ATR localizes to the photoreceptor connecting cilium and deficiency leads to severe photoreceptor degeneration in mice

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Ataxia-telangiectasia and Rad3 (ATR), a sensor of DNA damage, is associated with the regulation and control of cell division. ATR deficit is known to cause Seckel syndrome, characterized by severe proportionate short stature and microcephaly. We used a mouse model for Seckel disease to study the effect of ATR deficit on retinal development and function and we have found a new role for ATR, which is critical for the postnatal development of the photoreceptor (PR) layer in mouse retina. The structural and functional characterization of the ATR+/s mouse retinas displayed a specific, severe and early degeneration of rod and cone cells resembling some characteristics of human retinal degenerations. A new localization of ATR in the cilia of PRs and the fact that mutant mice have shorter cilia suggests that the PR degeneration here described results from a ciliary defect.

INTRODUCTION

Seckel syndrome is an autosomal recessive disorder characterized by intra-uterine growth retardation, severe proportionate short stature and marked microcephaly (1). The ataxia-telangiectasia and Rad3-related (ATR) gene has been associated with Seckel syndrome in most of the patients (ATR-Seckel) (1). ATR encodes a phosphatidylinositol-3-kinase-like kinase, which has distinct, but also overlapping functions with ataxia-telangiectasia-mutated (ATM) in co-ordinating the response to DNA damage (2). ATM is activated directly by the presence of DNA double strand breaks (3), while ATR is activated by the appearance of single stranded DNA as a result of replication problems or the processing of DNA double strand break (4). Both kinases work in an overlapping manner phosphorylating many of the same substrates.

In humans, a total deficiency of ATR is not compatible with life (5); however, ATR partial impairment causes Seckel syndrome. Although at least six different mutations can cause this disease, the most typical form is caused by a splicing defect that results in a reduced amount of ATR in the cell (OMIM 210600). Seckel syndrome is a rare and severe disease with complex syndromic manifestations. Few eye examinations have been conducted in Seckel patients but retinal abnormalities have been reported (6,7). However, the morphological and molecular characterization of such phenotypes is still unknown.

A mouse model for ATR-Seckel syndrome has previously been generated with the same splicing defect as in human disease using a humanized allele (ATRs/s) (8). The mutation leads to an unstable splice site, and tissues in the affected animals retain the enough quantity of ATR to allow survival. Many of the Seckel syndrome characteristics are displayed by the mutant mice but no ophthalmological examinations were previously reported in these mice. In order to evaluate whether ATR mutation leads to retinal degeneration, the...
structural and functional properties of the retina were studied in ATR mutant mice from postnatal day 10 (P10) until adulthood (P60). Due to the extremely low birth rate of ATR+/s (8), all experiments were carried out in heterozygous ATR+/s mice.

Here, we report a severe retinal degeneration in ATR+/s heterozygous mice specifically affecting photoreceptor (PR) cells, accompanied by a decrease in electroretinographic (ERG) amplitude for both rod and cone responses during postnatal development of the retina. A rapid degeneration of rods occurred initially and was followed by cone loss, leading to a complete absence of the PR layer in the adult. Extinguished ERG responses, greatly altered levels of typical markers for the healthy retina, such as rhodopsin, opsin and recoverin, as well as severe changes in retinal fundus morphology similar to that of retinitis pigmentosa (RP) (OMIM 268000) patients were observed in the affected adult mice.

The discovery of a new mouse model of retinal degeneration resembling human RP will be useful for the study of disease mechanisms in PR degeneration. Also, a new role for ATR in the postnatal development and maintenance of PRs is shown. The localization of ATR in the cilium of normal PRs, and the presence of shortened cilia in heterozygous mutant mice, point to a ciliary defect as the mechanism leading to the retinal degeneration described here, similar to other retinal degenerative diseases associated with defective PR cilia.

RESULTS AND DISCUSSION

ATR+/s adult mouse retina

In order to evaluate the appearance of the retina in ATR+/s mice, adult animals were subjected to funduscopy (Fig. 1). Fundus images of the central and peripheral regions showed marked retinal atrophy, dark pigment accumulation, thinning of retinal blood vessels and a bright pale optic disc, a sign of optic nerve atrophy, all of which are typical characteristics of human RP.

Histological characterization of ATR+/s adult mice retina (Fig. 2A and B) displayed a complete absence of the PR layer. The two different PR cell types, rods and cones, are usually differentially affected in retinal disease. In order to distinguish both cell types, specific immunostaining for rhodopsin (rods) and LM opsin (LM cones) was carried out (Fig. 2C–F). A lack of both rods and cones was seen in the outer nuclear layer (ONL). These data were further checked by western blot analysis of retinal extracts (Fig. 2G). Blots displayed a lack of rhodopsin signal in adult retinas, whereas the cone (LM) opsin signal was reduced but not totally absent. Images of retinal sections indicate some LM opsin labelling of cells other than cones in the ONL; this may explain the presence of some LM opsin signal in the blots of adult mice even though the ONL is completely degenerated (Fig. 2E–G).

Further characterization of the retinal degeneration was carried out using a PR marker, recoverin and the retinal pigment epithelium (RPE) marker RPE65 (see Supplementary Material, Fig. S1). While no recoverin signal was left in ATR+/s adult retinas, as expected from the absence of PRs, the RPE65 signal was mislocalized, due to some degree of RPE disorganization. This may account for the dark spots seen on funduscopy; a sign of pigment accumulation in degenerate RPE cells. In human RP, the RPE is affected secondarily to PR degeneration since RPE metabolism is linked to that of PRs through phagocytosis of outer segment (OS) discs and in recycling of retinoids.

As a result of the structural degeneration found in ATR+/s mouse retina evidence for functional visual impairment was sought by ERG. Representative scotopic (rod mediated) and photopic (cone mediated) flash ERG waves (Fig. 2H–M) displayed completely flat responses for both types of PR in mutant compared with wild-type (WT) adult mice. A severe form of retinal degeneration was present in adult ATR+/s mice in which both an altered fundus appearance and an absence of ERG responses resembled the late stages of human RP.

Progression of retinal degeneration and dysfunction

In order to evaluate the time course of the retinal degeneration in these animals, an examination of the structural and functional characteristics of ATR+/s retina was conducted during postnatal ages P10–P25 (Fig. 3 and see Supplementary Material, Fig. S2). Progressive demise of PRs and disorganization of the retina were visible from P15 to P25 as shown in the histological localization of rods, marked by rhodopsin (Fig. 3A–H), and of cones, marked by LM opsin (Fig. 3I–P). The accompanying decrease of rhodopsin and opsin quantities was checked by western blot (Fig. 3Q and R).

At P10, both rods and cones are developing to form the ONL and their visual pigment markers rhodopsin and LM opsin were localized in ATR+/s as well as in the WT retina (Fig. 3A, B, I and J), indicating that low levels of ATR do not influence embryonic or even postnatal development of
At P10, the rhodopsin signal was greatly reduced in ATR+/s when compared with WT retina consistent with the marked rod loss at this stage (Fig. 3C, D, Q and R) and by P20 (Fig. 3E, F, Q and R) no rod signal was present. Cone cell death started later, as some LM opsin signal was still present in the ONL at P20 (Fig. 3M, N, Q and R). The ONL became progressively thinner and by P25 it had disappeared from the affected retina (Fig. 3H and P). The inner layers of the retina remained unaffected into adulthood, suggesting a very specific pattern for cellular degeneration affecting the PR layer and underlying RPE.

The progressive loss of the ONL is characteristic of many different mouse models of RP and Leber’s congenital amaurosis (9–11). The ATR-Seckel mouse, although initially generated for the study of Seckel syndrome, is proposed here as a useful model for the study of retinal degenerative diseases and more specifically to dissect mechanisms leading to severe RP. Visual function in these mice, from P10 to P25 was evaluated using flash ERG. Both light and dark-adapted conditions were used to monitor cone and rod function, respectively, and a severe visual impairment was noted (see Supplementary Material, Fig. S2). The amplitude of scotopic and photopic waves was diminished, compared with those of WT mice, concomitant with the PR cell loss described above. Rod function was affected from P20 onwards, as shown by a decrease in the amplitude of scotopic waveforms, while cone function is preserved until P25, when the light-adapted signal was also decreased compared with WT mice recordings. The amplitudes of ERG responses from both cones and rods diminished progressively in ATR+/s mice and were completely extinguished in adult mice (Fig. 2H–M).

It is not unusual in retinal degenerative diseases for rods to be affected first and cones later. In some rod-specific disorders, loss of rod cells leads to cone cell death secondary to changes within the retinal environment. The progression of cellular degeneration characterized in ATR+/s mice is characteristic of a rod-cone dystrophy, as is typical in patients with RP in which degeneration of rods precedes that of cones, causing an initial loss of night vision followed by a progressive loss of day vision.
Apoptotic death of PRs

One possible explanation for PR death in ATR+/s mice is that UV light can trigger DNA damage sensed by ATR. PRs are light-sensitive cells specialized for light capture and are unusually prone to light-induced DNA damage, perhaps making them more sensitive to an ATR deficit than the other retinal cell layers. However, ATR+/s mouse retinas from P10 to P20 did not show any difference in the signal intensity of Ser139-phosphorylated histone H2AX, a widely used marker of DNA damage caused by UV-light, ionizing radiation or radiomimetic agents (see Supplementary Material, Fig. S3C–S3H). In P10 and P15 ATR+/s retina, nuclei from the PR layer were intensely stained when subjected to the TUNEL assay for the detection of apoptotic programmed cell death (see Supplementary Material, Fig. S3N–S3O). It is worth noting that nuclei from the other retinal layers were not labelled in the TUNEL assay, and these layers remained unaffected. Therefore, these data argue against light stress as the factor linking ATR deficit with PR death, but in favour of a specific role that ATR plays in PR survival following the start of phototransduction.

ATR localizes to the cilia

Given the effect of ATR mutation on retinal degeneration, we investigated the subcellular localization of ATR in PR cells. We performed co-immunostaining for ATR with both gamma-tubulin and acetylated alpha-tubulin as markers of the basal body and the ciliary axoneme, respectively, in P10 mouse retinas (Fig. 4). A strong ATR signal was shown in the cilia of WT PRs (Fig. 4A) that partially co-localized with gamma-tubulin and acetylated tubulin signals (Fig. 4D and I). The shape and size of the ATR signal resembled that of the cilia. In mutated animals, ATR and acetylated tubulin signals were very faint in the ciliary region (Fig. 4E, G, H and...
J). A statistically significant reduction of the ATR signal of \( \approx 78\% \) was seen (Fig. 4K). According to the data reported here, ATR is present in the PR cilium.

**Comparison to ATM\(^{-/-}\) retina**

ATR and ATM are both members of the PI3-kinase family, being structurally and functionally related serine-threonine kinases activated in response to DNA damage and involved in cell-cycle regulation (12). These two proteins share some regulatory factors and phosphorylate many of the same substrates. It is known that an ATR-dependent phosphorylation activates ATM in response to UV or replicative stress (13). In order to ascertain whether the retinal phenotype described here is specifically due to ATR deficiency or is also present in a mouse defective for ATM, we studied the distribution of ATM and ATR in adult mouse retinas and we evaluated the functional and morphological characteristics of the ATM\(^{-/-}\) mouse retina (14). In contrast to ATR, ATM did not show any ciliary distribution in adult mice (see Supplementary Material, Fig. S4A–S4D). Neither retinal histology nor opsin or rhodopsin signals were affected in adult ATM\(^{-/-}\) (P60) mutant animals (see Supplementary Material, Fig. S4E). ERG response showed some degree of impairment (see Supplementary Material, Fig. S4F and S4G). Diminished photopic b-waves point to some degree of functional disability rather than to retinal degeneration, since the morphology of the retinal layers was the same as in WT. ATM-related retinal dysfunction could be due to vascular deficiency, which has already been described in these mice (15) and is known to induce distinct changes in ATM\(^{-/-}\) ERG recordings. Moreover, idiopathic perifoveal telangiectasia has been reported in human patients with ATM allelic variants, leading to varying degrees of visual impairment and fundus abnormality (16).

In summary, complete absence of ATM is tolerated during retinal development and function until adulthood, when it produces a mildly dysfunctional phenotype, whereas a partial deficiency in ATR leads to a severe and early degeneration of PRs, pointing to a specific and essential role for ATR during retinal postnatal development.
A defect in OS formation may explain retinal degeneration caused by ATR deficiency

ATR is thought to function as a protein kinase that is fundamentally activated by replicative DNA damage. It starts a signalling cascade leading to the arrest of mitotic cell-cycle progression. The PR layer is composed of post-mitotic cells at the time when cell degeneration commences in \( ATR^{+/s} \) mice. In the original characterization of the ATR-Seckel mice (8), embryonic replicative stress was proposed to be the main cause of the disease phenotype. In our study of \( ATR^{+/s} \) mouse retina, we have found that a complete ONL is present at P10; thereafter no cell division takes place, implying that replicative stress is unlikely to be the trigger for retinal degeneration in \( ATR^{+/s} \) mice. However, a key developmental event in the mouse retina starting around P10 is the development of the OS. The OS is the light-sensitive segment of the PR; a specialized cilary protrusion containing the photopigment discs, connected to the PR inner segment (IS) through a specialized non-motile cilium named the connecting cilium. All protein destined for the OS must be transported from the IS through this connecting cilium (17).

A number of retinal diseases are related to defects in cilia formation, maintenance or function as reviewed in (18). Some of these diseases, named ciliopathies, are syndromes that affect organs other than the eye (19). In zebrafish models of genetic defects involving intraflagellar transport proteins (17), retinal development proceeds normally until the stage when OS starts to form, thereafter triggering PR degeneration apparently resulting from an inability to generate functional OS. In humans, defects in the ciliary transport machinery or in ciliary formation are a common cause of retinal degeneration. Human retinal ciliopathies display different rates and degrees of progression depending on the affected gene and mutation, but the timing of the PR degeneration reported here parallels that of a typical ciliary retinopathy.

Not all Seckel patients have an ATR mutation. In some cases, the disease is caused by a homozygous defect in the pericentrin gene, which encodes an integral component of the pericentriolar material known to interact with the nucleation component gamma-tubulin and PCM1. In patients affected by pericentrin mutation, the phenotypic presentation of the disease is the same as seen in the ATR-Seckel patients (20). In cells from these patients, the ATR-dependent checkpoint signalling is defective, linking a structural centrosomal protein with the ATR pathway. More interestingly for our study is the finding that pericentrin is located in the basal body of the PR cilium (21).

It has previously been reported that ATR, ATM, Chk1 and other components of the ATR checkpoint pathway localize to the centrosome (22). Moreover, a direct interaction between ATR and gamma-tubulin has also been shown in the nucleus, controlling the kinetics of microtubule nucleation in response to DNA damage. No direct interaction of ATM and gamma-tubulin could be demonstrated in the same work, pointing to a specific role of ATR in gamma-tubulin regulation. The role of the centrosome in mitotic cell division as well as in cilia formation has also been studied (23), which showed that the assembly of the non-motile primary cilium in vertebrate cells requires one of the centrioles, linking ciliary assembly proteins to the regulation of cell-cycle progression, which is a major function of ATR.

MAP2 has been found to be a substrate for ATR (24), so we focused our attention on this neuronal microtubule-binding protein. High-molecular weight forms of MAP2, such as MAP2a/b, are found in mature brain, whereas the low-molecular form of MAP2, MAP2c, is found in the developing brain. MAP2c has been related to neuron formation and elongation (25) and is found in PRs as well as hippocampal neurons. Both types of cell harbour the ‘immature’ form MAP2c throughout life, which was attributed to the plasticity of both types of neurons. In PR cells, there is a continuous growth of OS, since the discs containing photosensitive pigment have to be renewed to replace the shed discs, which are phagocytized by the RPE (25). It has also been reported that MAP2c triggers neurite formation through the stabilization of microtubules and affects actin organization during neurite initiation (26). This protein has also been involved in the regulation of cellular trafficking through competitive inhibition of the molecular motor kinesin (26,27). All these data make MAP2c a good candidate for linking ATR deficit to retinal degeneration.

Immunofluorescence characterization of MAP2 and peripherin was conducted on mouse retinas. MAP2 is a marker of the IS of PRs in which the MAP2 signal corresponds to that of MAP2c (25) and peripherin is a marker of the OS. We found that the amount of both proteins was reduced in \( ATR^{+/s} \) retinas compared with WT. The reduction in both signals was coincident with the progression of PR degeneration as expected with any specific PR component. Interestingly, a noticeable reduction in MAP2c and peripherin signals was seen in \( ATR^{+/s} \) retinas at P10 (Fig. 5A–I), prior to the time when the PR cell layer starts to degenerate and PR markers, such as LM opsin and rhodopsin, start to decrease (Fig. 3). The strongest decrease in the MAP2c signal was found in the IS and nascent OS of PRs (Fig. 5E–I). We conclude that MAP2c levels in the retina are affected by the ATR mutation and we propose a new role for ATR as either a direct or indirect regulator of MAP2c levels in the IS of PRs, linking ATR to microtubule dynamics during the initiation of OS development in mouse PR. Further work will be needed to dissect the molecular mechanism of such interaction.

ATR mutation causes shortening of the PRs’ cilia

In order to investigate P10 ciliary structure in the mutated animals, we used gamma-tubulin and acetylated tubulin as markers of the basal body and the cilium, respectively, in immunofluorescence experiments. In the \( ATR^{+/s} \) PR layer, we found a pronounced decrease in both markers (Fig. 5J and M). Magnified images of individual cilia showed clearly a shorter length than normal cilia (Fig. 5P). Incorrect PR ciliary length has previously been reported to cause PR degeneration due to aberrant OS formation (28).

Concluding remarks

A new mouse model for retinal degeneration displaying a complete loss of the PR layer and a very early loss of visual function is presented. The initial loss of rods, only afterwards being followed by cones, suggests an RP-like trait. Since a
decreased level of ATR is probably the most likely cause of the phenotype, we now propose a new role for this protein. ATR is strictly necessary for the correct postnatal development of the PR layer of the mouse retina. ATR deficit is associated with diminished levels of the microtubule-interacting protein MAP2c in the IS of PRs. Localization of ATR in the cilium of PRs and the fact that cilia in affected animals are markedly shortened suggest that the retinal degeneration in ATR<sup>+/−</sup> mice is a novel ciliopathy. We propose that the mechanism of degeneration involves a defect in OS development, leading to PR cell death in a manner similar to several other retinal degenerative diseases affecting PR cilia.

**MATERIALS AND METHODS**

**Animal handling**

All experiments described in this work have been performed in compliance with the Spanish and European Union laws on animal care in experimentation. Animal manipulation and experimental methods of our laboratory have been analysed and approved by the Committee of Animal Experimentation of CABIMER, Seville, Spain. All efforts were made to minimize the number of animals used and their suffering. We used ATR<sup>+/−</sup> mutant mice (on the C57BL/6 background) described in (8). Heterozygous mutants were used as the experimental subjects, and littermates were used as controls. For genotyping, DNA was extracted from a piece of mice tail using a DNA isolation kit (Qiagen) and subjected to PCR as described in (8). We used ATM<sup>2/2</sup> mice described in (14).

**Funduscropy**

Mouse retinas were evaluated in vivo using an advanced retinal-imaging microscope (MICRON III, Phoenix Research Laboratories, Inc.). First, the pupils were dilated with one drop each of 10% phenylephrine and 1% tropicamide. Then, animals were anaesthetized by subcutaneous injection of...
ketamine hydrochloride/xylocaine solution (80/12 mg/kg body weight). In addition, eyes were locally anaesthesitized with 0.1% tetracaine and 0.4% oxybuprocaine. Immediately after the animals were sedated, a generous amount of 1% methylcellulose was placed on the mouse corneas to keep the eye moist. The correct alignment of the eye and dilatation of the pupils were checked before putting the camera lens in contact with the cornea to visualize the retina. Finally, images of the central and peripheral regions of the retina were repeatedly captured with a three separate charge-coupled device camera.

**Electroretinography**

Full-field ERGs were recorded in a ColorDome Ganzfeld (Diagnosys LCC, MA, USA). To evaluate scotopic vision, mice were dark-adapted overnight and anaesthesitized by subcutaneous injection of ketamine/xylocaine (80/12 mg/kg body weight). The dilatation of the pupil and the local anaesthesia of the cornea were performed as we did for funduscopy. The rings of gold wire electrodes were put on the surface of the corneas previously treated with the eye wetting agent (1% methylcellulose). These gold wire electrodes were used as active electrodes to detect the retinal responses from both eyes. Stainless steel needles were used as reference (forehead) and ground (tail) electrodes. The band-pass filter cut-off frequencies were 0.312–300 Hz. A single pulse white-flash (6500 K) was used for stimulation with stimulus strength divided into six steps of 0.01, 0.05, 0.2, 1, 3 and 10 candela (cd) s/m². Fifteen responses were averaged in each step with a background illumination of 30 cd/m². Stimulus strength of 3, 5, 10, 15 and 20 cd s/m² was used to obtain the photopic responses.

**Antibodies**

**Primary antibodies**

Anti-Rhodopsin (rabbit, Abcam, ab59260), anti-Opsin L/M (rabbit, Millipore AB5405), anti-Recoverin (rabbit, Millipore AB5585), anti-RPE65 (mouse, Abcam ab13826), anti-Peripherin (rabbit, Millipore AB5405), anti-Recoverin (rabbit, Millipore AB5405), anti-RPE65 (mouse, Abcam ab13826), anti-ATR (ab2905, Abcam), anti-acetylated-tubulin (mouse, Abcam), anti-ATR and anti-gamma-tubulin antibodies. Confocal images were captured in a confocal Leica TCS SP5 with an HCX PL APO Lambda blue 63 × 1.4 OIL objective at 22°C. Eight bit images were used for quantification. A region of interest (ROI) was drawn on the IS and OS regions and the ATR and gamma-tubulin signals intensity into the ROI were measured as grey values using the MBF ImageJ software (National Institutes of Health, Bethesda, MA, USA). The ciliary length was measured as an acetylated tubulin signal in micrometre in a ROI drawn containing the IS and OS regions and using the same software. For quantification, the length of each individual cilia of the ROI was measured. The same threshold was applied to each image. The values were expressed as mean ± the standard error of the mean. The differences between groups were examined for statistical significance using the t-test. A P-value of <0.05 denoted the presence of a statistically significant difference.

**TUNEL assay**

Detection of apoptosis was performed in fixed retinas in 4% PFA of P10, P15 and P20 mice using the In Situ Cell Death Detection Kit, TMR red (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Then, tissue sections were observed with a confocal Leica TCS SP5 using an excitation wavelength of 540 nm and detection in the range of 580 nm.

**Western blot**

Eyes from P10, P15, P20, P25 and P60 C57BL/6NCrl WT and ATR−/− mice were dissected after cervical dislocation. For neural retina lens, vitreous and aqueous humour were removed, and the retina was peeled from RPE. Tissues were snap frozen in liquid nitrogen and stored at −80°C until use. Proteins were extracted in cold RIPA buffer plus protease and phosphatase inhibitor cocktails. The protein content was measured by the Bradford assay and the samples stored at −80°C. Thirty micrograms of each extract were separated in a denaturing 10% SDS–PAGE gel and the proteins transferred...
to a PVDF membrane and blocked using 5% NFDM or Superblock (Thermo) for 1 h. The primary antibody was incubated at 1 mg/ml overnight at 4°C. Membrane was probed with an HRP-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were detected by chemiluminescence using ECL plus (Amersham) and registered by film exposure and automatic development.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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