telomeres in mitotic $Atrx^{+/+}$:Ad-LacZ and $Atrx^{+/+}$:Ad-Cre myoblasts. Values represent percent total ± 95% confidence interval. $Atrx^{+/+}$:Ad-LacZ, 661 chromosomes ($n = 3$); $Atrx^{+/+}$:Ad-Cre, 1,096 chromosomes ($n = 3$). Original magnification, $\times 630$ (A); $\times 1,000$ (C and D).
was increased approximately 2-fold in myoblasts by adenoviral reintroduction of Ad-ATRX into Atrx f/y myoblasts (Supplemental Figure 5). After Ad-ATRX infection, we observed reduced levels of activated Chk1 compared with Atrx f/y 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). It is likely that significant chromatin changes occur in the 48 hours prior to ATRX reintroduction that dampen more robust rescue of the aberrant nuclei. Nonetheless, we did observe a 36% increase in the number of viable cells after Ad-ATRX infection (Supplemental Figure 5C), demonstrating partial rescue of the growth defect.

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

### Table 2

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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 f/ y Ad-Cre myoblasts accumulate DNA damage, and thus suggest that Atrx is involved in the processing or maintenance of late-replicating heterochromatin.

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 f/ y Ad-Cre myoblasts revealed an average 3-fold increase in phospho-ATM Ser1981 and a 1.5-fold increase of phospho-Chk1 Ser345, 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 Brca1 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 f/ y Ad-Cre myoblasts (Figure 7A and Supplemental Table 2). Interestingly, levels of the HR repair protein Rad51 were reduced by 2-fold in Atrx f/ y Ad-Cre compared with Atrx f/ y 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 f/ y Ad-Cre and Atrx f/ y Ad-LacZ myoblasts were pulsed with 10 J/m2 UV and harvested 24, 48, and 72 hours later. Under high-mitogen growth conditions, Atrx f/ y 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 f/ y 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 f/ y Ad-Cre myoblasts (Supplemental Figure 5). After Ad-ATRX infection, we observed reduced levels of activated Chk1 compared with Atrx f/ y Ad-Cre myoblasts, although p53 levels remained elevated and the percent total of aberrant nuclei
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![Figure 8](http://www.jci.org)

**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. Immunoblotts 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. Immunoblotts 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).

all 3 patient cell lines a marked increase in phosphorylation of p53 at Ser37 (Figure 8D), a target of the ATM/ATR DDR kinases (38).

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-ATM DDR pathway and increases the frequency of genomic instability.

**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 during postnatal muscle growth and regeneration. Conditional knockout of Atrx in skeletal muscle resulted in viable offspring. The Journal of Clinical Investigation

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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. Immunoblotts 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. Immunoblotts 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).

sequently, 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.

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,
despite the lack of any indication of a defective cell cycle profile (27). Other studies using Atrx shRNA treatment of HeLa cells or a Sertoli cell-specific Atrx conditional knockout mouse described a delay in G2/M progression along with increased numbers of dying cells and cells with chromosomal defects (e.g., micronuclei and DNA bridges; refs. 4, 31). Our present results implicate a role for Atrx in the progression through mid to late S phase, due to the following 2 lines of evidence: (a) in actively dividing Atrx<sup>−/−</sup> myoblasts, the localization pattern of Atrx was almost indistinguishable from that of late-replicating heterochromatin; and (b) flow cytometry cell cycle chase analysis revealed a distinct and transient delay going through mid to late S phase by Atrx<sup>−/−</sup>:Ad-Cre myoblasts. It must be noted that the subtle and transient nature of this phenotype cannot be detected by a static cell cycle profile of asynchronously dividing cells.

Together, these data suggest that Atrx is involved in the replication of heterochromatin and/or its structural reestablishment after DNA synthesis. Moreover, the proper assembly and maintenance of centromeric and/or telomeric heterochromatin is critical for genomic integrity and for cell survival (5, 51–54). Under conditions of significant growth, the probability of acquiring deleterious genomic lesions in Atrx-deficient cells is increased, and at some point, cell survival is compromised. This has previously been illustrated by forebrain-specific inactivation of Atrx, which yielded a 12-fold increase in TUNEL<sup>+</sup> cells at E11.5 (3). This developmental time point coincides with the moment when neural progenitors are undergoing rapid expansion. Similarly, at E17.5, when Sertoli cells are at their proliferative peak, a dramatic 40-fold increase in apoptosis was observed (4). Results from WT and Atrx-null ES cell culture experiments can be interpreted in a similar manner. Indeed, this may be a common feature of many chromatin remodeling proteins, as similar observations have been made for ISWI, SWI/SNF, and Mi-2 complexes (55–58). Consistent with a role for Atrx in genomic stability, we also observed a higher incidence of γ-H2AX staining, p53 accumulation, and activation of the ATM damage pathways in Atrx<sup>−/−</sup>:Ad-Cre myoblasts that translated into an abnormal cytologic phenotype. These abnormalities were most apparent during mitosis, which suggests that previous studies describing mitotic progression deficits in HeLa and Sertoli cells may have occurred secondary to problems in heterochromatin replication/assembly, as indicated by our experiments.

**Involvement of Atrx in telomere replication.** Atrx binding to pericentromeric heterochromatin is well documented, and recent studies have determined that this targeting is mediated through recognition of a bipartite histone mark (H3K4me<sup>0</sup>/H3K9me<sup>3</sup>) via the ADD domain (7–9). Surprisingly, the centromeres of Atrx<sup>−/−</sup>:Ad-Cre myoblasts remained intact, including H3K14ac localization at these structures (M.S. Huh, unpublished observation). A mounting body of recent evidence has suggested a functional role for Atrx at the telomere. In mouse ES cells, chromatin IPs using Atrx antibodies achieved a dramatic enrichment for telomeric repeats (20), and Atrx-Daxx complexes were demonstrated to be essential for the deposition of histone variant H3.3 onto telomeres (17, 19, 59). In Atrx-depleted mouse primary myoblasts, we observed telomeric aberrations characterized by a higher incidence of telomeric bridging, duplications, and merging. Loss of telomeric integrity is commonly associated with the disruption of processes involving replication and protection of telomeres (60), and G-rich telomeric tracts are particularly susceptible to the formation of G4 structures (61). Furthermore, chemical stabilization of G4 structures by RHPS4 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 replisome stalling in heterochromatin necessitates the recruitment of homology-directed DNA repair proteins (Rad51, Brc2, 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).

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. 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.

**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+y primary myoblasts, as described previously (71), and Ad-ATRX was used at an MOI of 1.

Live myoblasts were dissociated and cultured from the E12.5 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 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, H-300; Santa Cruz), Desmin (1:500 dilution, clone D3; Dako), myogenin (1:10 dilution, FSV; DSHB), HIC (1:20 dilution, MF20; DSHB), BrdU (1:500 dilution, clone B4; BD Biosciences), α-tubulin (1:1,000 dilution, clone DM1A; Sigma-Aldrich), γ-tubulin (1:500 dilution, ab11317; Abcam), p53 (1:500 dilution, 06-570; Millipore), γ-H2AX (1:250 dilution, 20E3; Cell Signaling), and CENP-A (1:500 dilution, CSA17; Cell Signaling). Images were taken using either an Axio Imager M1 (Zeiss), LSM 510 (Zeiss), or DMI6000 B (Leica) microscopy system.

Immunostaining and Western blots. Primary myoblasts were trypsinized and resuspended in GM. Myoblasts were cytospun (Cytospin4; Thermo Shandon) onto slides and fixed in 2% PFA or ice-cold methanol/acetic 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, H-300; Santa Cruz), Desmin (1:500 dilution, clone D3; Dako), myogenin (1:10 dilution, FSV; DSHB), HIC (1:20 dilution, MF20; DSHB), BrdU (1:500 dilution, clone B4; BD Biosciences), α-tubulin (1:1,000 dilution, clone DM1A; Sigma-Aldrich), γ-tubulin (1:500 dilution, ab11317; Abcam), p53 (1:500 dilution, 06-570; Millipore), γ-H2AX (1:250 dilution, 20E3; Cell Signaling), and CENP-A (1:500 dilution, CSA17; Cell Signaling). Images were taken using either an Axio Imager M1 (Zeiss), LSM 510 (Zeiss), or DMi6000B (Leica) microscopy system.

For Western blots, primary myoblasts were harvested and lysed in RIPA extraction buffer (50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.5% sodium dodecyl sulfate; 10 mM EDTA; 150 mM NaCl; and 50 mM NaF) supplemented with protease inhibitors (Complete mini; Roche) on ice. Protein lysates were separated on a 3%-8% or 4%-12% gradient SDS-PAGE (NuPAGE; Invitrogen) and transferred onto an Immobilon-P (Millipore) membrane. Western blots were probed with antibodies to AtrxX (1:10 dilution, F39; gift of D. Higgs, University of Oxford, Oxford, United Kingdom), α-tubulin (1:10,000 dilution, clone DM1A; Sigma-Aldrich), p53 (1:2,000 dilution, 1C12; Cell Signaling), p53 (ab6) (1:1,000 dilution; Abcam), pH3 (1:500 dilution, 06-570; Millipore), γ-H2AX (1:250 dilution, 20E3; Cell Signaling), and CENP-A (1:500 dilution, CSA17; Cell Signaling). Western blots were quantified by densitometry analysis using Image J software. See complete unedited 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 × 10^6 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 washed 3 times with PBS, and fresh GM was reapplied. Myoblasts were trypsinized and fixed on ice-cold 70% ethanol at 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 FACS 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 siATRXX (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 (SuperScriptIII; Invitrogen). cDNA was used for expression analysis of cell cycle genes by qPCR SuperArray (PAMM-020, SABiosciences). QPCR was performed on 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 × 10^4 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. 1998577-01H.

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Address correspondence to: David J. Picketts, Ottawa Hospital Research Institute, Regenerative Medicine Program, 501 Smyth Road, Ottawa, Ontario K1H8L6, Canada. Phone: 613.737.8989; Fax: 613.737.8803; E-mail: dpicketts@ohri.ca.


