CHAPTER 5. CELL VIABILITY AND CHROMOSOME REARRANGEMENTS AFTER GAMMA IRRADIATION

5.1 Introduction

When a genetic screen is conducted it is critical to analyse enough independent mutations to recover genes in the pathway of interest. The chance of recovering a mutation will depend on the complexity of the library. Ideally independent mutations in the same gene will be recovered since this will help to validate the role of that gene in the specific pathway. An average complexity of 5 mutations per gene is adequate for most purposes.

The complexity of the library will depend on the number of mutations per cell and the number of cells in the library. In this chapter, experiments were conducted to measure ES cell survival rates and mutation frequencies after irradiation. Cell viability data at a series irradiation doses enables the complexity of the mutation pools to be established. A whole mutation library is ideally composed of separated mutation pools. There are two advantages to do this.

First, independent mutations at the same locus can be distinguished quickly. It is known that irradiation can generate mutations at a rate of ~10^{-4} per locus per generation in mice (Asakawa *et al.* 2007; Malling *et al.* 1977). Assuming the same mutation rate, it can be assumed that approximately ten thousand clones can provide enough mutations to cover the whole mouse genome once. If a genetic screen is designed and implemented properly in mutation libraries, in which mutations cover the whole genome several times, individual mutant cells with different mutations at the same locus can be identified. A fast way to distinguish that these are different is to conduct a screen in separated mutation pools so that mutants are independent between pools. Mutant cells with the same gene mutated can be quickly recognized as independent if they are from separated mutant pools.

Second, screening in separated mutation pools prevents daughter cells with one mutant allele to be dominant among the isolated mutants. As I use *Blm*-deficient ES cells to generate homozygous mutations through the mechanism of loss of heterozygosity (LOH), LOH events may occur at any time during the cell culture. When a homozygous mutant cell occurs several generations earlier than the others, it will become dominating in the homozygous mutant population after several cycles. As a result, it will be more difficult to

identify most of the other mutants. If mutants are segregated and replicate within smaller mutant pools, a single mutant cell can only be dominant in one specific pool, without making it difficult to identify mutations in other mutant pools.

Experiments in this chapter also characterized the size and frequency of deletions produced by irradiation in 129 inbred mouse ES cells. It is known that irradiation generates chromosome rearrangements, for instance amplifications and deletions. Deletions are required to generate loss-of-function mutations. Deletions generated by irradiation had been examined in a number of species including *Drosophila* and mice by cytogenetic analysis (Brewen *et al.* 1973; Searle *et al.* 1974; Traut *et al.* 1971). More recently the negative selection cassette Herpes Simplex Virus type 1 thymidine kinase (*HSV-tk*) was targeted to specific sites in mouse ES cells and deletion sizes were measured after irradiation and negative selection (Chick *et al.* 2005; Thomas *et al.* 1998; You *et al.* 1997). However, these studies limited the analysis to specific loci. If any local genes are essential for cell viability, cells with these genes deleted cannot survive thus they will not be recovered for analysis, thus the deletion size may be biased. Additionally, it is not known the size and number of deletions that can be generated in a single ES cell genome. To answer this question, both wild type cells and *Blm*-deficient cells were irradiated at a series of dosages. The resulting chromosome deletions, as well as amplifications were analysed using a 200 kb resolution comparative genome hybridization (CGH) array. This technology is used in human genetics (Oostlander *et al.* 2004; van Beers *et al.* 2006). For instance array CGH was used to identify copy number variations among 270 healthy human beings, from four populations with ancestry in Europe, Africa or Asia (Redon *et al.* 2006). Considering the availability coverage and resolution, a 200 kb resolution array CGH was used in this study.

5.2 Results

5.2.1 Cell viability after irradiation

To measure cell viability after gamma irradiation, experiments were performed on cells with different genotypes and with a series of irradiation dosages. The AB1, AB2.2 and the *Blm*$^{-/-}$ cells were derived from the 129 S7/SvEv mouse. Here I consider the AB1 and AB2.2 cell lines to be as wild type cells, while the *Blm*-deficient ES cells are the NGG5-3 cells from Guo, G., derived from AB2.2 (129 S7/SvEv$^{\text{Brd-}Hprt\text{b-m2}}$) with the genotype *Blm*(m3/m4), *Gdf9*(m1/m3).

Cell viability was assessed by a colony survival assay. When cells grew to confluence on 90

mm feeder plates, they were collected by trypsinization and irradiated for different lengths of time and then re-plated onto 30 mm feeder plates. Since the percentage of cells surviving varied over several orders of magnitude, the cells were plated at different densities. Non-irradiated cells were plated at 500 cells per 30 mm well to determine their plating efficiency. After staining surviving colonies, plating efficiency was calculated as: Plating efficiency = Number of survival colonies/500. Plating efficiencies differed between independent experiments. Usually the plating efficiency was between 20-44% (Table 5-1).

The survival frequency of irradiated cells was calculated as: Survival frequency = Number of survival colonies/(Number of plated cells × plating efficiency). Survival frequency was normalized to 100% if the cells were unirradiated. Survival rates of the wild type cells and the *Blm*-deficient cells of different experiments are shown in Table 5-2 and Table 5-3, respectively and are summarized in Table 5-4. Survival rates of both types of cells were around 20% at 2 Gray, 10% at 3 Gray, 5% at 4 Gray, 3% at 5 Gray, 1% at 7 Gray and 0.1% at 10 Gray. In general, *Blm*-deficient cell are more resistant to irradiation at all doses, which can be seen in Figure 5-1.

Table 5-1 ES cell plating efficiency

Experiment	Number of survival colonies	Plating efficiency	Average plating efficiency
1	104	0.21	0.21
2	103	0.21	0.21
3	127	0.20	0.20
4	193	0.44	0.44
5	134	0.27	0.27
6	161	0.32	0.32
7	120	0.24	
	136	0.27	0.26
8	131	0.26	
	107	0.21	0.24
9	124	0.25	
	133	0.27	0.26
10	147	0.29	
	162	0.32	0.31
		Total average	**0.27**

ES cell plating efficiency was calculated in ten experiments. Experiments 7–10 have two replicated wells of cells. In each 30mm well, 500 cells were plated in normal M15 media and cultured for 10 days. Survival colonies were stained by methylene blue. The plating efficiency = Number of survival colonies/500. Survival rates of the wild type cells and the *Blm*-deficient cells were calculated in the experiments 4, 5, 6 and 10.

Table 5-2 Wild type ES cell survival rates following 0-10 Gray gamma irradiation

Dose (Gy)	Cells plated	Cells surviving	Plating efficiency	Survival rate	Average survival rate	SD
0	–	–	–	100%	100%	–
2	2000	107	31%	17%		
		111		18%		
		118		19%	18%	0.01
3	2000	49	31%	8%		
		56		9%		
		79		13%	9.9%	0.025
4	5000	65	31%	4.2%		
		68		4.4%		
		81		5.2%	4.6%	0.005
5	10^4	76	31%	2.5%		
		82		2.6%		
		96		3.1%	2.7%	0.003
7	5×10^4	80	32%	0.50%	0.50%	–
10	10^5	3	31%	0.01%		
		12		0.04%		
		18		0.06%		
	10^5	18	32%	0.06%		
		19		0.06%		
		25		0.08%	0.05%	0.0002

Survival rates of the wild type cells (AB1 and AB2.2) after irradiation at 2–10 Gray are shown. Survival frequency was normalized to 100% if the cells were unirradiated. Three–six independent experiments were performed at 2, 3, 4, 5 and 10 Gray. Standard deviations (SD) of the survival rates at these doses are also shown. One experiment was performed at 7 Gray. Plating efficiencies were calculated from experiment 6 and 10 (Table 5-1).

Table 5-3 ***Blm*-deficient ES cell survival rates following 0-10 Gray gamma irradiation**

Dose (Gy)	Cells plated	Cells surviving	Plating efficiency	Surviving rate	Average survival rate	SD
0	–	–	–	100%	100%	–
2	2000	220	44%	25%		
		220		25%		
		238		27%	26%	0.01
3	2000	157	44%	18%		
		159		18%		
		188		21%	19%	0.02
4	5000	175	44%	8.0%		
		186		8.5%		
		191		8.7%	8.4%	0.004
5	10^4	173	44%	3.9%		
		174		4.0%		
		184		4.2%	4.0%	0.001
7	5×10^4	221	27%	1.6%	1.6%	–
10	10^5	14	44%	0.03%		
		29		0.07%		
		60		0.14%		
	10^5	37	27%	0.14%		
		38		0.14%		
		40		0.15%	0.11%	0.0005

Survival rates of the *Blm*-deficient cells (NGG5-3) after irradiation at 2–10 Gray are shown. Survival frequency was normalized to 100% if the cells were unirradiated. Three–six independent experiments were performed at 2, 3, 4, 5 and 10 Gray. Standard deviations (SD) of the survival rates at these doses are also shown. One experiment was performed at 7 Gray. Plating efficiencies were calculated from experiment 4 and 5 (Table 5-1).

Table 5-4 Summary of ES cell survival rates following gamma irradiation

Dose (Gray)	0	2	3	4	5	7	10
WT (AB1/AB2.2)	100%	18±1%	9.9±2.5%	4.6±0.5%	2.7±0.3%	0.50	0.05±0.02%
***Blm*$^{-/-}$ (NGG5-3)**	100%	26±1%	19±2%	8.4±0.4%	4.0±0.1%	1.6	0.11±0.05%

The survival rates ± standard deviation (SD) of the wild type cells (AB1/AB2.2) and the *Blm*-deficient cells (NGG5-3) are compared at different doses of gamma irradiation. Survival frequency was normalized to 100% if the cells were unirradiated. Data from Table 5-2 and Table 5-3. n≥3 (except at 7 Gray).

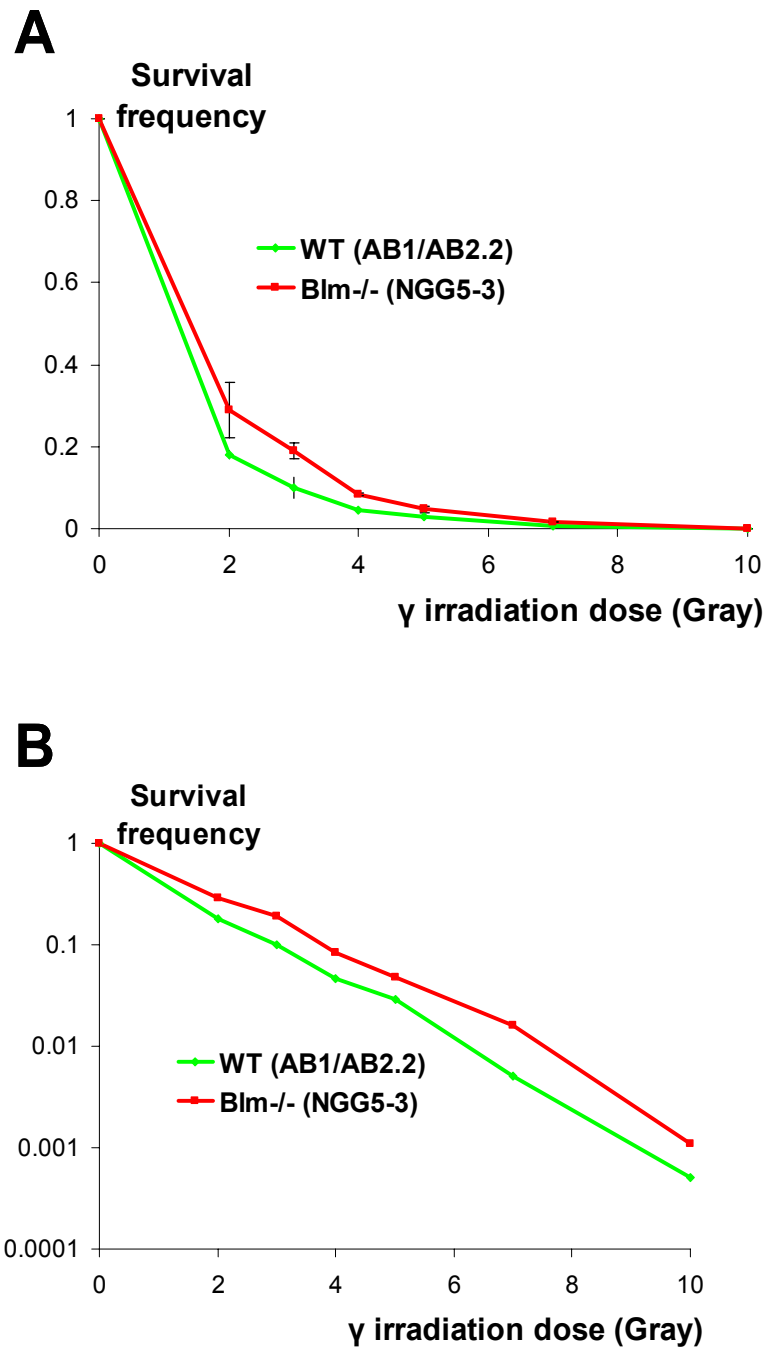

Figure 5-1 Gamma irradiation caused viability change in ES cells

Linear (**A**) and logarithmic (**B**) plots of survival rates. Error bars in (**A**) indicate standard deviation. WT, wild type ES cells (AB1 or AB2.2); *Blm*$^{-/-}$, *Blm*-deficient AB2.2 ES cells (NGG5-3).

5.2.2 Irradiation generates megabase sized chromosomal alterations

To measure large chromosome amplifications and deletions generated by gamma irradiation, both wild type (ZK6) and *Blm*-deficient cells (NGG5-3) were irradiated at 2, 5 and 7 Gray. The *Blm*-deficient NGG5-3 cell was also irradiated at 10 Gray. After irradiation, single colonies were isolated and expanded to extract DNA, which was analysed on the 200 kb resolution CGH array. The cost of array CGH is high, thus only total 11 samples of wild type cells and 15 samples of the *Blm*-deficient cells were analysed. The following samples were analysed: clones WT2G(4), WT5G(4) and WT7G(3) were wild type cells irradiated at 2, 5 and 7 Gray, respectively; clones Blm2G (3), Blm5G(3), Blm7G(3) and Blm10G(6) were the *Blm*-deficient cells irradiated at 2, 5, 7 and 10 Gray, respectively. The reference DNA used for these experiments was from ZK6 or NGG5-3 cells passaged immediately before irradiation.

To obtain convincing data, a dye-swap hybridization was necessary to be performed for each sample. By using the BlueFuse software, the Log_2(sample DNA signal)/(reference DNA signal) or the Log_2(reference DNA signal)/(sample DNA signal) of each BAC probe were measured. The CGH array contains 18,294 unique BAC probes, however, signals of BAC probes with high variations in the dye-swap arrays were excluded from the analysis. The Log_2(ratio)s of high quality signals from BAC probes were plotted against their position in the mouse genome. Considering the Log_2(sample DNA signal)/(reference DNA signal) is -1 ($Log_2(1/2)$) for a heterozygous deletion in a diploidy cell and -0.42 ($Log_2(3/4)$) for a deletion in a tetraploidy cell, it was necessary to set up threshold to distinguish deletions, duplications (amplifications) and unchanged DNA. Generally, we used the thresholds of 0.2999 and -0.2999 of Log_2(ratio) for nomal array analysis in our laboratory. These thresholds can leave buffer space between Log_2(ratio) of unchanged DNA and duplications/deletions. The Log_2(ratio) above 0.2999 indicates a duplication or an amplification. The Log_2(ratio) between -0.2999 and +0.2999 indicates unchanged DNA copy number. The Log_2(ratio) below -0.2999 indicates a deletion.

The array profile of the clones WT2G2, WT5G4, WT7G2, Blm2G2, Blm5G1, Blm7G1 and Blm10G1 are shown in Figure 5-2 to Figure 5-8. The copy number changes of these clones and the other clones are summarized in Table 5-5 and Table 5-6. In the wild type cells irradiated at 2–7 Gray (Table 5-5), 9% (1/11) clones carry large deletions. Deletions are usually heterozygous, ranging from 0.1 (detection limit of the array CGH) to 5 Mb. However, deletions as small as 0.1 Mb are detected through the copy number change of a single BAC

probe, thus this type of deletions are not very convincing. About one third of wild type cells surviving 7 Gray irradiation carry deletions, and none of the eight wild type cells surviving 2–5 Gray irradiation contains detectable deletions. About 27% (3/11) of the wild type cells irradiated at 2–7 Gray have duplications, ranging from 0.2–14 Mb. None of the four wild type clones surviving 2 Gray irradiation carries duplications.

Besides of the normal heterozygous deletions and duplications, array CGH can also detect other types of chromosomal rearrangements including whole chromosome gain/loss or segmental chromosomal gain/loss (Table 5-5 and Table 5-6). Thus, in the array analysis of the irradiated cells, these types of chromosomal rearrangement, which occurred spontaneously or induced by irradiation, can also be anticipated to be observed. The mutant cell WT2G2 may be a tetraploid because the Log_2(ratio) of the whole chromosomes 2 and 5 is near -0.2 and that of the chromosome 11 is near 0.2, suggesting the chromosomes 2 and 5 are probably 3N and the chromosome 11 is 5N. However, the Log_2(ratio) of 0.2 is also lower than 0.32, the value of Log_2(5/4), thus the WT2G2 cells might be mosaic. Similar chromosomal abnormalities were also observed in Blm5G1, Blm5G2, Blm5G3, Blm7G3, Blm10G4, Blm10G5 and Blm10G6. The chromosome Y in the mutant WT7G2, Blm7G1, Blm10G5 and Blm10G6 were lost and some one- to two-BAC-sized deletions in other chromosomes are associated with the loss of chromosome Y. The genomic loci of these BACs may not be correct thus need to be relocated to chromosome Y. On the contrary, the mutant of Blm2G3 has one more copy of chromosome Y, with the average Log_2(ratio) of about 1, than the *Blm*-deficient cells.

About 62% (7/11) of the wild type cells irradiated at 2–7 Gray does not carry any chromosome rearrangements. In the irradiated *Blm*-deficient ES cells (Table 5-6), 53% (8/15) of 2–10 Gray irradiated cells have heterozygous deletions, ranging from 0.3–46 Mb. The frequency of deletions is higher in 7 and 10 Gray irradiated cells (67%, 6/9) than in 2 and 5 Gray irradiated cells (33%, 2/6). Sixty-seven per cent (10/15) of 2–10 Gray irradiated *Blm*-deficient cells obtain duplications, ranging from 0.1–24 Mb. Considering deletions and duplications together, thirty-three per cent (5/15) cells contain the both types of chromosome rearrangements after 2–10 Gray irradiation, and 93% (14/15) of the 2, 5, 7 and 10 Gray irradiated *Blm*-deficient cells have either deletions or duplications or both.

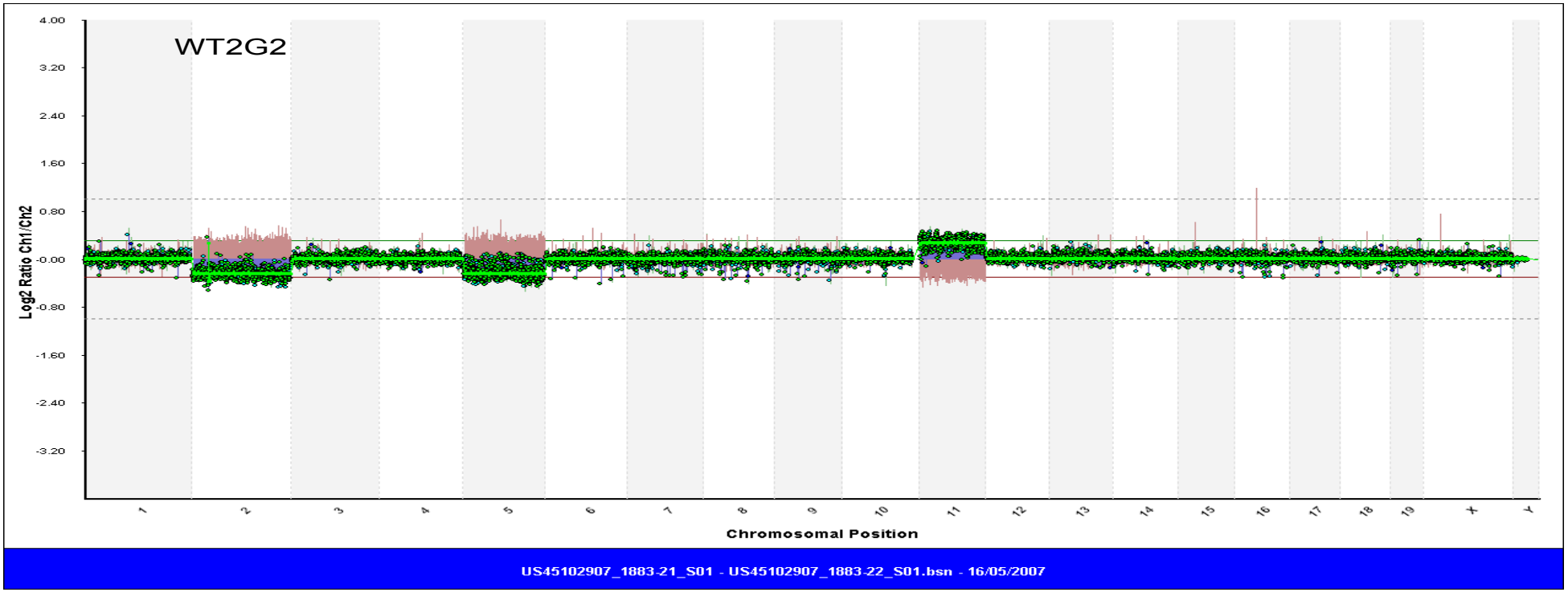

Figure 5-2 Array CGH analysis on WT2G2 – a wild type clone surviving 2Gy irradiation

BACs were aligned on the X axis in ascending order according to their genomic position in every chromosome. Chromosomes were also placed in ascending order followed by X and Y. A dot indicates the middle point position of a given BAC. The Y axis indicates the Log2(mutant DNA signal/reference DNA signal) of a given BAC probe. This Log_2(ratio) indicates a duplication (amplification) when it is above +0.29 or a deletion when it is below -0.29. Green lines were smoothly connected between the values of Log2(Mutant DNA signal/Reference DNA signal) of BAC clone probes. The red line indicates Log2(Reference DNA signal/Mutant DNA signal) of BAC clone probes from the reciprocal hybridization experiment. The grey dotted lines are for Log2(ratio) references of either +1 or -1. This clone might be a tetraploid because the Log2(ratio) of chromosomes 2, 5 and 11 are near either 0.3 or -0.3, indicating the signal ratio of sample to reference is close to 5/4 or 3/4. In the 4N genome, one copy of the chromosomes of 2 and 5 is probably lost and one copy of chromosome 11 is probably duplicated.

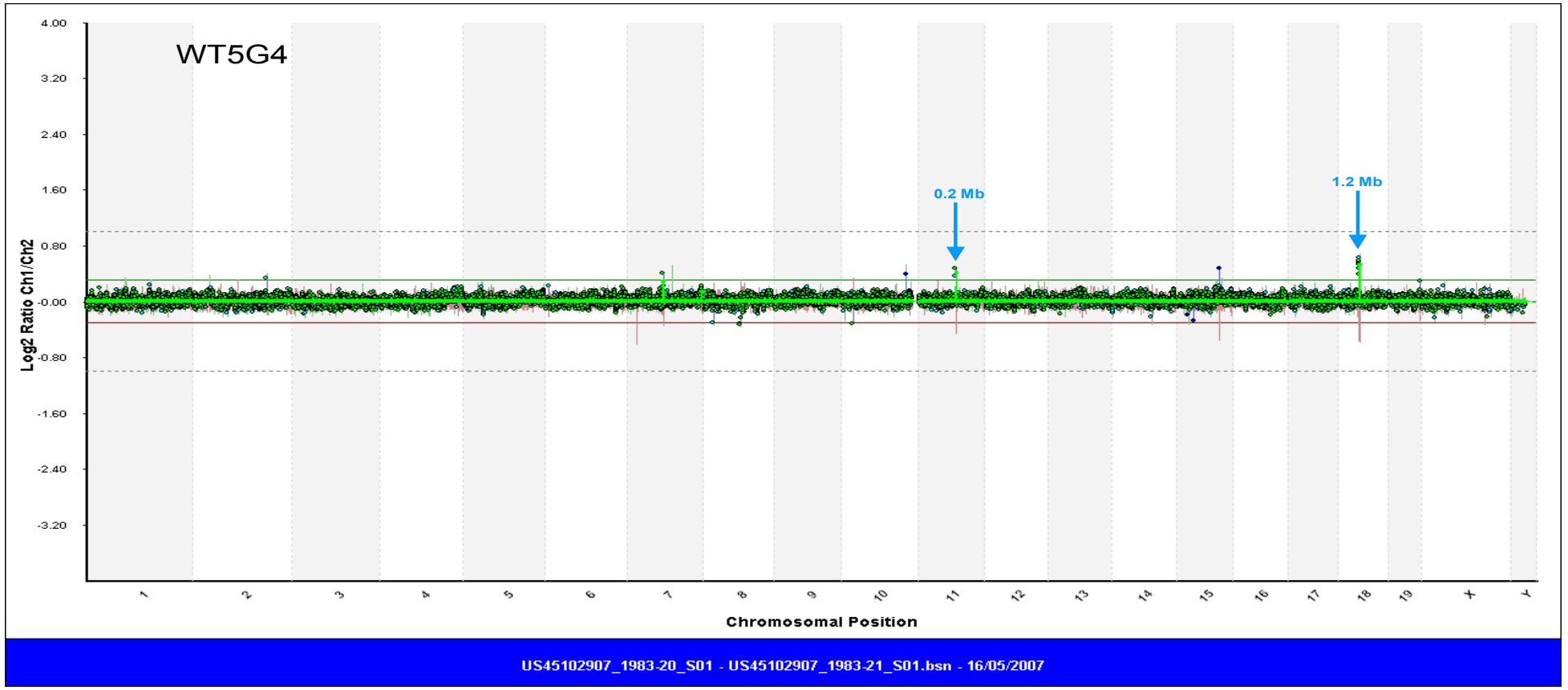

Figure 5-3 Array CGH analysis on WT5G4 – a wild type clone surviving 5Gy irradiation

The locations of two duplications of the clone WT5G4 are indicated by blue arrows. One is on chromosome 11 (0.2 Mb) and the other is on chromosome 18 (1.2Mb).

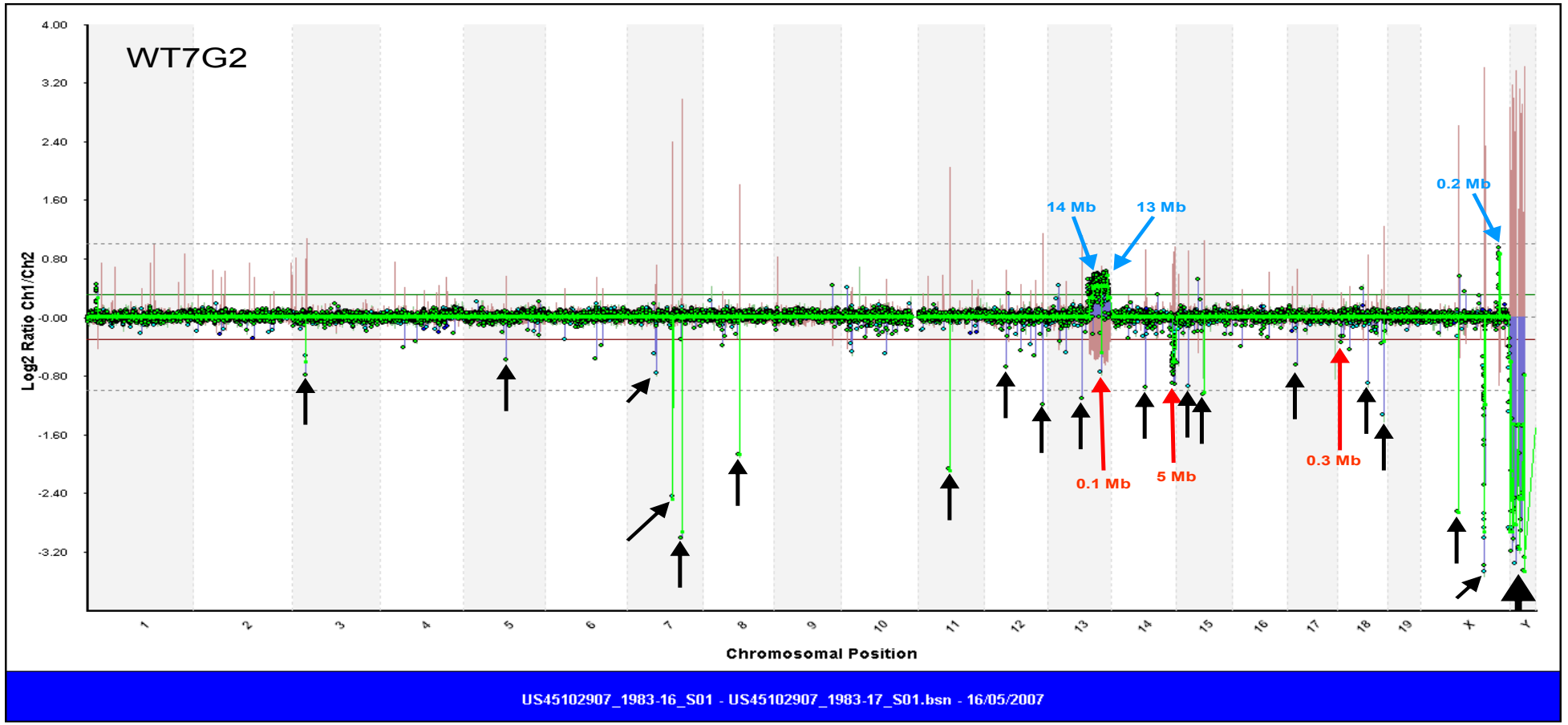

Figure 5-4 **Array CGH analysis on WT7G2 – a wild type clone surviving 7Gy irradiation**

The locations of the deletions and duplications of the clone WT7G2 are indicated by red arrows and blue arrows, respectively. Three heterozygous deletions are on chromosome 13 (0.1 Mb), 14 (5 Mb) and 18 (0.3 Mb). Two duplications are on chromosome 13 (13 and 14 Mb) and one is on chromosome X (0.2 Mb). A two-BAC-sized deletion on chromosome 3 (black arrow) and many one-BAC-sized deletions (black arrows) on chromosomes 5, 7, 8, 11, 12, 13, 14, 15, 17, 18 and X are probably associated with the loss of chromosome Y because the same pattern of BAC deletions were also observed in other mutant clones, which lost chromosome Y.

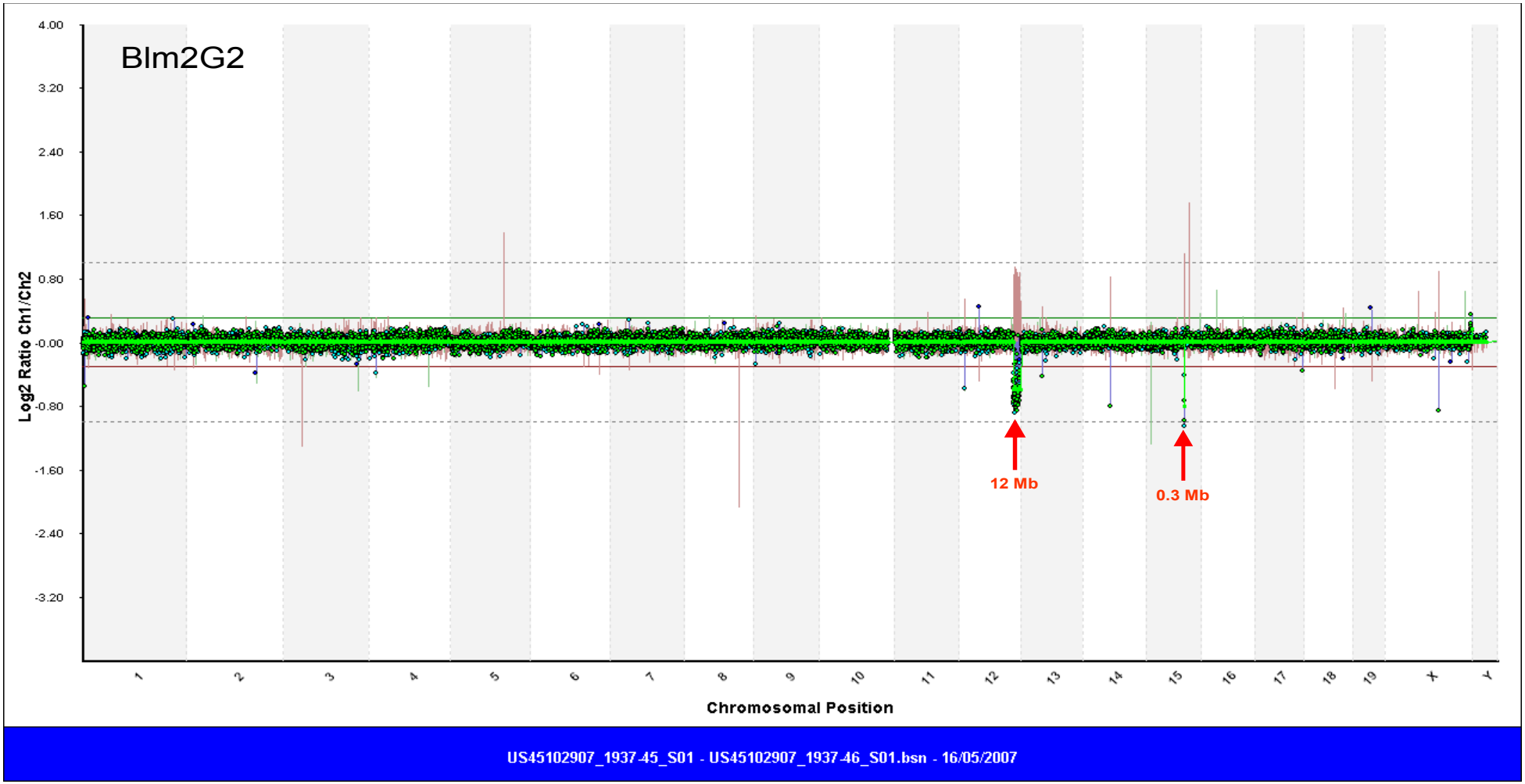

Figure 5-5 **Array CGH analysis on Blm2G2 – a *Blm*$^{-/-}$ clone surviving 2 Gy irradiation**

The locations of two heterozygous deletions of the clone Blm2G2 are indicated by red arrows. One is on chromosome 12 (12 Mb) and the other is on chromosome 15 (0.3 Mb).

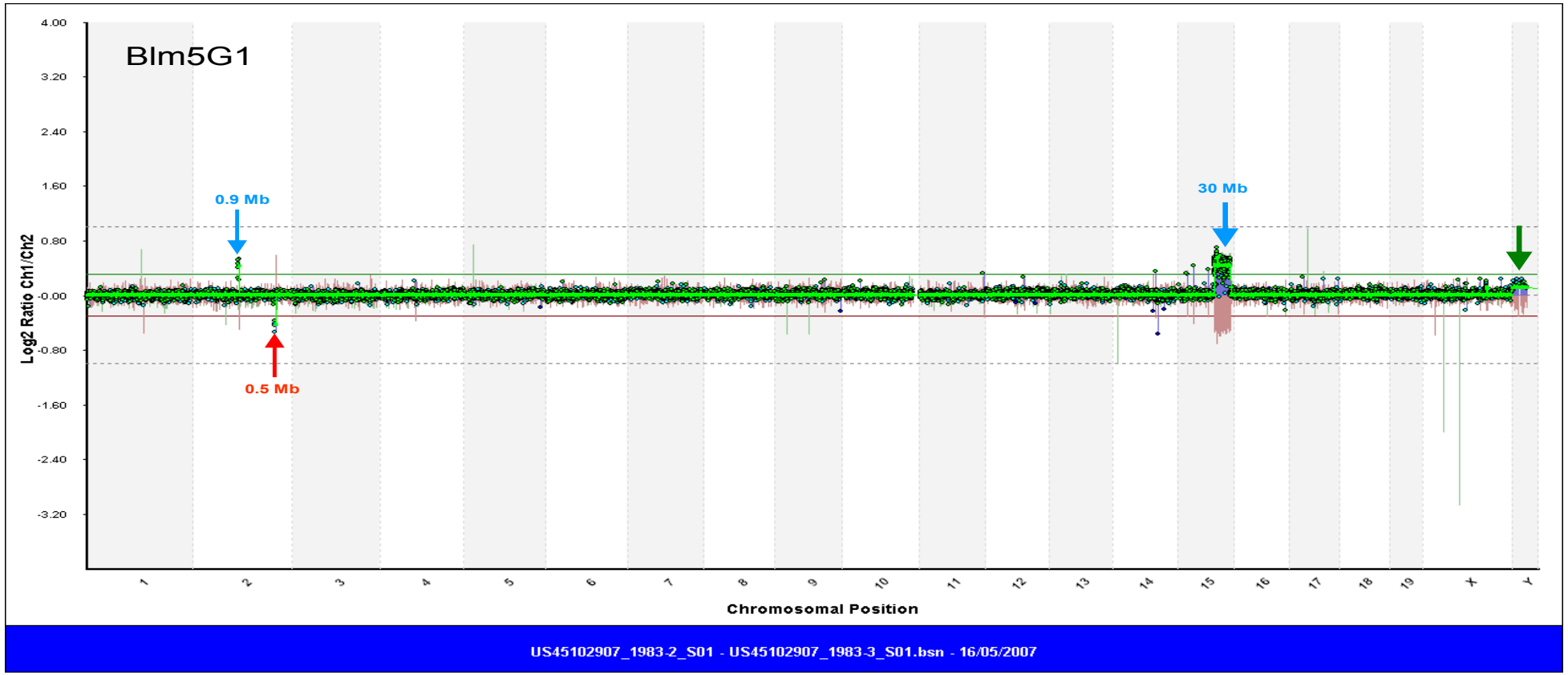

Figure 5-6 **Array CGH analysis on Blm5G1 – a *Blm*$^{-/-}$ clone surviving 5 Gy irradiation**

The locations of deletions and duplications of the clone Blm2G2 are indicated by red arrows and blue arrows, respectively. There are one heterozygous deletion on chromosome 2 (0.5 Mb) and two duplications on chromosome 2 (0.9 Mb) and chromosome 15 (30 Mb). A green arrow shows that the copy number of the clone is slightly higher on chromosome Y than that of the reference cells, indicating this clone is a mosaic population with the cells containing a normal chromosome Y and the cells containing a duplicated chromosome Y.

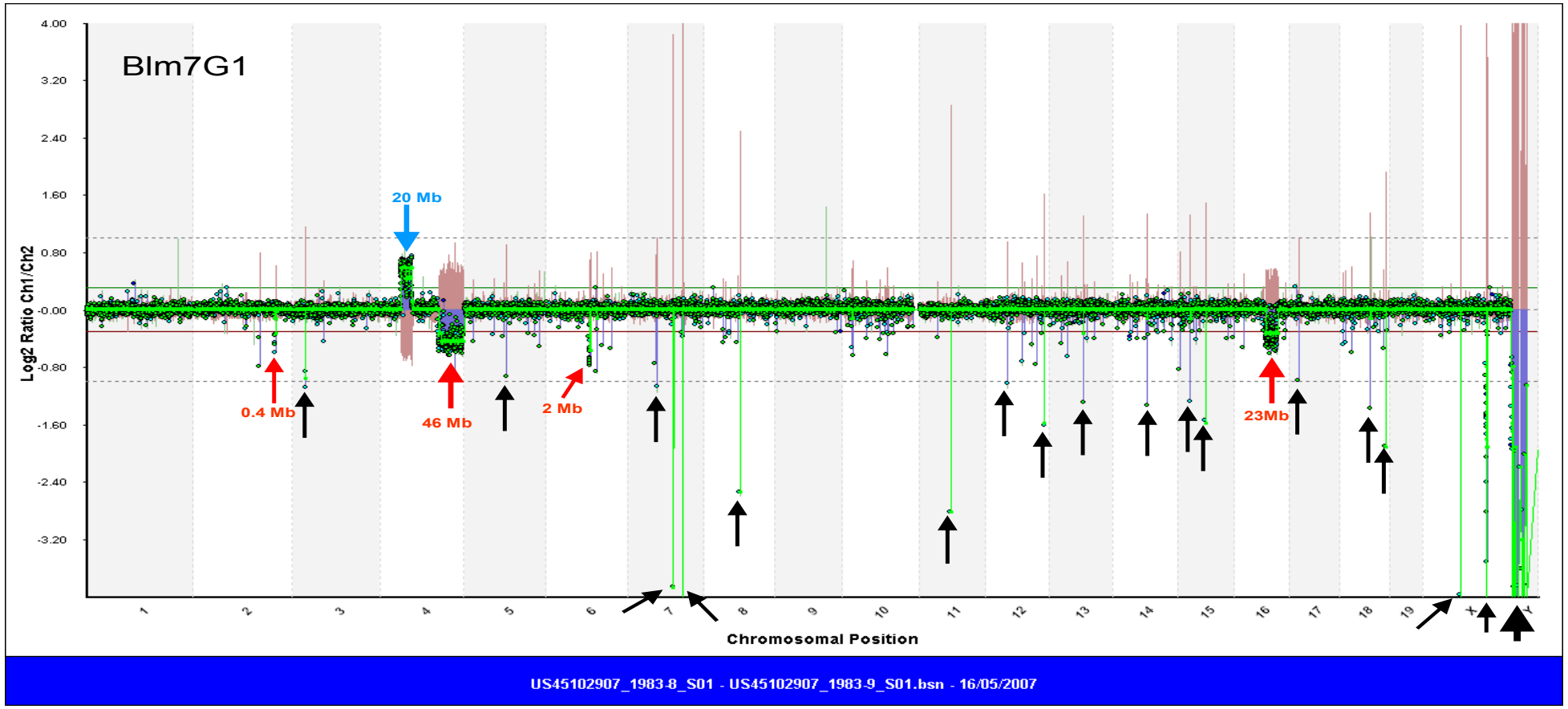

Figure 5-7 **Array CGH analysis on Blm7G1 – a *Blm*$^{-/-}$ clone surviving 7 Gy irradiation**

The locations of the deletions and duplications of the clone Blm7G1 are indicated by red arrows and blue arrows, respectively. Four heterozygous deletions are on chromosome 2 (0.4 Mb), 4 (46 Mb), 6 (2 Mb) and 16 (23 Mb). A duplication is on chromosome 4 (20 Mb). A two-BAC-sized deletion on chromosome 3 (black arrow) and many one-BAC-sized deletions (black arrows) on chromosomes 5, 7, 8, 11, 12, 13, 14, 15, 17, 18 and X are probably associated with the loss of chromosome Y because the same pattern of BAC deletions were also observed in other mutant clones, which lost chromosome Y.

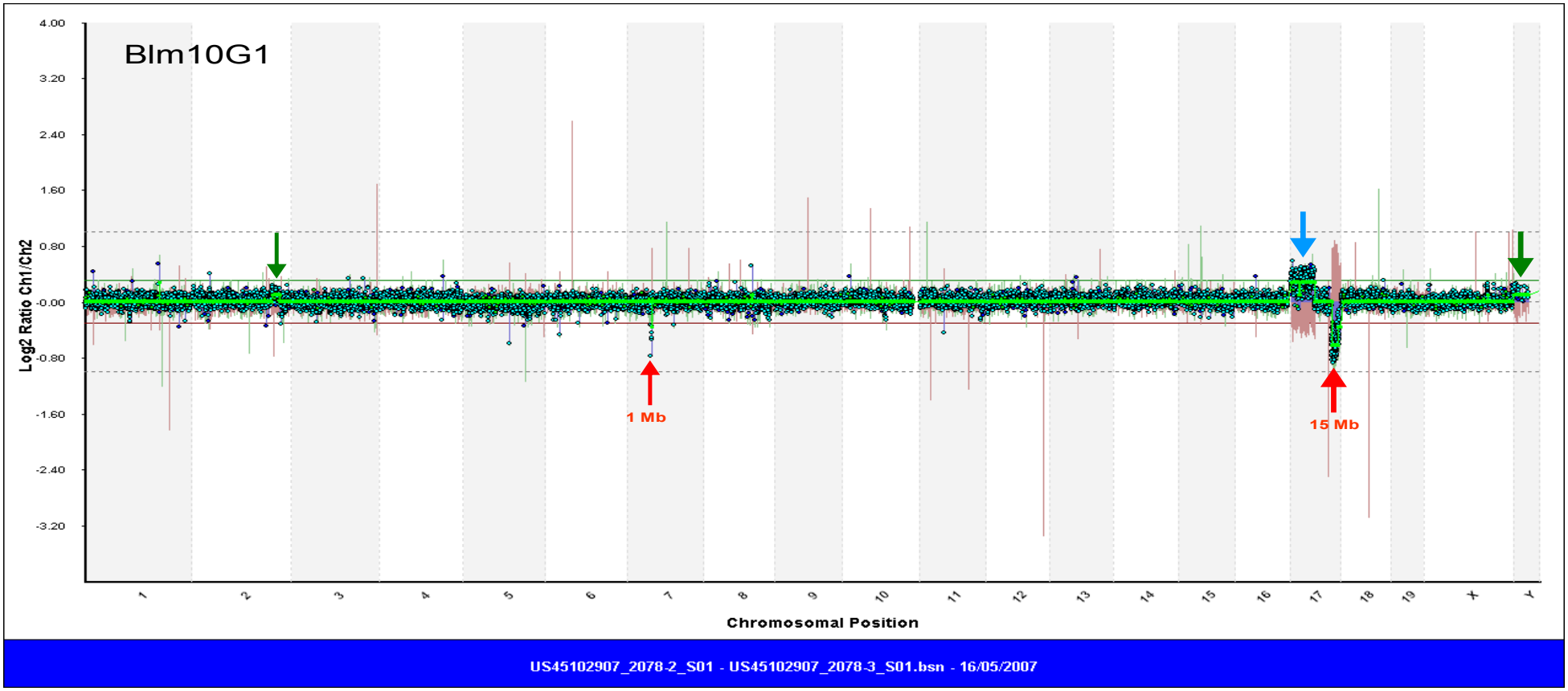

Figure 5-8 **Array CGH analysis on Blm10G1 – a *Blm*$^{-/-}$ clone surviving 10 Gy irradiation**

The locations of deletions of the clone Blm10G1 are indicated by red arrows. Two heterozygous deletions are on chromosome 7 (1 Mb) and chromosome 17 (15 Mb). A blue arrow indicates a segmental change of chromosome 17. The Log_2(ratio) of this region is near 0.3, suggesting the clone might be a tetraploid with a segmental duplication. Two green arrows show that the copy number of the clone is slightly higher in part of chromosome 2 and the whole chromosome Y, indicating this clone is a mosaic population similar to the mutant clone Blm5G1.

Table 5-5 Summary of chromosome changes in wild type cells caused by irradiation

Dose (Gray)	Clone	Number of deletions	Deletion range (Mb)	Abnormal chromosomes Log_2(ratio)<0	Number of duplications	Duplication range (Mb)	Abnormal chromosomes Log_2(ratio)>0
2	WT2G1	0	-	-	0	-	-
	WT2G2	0	-	Ch 2 and 5 (Log_2≈-0.2)	0	-	Ch 11 (Log_2≈0.2)
	WT2G3	0	-	-	0	-	-
	WT2G4	0	-	-	0	-	-
5	WT5G1	0	-	-	0	-	-
	WT5G2	0	-	-	0	-	-
	WT5G3	0	-	-	6	0.03(2), 0.05, 0.08, 0.2(2)	-
	WT5G4	0	-	-	2	0.2, 1.2	-
7	WT7G1	0	-	-	0	-	-
	WT7G2	3	0.1, 0.3, 5	Ch Y (loss)	3	0.2, 13, 14	-
	WT7G3	0	-	-	0	-	-

Data based on selective analysis by BlueFuse software. All BACs with either Log_2(ratio) between -0.29 and +0.29 or standard deviation above 0.2 in fluorescence swap experiments were excluded.

Table 5-6 Summary of chromosome changes in *Blm*$^{-/-}$ cells caused by irradiation

Dose (Gray)	Clone	Number of deletions	Deletion Range (Mb)	Abnormal chromosomes Log_2(ratio)<0	Number of duplications	Duplication Range (Mb)	Abnormal chromosomes Log_2(ratio)>0
2	Blm2G1	0	-	-	0	-	-
	Blm2G2	2	0.3, 12	-	0	-	-
	Blm2G3	0	-	-	7	0.1(2), 0.2(3), 1.5, 1.9	Ch Y (Log_2≈1)
5	Blm5G1	1	0.5	-	2	0.9, 30	Ch Y (Log_2≈0.2)
	Blm5G2	0	-	-	2	1.2, 1.7	Ch Y (Log_2≈0.2)
	Blm5G3	0	-	-	2	0.1, 0.9	Ch Y (Log_2≈0.2)
7	Blm7G1	4	0.4, 2, 23, 46	Ch Y (loss)	1	20	-
	Blm7G2	0	-	-	4	0.1, 0.5, 1.3, 2.1	-
	Blm7G3	2	2, 5.4	Ch 3, 4, 16, 17 and Y (Log_2≈-0.2)	1	0.1	Ch 5, 13 (Log_2≈0.2)
10	Blm10G1	2	1, 15	-	0	-	-
	Blm10G2	0	-	-	1	0.5	-
	Blm10G3	2	2, 13	-	1	0.5	-
	Blm10G4	3	2, 6, 7	Ch 2, 3, 4(part), 7(part), 13(part) and 17(part); (Log_2≈-0.2)	0	-	Ch Y (Log_2≈0.2)
	Blm10G5	2	6, 7	Ch Y (loss)	0	-	-
	Blm10G6	0	-	Ch Y (loss)	1	0.5	-

Data based on selective analysis by BlueFuse software. All BACs with either Log_2(ratio) between -0.29 and +0.29 or standard deviation above 0.2 in fluorescence swap experiments were excluded.

The deletion and duplication data of Table 5-5 and Table 5-6 are summarized in Table 5-7. The chromosomal abnormality is usually caused by other cellular factors instead of DNA strand breaks induced by irradiation. And chromosomal duplication and loss are so large that counting these abnormalities will distract the analysis of the sizes of the duplications and deletions induced by irradiation. Therefore, in these summarized data, abnormal chromosomes are not included. Analysis from both the wild type and the *Blm*-deficient (Table 5-7) cells exhibit that the average 0–2 deletions and 0–2 duplications were generated in each 2–10 Gray irradiated cell. Despite the similarity of the number of deletions between the two cell types, the *Blm*-deficient cells contain much larger deleted regions than that in the wild type cells. About 10–26 Mb DNA sequence was deleted after irradiating the *Blm*-deficient cells at 7 and 10 Gray but only ~3 Mb DNA sequence was deleted after irradiating the wild type cells at 7 Gray. Four out of four wild type cells surviving 2 Gray irradiation do not carry DNA duplications while the average length of duplicated regions per cell is 1.4 Mb in the *Blm*-deficient cells. There are 0.5 Mb (5 Gray) and 9.1 Mb (7 Gray) duplicated chromosome regions per wild type cell. And there are 12 Mb (5 Gray), 8.0 Mb (7 Gray) and 0.3 Mb (10 Gray) duplicated DNA regions in each *Blm*-deficient cell.

Table 5-7 **Summary of deletions and duplications in wild type cells and *Blm*-deficient cells generated by 2-10 Gray gamma irradiation**

A

Dose (Gray)	Number of samples	Average number of deleted regions ± SD	Average length per deletion (Mb)	Average length of deleted regions per cell (Mb)
2	4	0 ± 0	–	0
5	4	0 ± 0	–	0
7	3	1 ± 2	1.8	1.8

B

Dose (Gray)	Number of samples	Average number of duplicated regions ± SD	Average length per duplication (Mb)	Average length of duplicated regions per cell (Mb)
2	4	0 ± 0	–	0
5	4	2 ± 3	0.3	0.5
7	3	1 ± 2	9.1	9.1

C

Dose (Gray)	Number of samples	Average number of deleted regions ± SD	Average length per deletion (Mb)	Average length of deleted regions per cell (Mb)
2	3	1 ± 1	6.2	4.1
5	3	0 ± 1	0.5	0.2
7	3	2 ± 2	13	26
10	6	2 ± 1	6.6	9.8

D

Dose (Gray)	Number of samples	Average number of duplicated regions ± SD	Average length per duplication (Mb)	Average length of duplicated regions per cell (Mb)
2	3	2 ± 4	0.6	1.4
5	3	2 ± 0	5.8	12
7	3	2 ± 2	4.0	8.0
10	6	1 ± 1	0.5	0.3

A. Deletions in the wild type cells; **B**. Duplications in the wild type cells; **C**. Deletions in the *Blm*-deficient cells; **D**. Duplications in the *Blm*-deficient cells. SD: standard deviation.

5.3 Discussion

5.3.1 Survival frequency of ES cells after irradiation

As expected the survival rate fell as the dose of gamma irradiation increased. The rates obtained for wild type cells are consistent with published reports (You *et al.* 1997). These data enable me to extrapolate the number of independent clones in each pool of ES cells. Combined with sizes and frequency of deletions generated by a certain dose of irradiation, the genome coverage of the mutations can be calculated in this project.

Although cells with deficiency of DNA repair proteins, such as the human autosomal recessive disorder *xeroderma pigmentosum* (XP) cells can have increased sensitivity to killing exposure to a variety of DNA-damaging agents, including UV irradiation. The deficiency of the murine Bloom syndrome protein in ES cells exhibit slightly higher resistance to irradiation compared to the wild type cells. The repair of double-strand breaks (DSB), which is induced by irradiation, is usually executed through two major pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). Several other sub-pathways may also exist, since many proteins have been reported to be involved in DSB repair and the coordinated functions of these proteins are necessary for successful DSB repair (Lieber *et al.* 2003; West 2003). It is known that BLM responds quickly to DNA DSB by colocalizing with phosphorylated histone H2AX (γH2AX) and ATM kinases at sites of laser light-induced DNA double-strand breaks. However, the recruitment of BLM to the damaged sites is not dependent on the presence of ATM or any of RAD17, DNA-PKcs, NBS1, XRCC3, RAD52, RAD54 and WRN (Karmakar *et al.* 2006). In *BLM*-deficient cells, phosphorylated histone H2AX (γH2AX), Chk2 and ATM checkpoint proteins can colocalized in nuclear foci following DSB (Rao *et al.* 2007). In addition, microhomology elements are not used to DSB repair in *Blm*-deficient cells (Langland *et al.* 2002). After DSB repair, more mutations were found in Bloom syndrome deficient cell extracts. This result is consistent with my results shown by array CGH that *Blm*-deficient cells carry more duplications and deletions after irradiation.

5.3.2 Megabase duplications and deletions generated by irradiation

After irradiation at certain dosage, a higher proportion of the *Blm*-deficient cells carry chromosome duplications and deletions compared with wild type cells (Table 5-5, Table 5-6) although the average number of duplications and deletions is similar between two genetic backgrounds. The average 0–2 deletions and 0–2 duplications were generated in each of the wild type and the *Blm*-deficient (Table 5-7) cells after irradiation at 2–10 Gray. The *Blm*-

deficient cells contain larger deleted regions than that in the wild type cells. About 10–26 Mb DNA sequence was deleted after irradiating the *Blm*-deficient cells at 7 and 10 Gray but only ~3 Mb DNA sequence was deleted after irradiating the wild type cells at 7 Gray. When the irradiation dose is between 2 to 7 Gray, deletion and duplication can be very large, for example there is a 46 Mb deletion and a 28 Mb duplication in Blm7G1. These large mutations make the process to map the mutated genes harder than that mutated by smaller mutations. Although the number of samples is relatively low, these deletion/duplication data roughly reflected the frequency and the size of deletions induced by irradiation. I decided to use 10 Gray to make mutation libraries as it generates reasonable size of mutations (~10 Mb per cell) at a high percentage (67%, 4/6). Thus, there is no need to select for mutations before screening by 6TG. Ten Gray irradiated cells retain around 10 Mb deleted regaions, so 450 independent survival clones will be enough to make mutations cover the whole mouse genome.

5.3.3 Chromosomal abnormalities

Besides of the heterozygous deletions and duplications induced by irradiation, whole chromosome gain/loss or segmental chromosomal gain/loss were also observed in the mutants. These can be either spontaneous or irradiation-induced. The copy number change of the whole chromosomes might be spontaneous. Chromosomes can be lost or gained in tetraploid cells, resulting in three copies or five copies of individual chromosomes. The array Log_2(ratio) of these chromosome gain should be able to be distinguished with trisomies. However, it will be hard to do so if a trisomy occur in the process of cell culture and lead to a mosaic cell population. Trisomy 8 and trisomy 11 are commonly observed in a large proportion of cultured ES cell lines (Sugawara *et al.* 2006) and thus were expected to be seen in the mutant cells. The clone WT2G2 might have a trisomy 11. Chromosome Y can be lost in diploid cells, which was also observed by my colleagues in their projects.

On the other hand, irradiation can generate large segmental chromosome gain and loss. During S/G2 phase, a deletion or a duplication of a segmental chromosome can result in 3N or 5N of the region and be kept in a tetraploid cell. If such a cell divides normally, a mosaic colony can be generated so that the Log_2(ratio) of the changed region is nearer to zero compared to those in pure colonies.