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Cellular and clinical impact of haploinsufficiency for genes involved in ATR-signalling.

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Running title: ATR, haploinsufficiency, microcephaly.

Abstract.

Ataxia telangiectasia and Rad3-related (ATR), a kinase that regulates a DNA damage response pathway, is mutated in ATR-Seckel Syndrome (ATR-SS), a disorder characterised by severe microcephaly and growth delay. Impaired ATR-signalling is also observed in cell lines from additional disorders characterised by microcephaly and growth delay, including non-ATR SS, Nijmegen Breakage Syndrome and MCPH1-dependent Primary Microcephaly. Here, we examined ATR-pathway function in cell lines from three haploinsufficient contiguous gene deletion disorders, a subset of Blepharophimosis-Ptosis-Epicanthus Inversus Syndrome, Miller-Dieker Lissencephaly Syndrome and Williams-Beuren Syndrome, in which the deleted region encompasses *ATR, RPA1* or *RFC2* respectively. These three genes function in ATR-signalling. Cell lines from these disorders displayed an impaired ATR-dependent DNA damage response. Thus, we describe ATR-signalling as a pathway unusually sensitive to haploinsufficiency and identify three further human disorders displaying a defective ATR-dependent DNA damage response. The striking correlation of ATR-pathway dysfunction with the presence of microcephaly and growth delay strongly suggests a causal relationship.

Introduction.

Ataxia telangiectasia and Rad3-related (ATR) protein is a central component of a DNA damage response signalling pathway [1,2]. *ATR* (MIM601215) is mutated in two families displaying Seckel Syndrome (SS) (MIM 210600), a disorder characterised by severe microcephaly, proportionate dwarfism and dysmorphic facial features [3-5]. SS is clinically and genetically heterogeneous [6]. Significantly, cell lines derived from additional SS patients, although not harbouring mutations in *ATR*, display ATR-signalling defects [6]. Thus, SS can be attributed to defects in ATR-signalling with a sub-set of patients, designated ATR-SS, having mutations in *ATR* itself. Additionally, three other disorders characterised by microcephaly and growth delay, Nijmegen Breakage Syndrome (MIM251260), Fanconi anaemia (MIM227650) and MCPH1-defective Primary Microcephaly (MIM251200), display impaired ATR-signalling responses [7,8]. Together, these findings suggest that impaired ATR signalling can impact upon development conferring microcephaly and growth delay.

ATR is a phosphoinositol 3-kinase-like kinase (PIKK) that is activated by single stranded (ss) regions of DNA generated following replication fork stalling or during the repair of bulky lesions [9]. ATR interacts with ATRIP (ATR-interacting protein) and is recruited to ssDNA regions, in part by ATRIP's ability to bind to Replication Protein A (RPA), a complex of three subunits, Rpa1-3 [9-11]. The Rad17/Rfc2-5 complex, together with a complex involving Rad9, Rad1 and Hus1, also functions to enhance ATR-signalling either by impacting upon ATR recruitment or activation [12]. Thus, these additional proteins are required for the ATR-signalling response and therefore represent potential candidate genetic defects for SS [6]. Hence, defects in these genes may confer 'Seckel-like' clinical features.

High resolution genetic mapping studies have led to the characterisation of contiguous gene deletion disorders caused by heterozygous microdeletions resulting in haploinsufficiency for a single or, more usually, several genes [13,14]. It is likely that the clinical manifestations of these disorders arise from the combined impact of haploinsufficiency for multiple genes [15]. It is also possible that there are critical genes or pathways sensitive to haploinsufficiency either alone or when combined with haploinsufficiency for other genes. Whilst investigating disorders exhibiting microcephaly and growth delay, we were struck by the observation that the microdeletion in three such disorders involved haploinsufficiency for genes involved in ATR-pathway function.

Belpharophimosis-Ptosis-Epicanthus Inversus Syndrome (BPES, MIM110100), which is characterised by a small eye sockets (Blepharophimosis), drooping eyelids (ptosis) and upward folding inner eyelids (epicanthus inversus), is an autosomal dominant disorder caused by mutation of the putative forkhead transcription factor *FOXL2* (MIM605597) [16,17]. Seventeen cases of BPES with heterozygous interstitial deletions of various sizes on chromosome 3q leading to loss of *FOXL2* have been documented (reviewed in de Rue at al [18]). Of these, thirteen patients were also reported to exhibit microcephaly and growth retardation, clinical features not normally associated with BPES. Recently, the microdeletion in one such patient was carefully mapped and shown to encompass *ATR*, which localises to the same region[18]. It was, therefore, proposed that the non-BPES features observed in such patients might be due to haploinsufficiency for *ATR* [18].

Hemizygous deletions on chromosome 17p also confer microcephaly and growth delay [19]. *PAHFAH1B1/Lis1* (MIM601545) encodes a protein, Lissencephaly 1 (Lis1) that functions in neuronal migration [20]. Mutations in or heterozygous

deletions of *PAHFAH1B1/Lis1* alone causes Isolated Lissencephly Sequence (ILS), a disorder typified by reduced cerebellar invagination resulting in a "smooth brain" (lissencephaly) [21]. Larger deletions identified in some ILS patients confer a more severe grade of lissencephaly associated with craniofacial abnormalities (ILS+). Even larger deletions extending from the *PAHFAH1B1/Lis1* gene to the telomere are observed in Miller-Dieker Lissencephaly Syndrome (MDLS, MIM247200), a disorder characterised by the most severe grade of lissencephaly, craniofacial abnormalities, microcephaly and growth retardation. *RPA1* (MIM179835), the largest subunit of RPA, is heterozygously deleted in MDLS and ILS+ but not in ILS [19].

Finally, Williams-Beuren Syndrome (WBS, MIM194050) is caused by hemizygous deletion of chromosome 7q11.23 resulting in haploinsufficiency of multiple genes [22]. WBS is characterised by facial dysmorphia, microcephaly, growth retardation and supravalvular aortic stenosis (SVAS). Hemizygous deletion or mutations in *ELN* (MIM130160) alone, the gene encoding elastin, a structural component of arteries, causes SVAS (MIM185500) [23]. WBS patients, in contrast, have larger deletions encompassing Replication factor C2 (*RFC2;* MIM600404), a subunit of Replication factor C (RF-C)[24]. RF-C loads proliferating cell nuclear antigen (PCNA) onto chromatin during DNA replication and four of its five subunits, Rfc2-5, form a complex with Rad17 that functions in ATR-signalling [11,25-27].

The association of severe microcephaly and growth retardation with heterozygous loss of genes encoding proteins involved in the ATR-signalling pathway, prompted us to examine whether haploinsufficiency for these genes might impact upon the ATR-signalling response. We, therefore, examined cell lines obtained from these contiguous gene disorders for their ability to effect the ATR-dependent DNA damage response. We employed sensitive assays capable of detecting

defective ATR-pathway function that we had previously established to examine SS cell lines. Strikingly, we observed defects in ATR-signalling in all three disorders demonstrating that ATR-signalling is sensitive to haploinsufficiency.

Materials and methods

Cell lines.

Lymphoblastoid cell lines (LBLs) were cultured in RPMI 1640 with 15% foetal calf serum. GM02188 (wild type, WT) and DK0064 (ATR-SS) have been described previously [6]. VD9396 (BPES-ATR+/-) LBLs were obtained from J.M. van Hagen [18]. ILS, ILS+ and MDLS LBLs (DR00-063a1 (Con-MR), LP99-017 (ILS A), LP94-013 (ILS B), LP90-017 (ILS+ A), LP99-086 (ILS+ B), LP91-026 (ILS+ C), L95-059 (MDLS-A), LP92-005 (MDLS-B), LP90-006 (MDLS-C), LP88-002 (MDLS-D) have been described previously and were obtained from W.B. Dobyns [19]. WBS and respective parental LBLs (GM14183 (WT-I), GM14182 (WBS-I), GM14295 (WT-II), GM14297 (WBS-II) were obtained from Corriel Cell Repository (NJ, USA).

Antibodies.

Antibodies were obtained as follows, α–ATR (N19), α–Lis1 (H-300) and α–β-tubulin (H235), Autogen Bioclear (Whiltshire, UK); α–RPA1 (Ab-1), α-H2AX (DR-1016) and α–NBS1 (Ab-1), Merck (Nottingham, UK); α–γH2AX, α-pS10 Histone H3 and α–ATRIP, Upstate Technology (Hampshire, UK) and α-pSer317-Chk1, New England Biolabs (Herts, UK).

Western blotting and Chromatin extraction.

Western blotting was carried out as described previously [8]. For γ-H2AX analysis, a chromatin extraction step was included. Briefly, $1x10^7$ cells were washed once in PBS and re-suspended in 100 µl hypotonic buffer (10mM HEPES pH 7.5, 5mM KCl, 1.5mM $MgCl_2$, 1mM DTT, 10mM NaF, 1mM Na_2VO_3, 10mM β-glycerolphosphate, 0.5% IPEGAL and Protease Inhibitor Cocktail from Sigma, Poole, UK). Lysates were incubated on ice for 15 mins, pelleted and washed twice (200 µl each) in hypotonic buffer. The pellet was treated with hypertonic buffer (hypotonic buffer with 0.5M

NaCl) and incubated on ice for 15mins. Following washing in hypertonic buffer, the chromatin pellets were re-suspended in 100 µl of SDS-PAGE loading buffer (with 5% SDS, 10% β-mercaptoethanol) and sonicated. 10 µl of the chromatin fraction was separated on 17% SDS-PAGE.

G2/M checkpoint arrest.

Cells were irradiated with 5 J/m^2 UV-C and immediately seeded into complete medium with 1.5 µM nocodazole and incubated for 24 hrs before being cytospun onto poly-D-lysine coated slides and processed for immunofluorescence with α-pS10-Histone H3 and counterstaining with DAPI.

Nuclear fragmentation.

Cells were treated with 5 mM HU in the presence of 1.5 µM nocodazole for 24 hrs and processed as described previously [6].

Transfection.

3 x 10^5 cells/ml in 3 mls were transiently transfected with 2 µg of pcDNA3-*ATR*, pcDNA3.1-*RPA1* or pcDNA3.1-*RFC2* using Genejuice (Novagen), according to the manufacturers instructions, and incubated for 24 hrs prior to nuclear fragmentation processing. For complementation of the G2/M checkpoint, cells were incubated for 24 hrs and re-transfected for a further 24 hrs before processing.

RNAi transfection.

The control wild type LBL GM02188 was used for siRNA experiments. Cells were transfected once with 10 nM of respective siRNA duplex using SiPort™ NeoFX™ transfection reagent according to the manufacturers instructions (Ambion, Huntington, UK). Oligonucleotides (sense) used were ATR 5'-AAC CUC CGU GAU GUU GCU UGA -3', RPA1 5'-AAA CCA UCC ACG AAG CUU AUA GGC C -3',

Lis1 5’-UUG AUU UGG CCG UAC CAU ACG UAC C-3’ and a control oligo directed to GFP 5’-AAC ACU UGU CAC UAC UUU CTC.

Results.

ATR-signalling is impaired in a BPES cell line haploinsufficient for ATR.

A previous study reported heterozygosity for *ATR* due to an interstitial deletion on 3q in a boy with BPES who displayed non-BPES clinical features that included mental retardation, microcephaly and growth delay [18]. A representation of the deletion is shown in Fig 1A. We obtained a lymphoblastoid cell line (LBL) derived from this patient and by immunoblotting observed approximately two fold reduced ATR protein levels compared to a control LBL (ATR-BPES+/- Fig 1B). The level of ATRIP was also reduced approximately two fold, consistent with reports that ATR and ATRIP are co-regulated [10]. Nbs1 served as a loading control and was expressed at similar levels in both lines (Fig 1B). An early step in the DNA damage response regulated by ATR is phosphorylation of the histone H2A variant, H2AX on serine 139 (termed γH2AX), which can be detected by immunoblotting of chromatin bound proteins using α-γH2AX antibodies [28]. Following exposure to hydroxyurea (HU), an agent that causes replication fork stalling, a strong γH2AX signal was observed in control LBLs indicating activation of ATR-dependent damage response signalling. However, in marked contrast, detectable γH2AX was not observed in the ATR-SS LBLs, nor in LBLs derived from the BPES-ATR+/- patient (Fig 1C). Chk1 represents an important phosphorylation target of ATR that is required for the stability of arrested replication forks and cell cycle arrest [29-33]. HU-induced phosphorylation of Chk1 on serine 317, a known ATR target site, was also significantly diminished in LBLs from the BPES-ATR+/- patient, similar to ATR-SS LBLs (Fig 1D)[6]. An important end point of ATR activation regulated by Chk1 is onset of G2/M checkpoint arrest, which serves to prevent cells harbouring DNA damage from entering mitosis [6]. To monitor G2/M checkpoint arrest, the percentage of mitotic cells was examined in untreated cells or

24 h post irradiation with UV (5 Jm^{-2}). Control LBLs showed a marked reduction in mitotic cells due to arrest at the G2/M checkpoint whilst ATR-SS and BPES-ATR+/- cells showed a similar mitotic index to that observed in the absence of UV treatment (Fig 1E). A further hallmark of impaired ATR-signalling is the presence of cells with nuclear fragmentation (NF) following treatment with HU [6]. ATR-SS and BPES-ATR+/- LBLs showed markedly elevated levels of cells displaying NF following HU treatment in contrast to control LBLs (Fig 1F). To verify that the failure to effect G2/M checkpoint arrest and the damage induced NF phenotype of BPES-ATR+/- cells were directly attributable to an impaired ATR response, the cells were transfected with *ATR* cDNA and re-examined for these phenotypes. Significant UV-induced G2/M arrest (Fig 1G) and reduced NF (Fig 1H) was observed following transfection with *ATR* cDNA.

Collectively, these results provide strong evidence that haploinsufficiency for ATR in the BPES-ATR+/- cell line confers an impaired response to DNA damage that is similar in magnitude to that observed in an ATR-SS cell line.

Cells haploinsufficient for RPA1 show deficient ATR-dependent DNA damage response signalling.

A representation of the deletion on chromosome 17p observed in MDLS is shown in Fig 2A. The size of the heterozygous deletions on chromosome 17p in a panel of LBLs derived from patients with ILS, ILS+, MDLS and a control patient (Con-MR) are shown in Fig 2B. ILS *A* and ILS *B* were derived from patients with mild isolated lissencephaly sequence (ILS) due to microdeletions involving *Lis1*only. ILS+ *A*, ILS+ *B* and ILS+ *C* were obtained from patients with more severe ILS and craniofacial abnormalities. MDLS-*A*, MDLS-*B*, MDLS-*C* and MDLS-*D*, which have deletions

extending from *Lis1* to the telomere, were derived from MDLS patients with the severest grade of lissencephaly together with microcephaly and growth retardation. Con-MR, which has a heterozygous telomeric deletion that does not involve either *RPA1* or *Lis1*, was derived from a mildly mentally retarded patient (Fig 2B). This patient does not exhibit MDLS, lissencephaly, microcephaly or growth delay. The location of *RPA1* is shown in Fig 2B, demonstrating that this gene is deleted in some but not all the LBLs in the panel. To examine whether haploinsufficiency for *RPA1* correlated with impaired ATR-dependent damage response signalling, we examined the response to DNA damage in this panel of LBLs. Firstly, we examined Rpa1 expression in whole cell extracts derived from Con-MR, ILS *A*, ILS *B*, compared to MDLS-*A*, -*B* and -*C*. All three MDLS LBLs displayed reduced levels of Rpa1 compared to the Con-MR, ILS *A* and ILS *B* LBLs consistent with the deletion mapping (Fig 2B). Next we examined HU-induced γH2AX formation in representative LBLs either haploinsufficient for *RPA1* or with both copies of the gene. Impaired HU-induced γH2AX was observed in the ATR-SS, ILS+ *A* and MDLS *A* LBLs, the latter two of which exhibit haploinsufficiency for *RPA1* (Fig 2B & Fig 2D). Normal HU-induced γH2AX was observed in wild type control LBLs (WT) and in those cell lines where the deletion that did not encompass *RPA1* (Con-MR, ILS *A*, ILS *B*) (Fig 2B & Fig 2D). Similarly, MDLS *A* LBLs also exhibited impaired HU-induced Chk1-pSer317 formation, unlike Con-MR and ILS *A* LBLs (Fig 2E). We also examined the ability to activate UV-induced G2/M checkpoint arrest and observed impaired arrest specifically in the seven LBLs with haploinsufficiency for *RPA1* (Fig 3A & Fig 2B). Furthermore, we examined HU-induced NF and observed elevated levels specifically in those LBLs from patient's haploinsufficient for *RPA1* (Fig 3B & Fig 2B).

To verify that the G2/M checkpoint and NF phenotypes were a consequence of haploinsufficiency for *RPA1*, these damage response phenotypes were examined following transfection of two MDLS LBLs (MDLS *A* and MDLS *C*) with *RPA1* cDNA. Expression of *RPA1* cDNA resulted in recovery of G2/M checkpoint arrest and a diminished number of cells displaying HU-induced NF in both LBLs (Fig 3C & D).

Mildly decreased expression of RPA1 and ATR but not Lis1 using siRNA affects ATR-pathway function following DNA damage.

As an alternative way to determine whether the ATR-dependent DNA damage response might be sensitive to small perturbations in ATR or Rpa1 expression, we utilised a single round of transfection with a low concentration (10nM) of siRNA oligonucleotides to mildly reduce the expression of Lis1, Rpa1 and ATR in the control LBLs. We obtained an approximately two-fold reduction in Lis1, Rpa1 and ATR expression in control LBLs (Fig 4A). This reduction in Rpa1 and ATR protein levels conferred a failure to activate G2/M checkpoint arrest following UV-irradiation (Fig 4B). In contrast, the clearly observable reduction in Lis1 expression following siRNA did not impact upon UV-induced G2/M checkpoint arrest (Fig 4B). Similarly, mild siRNA mediated reduction in the expression of ATR and Rpa1 caused elevated levels of NF after HU treatment, which was not observed following siRNA using oligonucleotides directed against Lis1 or GFP (Fig 4C).

These results provide strong evidence suggesting that even a mild decrease in protein levels of either ATR or Rpa1 impacts upon the ability of cells to mount a normal ATR-signalling response following exposure to DNA damage.

Impaired ATR-dependent DNA damage response signalling in Williams-Beuren Syndrome: correlation with haploinsufficiency for RFC2.

The heterozygous interstitial deletion on chromosome 7q11.23 seen in WBS is shown in Fig 5A. The deletion encompasses multiple genes including *RFC2*, a subunit of the Rad17/Rfc2-5 complex that plays a role in the ATR-dependent pathway (Fig 5B). Fig 5B also shows the microdeletion observed in a typical SVAS patient that does not encompass *RFC2* but includes *ELN*. Whilst a hypomorphic mutation in *Saccharomyces cereviaiae RFC2* (*rfc2-1*) has been shown to impair normal cell cycle checkpoint activation, a direct demonstration of a similar response in humans cells has not been described [34]. To examine whether haploinsufficiency for *RFC2* might cause impaired ATR-dependent damage response, we examined two WBS LBLs (WBS-I & WBS-II) and LBLs derived from one respective clinically normal parent (WT-I & WT-II). Significantly, defective HU-induced Chk1-pSer317 formation was also seen in WBS LBLs (WBS-I and WBS-II), compared to their respective clinically normal parental LBLs (WT-I and WT-II) (Fig 5C). Correspondingly, both WBS LBLs failed to induce any significant G2/M checkpoint arrest following exposure to UV in contrast to the efficient checkpoint arrest observed in both parent lines (Fig 5D). Additionally, both WBS LBLs but neither parental line showed induction of NF after exposure to HU (Fig 5E). Elevated levels of G2/M checkpoint arrest (Fig 5F) and reduced HU-induced NF (Fig 5G) were observed in both WBS LBLs following transfection with *RFC2* cDNA. Together, these data provide strong evidence that haploinsufficiency for *RFC*2 also confers an impaired ATR-dependent signalling response.

Discussion

Here, we examined cell lines derived from patients with BPES-ATR+/-, MDLS and WBS, three distinct contiguous gene deletion disorders exhibiting microcephaly and growth delay. In all cases, the heterozygous genomic deletions encompass a gene that is critical for the ATR-signalling response, namely *ATR* itself (BPES-ATR+/-), *RPA1* (MDLS) or *RFC2* (WBS). Patient-derived cell lines haploinsufficient for any of these genes displayed an impaired response to DNA damage using assays that have been selected to identify defective ATR-signalling. Indeed, the defect was similar to that displayed by an SS cell line with a homozgyous, hypomorphic mutation in *ATR* (ATR-SS) [3,6]. Our findings, therefore, provide strong evidence that haploinsufficiency for ATR as well as additional components of the ATR-dependent signalling pathway, confers an impaired ability to respond to DNA damage or replication fork stalling. Our assays were established to identify ATR-signalling defects in SS and employ relatively modest DNA damaging treatments to allow the detection of potentially hypomorphic mutations [3,6]. Indeed, we have observed that following more dramatic treatments (eg. high UV doses), the defect in ATR-SS cells is overridden, presumably due to the induction of a sufficient damage response signal by the residual protein [3]. This is distinct to other DNA damage response assays where high doses are often utilised to overload the pathway and expose a repair defect [35]. Although our assays are optimised to detect a subtle deficiency and may not completely reflect the role of ATR during development, they represent modest treatments that may not be entirely distinct to those occurring during cellular growth and development. Thus, the impact of haploinsufficiency appears to represent a unique phenotype of ATR-signalling in contrast to other DNA damage response pathways.

Impaired ATR-signalling is associated with microcephaly and growth delay in humans [3,7,8,36]. The correlation of microcephaly, growth delay and impaired ATR-pathway dysfunction in the haploinsufficiency disorders presented here provides further evidence suggestive of a causal relationship. Since ATR-SS is a recessive disorder, *ATR*-haploinsufficiency alone is unlikely to confer a clinical phenotype. Rather, the clinical features observed in these haploinsufficiency disorders are probably a consequence of combined haploinsufficiency for ATR-signalling genes and additional genes that may impact upon neuronal development and cell proliferation. Indeed combined heterozygosity of *Lis1* and *14-3-3ε* has been shown to influence the severe clinical features in MDLS [37]. Human brain size has increased dramatically during evolution requiring enormous and rapid proliferation from a small number of precursor stem cells [38]. Moreover, developing neurons incur high levels of oxidative DNA damage placing a significant load on the damage response pathways. Thus, the developing brain may have a high requirement for the ATR-signalling pathway necessitating a diploid content of component proteins, particularly if further stress is imposed by haploinsufficiency of other genes. Mice, as models for haploinsufficiency disorders have been used to investigate neuronal migration but may be limited in their application concerning microcephaly due to the relatively smaller size of murine brains compared to humans [39,40].

Finally, it should be noted that defective ATR-pathway function has not previously been described in any of the contiguous gene deletion disorders investigated here (BPES-ATR+/-, MDLS and WBS). Since increased life expectancy due to improved medical supervision is now a feature of conditions such as MDLS, a defective DNA damage response in this context may be relevant to potential tumour development [41]. Furthermore, a defective DNA damage response can adversely affect

treatment of malignancy by standard chemotherapeutic or bone marrow transplantation regimens [42,43]. It is unclear whether any of the conditions investigated here are tumour predisposition conditions, although isolated reports of malignancy in MDLS and particularly WBS patients exist [41,44-46]. Interestingly, hypomorphic mutations in RFC subunits are associated with genetic instability in yeast and a heterozygous missence mutation in *Rpa1* results in increased levels of lymphoid malignancy in mice [34,47,48]. Defective ATR function has been described in various cancer-types [49-51]. Furthermore, it has been suggested that ATR may act as a tumour suppressor, when haploinsufficient, under certain circumstances [52].

A recent study reported that copy number variation of DNA sequences is a common genomic trait [53]. Understanding the impact of haploinsufficiency is likely to be important to assess inter-individual genetic variation as well as the basis underlying haploinsufficiency disorders. In conclusion, we identify ATR-signalling as a response sensitive to haploinsufficiency at the cellular level with a provocative clinical link to microcephaly and growth delay. This provides novel insight into the impact of ATR-pathway haploinsufficiency and further strengthens the link between defective ATR-pathway function and the development of microcephaly and growth retardation in humans.

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Figure legends

Figure 1. BPES-ATR+/- cells display an impaired ATR-dependent damage response.

a). Chromosome 3 karyotype of the BPES-ATR+/- patient showing the heterozygous deletion, del(3)(q23,q25). ATR localises to 3q22-q24.

b). Whole cell extract (WCE) (100µg) from wild type (WT) and BPES-ATR+/- LBLs was analysed by immunoblotting using α–ATR, ATRIP and NBS1 antibodies. Reduced expression of ATR and ATRIP is seen in BPES-ATR+/- cells specifically. Nbs1 served as a loading control and was expressed at normal levels.

c). Wild type (WT), ATR-Seckel syndrome (ATR-SS) and BPES-ATR+/- LBLs were exposed to 100 or 500 µM hydroxyurea (HU) for 1 hr prior to chromatin fractionation. ATR-SS and BPES-ATR+/- cells display reduced γ-H2AX compared to WT cells. Blots were re-probed using α-H2AX to confirm loading.

d). Wild type (WT), ATR-Seckel syndrome (ATR-SS) and BPES-ATR+/- LBLs were exposed to 500 µM hydroxyurea (HU) for 1 hr prior to extraction. ATR-SS and BPES-ATR+/- cells display reduced Chk1-pSer317 formation compared to WT cells. Blots were re-probed using α-Chk1 to confirm loading.

e). ATR-SS and BPES-ATR+/- LBLs show defective UV-induced (5 J/m^2) G2/M checkpoint arrest 24 hrs post irradiation. Arrest in WT LBLs is seen as a decrease in the mitotic index (MI) following UV-irradiation.

f). ATR-SS and BPES-ATR+/- LBLs, unlike WT cells, show increased Nuclear Fragmentation (NF) following 24 hrs treatment with HU (5mM).

g). UV-induced G2/M defect in BPES-ATR+/- LBLs is complemented following transfection with *ATR* cDNA. BPES-ATR+/- cells, either untransfected (UNT) or

transfected (*ATR*) with pc-DNA3-*ATR*, were UV-irradiated (5 J/m^2) and the MI determined after 24 hrs.

h). HU-induced NF in BPES-ATR+/- is complemented following transfection with *ATR* cDNA. BPES-ATR+/- LBLs either untransfected (UNT) or transfected (*ATR*) with pc-DNA3-*ATR* were untreated (control: Con) or treated with HU (5 mM) 24 hrs post-transfection. NF was analysed 24 hrs post-treatment with HU.

Figure 2. MDLS cells display an impaired ATR-dependent DNA damage response.

a). Chromosome 17 karyotype of Miller Dieker Lissencephaly Syndrome highlighting the heterozygous deletion at 17p13.3.

b). Deletion mapping in the panel of LBLs from ILS, ILS+ and MDLS patients. The dashed line indicates the deleted region all of which are heterozygous. Con-MR is a control (Con) LBL from a mildly mentally retarded (MR) patient that does not exhibit lissencephaly, microcephaly, growth retardation or Miller-Dieker Lissencephaly syndrome but has a hemizygous telomeric deletion that does not involve either *RPA1* or *PAFAH1B1/Lis1*. ILS *A* and ILS *B* are patients with low grade ILS due to microdeletions involving *PAFAH1B1/Lis1*only. ILS+ *A*, + *B* and + *C* denote patients with larger deletions, a more severe ILS and additional craniofacial abnormalities, whilst MDLS-*A*, -*B*, -*C* and -*D*, are patients with the largest deletions who exhibit the most severe grade of lissencephaly along with microcephaly and growth retardation. The position of *PAFAH1B1/Lis1* and *RPA1* is highlighted.

c). Western blot analysis of Rpa1 expression from whole cell extracts (WCEs) from Con-MR, ILS *A,* ILS *B* and MDLS-*A*, MDLS-*B* and MDLS-*C* showing reduced expression of Rpa1 specifically in the three MDLS cell lines.

d). Defective HU-induced γ-H2AX formation segregates with *RPA1* haploinsufficiency. Cells were treated as in Fig 1C. Defective γ-H2AX formation is seen in ATR-SS, ILS+ *A* and MDLS-*A* cells compared to the normal response in wild type (WT), Con-MR and ILS *A* and ILS *B* cells.

e). Impaired HU-induced Chk1-pSer317 is seen in MDLS *A* LBLs compared to those of Con-MR and ILS *A*. Cells were treated with 500 μM hydroxyurea (HU) for 1 hr prior to extraction and re-probed using α-Chk1 to confirm loading.

Figure 3. Haploinsufficiency of *RPA1* specifically segregates with a defective ATR-dependent DNA damage response.

a). Defective ATR-dependent G2/M checkpoint arrest segregates with *RPA1* haploinsufficiency. Con-MR, ILS *A* and ILS *B* cells show a reduction in mitotic index (MI; % mitosis) at 24 hrs following UV-irradiation (5 J/m^2) indicating G2/M checkpoint arrest. ILS+ *A*, ILS+ *B*, ILS+ *C*, MDLS-*A*, -*B*, -*C* and -*D* cell lines failed to show a decrease in MI post UV treatment.

b). Increased HU-induced NF segregates with *RPA1* haploinsufficiency. No increase in HU-induced NF is seen in Con-MR, ILS *A* or ILS *B* cells. In contrast, ILS+ *A*, ILS+ *B*, ILS+ *C* and MDLS-*A*, -*B*, -*C* and –*D* cells show elevated HU-induced NF.

c). The ATR-dependent UV-induced G2/M defect in MDLS cells is complemented following transfection with *RPA1* cDNA. MDLS-*A* and MDLS-*C* cells, either untransfected (UNT), or transfected (*ATR*) with pc-DNA3-*ATR* were not irradiated (control; Con) or UV-irradiated (UV; 5 J/m^2) and the MI determined after 24 hrs. *RPA1* cDNA specifically corrected the G2/M checkpoint defect of these cells as seen by the reduced UV-induced MI following transfection (*RPA1* UV) compared to untransfected irradiated cells (UNT UV).

d). The increased HU-induced NF seen in MDLS is complemented following transfection with *RPA1* cDNA. MDLS-*A* and -*C*, either untransfected (UNT), or transfected (*RPA1*) with pc-DNA3-*RPA1* were untreated (control; Con) or treated with HU (HU; 5mM) 24 hrs post-transfection. A reduction in HU-induced NF is specifically seen following transfection of MDLS LBLs with *RPA1* cDNA (*RPA1* HU) compared to the untransfected (UNT HU) cells.

Figure 4. Inefficient siRNA of ATR or Rpa1 impairs the ATR-dependent DNA damage response.

a). Wild type (WT) LBLs were transfected once with a low concentration (10nM) of siRNA oligos for *GFP*, *Lis1*, *RPA1*, or *ATR* and analysed for expression of Lis1, Rpa1 and ATR by western blotting, using β-tubulin as a loading control, 24 hrs post-transfection.

b). WT LBLs transfected with the indicated siRNA oligos were analysed for UV-induced G2/M checkpoint arrest by monitoring MI (UNT; untransfected). Tranfection with siRNA oligos against *ATR* or *RPA1* impaired G2/M arrest after UV. In contrast, following transfection with oligos against *GFP* and *Lis1*, an intact G2/M arrest was observed.

c). WT LBLs transfected with the indicated siRNA oligos were analysed for HU-induced NF (UNT; untransfected). Transfection with siRNA oligos against *ATR* and *RPA1* caused HU-induced NF in contrast to the lack of impact of siRNA oligos against *GFP* and *Lis1*.

Figure 5. WBS cells show an impaired ATR-dependent DNA damage response.

a). Chromosome 7 karyotype from Williams-Beuren syndrome (WBS) showing the location of the submicroscopic heterozygous interstitial deletion on chromosome 7q11.23.

b). Deletion mapping of a WBS and Supravalvular aortic stenosis (SVAS) patients showing the position of Elastin (*ELN*) and Replication factor C 2 (*RFC2*). The dashed line indicates the size of the heterozygous deletion.

c). Impaired HU-induced Chk1-pSer317 is seen in WBS LBLs (WBS-I and WBS-II) compared to those of the respective clinically normal parent (WT-I and WT-II). Cells were treated with 500 μM hydroxyurea (HU) for 1 hr prior to extraction and re-probed using α-Chk1 to confirm loading.

d). WBS LBLs exhibit impaired UV-induced G2/M checkpoint arrest. The MI was determined 24 hrs post UV-irradiation (5 J/m^2). WBS-I and WBS-II LBLs show defective UV-induced G2/M arrest unlike wild type LBLs from their respective parents (WT-I and WT-II).

e). Increased HU-induced NF is seen in WBS cell lines. LBLs were treated with HU (5 mM) and examined for NF 24 hrs following treatment. Both WBS-I and WBS-II LBLs show increased HU-induced NF unlike wild type LBLs from their respective parents (WT-I and WT-II).

f). UV-induced G2/M defect in WBS LBLs is complemented following transfection with *RFC2* cDNA. WBS-I and WBS-II cells, either untransfected (UNT), or transfected (*RFC2*) with pc-DNA3-*RFC2* were UV-irradiated (UV; 5 J/m^2) and the MI determined after 24 hrs. A reduction in MI following UV-irradiation is observed following transfection of WBS LBLs with *RFC2* cDNA transfection (*RFC2* UV) compared to the untransfected irradiated cells (UNT UV).

g). The increased HU-induced NF seen in WBS LBLs is complemented following transfection with *RFC2* cDNA. WBS-I and WBS-II, either untransfected (UNT), or transfected (*RFC2*) with pc-DNA3-*RFC2* were treated with HU (5 mM) 24 hrs post-transfection. A reduction in HU-induced NF is seen following transfection of WBS LBLs with *RFC2* cDNA (*RFC2* HU) compared to the untransfected HU-treated (UNT HU) cells.

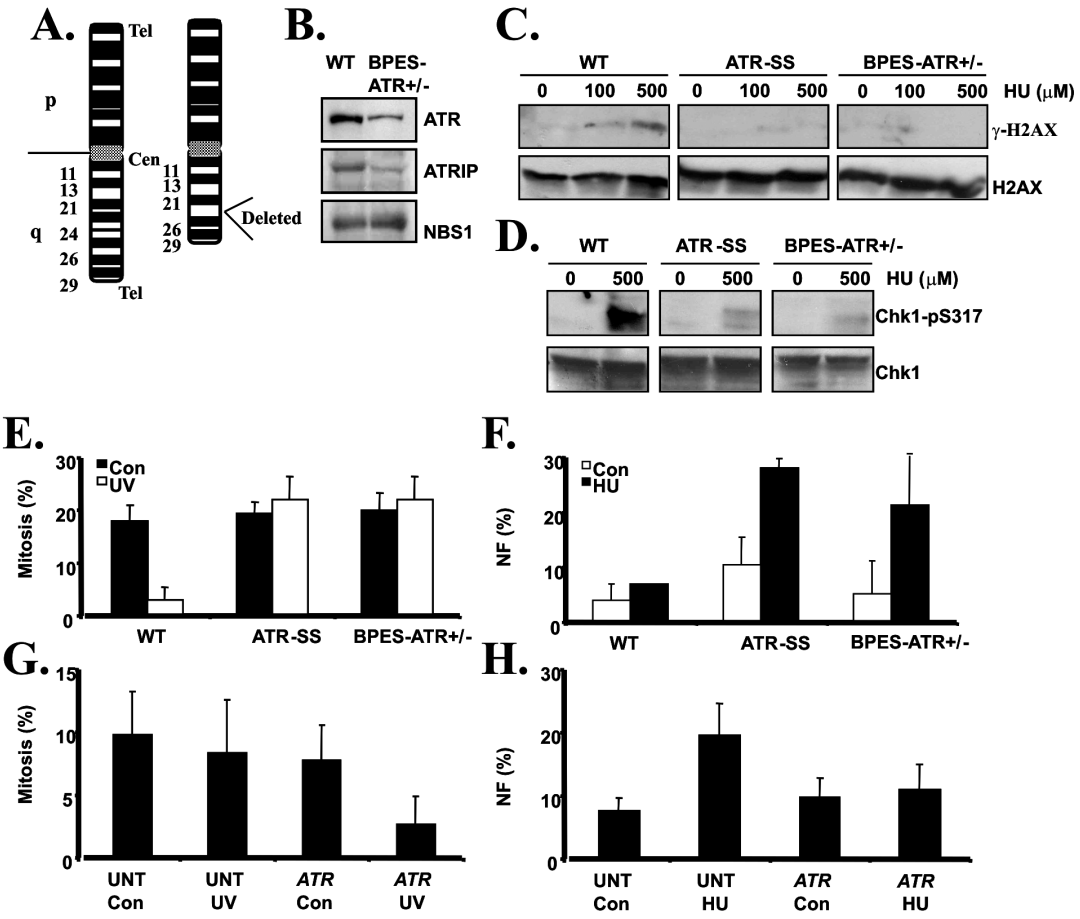
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Figure 1.

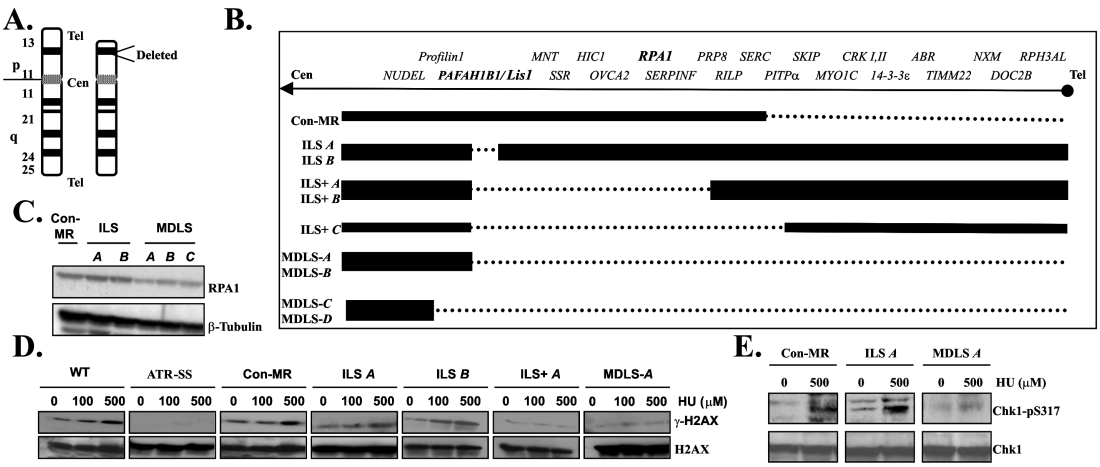
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Figure 2.

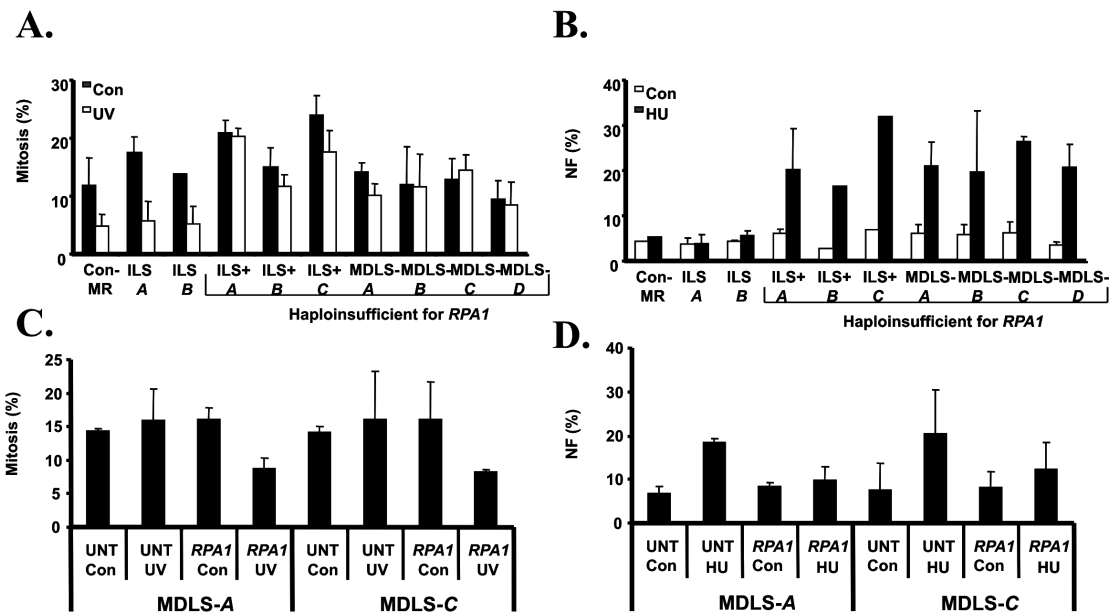
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Figure 3.

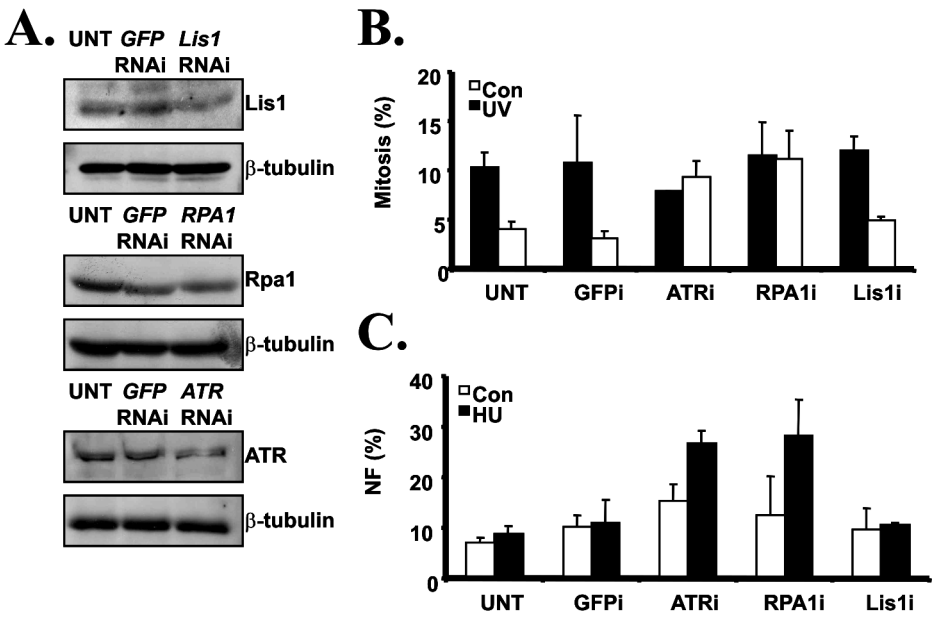
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Figure 4.

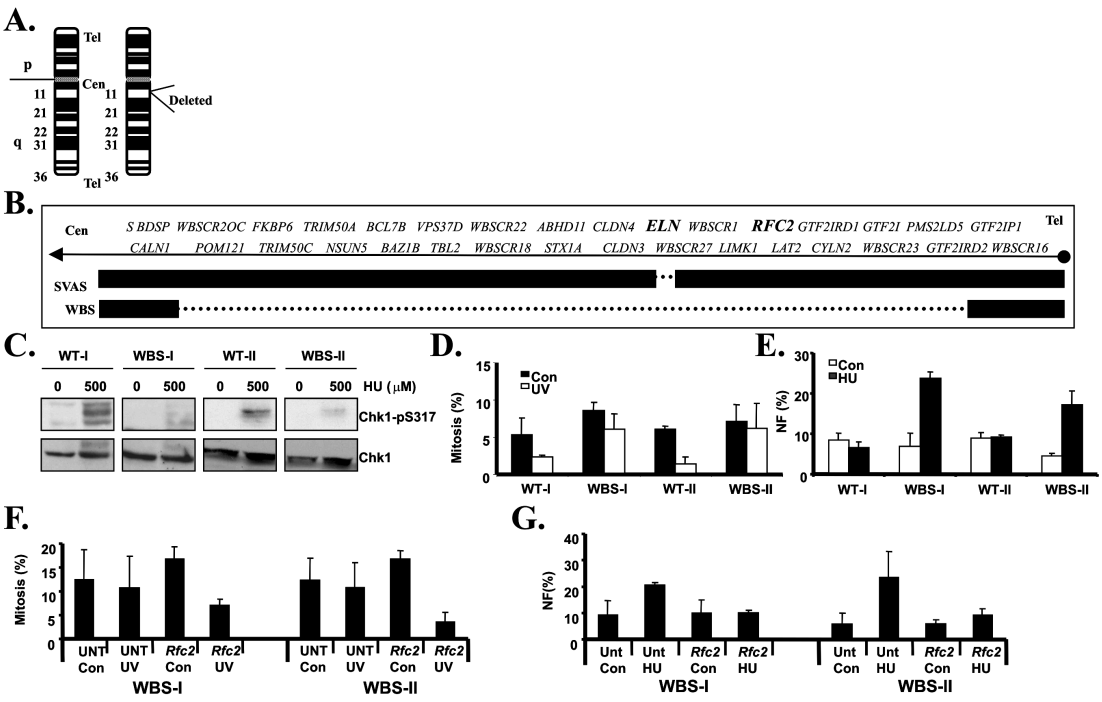
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Figure 5.