ONLINE SUPPLEMENTAL INFORMATION

SUPPLYMENTAL FIGURE LEGENDS

Supplemental Fig. 1. MEFs lacking p65 maintain the ability to enter senescence. (**A**) Growth of *p65+/+* and *p65-/-* primary MEFs analyzed by cell counting. (**B**) BrdU incorporation analysis. Early passage (P2) and senescent (P11) MEFs were pulsed with BrdU for 4 hours, and subsequently harvested and stained with a BrdU antibody. The DNA content were counterstained with 7-AAD and then analyzed by Fluorescence Activated Cell Sorting. (**C**) Cell numbers of *p65+/+* and *p65-/-* MEFs passaged from a primary to an immortalized state. (**D**) senescence associated β-galactosidase (SA β-GAL) staining of primary *p65+/+* and *p65-/-* MEFs at P4 (rapidly proliferating) and P12 (senescent). (**E**) Percentage of positive SA β-GAL cells from various passages. (**F**) Analysis of *p65+/+* and *p65-/-* MEFs transitioning from a primary to an immortalized state using a 3T9 protocol.

Supplemental Fig. 2. Stable IκBα-SR and p65 expression in MEFs. (**A**) Primary *p65+/+* MEFs were infected with either an empty vector pBabepuro (V) or IκBα-SR expression retrovirus (SR) at P3 and Westerns were done probing IκBα. Whole cell lysates were collected from primary (PRM) cells or cells after immortalization (IMT) and Westerns were repeated for IκBα to confirm that IκBα-SR remained stably expressed in immortalized cells. (**B**) Primary *p65-/-* MEFs were infected with either an empty vector pBabepuro (V) or a pBabep65 (p65) retrovirus and Westerns were subsequently performed to probe for p65 expression.

Supplemental Fig. 3. p65 regulates genomic stability in primary MEFs. (**A**) Representative picture of chromosomes from metaphase arrested *p65+/+* and *p65-/-* primary cells. (**B**) Summary table of detailed SKY results depicting chromosomes in metaphases from *p65+/+* and *p65-/-* cell lines at P42. (**C**) Representative SKY results showing Marker Chromosomes (M. Chr.) in immortalized *p65-/-* cells.

Supplemental Fig. 4. Oxidative DNA damage is not increased in primary *p65-/-* MEFs. (**A**) Representative FLARE-comet assay results of early (P6) and late (P12) passage primary *p65+/+* and *p65-/-* cells. NC: negative control. (**B**) Average oxidative DNA damage score of primary *p65+/+* and *p65-/-* cells at different passages. Tail length and percentage of tail DNA contents were calculated with Cometscore software. DNA damage index was calculated as described in supplemental materials and methods. Data are representatives of 3 independent experiments (**A, B**) and derived from at least 100 nuclei per experiment (**B**).

Supplemental Fig. 5. p65 regulates DNA repair in primary MEFs. (**A**) Primary MEFs were irradiated with graded γ-rays as indicated. Thirty minutes after irradiation, whole cell extracts were prepared from irradiated cells and γ-H2AX levels were measured by Western blotting. Blots were stripped and subsequently reprobed for p65 and α-tubulin. (**B**) Primary MEFs at passage 4 - 6 were treated with 0.25μM doxorubicin (dox) for 4 hrs. Following this treatment, cells were grown in fresh media without doxorubicin. At indicated time points, whole cell extracts were prepared and Western blots were consecutively probed for γ-H2AX, p65 and α-tubulin. The time points of r1 – r6 indicate that cells were released from dox treatment for 1 hr to 6 hrs. (**C**), (D) Early passage primary MEFs were irradiated with 8Gy of γ-ray, and at indicated

time points cells were stained for phospho-ATM (p-ATM) (C) or phospho-BRCA1 (p-BRCA1) (D) and then counter stained with Hoechst to visualize nuclei. Data is representative of three independent experiments.

Supplemental Figure 6. p65 regulates DNA repair in vivo. (**A, B**) Frozen sections from *TNF-a-/-;p65*+/+ or *TNF-a-/-;p65-/-* spleens (**A**) and kidneys (**B**) were stained for γ-H2AX and then counter stained with Hoechst to visualize nuclei. (**C, D**) Six to eight week old *TNF-a-/-;p65*+/+ and *TNF-a-/-;p65-/-* mice were irradiated with 8Gy of γ-ray. At indicated time points, irradiated or non-irradiated mice were sacrificed and frozen sections from livers (**C**) and kidneys (**D**) were prepared and stained for γ-H2AX. Sections were counterstained with Hoechst to visualize nuclei. Data is representative of 3 independent experiments (**A** - **D**).

Supplemental Fig. 7. γ-irradiation induces cellular senescence of primary MEFs cultured in 3% oxygen. SA β-GAL staining was performed on primary *p65*+/+ and *p65-/-* MEFs to confirm cellular senescence after cells were treated with 8Gy γ-irradiation in 3% oxygen culture conditions.

Supplemental Fig. 8. Stable expression of IκBα-SR in hTERT expressing human foreskin fibroblasts. (**A**) Western analysis probing for IκBα-SR (SR) in HF-TERT cells before senescence (PRM: Primary) and after immortalization (IMT: immortalized). (**B**) NF-κB function was analyzed in IκBα-SR expressing HF-TERT cells by transfecting in triplicate HF-TERT/V and HF-TERT/SR cells with an NF-κB responsive luciferase reporter and subsequently treating cells with 5ng/ml TNF-α for 6 hrs after which a standard luciferase assay was performed. The

reduction of NF-κB activity in HF-TERT/SR cells indicates functional IκBα-SR. (**C**) Growth profile of HF-TERT/V and HF-TERT/SR after cells induced into senescence with 400μM H_2O_2 for 10 days and then maintained in culture for >50 weeks without further H_2O_2 treatment. (**D**) Cells were senesced as explained in (**C**) and then intermittently treated with H_2O_2 until immortalization was established. EXP2: experiment 2. (**E**) Representative karyotyping of HF-TERT/V (P104) and HF-TERT/SR cells (P104 and immortalized).

SUPPLEMENTAL METHODS

Materials. Antibodies to IκBα was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), α-tubulin from Sigma (St. Louis, MO), p65 from Rockland Immunochemicals Inc. (Gilbertsville, PA), γ-H2AX (JBW301) from Upstate (Charlottesville, VA), phosphor-ATM [S1981] and phosphor-BRCA1 [S1423] from Novus Biologicals (Littleton, CO), and X-GAL from Sigma. Primer sequences are shown in Supplemental table 2.

Mouse maintenance and genotyping. Balb/c, *TNF-α*, *p50* and *Bcl-3* null mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Animals were housed at the Ohio State University BRT Research Facility under sterile conditions with constant temperature and humidity and fed a standard diet. All treatments of mice were in accordance with the guidelines of the Institutional Animal Care and Use Committee. The *p50* and *Bcl-3* null mice were backcrossed into a C57BL/6 background. C57BL/6 *p65*+/- mice were backcrossed into a Balb/c background. All backcrossing was performed for more than 6 generations. All genotyping was performed by PCR prepared from tail DNA. *Bcl-3* and *p50* genotyping was carried out as

described by Jackson Laboratories. Primers and conditions for *p65* genotyping can be obtained upon request.

MEF preparation and cell culture. Heterozygous mice were mated and pregnancies checked by vaginal plugs. MEFs were isolated at day 13.5 post-coitus. Isolated MEFs were cultured until confluent and then frozen in liquid nitrogen as P1 stocks. All cells were cultured with DMEM with high glucose (Invitrogen, Carlsbad, CA) plus 10% FBS (Hyclone, Logan, Utah). Immortalization was performed by trypsinizing and replating $3X10^5$ (3T3 protocol) or $9X10^5$ (3T9 protocol) cells/6cm dish every three days. The cumulative increase of cell numbers were calculated and represented in the figures.

BrdU incorporation Analysis. An FITC conjugated anti-BrdU kit was obtained from BD Biosciences (San Jose, CA). Cells were pulsed with 10μM BrdU for 4 hours and subsequently harvested and stained for BrdU. Cells were then counter stained for DNA content with 7-AAD as recommended by the manufacturer. Fluorescence Activated Cell Sorting was performed with a FACS Calibur flow cytometer.

Retrovirus preparation and infection. pBabepuro retroviral vectors were used to express a degradation resistant IκBα-super repressor (pBabe-SR), *p65*. The pBabe-LacZ vector was constructed by subcloning a full length LacZ gene fragment into the SnaB I site of pBabepuro. Retroviruses were prepared by calcium mediated gene transfection kit supplied by promega (Madison WI). Infection of MEFs was performed by applying 4-5cfu/cell virus in a total volume of 4ml full culture medium containing 4μg/ml polybrene for 8 hours.

Quantitative real time PCR. Total RNA was extracted with Trizol reagent as recommended by the manufacturer (Invitrogen, Carlsbad, CA). Genomic DNA was extracted with a standard DNA extraction protocol. For reverse transcription (RT) real time PCR, total RNA was reverse transcribed with a SuperScript® III RT kit**, (**Invitrogen, Carlsbad, CA). SYBR Green real time PCR reagent was purchased from Bio-Rad (Hercules, CA). Real time PCR was performed as recommended by the manufacturer. The amplification efficiency was calculated with different folds of diluted samples as a standard. Relative copy numbers were calculated with the amplification efficiency derived from a standard curve and the sample application was normalized to GAPDH.

Western blotting. Protein extraction, immunoblotting were performed as previously described except that 1mM Na_3VO_4 was added into the extraction buffer (Guttridge et al, 1999). Expression levels were quantified by NIH image software. TE = band area × (mean O.D. – background O.D). CE = $TE_{\gamma\text{-H2AX}}/TE_{\text{tubulin}}$. TE: total expression; CE: comparative expression. Data in panels (**C)** and (**D)** are representative of three independent experiments.

Immunohistochemistry. Staining was performed as described previously (Sedelnikova et al, 2002). For γ-H2AX staining on tissue sections, the MOM blocking reagent was used as recommended by the manufacturer (Vector Lab, Burlingame, CA).

Fragment length analysis using repair enzymes (FLARE)-Comet Assay and alkaline comet assay. The FLARE-Comet assay kit was purchased from Trevigen (Gaitherburg, MD). FLARE-

Comet analysis was performed as recommended by the manufacturer. Nuclei from early passage (P4 – P6) cells that were not treated with formamidopyrimidine-DNA glycosylase (FPG) were used as a negative control. Comet tail length and percentage of DNA content in comet tails were calculated with Cometscore software (www.tritekcorp.com). The DNA damage index was calculated with a formula as DNA damage index = comet tail length × % of tail DNA content / 10000. Alkaline comet assay was performed the same as FLARE-comet assay without FGP treatment.

Growth analysis and senescence associated β-galactosidase (SA β-GAL) staining. For growth analysis, 10^4 cells were plated in triplicate into 24 well plate and the cells were trypsinized and counted every day. The SA β-GAL staining was performed as previously described (Serrano et al. 1997).

Immortalization of human primary fibroblasts after H_2O_2 treatment. Human primary foreskin fibroblasts (HF) were first infected with a pBabe-hygro-hTERT retrovirus, and then selected with hygromycin (HF-TERT). The cells were subsequently infected with retroviruses carrying either an empty vector (pBabe-puro) or the IκBα-SR (pBabe-puro- IκBα-SR). These infected cells were then selected in full culture media containing 2μg/ml puromycin for 5 days and maintained in the same media containing 1μg/ml puromycin. The immortalization of these cells was confirmed by continuous culturing for more than 100 passages. The H_2O_2 concentration used for inducing cellular senescence of these cells was empirically determined by using a dose of 400μM that induced complete senescence with minimal apoptosis after 10 days.

After the initial induction of cellular senescence, cells were further treated with 200μM of H_2O_2 for 3 days per week for 4 weeks and then the treatment was continued with 100μM H_2O_2 every day until cells reached immortalization.

Statistical analysis. Results are shown as mean ± s.d. *p* value was calculated by two tailed student's *t*-test. $P < 0.05$ was considered as significant.

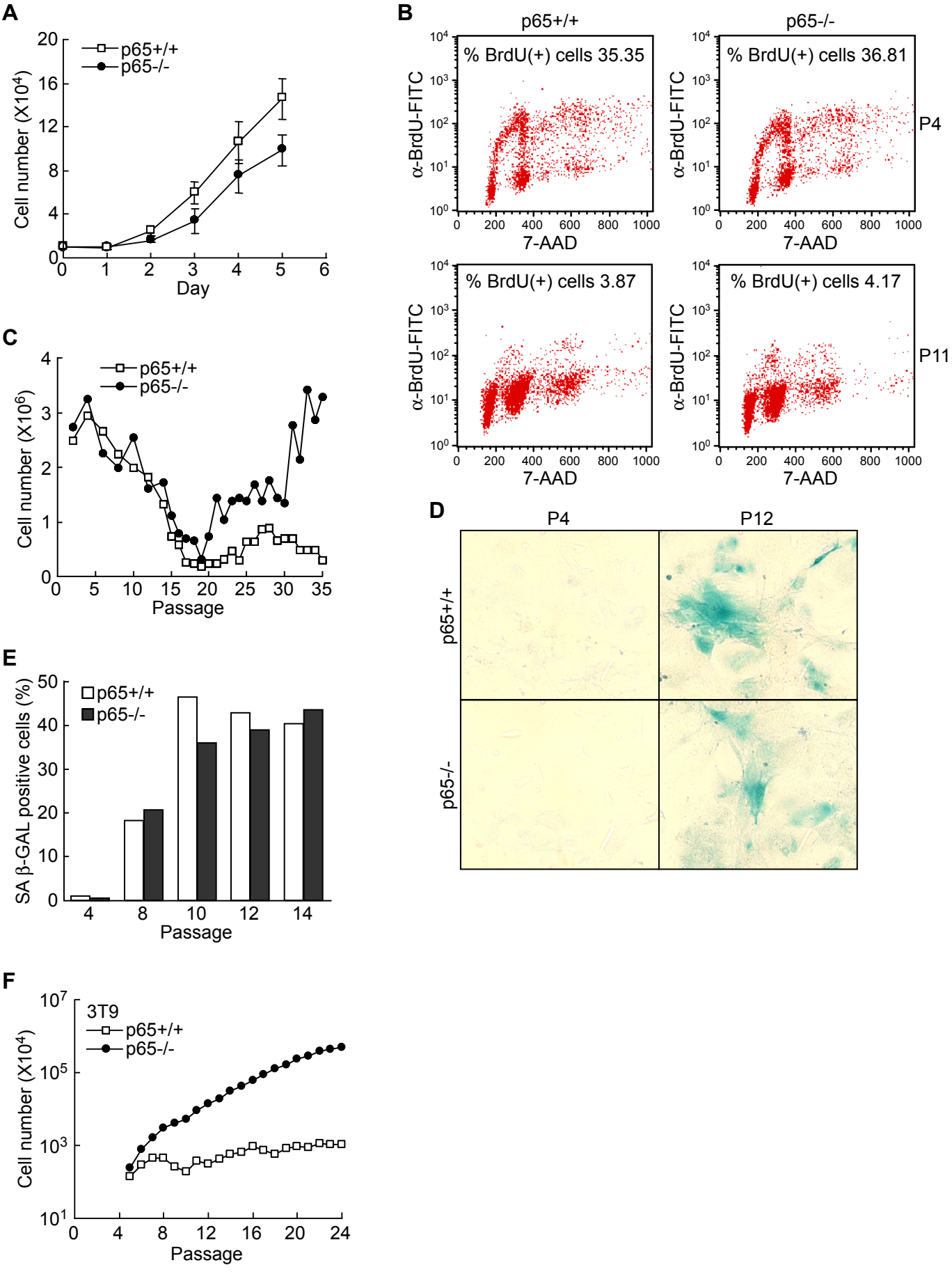
SUPPLEMENTAL REFERENCES

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88, 593-602 (1997).

Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS, Jr. (1999) NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol Cell Biol* **19**(8)**:** 5785-5799

Sedelnikova OA, Rogakou EP, Panyutin IG, Bonner WM (2002) Quantitative detection of (125)IdU-induced DNA double-strand breaks with gamma-H2AX antibody. *Radiat Res* **158**(4)**:** 486-492

Wang_Supplemental Figure 1



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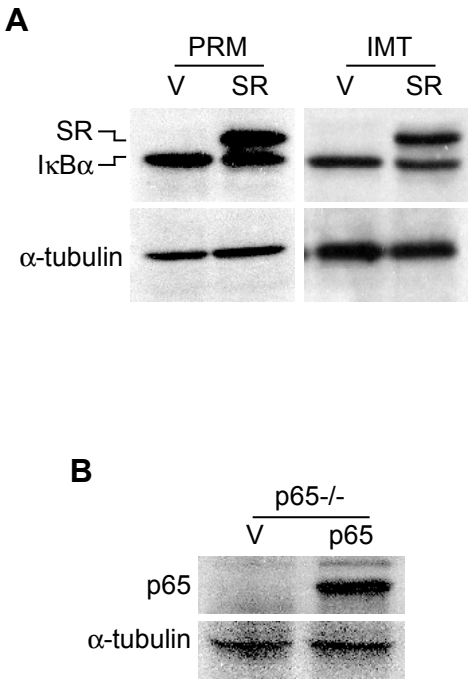

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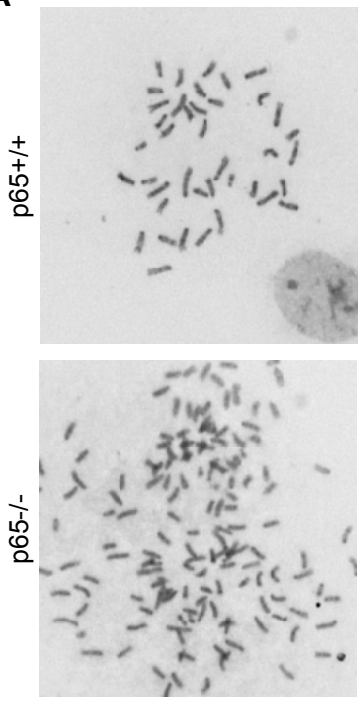

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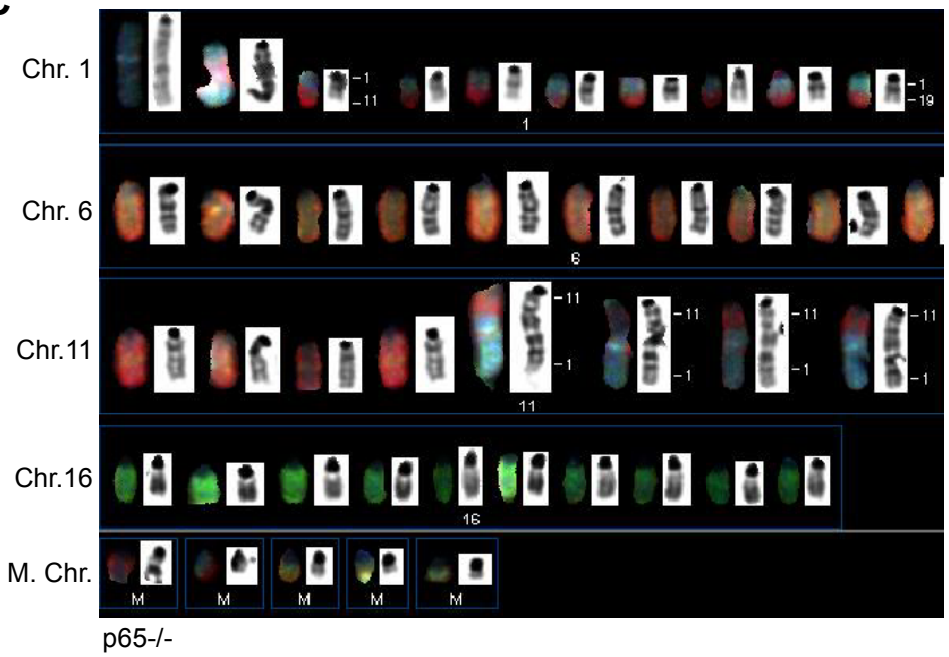
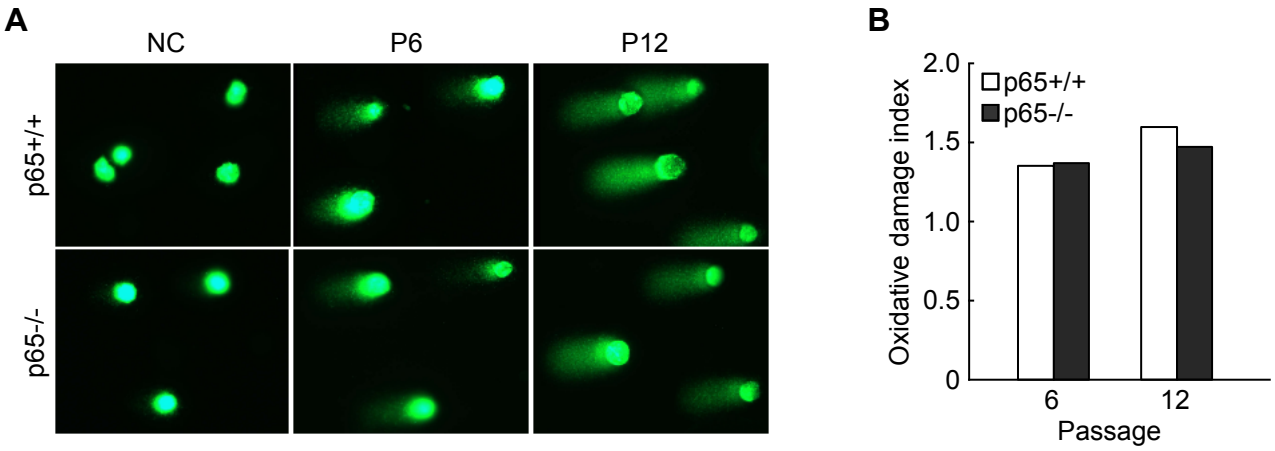


B

Summary of SKY analysis in immortalized *p65*+/+ and *p65-/-* MEFs P = 42

Genotype	Chromosome translocations				Aneuploidy				Marker Chr.	Total metaphases
	none	1-2	3-4	>5	3N	4N	5N	>8N		
p65+/+	14	16	0	0	10	12	8	0	0/30	30
p65-/-	0	0	2	21	0	2	19	2	5/23	23

Wang_Supplemental Figure 4



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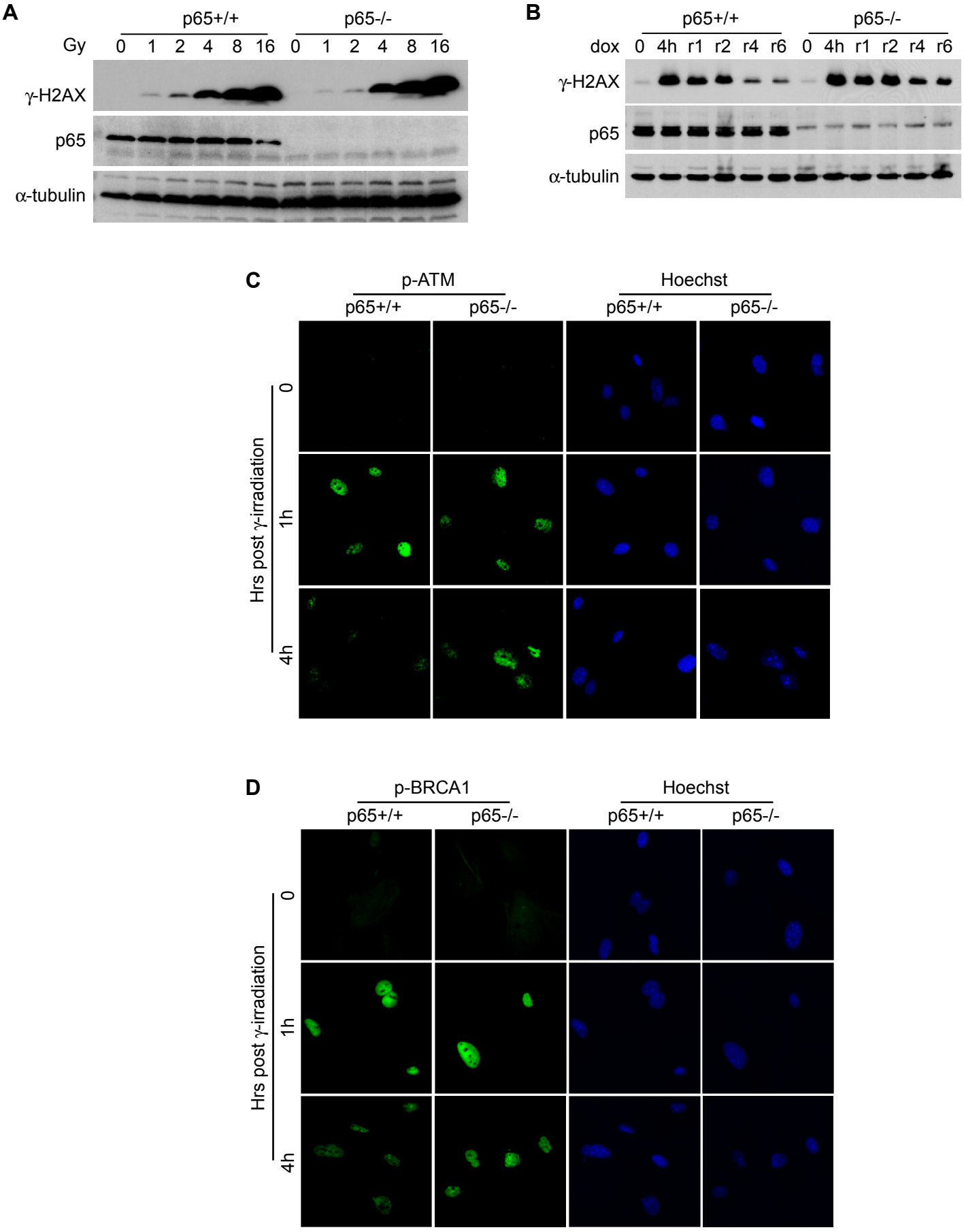

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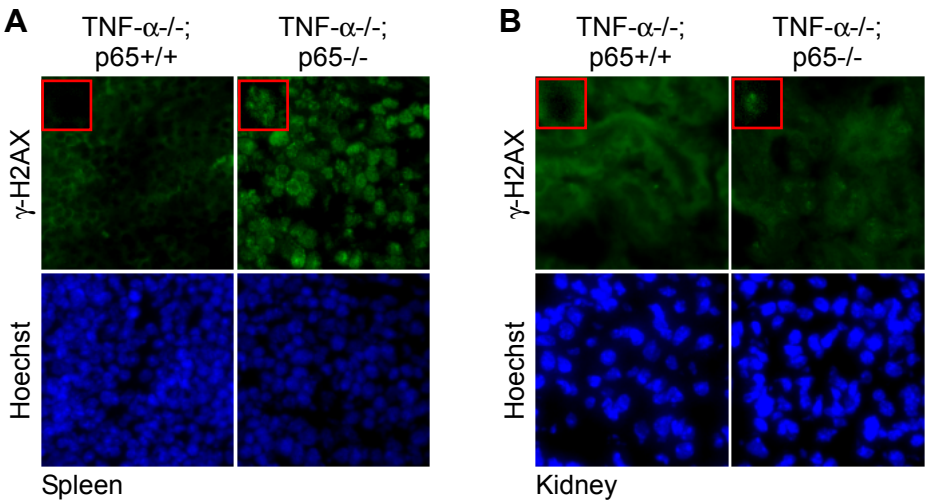

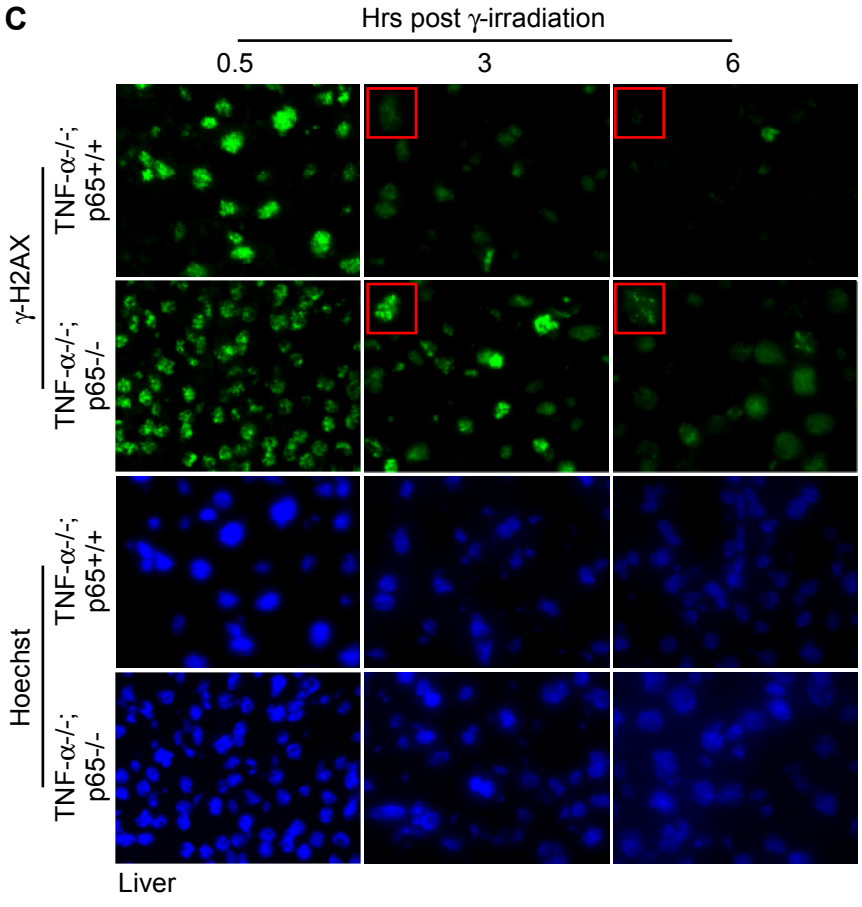

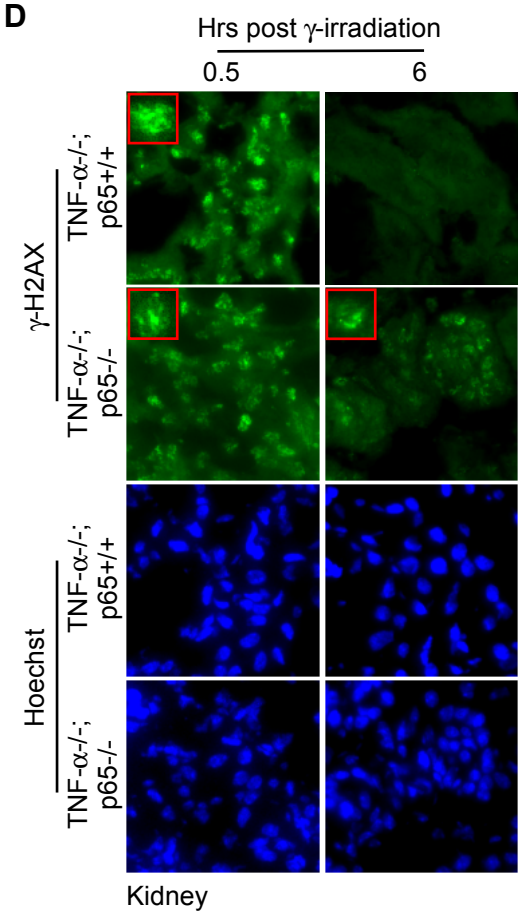

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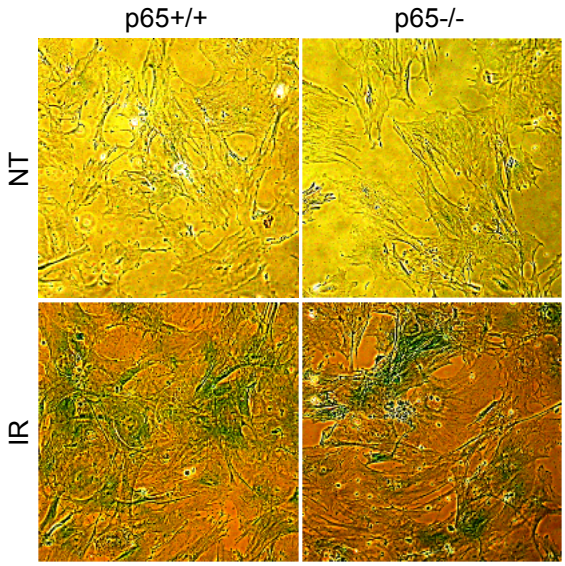

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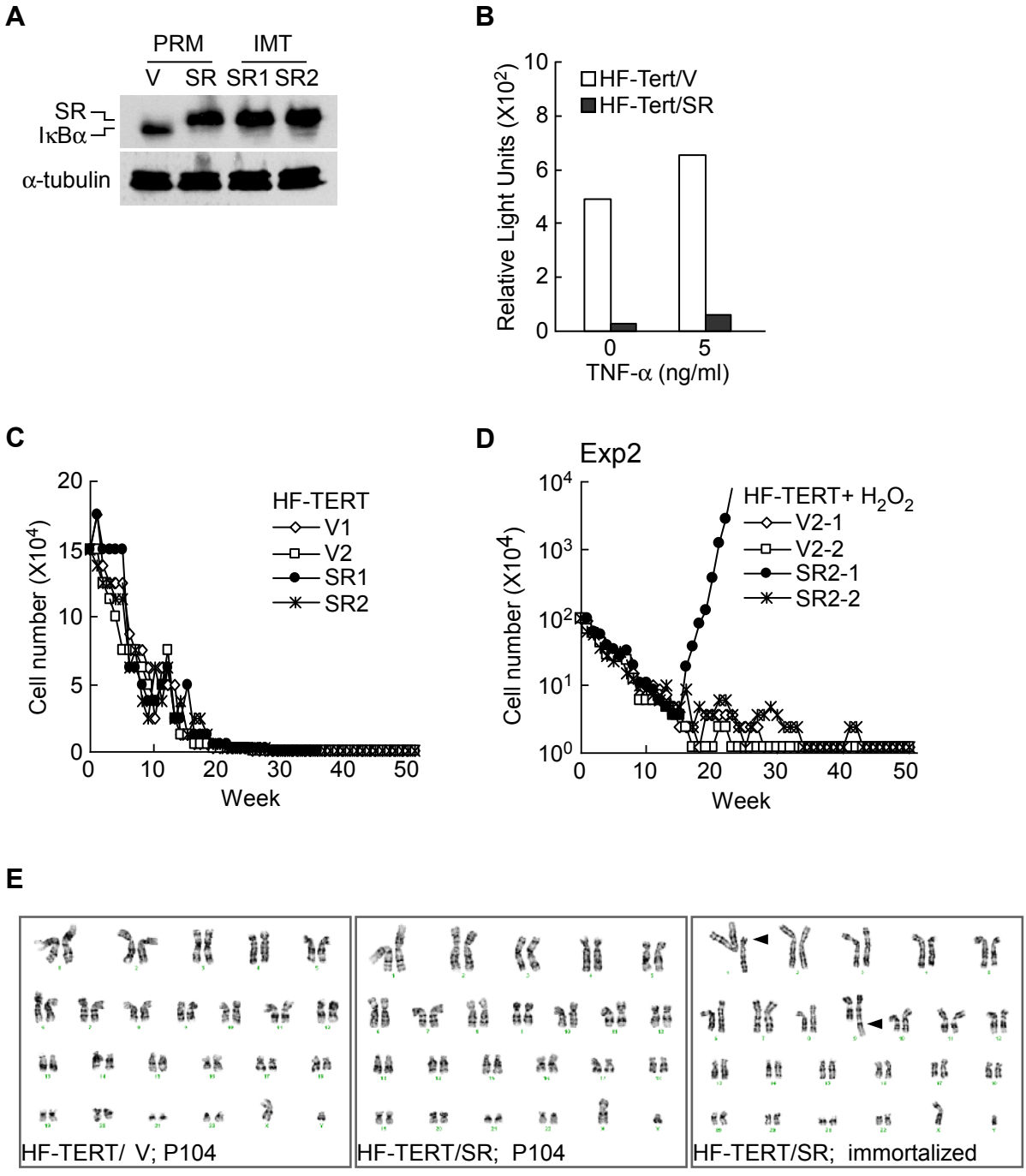

Supplemetal Table 1: Partial list for the recurrent chromosome 9q34 aberrations in human malignancies (Derived from Mitelman breakpoint report; http://www.ncbi.nlm.nih.gov/)

Abnormality	Morphology	Topography	Cases	Genes
ins(22;9)(q11;q34q34)	Chronic myeloid leukemia		15	BCR/ABL1,BCR+
ins(9;22)(q34;q11q11)	Chronic myeloid leukemia		13	BCR/ABL1
t(1;9)(p32;q34)	Chronic myeloid leukemia		2	
t(1;9;22)(p22;q34;q11)	Chronic myeloid leukemia		2	BCR+
t(1;9;22)(p34;q34;q11)	Chronic myeloid leukemia		2	BCR/ABL1
t(1;9;22)(p36;q34;q11)	Chronic myeloid leukemia		12	BCR/ABL1,BCR+
t(3;9;22)(p21;q34;q11)	Chronic myeloid leukemia		13	BCR/ABL1
t(4;9)(p12;q34)	Chordoma	Skeleton	2	
t(4;9;22)(p16;q34;q11)	Chronic myeloid leukemia		5	BCR/ABL1
t(4;9;22)(q21;q34;q11)	Chronic myeloid leukemia		7	BCR+
t(5;9;22)(q13;q34;q11)	Chronic myeloid leukemia		10	BCR/ABL1,BCR+
t(6;9)(p23;q34)	Acute myeloblastic leukemia (FAB type M2)		32	
t(6;9)(p23;q34)	Acute myeloid leukemia, NOS		22	
t(6;9)(p23;q34)	Acute myelomonocytic leukemia (FAB type M4)		24	
t(6;9;22)(p21;q34;q11)	Chronic myeloid leukemia		12	BCR+,BCR/ABL1
t(9;22)(q34;q11)	Acute lymphoblastic leukemia/lymphoblastic lymphoma		815	BCR/ABL1,BCR+
t(9;22)(q34;q11)	Acute myeloblastic leukemia (FAB type M2)		45	BCR/ABL1,BCR+
t(9;22)(q34;q11)	Acute myeloblastic leukemia (FAB type M1)		42	BCR/ABL1,BCR+
t(9;22)(q34;q11)	Acute myeloid leukemia, NOS		55	BCR/ABL1,BCR+
t(9;22)(q34;q11)	Bilineage or biphenotypic leukemia		69	BCR/ABL1
t(9;22)(q34;q11)	Chronic myeloid leukemia, t(9;22)		1668	ABL1+,BCR/ABL1,BCR+
t(9;22;11)(q34;q11;q13)	Chronic myeloid leukemia, aberrant translocation		13	BCR/ABL1,BCR+,GSTP1+
add(9)(q34)	Adenocarcinoma	Kidney	7	
add(9)(q34)	Adenocarcinoma	Ovary	11	
add(9)(q34)	Adenocarcinoma	Lung	3	
add(9)(q34)	Adenocarcinoma	Breast	4	
add(9)(q34)	Astrocytoma, grade III-IV	Brain	4	
add(9)(q34)	Endometrial stromal sarcoma	Uterus, corpus	2	
add(9)(q34)	Ependymoma	Cerebellum	2	
add(9)(q34)	Leiomyosarcoma	Soft tissue	2	
add(9)(q34)	Malignant melanoma	Eye	3	
add(9)(q34)	Malignant peripheral nerve sheath tumor/Triton	Soft tissue	2	
add(9)(q34)	Mantle cell lymphoma		6	
add(9)(q34)	Mature B-cell neoplasm, NOS		11	
add(9)(q34)	Multiple myeloma		16	
add(9)(q34)	Osteosarcoma, NOS	Skeleton	5	
add(9)(q34)	Seminoma/Dysgerminoma	Testis	2	
add(9)(q34)	Squamous cell carcinoma	Larynx	2	
add(9)(q34)	Undifferentiated carcinoma, large cell	Lung	2	

Supplemental Table 2. Primer sequences and PCR conditions:

LacZ gene full length	forward	CGGGATCCGAGTTTGTCAGAAAGCAGACCAA
	reverse	CCCAAGCTTAGGAGGAAGTCACCATGTCGTTTACTTTGACCA
	PCR	95ºC, 30''; 63ºC, 30'', 72ºC, 4'; Repeat 30 cycles
LacZ real time PCR(DNA)	forward	TGATTTCAGCCGCGCTGTACT
	reverse	TTCGGGTTTTCGACGTTCAGA
GAPDH real time PCR (DNA)	forward	TCTCATGCCGTGTGAAATGC
	reverse	GCACACCGTCTGAGGACGTT
All Real Time PCR		95ºC, 10' then (95ºC, 30''; 60ºC, 30''; 72ºC, 30''; Repeat 40 cycles)