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## Inhibition of *Atm* and/or *Atr* disrupts gene silencing on the inactive X chromosome

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### Abstract

ATM and ATR are well documented for their roles in maintaining the integrity of genomic DNA by responding to DNA damage and preparing the cell for repair. Since ATM and ATR have been reported to exist in complexes with histone deacetylases, we asked whether *Atm* and *Atr* might also uphold gene silencing by heterochromatin. We show that the *Atm/Atr* inhibitor 2-aminopurine causes the inactive X chromosome to accumulate abnormal chromatin and undergo unwanted gene reactivation. We provide evidence that this gene expression from the inactive X chromosome is not a byproduct of the accumulation of DNA breaks. Individually inhibiting *Atm* and *Atr* by either small interfering RNA or the expression of dominant-negative *ATM* and *ATR* constructs also compromised X-inactivation. *Atm* and *Atr*, therefore, not only function in responding to DNA damage but perhaps also are involved in gene silencing via the maintenance of heterochromatin.

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Ataxia telangiectasia mutated (ATM) and ATR (ATM and Rad3 related) are protein kinases with partially overlapping functions that are key components of DNA damage response pathways [1]. In response to DNA double strand breaks (DSBs) ATM becomes an active kinase [2] that phosphorylates proteins involved in cell cycle arrest and DNA repair [1]. In response to other types of DNA damage including UV damage and single strand breaks during DNA replication, ATR appears to play a similar role, phosphorylating a number of the same targets [1]. ATM is mutated in the disease Ataxia-telangiectasia (A-T) [3], and A-T patients and *Atm*<sup>-/-</sup> mice display a variety

of symptoms including chromosomal breaks and translocations, and a predisposition to cancer [4–10]. Targeted *Atr* disruption in mice leads to chromosomal fragmentation and early embryonic lethality [11]. Consistent with their roles in DNA damage checkpoints, *ATM*- and *ATR*-deficient cells are sensitive to genotoxic challenges [12].

Chromatin proteins play important roles in gene expression and DNA repair. The importance of chromatin composition on gene expression is exemplified by X-inactivation, where one of the two X chromosomes in female mammalian cells is transcriptionally silenced by heterochromatin [13–16]. In the ATR pathway, a histone acetyltransferase-containing protein complex termed TFTC preferentially binds UV-damaged DNA and acetylates histone H3 in vitro [17]. ATM interactions with the histone acetyltransferase hMOF [18], histone H2AX [19], 52

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53 and the histone acetyltransferase TIP60 [20] are important  
54 for the repair of DSBs. ATM kinase was shown to be acti-  
55 vated when cells are exposed to agents that alter chromatin  
56 structure but do not cause detectable DSBs; this led to the  
57 proposal that DSBs activate ATM via chromatin changes  
58 [2].

59 In *Drosophila*, loss of *Atm* reduces the levels of hetero-  
60 chromatin protein 1 (HP1) at telomeres and increases  
61 HP1 abundance at an internal euchromatic region [21]. In  
62 mammalian cells, ATM and ATR associate with histone  
63 deacetylases even in the absence of DNA damaging agents  
64 [22,23]. Here we show that disruption of *Atm* and *Atr* func-  
65 tion destabilizes the silencing of a reporter gene on the  
66 mouse inactive X chromosome (Xi). In contrast, the  
67 reporter gene was not reactivated when cells with function-  
68 ally intact *Atm* and *Atr* genes were subjected to consider-  
69 able DNA damage.

## 70 Materials and methods

71 Preparation of the reporter cell line with GFP on the Xi has been  
72 described [24,25]. 2-Aminopurine (Sigma Chemical Co., Saint Louis) was  
73 applied at 5 mM to cell culture for 1 day [for immunostaining or gene  
74 reactivation with 5-aza-cytidine (5-AZ), Sigma Chemical Co., Saint Louis]  
75 for 3 days (gene reactivation without 5-AZ). Caffeine (Sigma Chemical  
76 Co., Saint Louis) was applied at 5 mM to cell culture for 3 days.

77 RNA FISH was performed as described in [26] using a 50-mer DNA  
78 probe complementary to map position 3064–3113, which is in Repeat C of  
79 *Xist*. Immunostaining was performed as described previously [27], with  
80 modifications. Briefly, for macroH2A staining, murine embryonic fibro-  
81 blasts were grown on coverslips, and then treated with methanol for fixa-  
82 tion, followed by TBST (0.1 M Tris 8.0, 0.15 M NaCl, and 0.5% Triton  
83 X-100) washes. Cells were immunostained with anti-macroH2A (Upstate  
84 Inc., Lake Placid) or anti-acetyl H4 (Serotec, UK) in 3% TBST milk at  
85 4 °C overnight. After TBS wash, FITC-conjugated secondary antibody  
86 (Sigma, Saint Louis) was applied for 30 min. DAPI (Vector Laboratories,  
87 Burlingame) was used for counterstaining. Metaphase spread chromo-  
88 somes were prepared by harvesting colcemid-treated cells followed by  
89 centrifugation onto coverslips. NIH image 1.63 software ([http://rsb.in-](http://rsb.info.nih.gov/nih-image/Default.html)  
90 [fo.nih.gov/nih-image/Default.html](http://rsb.info.nih.gov/nih-image/Default.html)) was used to quantify the FITC signal  
91 of immunostained mitotic chromosome spreads. For each individual  
92 chromosome in a spread, the number of pixels occupied by each chromo-  
93 some (DAPI) and the number of pixels displaying FITC signal were  
94 determined.

95 SiRNA was made with Silencer Construction (Ambion, Austin) and  
96 transfections were performed as previously described [25]. Human coun-  
97 terpart of SiRNA sequence for *Atm* was in [28] and for *Atr* in [29]. SiRNA  
98 sequences were as follows: *Atr/musAntisense* (AAA CCA AGA CAG  
99 ATT CTC TGC CCT GTC TC), *Atr/musSense* (aaG CAG AGA ATC  
100 TGT CTT GGT CCT GTC TC), *Atm/musAntisense* (AAC ATA CTA  
101 CTC AAA GAC ATT CCT GTC TC), *Atm/musSense* (aaA ATG TCT  
102 TTG AGT AGT ATG CCT GTC TC), and *SMC1/Antisense* (AAG ACT  
103 TGA AGG AGA AGA TGA CCT GTC TC), *SMC1/Sense* (aaT CAT  
104 CTT CTC CTT CAA GTC CCT GTC TC). The control siRNA sequence  
105 is AAG GGC CTT CAG TAT GTC CTT CCT GTC TC, which is against  
106 *Bracl* with a mismatch that prevents RNA interference. Nine microliter of  
107 oligofectamine (Invitrogen, Carlsbad) and 0.12 μM siRNA (final concen-  
108 tration) were transfected into 6-well plates at  $2 \times 10^5$  cells/well and assayed  
109 using flow cytometry 3 days later. To quantitate the levels of *Atm* and *Atr*  
110 transcripts in siRNA-treated cells, reporter cells were, in parallel, sub-  
111 jected to siRNA against *Smc1*. GFP-expressing cells from each of the three  
112 siRNA reactions were flow sorted and subjected to real-time RT-PCR for  
113 either *Atm* and *Gadph* or *Atr* and *Gadph*. The PCR primers used for these  
114 real-time RT-PCRs were *Atr*-reverse (TGT TCA CCC ATT CAA TAA

TCC CAC), *Atr*-forward (TAA AAG GCT TGT AGA AGA CCC GAC),  
115 *Atm*-reverse (GTG CGC AGA CAG CAG AGT TCT CCA CGA TTC),  
116 *Atm*-forward (GAA GGC CTG GAT GCT GTG AAT CTG TGG GTT),  
117 and *Gadph* reverse (CAT ACC AGG AAA TGA GCT TG), and *Gadph*  
118 forward (ATG ACA TCA AGA AGG TGG TG). The conditions for RT-  
119 PCR were 95 °C for 2 min, then 40 cycles of 95 °C for 15 s, 56 °C for 15 s,  
120 and 72 °C for 30 s. For the reverse transcription reaction we used the  
121 reverse primers and M-MuLV reverse transcriptase (New England Bio-  
122 Labs, Beverly, MA) following manufacturers instructions. Real-time PCR  
123 was carried out by using iQ Syber-Green reaction mix from Bio-Rad  
124 (Hercules, CA) following manufacturers instructions. *Atm* and *Atr*  
125 mRNA levels thus obtained from siRNA treated cells were normalized  
126 against the *Gadph* mRNA levels in the same reactions and then expressed  
127 as a percent of the transcript level for *Atm* or *Atr* in cells treated with siRNA  
128 directed against *Smc1*.  
129

130 Transient transfection with *ATR* kinase-dead ( $D^{2475} \rightarrow A$ ) [30] and  
131 *ATM* kinase-dead ( $D^{2870} \rightarrow Ala$  and  $N^{2875} \rightarrow K$ ) [31] constructs was per-  
132 formed using Fugene 6 (Roche Applied Science, Indianapolis). Three  
133 microliters of fugene 6 + 1 μg plasmid was used in transfections using 6-well  
134 plates at  $2 \times 10^5$  cells/well and assayed using flow cytometry 3 days later.

## 135 Results

### 136 Exposure of female cells to 2-aminopurine destabilizes 137 X-inactivation

138 The mouse inactive X chromosome (Xi) was chosen for  
139 identifying heterochromatin defects because of its nearly  
140 uniform histone H4 hypoacetylation [32]. In addition, the  
141 stability of gene silencing can be assayed in mouse reporter  
142 cell lines by scoring the reactivation of an X-linked GFP  
143 transgene that is normally silenced by the heterochromatin  
144 of the Xi [33]. Metaphase spread chromosomes were pre-  
145 pared from a transformed wild type mouse female embry-  
146 onic fibroblast (MEF) cell line (WT:F#104) and  
147 fluorescently immunostained using antibodies that recog-  
148 nize acetylated histone H4 [32]. Forty-six spreads prepared  
149 from an untreated (control) cell line all displayed an almost  
150 uniformly hypoacetylated chromosome (Figs. 1A and B). It  
151 was previously established that the hypoacetylated chro-  
152 mosome in female cells is the Xi [32]. When the same cell  
153 line was treated with 2-aminopurine (2-AP), an inhibitor  
154 of *Atm* and *Atr* [34,35], the Xi displayed an increased level  
155 of acetylation although it was still less acetylated than the  
156 other chromosomes (Figs. 1C and D). Using NIH-IMAGE to  
157 quantify the fluorescent signal, we found that in untreated  
158 cells the Xi displayed <2% fluorescent signal while all other  
159 chromosomes in the same spreads displayed >40% acetyla-  
160 tion (Fig. 1E). In contrast, in 2-AP treated cells the Xi con-  
161 sistentlly displayed >10% acetylation although its level of  
162 acetylation was still below the level of the other chromo-  
163 somes (Fig. 1E). Two other female MEF lines were treated  
164 with 2-AP and found to exhibit a similar increase in histone  
165 H4 acetylation on the Xi (data not shown). Two markers of  
166 the Xi, macroH2A [36] and *Xist* RNA [37] in localized to  
167 untreated interphase WT:F#104 cells (Figs. 1F and H)  
168 and in the 2-AP treated cells (Figs. 1G and I). Thus, the  
169 normally hypoacetylated Xi had acquired acetylation upon  
170 2-AP-treatment but maintained high concentrations of  
171 macroH2A and *Xist* RNA.

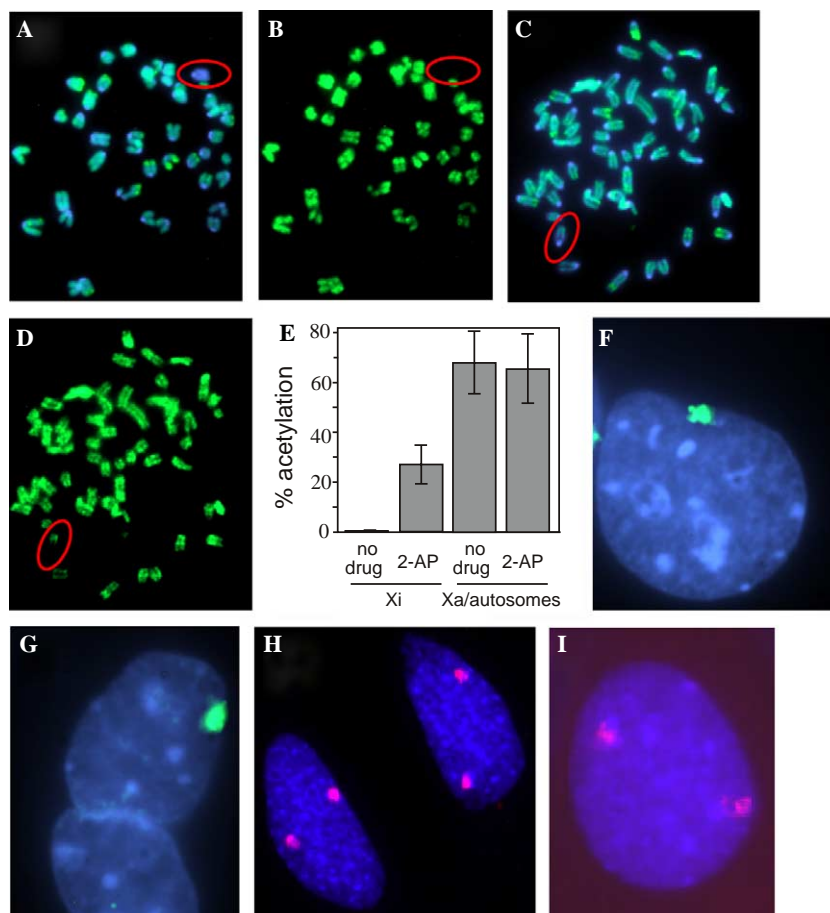


Fig. 1. Acetylation of histone H4 on the inactive X chromosome in response to 2-aminopurine. (A–E) Mitotic spreads of the transformed MEF line WT:F#104 were immunostained for lysine-acetylated histone H4 (FITC, green). Chromosomal DNA was stained with DAPI. (A,B) Untreated cells displaying acetyl-H4 (green) and DNA (blue) signal (A), or acetyl-H4 signal alone (B). The red circle indicates the position of the inactive X chromosome (Xi) which lacks acetylated H4. (C,D) 2-AP treated cells displaying acetyl-H4 and DNA (C), or acetyl-H4 alone (D). The red circle indicates the position of the hypoacetylated X chromosome. (E) NIH image quantitation of the FITC signal from five 2-AP treated and five untreated mitotic chromosome spreads immunostained for acetyl-H4. All five untreated spreads and none of the five 2-AP treated spreads displayed one or more chromosomes with <10% acetylation. (F,G) Immunostaining of WT:F#104 cells using an antibody against macro-H2A (green) of interphase MEF cells that are untreated (F) or 2-AP treated (G) showing that the inactive X chromosome is still present in 2-AP treated cells. (H, I) RNA FISH against Xist RNA in a tetraploid mouse cell line (WT:F#106) that is untreated (H) or 2-AP treated (I). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

172 Since histone acetylation is associated with gene trans-  
 173 cription and histone hypoacetylation with silencing [38],  
 174 the reacylation on the Xi raised the question as to  
 175 whether 2-AP treatment disrupted gene silencing. We as-  
 176 sayed for the reactivation of a GFP reporter gene that is  
 177 located on the Xi of a female transformed fibroblast line  
 178 ( $Xa^{AXist}Xi^{GFP-1}$ ) derived from a mouse embryo of the  
 179 same genotype [33]. The cells were obtained by mating a  
 180 male mouse that carries an X-linked GFP-expressing trans-  
 181 gene [39] under the control of a chicken  $\beta$ -actin promoter  
 182 and cytomegalovirus enhancer [40] to a female mouse that  
 183 is heterozygous for a deletion in the X-linked *Xist* gene [24].  
 184 The presence of the *Xist* mutation causes the X chromo-  
 185 some carrying the GFP transgene to be inactivated in all  
 186 cells of female embryos [41]. The GFP transgene is subject-  
 187 ed to X-inactivation and allows the destabilization of gene  
 188 silencing in response to interventions such as exposure to  
 189 chemical inhibitors or siRNA that can be quantitated by

flow cytometry [25,33]. Treatment of  $Xa^{AXist}Xi^{GFP-1}$  cells 190  
 with 2-AP resulted in a ~5-fold GFP reactivation, as com- 191  
 pared with the untreated cells ( $p = 0.0029$ ) (Fig. 2A). 192  
 Treatment of  $Xa^{AXist}Xi^{GFP-1}$  reporter cells with caffeine, 193  
 another inhibitor of Atm and Atr that produces similar ef- 194  
 fects on cells as 2-AP [29], also increased reactivation 195  
 (Fig. 2B). 196

Inhibition of ATM or ATR renders cells hypersensi- 197  
 tive to DNA damaging agents [12]. If these proteins also 198  
 maintain the heterochromatin of the Xi, then exposure to 199  
 2-AP should also render the Xi heterochromatin hyper- 200  
 sensitive to chromatin-altering agents, such as 5-aza-cyti- 201  
 dine (5-AZ), an inhibitor of the DNA methylation that 202  
 helps maintain Xi heterochromatin. In agreement with 203  
 this prediction, simultaneous treatment of cells with 2- 204  
 AP and 5-AZ produced a cumulative toxicity to cells. 205  
 Time of exposure of cells to 2-AP and 5-AZ was 206  
 consequently reduced from 3 days to 1 day resulting in 207

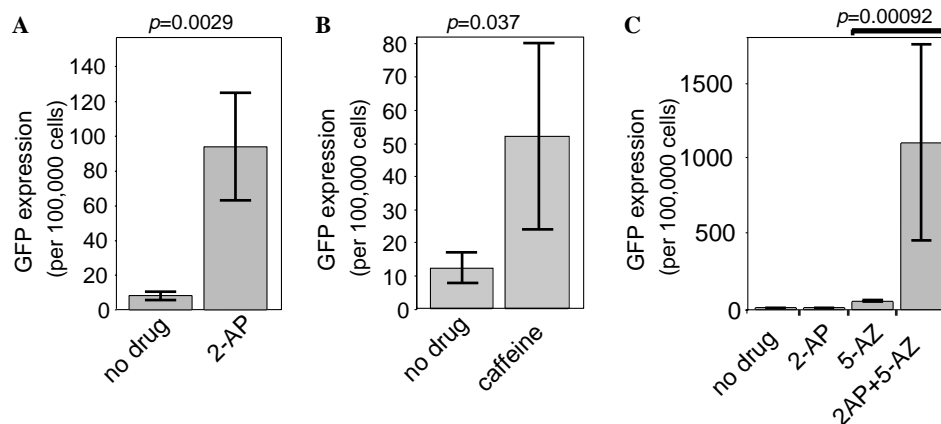


Fig. 2. Reactivation of a GFP transgene on the Xi after 2-AP exposure. Mean values of their 95% confidence intervals are reported. (A) 2-AP treatment for 3 days. (B) Caffeine treatment for 3 days. (C) Simultaneous 1 day treatment of reporter cells with 2-AP and 5-AZ. Similar results were obtained using another independently derived reporter cell line,  $Xa^{AXist}Xi^{GFP-2}$  [25] (data not shown).

208 reduced rates of GFP reactivation in response to individ-  
 209 ually treating reporter cells with 2-AP or 5-AZ (Fig. 2C).  
 210 However, even at this brief exposure, a >20-fold increase  
 211 in GFP gene reactivation was observed in cells treated  
 212 with both 2-AP and 5-AZ over cells exposed to 5-AZ  
 213 alone ( $p = 0.00092$ ) (Fig. 2C).

#### 214 Individually inhibiting *Atm* or *Atr* compromised 215 *X*-inactivation

216 To determine whether both *Atm* and *Atr* are involved in  
 217 gene silencing, they were individually inhibited in  
 218  $Xa^{AXist}Xi^{GFP-1}$  cells. It was previously shown that trans-  
 219 fection of cells with plasmids expressing ATM proteins  
 220 bearing missense mutations that abolish ATM kinase func-  
 221 tion also abolishes the kinase activity of the native wild  
 222 type ATM protein and renders cells hypersensitive to ion-  
 223 izing radiation [42]. Similarly, expression of an ATR pro-  
 224 tein rendered kinase-inactive by a point mutation had a  
 225 dominant-negative effect on the wild type protein and ren-  
 226 dered cells defective for ATR kinase activity [30] and  
 227 hypersensitive to UV irradiation [43]. GFP reactivation  
 228 was significantly induced when  $Xa^{AXist}Xi^{GFP-1}$  cells were  
 229 transiently transfected with plasmids expressing kinase-in-  
 230 active missense mutants for *Atm* (ATM-kd, [31]), or *Atr*  
 231 (ATR-kd, [30]) as compared with vector alone (ATM-kd,  
 232  $p = 0.0048$  and ATR-kd,  $p = 0.00088$ ) (Fig. 3A). Gene  
 233 reactivation from the Xi was also significantly elevated in  
 234 cultures treated with small interfering RNA against *Atr*  
 235 ( $p = 0.025$ ) or *Atm* ( $p = 0.035$ ) (Fig. 3B). Real-time RT-  
 236 PCR of *Atm* transcript sequence revealed that the siRNA  
 237 directed against *Atm* in this study reduced *Atm* transcript  
 238 levels to slightly less than 20% of the normal level. Surpris-  
 239 ingly, the siRNA directed against *Atr* only caused a modest  
 240 24% reduction in the *Atr* transcript level, despite this inhi-  
 241 bition causing a greater level of GFP reactivation than *Atm*  
 242 inhibition (see Materials and methods for more details on  
 243 mRNA quantitation).

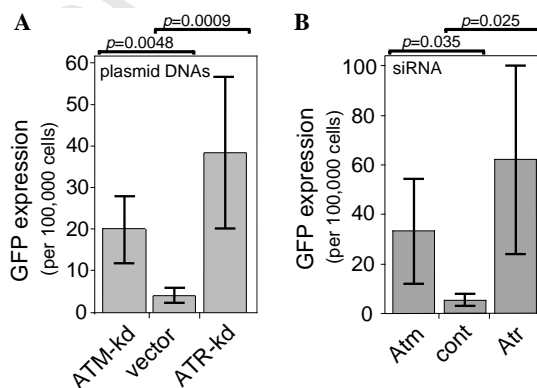


Fig. 3. Disruption of *Atm* or *Atr* function reactivates a GFP transgene located on the Xi. Mean values of their 95% confidence intervals are reported. *P* values were obtained using the Kruskal–Wallis test. (A) Transient transfection of kinase-dead (kd) dominant-negative mutants of *ATM* and *ATR* (both kinase dead genes are in the vector pCDNA) and the pCDNA vector alone. (B) SiRNA knockdown of *Atm* or *Atr* expression.

#### *γ*-Irradiation does not reactivate the GFP reporter gene 244

It has been proposed that DSBs loosen the chromatin 245  
 around the break [2]. *Atm* or *Atr* inhibition might therefore 246  
 have incurred DNA damage that reactivated the GFP 247  
 gene. Reporter cells were exposed to  $\gamma$ -irradiation and then 248  
 assayed for GFP reactivation. No increase in GFP reactivation 249  
 was observed, even at 10 Gy of  $\gamma$ -irradiation 250  
 (Fig. 4). 251

The checkpoint/repair protein BRCA1 [44] concentrates 252  
 on the Xi in non-mitotic cells ([25] and Figs. 5A and B) and 253  
 helps maintain Xi heterochromatin [25]. To determine 254  
 whether *Atm* protein also concentrates on the Xi, female 255  
 human lymphocytes stably expressing an ATM-GFP fusion 256  
 protein were examined.  $\gamma$ -Irradiation resulted in a 257  
 punctate ATM pattern (Figs. 5C and D) due to ATM 258  
 localizing to sites of DNA damage [2]. In the absence of 259  
 $\gamma$ -irradiation, no localized concentration of ATM protein 9  
 was observed (Figs. 5E and F), indicating that ATM did 261  
 not concentrate on the Xi. 262

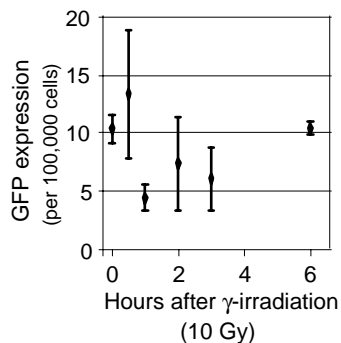


Fig. 4.  $\gamma$ -Irradiation fails to reactivate the GFP transgene. Reporter cells were exposed to 10 Gy and GFP reactivation was recorded at the times after irradiation indicated. Standard deviations of three independent experiments are shown.

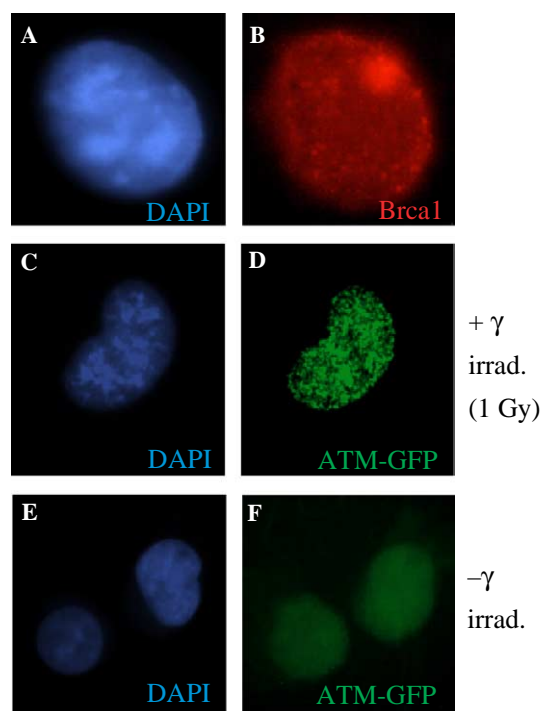


Fig. 5. ATM does not associate with the Xi at high concentrations. (A,B) Mouse fibroblasts stained with DAPI (A) and fluorescently immunostained for Brca1 protein (B). (C,D) Human lymphocytes stably expressing ATM-GFP fusion protein exposed to 1 Gy of  $\gamma$ -irradiation and stained with DAPI (C), and examined for GFP expression (D). (E,F) Unirradiated human lymphocytes stably expressing ATM-GFP fusion protein stained with DAPI (E) and examined for GFP expression (F).

## 263 Discussion

264 We provide evidence that both *Atm* and *Atr* participate  
 265 in gene silencing by maintaining heterochromatin on the  
 266 Xi. Treatment of mouse fibroblast cells with 2-AP caused  
 267 histone acetylation to appear on the Xi and resulted in  
 268 reactivation of a GFP reporter gene on the Xi. Individually  
 269 interfering with the *Atm* or *Atr* function by two different  
 270 methods, siRNA and the expression of dominant-interfer-  
 271 ing kinase-dead *ATM* and *ATR* genes, also caused GFP  
 272 reactivation. Our failure to detect an increase in gene

273 reactivation in response to ionizing radiation argues that  
 274 the observed gene reactivation is not caused by DNA dam-  
 275 age that accumulates when *Atm* or *Atr* is inhibited. Our  
 276 findings extend the roles of *Atm* and *Atr* to the mainte-  
 277 nance of gene silencing by heterochromatin and raise the  
 278 possibility that developmental defects seen in A-T patients  
 279 may be caused in part by chromatin abnormalities and  
 280 aberrant gene expression.

281 In view of the evidence for roles in the maintenance of  
 282 heterochromatin, *Atm* and *Atr* may function in a hetero-  
 283 chromatin checkpoint pathway that senses chromatin de-  
 284 fects and helps restore normal heterochromatin structure.  
 285 This may involve HDAC-2 which interacts with *ATR* in  
 286 unirradiated cells [22] or the BACS complex [23] which in-  
 287 cludes *ATM*, *ATR*, *HDAC1*, and *BRCA1* (which has been  
 288 shown to control Xi heterochromatin [25]). Alternatively  
 289 chromatin defects may induce *ATM* and *ATR* to interact  
 290 with factors that restore heterochromatin.

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 296 chael Kastan (St. Jude Children's Research Hospital,  
 297 Memphis, TN), respectively. The human lymphoblastoid  
 298 cell line expressing *ATM*-GFP was kindly provided by Sus-  
 299 an Lees-Miller (University of Calgary, CA) and Kum Kum  
 300 Khanna (Queensland Institute of Medical Research, AU).  
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