Supplementary Figure 1. XLFΔ/ΔATM−/− mice are significantly smaller than their littermate single mutant mice.

Panels A and B list the number of live born mice obtained at (P7) from crosses between XLFΔ/Δ ATM+/− mice (A) or XLFΔ/Δ ATM+/C mice (B). Panel C lists the total body weight (gram) of adult mice (P21-P28) from the different genotypes indicated. The data represents the average and the standard deviation from at least 3 mice of each genotype of each gender.
Supplementary Figure 2. XLFΔ/ΔATM−/− pro-B cells have reduced V(D)J recombination activity on chromosomally integrated substrates.

The histograms show flow cytometric analyses of GFP+ cells within the gated live-cell population (based on forward and side scatter) after 0, 2 or 4 day STI571 treatment of cell lines containing a clonally single integrated pMX-INV substrate (diagramed in Figure 2E and 2F).
Supplementary Figure 3: Analysis of endogenous Igκ rearrangement in pro-B cell lines.

(A) Schematic of the murine Jκ locus and the location and size of expected unjoined Jκ coding ends (CEs). (B and C) are two independent Southern blot analyses of genomic DNA (25-40µg) isolated from Rag2⁻/⁻, Artemis⁻/⁻, WT and XLFΔΔATM⁻/⁻ v-abl transformed pro-B cell lines 0, 48, or 96 hours after STI treatment. Genomic DNA was digested with EcoRI (RI) and ScaI (S) and probed with the JkIII probe at 3’end of Jk5.
### A.

- **WT**
- **AID^{-/-}**
- **XLFDelta**
- **ATM^{-/-}**
- **XLFDeltaATM^{-/-}**
- **HL+/K**

### B.

|                  | Direct | MH-mediated | Insertions | Total sequences |
|------------------|--------|-------------|------------|----------------|
|                  | 1-2nt | 3-4nt | 5+nt | Total MH |                |
| **XLFDeltaATM^{-/-} HL** |       |       |       |          |                |
| Mouse #1         | 3 (8%) | 19    | 9    | 5 33 (83%) | 4 40           |
| Mouse #2         | 1 (3%) | 11    | 13   | 7 31 (86%) | 4 36           |
| Mouse #3         | 1 (5%) | 4     | 7    | 8 19 (95%) | 20             |
| Mouse #4         | 1 (6%) | 1     | 5    | 9 15 (88%) | 1 17           |
| Avg ± SD        | 6 (6 ± 2%) |       |       | 98 (88 ± 5%) | 113           |
| **XLFDelta**     |       |       |       |          |                |
| Mouse #1         | 9 (21%) | 16    | 11   | 7 33 (69%) | 6 48           |
| Mouse #2         | 4 (10%) | 14    | 11   | 11 35 (74%) | 8 47           |
| Mouse #3         | 3 (8%) | 21    | 11   | 4 25 (66%) | 11 38           |
| Mouse #4         | 6 (20%) | 6     | 10   | 8 24 (73%) | 3 33           |
| Avg ± SD        | 22 (15 ± 7%) |       |       | 128 (75 ± 6%) | 181           |
| **ATM^{-/-}**    |       |       |       |          |                |
| Mouse #1         | 4 (20%) | 10    | 2    | 2 14 (70%) | 2 20           |
| Mouse #2         | 9 (19%) | 19    | 8    | 5 32 (68%) | 6 47           |
| Mouse #3         | 3 (25%) | 6     | 2    | 1 9 (75%) | 0 12           |
| Avg ± SD        | 16 (23 ± 2%) |       |       | 55 (71 ± 4%) | 79           |
| **HL+/Knock in** |       |       |       |          |                |
| Mouse #1         | 9 (31%) | 9     | 3    | 1 13 (44%) | 7 29           |
Supplementary Figure 4. XLFΔΔATM⁻⁻ B cells have CSR defects beyond those in ATM⁻⁻ or XLFΔΔ B cells.

(A) Additional independent Flow cytometry analyses to those shown in Figure 3 of purified CD43⁻ spleocytes after 4-day stimulation of the indicated splenic B cells with anti-CD40 and IL-4 as described. (B) Detailed analyses of the Sµ-Sγ1 junctions from each genotype summarized in Fig. 2D. The analyses were performed as previously described. (C) CD43⁻ spleocytes were purified from WT, XLFΔΔ, ATM⁻⁻ and XLFΔΔATM⁻⁻ mice and set up for anti-CD40/IL4 stimulation at 0.5x10⁶/ml. Two days later, the cell concentrations were counted and the average and standard deviations from at least 3 independent mice of each genotype were presented. The results show that there was no major proliferation defect of XLFΔΔATM⁻⁻ B cells compared to the other genotypes.
Zha S et al Sup. Fig 5

A. STI Pre treat 2 Day 4 Day 2 Day 4 Day

| ATM Inhibitor | - | - | - | + | + |
|---------------|---|---|---|---|---|
| XLFΔH2AXF/F 42 | -2.76 | -15.2 | -39.2 | -7.43 | -9.73 |
| XLFΔH2AXΔ- 42 | -0.78 | -4.35 | -14 | -3.22 | -5.99 |
| XLFΔH2AXF/F 29 | -2.08 | -10.5 | -29.5 | -4.25 | -9.02 |
| XLFΔH2AXΔ- 29 | 0.084 | -3.25 | -12.2 | -2.61 | -4.75 |
| XLFΔH2AXF/F 08 | -2.92 | -17.6 | -45.9 | -9.18 | -16.3 |
| XLFΔH2AXΔ- 08 | 0.048 | -2.79 | -6.3 | -2.71 | -5.22 |
Supplementary Figure 5. XLFΔΔH2AX⁺⁻ v-abl transformed pro-B lines have V(D)J recombination defects.

(A) The histograms show the flow cytometric analyses of GFP⁺ expression in gated live-cells (based on forward and side scatter) in matched XLFΔΔH2AX⁻⁻ and XLFΔΔH2AX⁺/+ cell lines with identical clonal single integrations of the pMX-INV substrate after 0, 2 or 4 days of STI571 induction. Panel A includes result from clones 29 and 42 for which the Southern blotting analyses are shown in Fig 4B, and clone 8 which the Southern Blotting Analyses are shown in panel B. (B) Southern blot analyses of the rearrangement status of a clonally single-integrated inversional V(D)J recombination substrate (pMX-INV) in matched XLFΔΔH2AX⁻⁻ and XLFΔΔH2AX⁺/+ lines (clone 8). For the upper gel, DNA was digested with EcoRV and Ncol and probed with the C4 probe. For the lower gel, DNA was digested with EcoRV and probed with the same C4 probe. See Figure 2 for details on the substrates and analyses of additional clones (clone 29 and 42).

**Methods:** To induce cre recombination between the flanking loxP sites, Abelson transformed cell lines (XLFΔΔH2AX⁺/+ or XLFΔΔATM⁺/+C⁻/⁻) were washed in ADCF-Mab serum-free media (Hyclone) three times and re-suspended at a final concentration of 5x10⁶/ml with 50μg/mL purified Tat-Cre and 50μg/mL Polymyxin B Sulfate (Sigma) for 45min at 37°C. Transduction was stopped by adding culture media containing 10% FCS. Single clones were isolated and genotyped for Cre recombination. Tat-CRE recombinant protein was purified from bacteria (BL21, Stratagene) containing pTriEx(Novagen)-His-TAT-NLS-Cre plasmid with Ni-NTA column (QIAGEN).
A. 

B. 

C. 

D. 

Zha S et al Sup. Fig 6
Supplementary Figure 6. Assay for Coding end by TdT-assisted PCR

This figure summarizes the results of TdT assisted PCR assays that provide a more sensitive means to detect unjoined coding ends as described elsewhere. (Panel A) Schematic of the TdT-assisted PCR strategy for assessment of pMX-INV coding ends. Briefly, two micrograms of genomic DNA was treated with Terminal deoxynucleotidyl transferase (TdT, New England Biolabs) according to the manufacturer’s protocol in the presence of 5μM dATP. The reaction was terminated by heating to 70°C for 15min. Two percent of the polyadenylation reaction was used for primary amplification (15 cycles) using primer IRES-REV5 (5’-CTC GAC TAA ACA CAT GTA AAG C-3’) and primer T17-UNIV (5’-GTA AAA CGA CGG CCA GTT TTT TTT TTT TT-3’). Two percent of the products of the primary amplification and serial 1:5 dilutions were used as templates for a secondary amplification step (17 cycles) using primers IRES-REV4 (5’-AGT GTA GAA TTC CCC TTG TTG AAT ACG CTT G-3’) and UNIV (5’-AGA GCT GGA TCC GTA AAA CGA CGG CCA GT-3’). One half of each secondary reaction was analyzed on 1% Agarose 1x TBE gel transferred onto Zeta-Probe GT membrane (BioRad), and hybridized with 32P-labelled I4 oligonucleotide probe (5’-TAA GAT ACA CCT GCA AAG GCG-3’). (Panel B) Southern blot analysis with C4 probe of EcoRV digested genomic DNA from the indicated Clone 29 XLFΔ/ΔH2AXF/F and the XLFΔ/ΔH2AX-/- pro-B cell lines that contain the identical single integration of a pMX-INV substrate. The same DNA from Clone 29 XLFΔ/ΔH2AXF/F and XLFΔ/ΔH2AX-/- pro-B cell lines were used in TdT assistant PCR shown in Panel C. (Panel C&D) TdT assistant PCR results from ATM inhibitor treated clone 29 (panel C) and clone 42 (panel D) XLFΔ/ΔH2AXF/F and the XLFΔ/ΔH2AX-/- v-abl pro-B cells.

Summary of findings in this figure: First, although there is no little or no detectable CE from XLFΔ/ΔH2AX-/- v-abl pro-B cells as assayed by Southern blotting (Panel B), CE can be clearly detected in the same cells by this more sensitive TdT assistant PCR assay (Panel C). Therefore, unjoined coding ends are being generated in XLFΔ/ΔH2AXF/F v-abl pro-B cells. Second, while Southern blotting revealed substantially greater levels of CEs in ATM inhibitor treated XLFΔ/ΔH2AXF/F cells compared to those found in ATM inhibitor treated XLFΔ/ΔH2AXF/F cells (Panel B), less CE signals were detected in ATM inhibitor treated XLFΔ/ΔH2AXF/F cells versus ATM inhibitor treated XLFΔ/ΔH2AXF/F cells by the TdT assistant PCR assay (Panel C). This finding suggests that the CEs in ATM inhibitor treated XLFΔ/ΔH2AXF/F cells may be relatively refractory to TdT activity, consistent with a sealed hair-pin structure or some other end modification.
Supplementary Figure 7: Assessment of coding end structure by urea denaturing Southern blotting analyses.

(A) Schematic of native (N) and denatured (D) pMX-INV recombination substrate following digestion with EcoRV: unrearranged (UR), rearranged (CJ+SJ, same size as UR), hair-pin-sealed coding ends (hCE) and open coding ends (oCE). GFP coding region (red and green) will be inverted after rearrangement. Note that a sealed hair-pin structure (covalent bond) will keep the two CE strands together under denaturing condition [hCE(D)], while the same condition will separate these two CE strands when the hair-pin is opened [oCE(D)]. (B and C) Southern blot analysis of native (N) and denatured (D) EcoRV-digested genomic DNA from XRCC4−/−, Artemis−/−, XLFΔΔ (clone 42 and clone 29, with and without treatment of ATM inhibitor, respectively) pro-B cells treated with STI571 for 0 or 48 hours, and probed with the C4 probe. The configurations of the native (N) and denatured (D) fragments as shown in panel A are indicated. At the right side of panel B, native (N) and denatured (D) pMX-INV recombination substrate following digestion with EcoRV and EcoRV+Ncol is shown as a control. Note that a band corresponding to the hair-pin coding ends is induced by STI treatment similarly in Artemis−/− and ATM inhibitor treated XLFΔΔ pro-B lines.

Methods: Denaturing agarose electrophoresis gels was carried out as described with minor modifications24. Briefly, 40μg of genomic DNA was digested overnight with EcoRV in a 400μl volume and concentrated to 30μl. The DNA was resuspended with the addition of 5 volumes of a solution containing 8M urea, 1%NP-40, 1mM Tris pH 8.0 and 0.5 mg/ml bromophenol blue. This DNA solution was divided into two portions, with one half heated at 90°C for 8 min to denature genomic DNA; while the other half was incubated on ice. The heated DNA samples were placed on ice prior to electrophoresis on a 1.2% agarose 1xTAE gel with 1M urea at 50 V for ~20hrs at 4°C in 1xTAE buffer containing 1M urea. After electrophoresis, the gel was soaked in 1xTAE buffer without urea twice for 5 min, then denatured in a buffer containing 0.6M NaCl and 0.4M NaOH for 30 min. Southern blotting was performed with the C4 probe.
Supplementary Figure 8. Analysis of DNA-PK Activity in XLF&ATM double deficient cells.

Wild type (WT), XLFΔ/Δ, ATMΔ/Δ and XLFΔ/ΔATMΔ/Δ pro-B cells were cultured with or without 20µM DNA-PK inhibitor NU7026 (Tocris Bioscience) (Panels A and C) or 5 µM DNA-PK inhibitor NU7441 (Tocris Bioscience) (Panels B) for 1 hour, exposed to either 0 or 10 Gy γ-irradiation as indicated and harvested 6 hours later. Cells were lysed for 10min at 4°C in a buffer containing 50mM Tris Cl PH7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% NaDOC, 1mM DTT, and Complete Protease Inhibitor Cocktail Tablets (Roche), PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche). Then NaCl concentration was brought up to 400mM before sonication. Protein concentration was measured by DC Protein Assay (Bio-Rad). α-Tubulin was blotted as loading controls (Sigma Aldrich). Total H2AX and γH2AX, total KAP-1 and phospho-KAP-1, were assayed by running the same amount of samples on separate gels (paned C) or by stripping and reprobing the same membrane sequentially (Panels A and B). Primary antibodies were used at the following dilutions: anti-H2AX (1:1,000, Millipore), anti-γH2AX (1:1,000, Millipore), anti-KAP-1 (1:1,000, Cell Signaling), anti-phospho-KAP-1 (S824) (1:1,000, Bethyl Laboratories).
**Zha S et al Sup. Table 1**

|                  | Thymocytes | DN Thymocytes | DP Thymocytes | Splenocytes | IgM+ Splenic B cells |
|------------------|------------|---------------|---------------|-------------|----------------------|
| **Cell number (x10^6)** |            |               |               |             |                      |
| **WT (n=5)**     | 190.4±53.0 | 6.5±5.0       | 152.6±48.0    | 69.6±9.5    | 36.4±7.7             |
| **XLFA/Δ (n=5)** | 61.2±19.5  | 1.9±1.1       | 47.9±15.2     | 28.4±9.0    | 12.3±6.3             |
| **ATM^−/− (n=4)**| 89.5±14.8  | 4.1±1.6       | 79.0±13.9     | 47.5±6.9    | 20.2±4.6             |
| **XLFA/ΔATM^−/− (n=4)** | 8.8±2.5 | 1.3±0.6       | 6.4±1.8       | 10.5±3.3    | 0.8±0.2              |
| **XLFA/ΔATM^−/− HL (n=3)** | 8.3±0.6 | 1.8±0.5       | 6.2±0.5       | 19.3±1.0    | 8.1±1.1              |
| **Rag2^−/− (n=3)** | 5.3±1.2   | 4.8±0.9       | 0.1±0.0       | 14.5±4.2    | -                    |

**Supplementary Table 1. Cellularity of lymphoid organs.**

Total thymocyte and splenocyte numbers, as well total DN and DP thymocyte and IgM+ splenic B cell numbers. Each value listed represents the average ± standard deviation from at least three mice between 4 to 12 weeks of age.
### Supplementary Table 2. Extrachromosomal V(D)J recombination efficiency in v-abl transformed pro-B lines of different genotypes.

Substrates that test for either coding join formation (pJH290) or RS join formation (pJH200) were transiently transfected into test cells together with full length RAG1 and RAG2 expression vectors and assayed as described\(^6\). Approximately, 30x10\(^6\) cells were used for each transfection. The numbers in individual columns indicate the relative V(D)J recombination level of a given line normalized to the value obtained with WT cells, which was set as 1 (three WT, two ATM\(^{-/-}\), two XLF\(^{Δ/Δ}\) and three XLF\(^{Δ/Δ}\)ATM\(^{-/-}\) cell lines were used in these experiments). Note that experiments #1-3 used the same cells (with the same integrations) as shown in Figure 2C for chromosomal integrated substrates.

|                  | Exp1 | Exp2 | Exp3 | Exp4 | Exp5 | Exp6 | Exp7 |
|------------------|------|------|------|------|------|------|------|
| **JH290 Coding**|      |      |      |      |      |      |      |
| WT               | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| XLFD\(^{Δ/Δ}\)   | 0.37 | 0.73 | 0.96 | 0.61 | 1.35 | 1.04 | 0.51 |
| ATM\(^{-/-}\)    | 1.66 | 0.76 | 0.66 | 0.99 | 0.78 | 0.39 |
| XLFD\(^{Δ/Δ}\)ATM\(^{-/-}\) | 0.22 | 0.23 | 0.57 | 0.36 | 0.77 | 0.59 | 0.49 |
| XRCC4\(^{-/-}\)  | <0.120 | 0.06 |      |      |      |      |      |
| no-Rag           | <0.018 | <0.031 | <0.013 |
| **JH200 Signaling**|      |      |      |      |      |      |      |
| WT               | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| XLFD\(^{Δ/Δ}\)   | 0.39 | 0.59 | 0.99 | 1.44 | 1.57 | 0.70 |
| ATM\(^{-/-}\)    | 0.29 | 1.00 | 0.85 |      |      |      | 1.10 |
| XLFD\(^{Δ/Δ}\)ATM\(^{-/-}\) | 0.42 | 0.37 | 0.62 | 0.23 | 0.77 | 0.29 |
| XRCC4\(^{-/-}\)  | <0.062 | <0.080 | <0.013 |
| no-Rag           | <0.0067 | <0.021 | <0.009 |